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The brain-pituitary-gonadal axis in the rainbow trout, *Salmo gairdneri*

II. Direct effect of gonadal steroids on the gonadotropic cells*

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Summary. In a cytophysiological study it was investigated whether in juvenile trout gonadal steroids stimulate the gonadotropic (GTH)-cells directly or indirectly via the brain. Pituitaries of donor animals were transplanted into the caudal musculature of testosterone-treated and non-testosterone-treated host fish. Testosterone treatment caused an increase in GTH-content in the in situ pituitaries and in the grafts. Accordingly, the gonadotrops displayed ultrastructural changes such as the appearance of well-developed Golgi systems and large globules. The stimulation of the morphological development of gonadotrops and of synthesis and storage of GTH in the allografted pituitaries indicates that testosterone affects the GTH-cells directly. In untreated juvenile trout the gonadotropin content of the pituitary and the gonadotropin concentration in the plasma vary with the time of year. This variation and the role of testosterone and gonadotropin-releasing hormone on the release of GTH are discussed.

Key words: Gonadotrops – Gonadotropin secretion – Steroids – Brain-pituitary-gonadal axis – Juvenile teleost

In some teleost species, e.g. salmonids, two different types of gonadotropins have been demonstrated (Idler and Ng 1979; Idler 1982). The one, the maturational hormone, is composed of two subunits, the α- and β-protein chain (Burzawa-Gérard 1982; Breton 1981), and has a high carbohydrate content. This glycoprotein induces gametogenesis and sex-hormone production and is involved in the processes of vitellogenesis, oocyte maturation, ovulation and spermiation. The other, referred to as vitellogenic gonadotro-

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pin, is a protein with a low carbohydrate content. So far, the only action ascribed to this hormone in female fish is the stimulation of vitellogenin incorporation into the oocytes.

In juvenile rainbow trout, *Salmo gairdneri*, administration of sex steroids induces synthesis and storage of the glycoprotein gonadotropic hormone (GTH) (for review, see Gielen et al. 1982b). This accumulation of GTH is accompanied by an accelerated development of the gonadotropic cells, including enlargement of the cells and their nuclei, an increase of granular endoplasmic reticulum, and the appearance of well-developed Golgi systems, more and enlarged secretory granules and large globules (Gielen et al. 1982a, b).

A similar stimulatory action of steroids on the morphological development and synthetic activity of GTH-cells has been observed in gonadectomized immature sockeye salmon, *Oncorhynchus nerka* (van Overbeeke and McBride 1971), in Atlantic salmon parr, *Salmo salar* (Crim and Peter 1978), and in European silver eels, *Anguilla anguilla* (Olivereau and Chambolle 1978, 1979; Olivereau et al. 1979; Olivereau and Olivereau 1979a, b; Olivereau and Nagahama 1982). Furthermore, administration of more or less purified GTH to juvenile fish also leads to an accelerated development of the gonadotrops, as was demonstrated by Olivereau (1967) for the European eel (*A. anguilla*), by Ueda and Takahashi (1978) for the Japanese eel (*A. japonica*), and for the rainbow trout (*S. gairdneri*) by Gielen et al. (1982a, b). The latter authors showed that the stimulatory effect was mediated by gonadal hormones, synthesized as a result of the GTH-treatment.

Growth to sexual maturity implies the development of the brain-pituitary-gonadal axis. From the studies mentioned above it is obvious that gonadal steroids play an important role in the development of this component of the endocrine system. However, it is not clear whether the steroids carry out this function by affecting gonadotropin-releasing hormone (GnRH)-centres in the brain, the GTH-cells, or both.

In the present study we investigated the direct effect of an exogenous steroid hormone on the gonadotrops using an in vivo culture system. Pituitaries of male and female juvenile donor animals were transplanted into the caudal musculature of testosterone- and non-testosterone-treated host animals of both sexes. The effects of these treatments on the in situ and grafted pituitaries were assessed by an ultrastructural study of the GTH-cells and by analyzing the levels of gonadotropin in pituitary extracts and plasma. In a second experiment only the GTH-content of the pituitaries and the GTH-concentration in the plasma were analyzed.

Materials and methods

Juvenile rainbow trout, *Salmo gairdneri*, hatched in February-March, 1981, were obtained from a trout hatchery in Apeldoorn (The Netherlands). Fish with a body weight of 30–60 g were kept in 200-l aquaria with running tap-water of $\pm 12^\circ$ C and under a simulated natural photoperiod. Two experiments were carried out: The first one started in November of the year of hatching, the second one in January of the following year. The experimental groups are listed in Table 1.

Before operation, injection and decapitation, the fish were anaesthetized in 2-phenoxy-aethanol 0.03%.

Transplantation of pituitaries

A single whole pituitary gland from male or female juvenile donor animals was transplanted to host animals. After removal of the pituitary from the donor fish, the gland was immediately inserted into a pouch made in the caudal musculature of the recipient. Four weeks after the transplantation the recipients were sacrificed together with sham-operated fish, and sex was recorded. The four possible combinations of sexes between donor and acceptor animals were at randomly distributed. Animals serving as untreated controls were autopsied two weeks after the beginning of the experiment.

Testosterone treatment

One day prior to the pituitary implantation host animals received testosterone or vehicle. Sham-operated animals only received testosterone. Testosterone was dissolved in molten cocoa butter, drawn into syringes, which were kept in warm water ($\pm 45°$ C), and injected intraperitoneally at a concentration of 1 µg/g body weight.

Preparation of pituitaries for electron microscopy

At the end of the first experiment the pituitaries of five fish from each group of animals were fixed in a 1:1 mixture of 2% paraformaldehyde and 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 0° C. After 1 h the pituitaries were postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer at 0° C. Subsequently, the glands were dehydrated in graded alcohols and propylene oxide and embedded in Epon. Ultrathin sections were cut on a Reichert OMU 3 ultramicrotome, collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate (Reynolds 1963), and examined with a Zeiss EM 10 electron microscope.

Preparation of pituitaries and blood samples for determination of hormone content

For gonadotropin determination pituitaries were quickly frozen over solid carbon dioxide and stored at $-20°$ C. Following storage they were individually homogenized in 200 µl phosphate-buffered saline (PBS 0.01 M; pH 7.6) at 0° C. Samples were then centrifuged for 20 min ($5000 \times g$), supernatants were collected and frozen, the pellets resuspended in 200 µl PBS and stored overnight at 4° C. After 12 h the latter samples were mixed and recentrifuged, supernatants collected and pooled with the initial ones. They were immunoassayed for GTH-content.

Prior to decapitation blood samples were taken with heparinized syringes and the plasma was stored at $-20°$ C for GTH and testosterone analysis.

GTH- and testosterone radioimmunoassay

For GTH measurement a double antibody radioimmunoassay was used.

Highly purified salmon glycoprotein gonadotropin (Con-A II fraction provided by Dr. Idler, St. John's, Canada) was used for labelling and as standard hormone. Labelling with ^{125}I was carried out according the Chloramine-T method of Greenwood et al. (1963). Labelled hormone was purified on a Sephadex-G 100 column.

As the primary antibody an antiserum against SG-G 100 salmon gonadotropin (Dr. Donaldson, Vancouver, Canada) prepared in a similar way as described by Goos et al. (1976) was used in a final dilution of $0.5 \cdot 10^{-5}$. As second antibody a donkey-anti-rabbit serum (Wellcome) was used in a final dilution of 1:240. The assay includes a 48 h preincubation at 4° C of standard hormone and samples (50 µl) with the first antibody (250 µl), and a subsequent 48 h incubation at 4° C with the label (200 µl, 10000 cpm). 24 h after adding 100 µl of the second antiserum (inbubation at room temperature) 2 ml buffer were added and the tubes centrifuged at $5000 \times g$ (20′, 4° C). The supernatants were aspirated off and the precipitates counted in a gamma counter.

All dilutions were carried out with 0.05 M sodium barbital buffer (pH 8.6) containing 0.02% bovine serum albumin and 0.01% sodium azide.

The RIA for testosterone was carried out according to the method described by van Landeghem et al. (1981), which can be summarized as follows: To 25 µl or 50 µl aliquots of plasma 25 µl or 50 µl 0.1 M sodium hydroxide, respectively, were added. Following ether extraction the extracts were incubated with the antibody (4° C, 24 h). Bound and free steroids were separated using dextran-coated charcoal. The cross reactivity of the antiserum, read at 50% of the initial binding, was 16.2% for 5α-dihydrotestosterone, 2.8% for androstenedione, and 3% for 11-ketotestosterone.

Calculation of both radioimmunoassays was performed after logit-log transformation. The amount of GTH is expressed in assay units (AU) instead of weight units (ng), because of the heterologous nature of the assay.

Statistical procedure

After logarithmic transformation of the values Bartlett's test for homogeneity of variances (Sokal and Rohlf 1969) was applied. Only when the variances within the experimental groups were significantly heterogeneous an approximate test for differences between means recognizing this heterogeneity was used. Otherwise, a *t*-test was used.

Results

At the end of the experiments the grafted pituitaries could readily be recognized under a dissecting microscope. They were well vascularized and frequently firmly attached to the surrounding tissue. In some cases, however, only poorly vascularized, necrotic pituitary fragments could be found, indicating that the hypophysis was not successfully grafted.

Histologically, in the successfully grafted pituitaries large accumulations of glandular hypophysial cells could be observed. Often infiltration of connective and muscle tissue was recognized. In the unsuccessfully grafted pituitaries most hypophysial tissue was ischemic and degenerated. In these degenerated grafts no cells could be identified as gonadotrops as defined by ulstrastructural characteristics. Animals carrying an unsuccessfully grafted pituitary were discarded from the experiment.

The ultrastructural features of GTH-cells identified in the pars distalis of the adenohypophysis in untreated fish were as follows. Most cells contained abundant secretory granules with a diameter between 124 nm and 285 nm (average diameter 196 nm), bundles of microfilaments and irregular cisternae of granular endoplasmic reticulum (GER). The contents of the secretory granules varied in electron density. Most granules were circular in outline, but some were oval or even more elongated. This latter phenomenon seemed to result from granular fusion. Identical GTH-cells were observed in the in situ and grafted hypophyses of non-testosterone treated animals (Fig. 1).

The GTH-cells in the in situ pituitaries and grafts of testosterone-treated fish differed from those in the in situ and the ectopic pituitaries from non-testosterone-treated animals or in the hypophysis of untreated fish (Figs. 2, 3). More cells could be identified as gonadotrops, most of them being enlarged as were their nuclei. Their Golgi apparatus was well developed. The most striking difference, however, was the presence of large globules (diame-

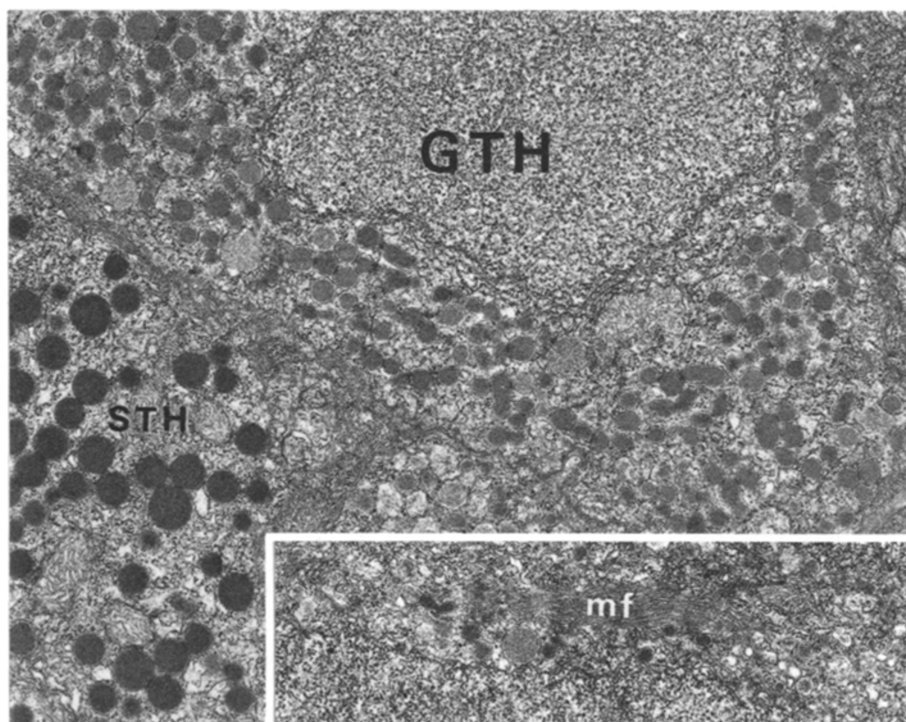


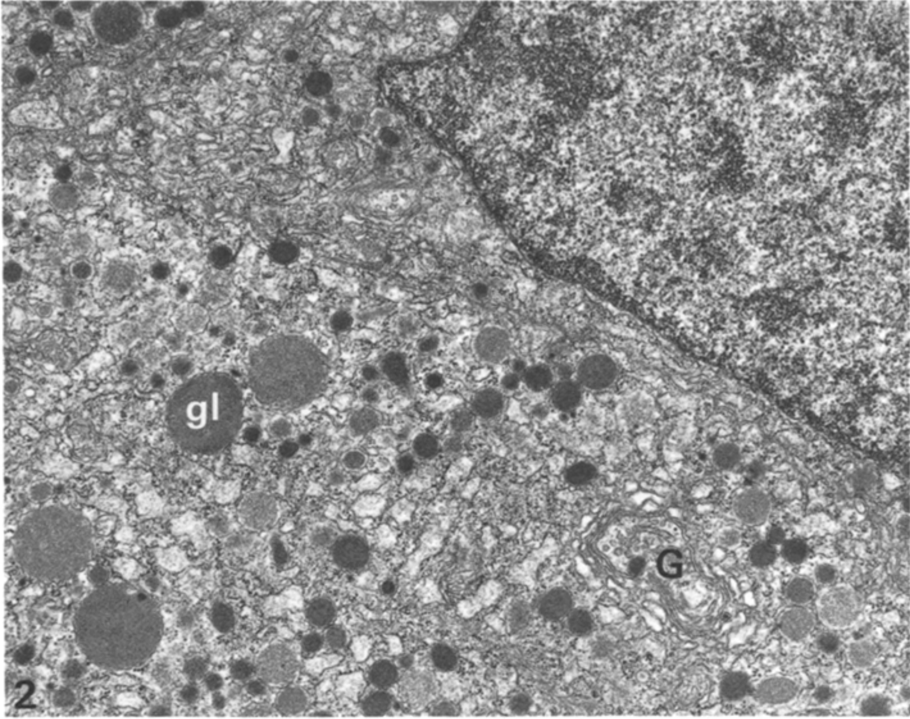
Fig. 1. GTH-cells in the in situ adenohypophysis of a non-testosterone-treated grafted juvenile rainbow trout. The *inset* shows microfilaments in a similar GTH-cell. ×15000. *GTH* gonadotropic cell, *STH* somatotropic cell, *mf* microfilaments

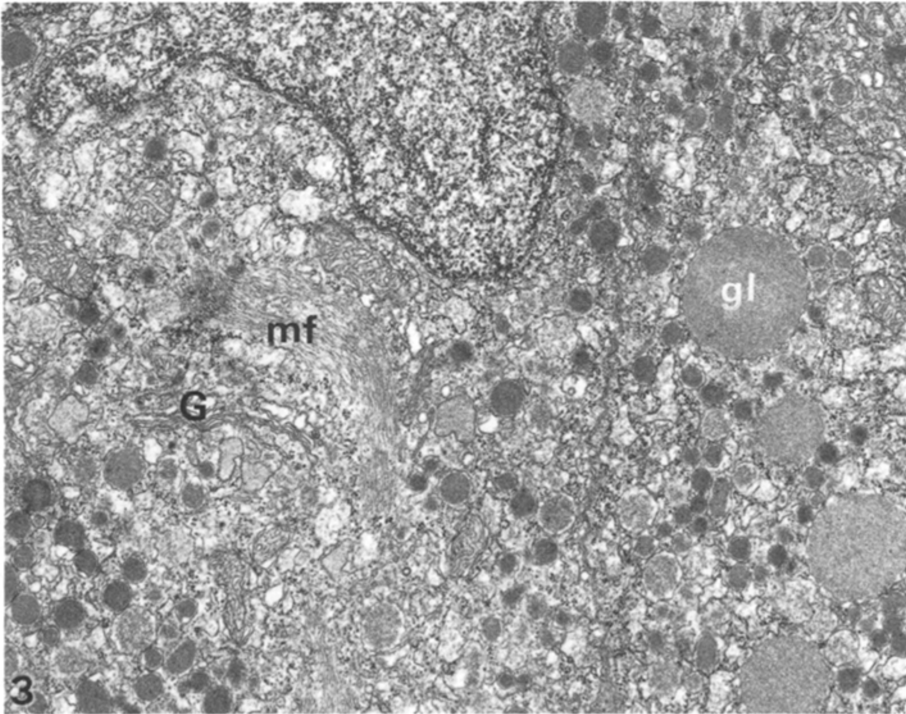
ter between 429–1 000 nm, average diameter 657 nm) in many GTH-cells. The density per cell of the secretory granules, and their diameter (124–314 nm, average diameter 206 nm) did not differ from those in the GTH-cells of control animals. Gonadotrops of non-grafted animals treated with testosterone exhibit similar morphological characteristics.

GTH-levels (Table 1)

According to the morphological development of the gonadotrops in the in situ and grafted pituitaries of animals treated with testosterone, both the in situ and the implanted glands had increased GTH-content. In situ and ectopic pituitaries of non-testosterone-treated recipients contained very low amounts of gonadotropin, similar to pituitaries of untreated fish.

Although there is a difference between the absolute GTH-content of the pituitaries in the two experiments, the relative differences between the experimental groups in both experiments are identical, i.e., in situ and grafted pituitaries contain more GTH after testosterone treatment. This does not account for the GTH-levels in the plasma. At the end of the first experiment, untreated animals, non-testosterone-treated fish carrying





Figs. 2, 3. GTH-cells from, respectively, an in situ pituitary and an allografted pituitary of a testosterone-treated juvenile trout. × 15000. *G* Golgi apparatus, *gl* large globule, *mf* microfilaments

Table 1. Gonadotropin content in plasma and pituitary gland of juvenile trout under different experimental conditions*

Gonadotropin (GTH)	Experimental groups			
	Untreated	Grafted	Grafted + Testosterone	Non-grafted + Testosterone
I: Number of animals	(11)	(18)	(17)	(11)
GTH				
AU/ml plasma	0.98±14[a]	0.97±0.04	1.23± 0.08[b,c]	0.98±0.15
AU/pituitary in situ	0.74±0.04[a]	0.69±0.04	112 ± 9[d]	96 ±9[d]
AU/pituitary graft		0.70±0.04	81 ±18[d]	
II: Number of animals	(12)	(16)	(16)	(12)
GTH				
AU/ml plasma	0.43±0.03	0.76±0.04[b]	0.93± 0.07[b,c]	0.78± 0.09[b]
AU/pituitary in situ	1.26±0.05	1.26±0.08	180 ±34[d]	136 ±31[d]
AU/pituitary graft		1.11±0.16	93 ±31[d]	

* Mean values±SEM
I, II Experiment carried out in November-December and January-February, respectively
[a] Untreated controls of experiment I *vs* untreated controls of experiment II. $P<0.001$
[b] Significantly different compared to the untreated controls. $0.02<P<0.01$ in experiment I; $P<0.001$ in experiment II
[c] Significantly different compared to the grafted animals. $0.001<P<0.01$ in experiment I; $0.02<P<0.05$ in experiment II
[d] Significantly different compared to control animals. $P<0.001$

a pituitary graft and non-grafted animals treated with testosterone had a similar plasma GTH-content. In the grafted fish treated with testosterone, however, the GTH-level was increased. In the second experiment not only the grafted animals treated with testosterone, but also the non-testosterone-treated recipients and non-grafted fish treated with testosterone showed higher plasma GTH-levels compared to the untreated animals. In accordance with the first experiment allografted animals treated with testosterone reached the highest levels of GTH. However, compared to the non-grafted fish treated with testosterone, in both experiments these levels are not significantly different: $0.05<P<0.1$.

Testosterone levels (Table 2)

Four weeks after implantation, the cocoa butter pellet containing testosterone was still able to cause an elevated testosterone level in the plasma. There was no difference between male and female animals. The plasma levels in both untreated controls and non-testosterone-treated grafted animals differed between the two experiments, i.e., detectable levels in the first experiment (mid-December) and undetectable or very low levels in mid-February. In males plasma testosterone levels were higher than in females.

Table 2. Plasma testosterone level in juvenile trout under different experimental conditions*

Testosterone pMol/ml plasma		Experimental groups			
		Untreated	Grafted	Grafted + Testosterone	Non-grafted + Testosterone
I	♂	1.32 ± 0.30^{a} (6)	1.27 ± 0.30^{b} (10)	26.6 ± 7.4^{c} (17)	20.1 ± 5.9^{c} (11)
	♀	0.88 ± 0.13^{a} (5)	0.07 ± 0.02^{b} (8)		
II	♂	0.16 ± 0.06 (5)	0.28 ± 0.06 (8)	9.8 ± 3.0^{c} (16)	6.9 ± 2.4^{c} (12)
	♀	n.d. (7)	n.d. (8)		

* Mean values ± SEM, (n) = number of animals
I, II Experiment carried out in November-December and January-February, respectively
n.d. Not detectable

[a] Untreated controls of experiment I *vs* untreated controls of experiment II. $P < 0.001$
[b] Grafted males vs grafted females. $P < 0.001$
[c] Testosterone-treated animals *vs* grafted and untreated animals. $P < 0.001$

Discussion

Implantation of an extra pituitary gland into juvenile trout has been shown to be a useful in vivo culture method. Neither the morphology of the GTH-cells, nor the glycoprotein gonadotropin content of the in situ and allografted pituitaries displayed differences when compared to hypophyses in untreated fish. In addition, corresponding to the observations on intact juvenile trout (cf. Gielen et al. 1982a, b), gonadal steroids, such as testosterone, stimulated the morphological development of gonadotropic cells, and the synthesis and storage of the glycoprotein GTH. This occurred not only in the in situ pituitary but also in the ectopic gland. The accelerated development of gonadotrops in pituitaries cultured in the tail musculature of testosterone-treated juvenile fish, and the accompanying enhanced production of GTH strongly indicates that the brain is not necessarily involved in these processes. On the other hand, although not very likely, circulating hormones secreted by brain centres that are possibly affected by testosterone could be responsible for the stimulatory effect on the grafted pituitary. However, in vitro experiments using both organ cultures and dispersed pituitary cell-culture systems support the idea of a direct stimulatory action of steroids on the gonadotrops (Fåhraeus-van Ree et al. 1982, 1983). Admittedly, this does not mean that hypophysiotropic factors such as the gonadotropin-releasing hormone (GnRH) are not involved in the development and synthetic activity of GTH-cells under natural circumstances, since in vivo and in vitro experiments on juvenile trout clearly indicated that GnRH not only causes an enhanced release of gonadotropin but also induces its synthesis (Fåhraeus-van Ree et al. 1982, 1983; Goos et al. 1982).

With respect to the ontogeny of the brain-pituitary-gonadal axis in juvenile trout, van den Hurk et al. (1982) have shown that gonads have the capacity to synthesize steroid hormones at very early stages. Moreover,

these gonads respond to exogenous GTH by secreting their hormones (Ng and Idler 1980; Gielen et al. 1982a, b; Magri et al. 1982). Subsequently, the steroids stimulate the morphological development of the gonadotrops and the production of GTH (cf. Gielen et al. 1982b; van den Hurk 1982).

Although the question, as put forward in the Introduction has been answered, some results concerning the release of GTH require a more detailed discussion. Gielen et al. (1982a, b) have observed that in juvenile trout exogenous testosterone does not affect the GTH-concentration in the plasma. This is confirmed in the first experiment of the present series; in November-December testosterone induced a strong increase in pituitary GTH-content of non-grafted fish, but did not alter the GTH-concentration of the plasma (cf. Table 1). However, in the second experiment a limited, but significant rise in plasma GTH-level followed the testosterone treatment. Thus, it seems that in January-February testosteron did not prevent a slight increase in plasma GTH-concentration. On the other hand, injection of a synthetic GnRH into testosterone-treated juvenile trout results in a much more prominent increase of the plasma GTH-content (Goos et al. 1982). This means that GTH-cells of testosterone-pretreated juvenile trout not only have a release mechanism but also are sensitive to GnRH. Possible explanations for the limited release of GTH by the testosterone-treated fish in the present investigations might be that testosterone prevents a full GTH-release or that in such juvenile trout the brain does not yet produce GnRH. The latter, however, is not likely since a GnRH-like activity could be demonstrated in brain extracts of immature trout (Crim and Evans 1980; Gielen and Jansen, unpublished). Thus, it seems that in juvenile trout GnRH-centres in the brain do not have the capacity to secrete sufficient GnRH to allow for a more than restricted GTH-release.

In both experiments, although not significantly different, there is a difference between the plasma GTH-level of non-testosterone-treated grafted animals and testosterone-treated recipients. Furthermore, in the second experiment the plasma GTH-content is significantly higher in the "grafted" group when compared with the "untreated" one. These observations on ectopic pituitaries indicate that gonadotrops can release their hormone, independently of a direct connection with the brain.

The only difference between the two experiments of the present study was the time of year in which they were carried out. Regardless, exogenous testosterone caused a much higher plasma testosterone level at the end of the first than at the end of the second experiment. Probably, the turnover of testosterone had been higher during January-February than during November-December. Such a difference in testosterone turnover might partly explain the difference in plasma levels between the animals not treated with testosterone in the first and second experiments; in the males the levels are higher in mid-December than in mid-February, and the same accounts for the females.

The low turnover of testosterone in November-December may be the reason why the GTH-content of the pituitaries of the "untreated" and "grafted" animals is relatively low and their plasma GTH-level relatively

high (cf. Table 1). Similarly, the increased turnover of testosterone in January-February can help to explain the increased GTH-content of the pituitaries and the decreased concentrations of GTH in the plasma.

Comparison of the plasma levels of untreated animals from the two experiments shows a one-hundred percent difference. Also the pituitary GTH-content is not similar in the two experiments, and moreover, the GTH-content of the pituitary is negatively correlated with the GTH-level in the plasma. Goos et al. (1982) also observed a variation in basic plasma GTH-levels in juvenile trout, which they described as a seasonal variation. Although not thoroughly investigated, there also seem to be variations in the ultrastructural appearance of the GTH-cells of juvenile trout throughout the year (Gielen, unpublished). In the present study pituitaries of untreated fishes fixed in December contain gonadotrops filled with secretory granules of varying electron density (Fig. 1). This does not accord with the description of GTH-cells from juvenile trout, autopsied in January and March (Gielen et al. 1982a, b). Such cells contain very few and smaller secretory granules. In contrast, the present results indicate a stronger storage of GTH in February than in December. Leunissen et al. (1982) have demonstrated that in adult rainbow trout immunoreactive GTH is present in globules and granules. However, this does not mean that all secretory vesicles contain immunoreactive GTH. It cannot be excluded that, especially in juvenile trout, the amount of immunoassayable gonadotropin stored in the pituitary is not reflected by the amount of secretory granules. On the other hand, the presence of increased numbers of GTH-cells with abundant secretory granules and globules, as a result of steroid treatment, corresponds well with an increased hormone content (cf. present study; Gielen et al. 1982a, b). Further investigations are needed to clarify the seasonal variations in pituitary GTH-content, GTH-release and ultrastructural appearance of the GTH-cells in juvenile trout.

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