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The Brain–Pituitary–Gonadal Axis in the Rainbow Trout, *Salmo gairdneri*

III. Absence of an Inhibiting Action of Testosterone on Gonadotrophin Release in Juveniles

J. TH. GIELEN[1] AND H. J. TH. GOOS[2]

Zoological Laboratory, Research Group for Comparative Endocrinology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

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In juvenile rainbow trout the effects of exogenous testosterone and of a synthetic gonadotrophin-releasing hormone (GnRH) on the secretion of gonadotrophin (GTH) were investigated. Treatment with implanted testosterone resulted in an accumulation of GTH in the pituitary, but did not affect the concentration of GTH in the plasma. After the testosterone implants were removed, the levels of testosterone in the circulation dropped to undetectable or control values, but the concentration of GTH in the plasma did not increase. These results indicate that testosterone stimulated the synthesis and storage of GTH, and did not prevent the release of this hormone. The synthetic GnRH, des-Gly^{10}[D-Ala^{6}]LH-RH ethylamide (LH-RHa) stimulated the release of GTH when injected into testosterone-pretreated fish, indicating that accumulated GTH is present in a releasable pool.

In adult rainbow trout, *Salmo gairdneri*, the secretion of gonadotrophin, i.e., the synthesis, storage, and release of a glycoprotein gonadotrophic hormone (GTH), varies during the annual reproductive cycle (Peute *et al.*, 1978; Bromage *et al.*, 1982; Zohar, 1982). As in other teleosts, in the rainbow trout these seasonal variations are to a certain extent the result of the influence of gonadal steroids and gonadotrophin-releasing hormone (GnRH) on GTH secretion (for reviews, see Ball, 1981; Peter, 1982a, b). Depending on the stage of the reproductive cycle, the regulation of GTH secretion in adults is under the influence of a negative feedback between the gonads and the GTH cells of the pituitary (Billard, 1978; Van Putten *et al.*, 1981; Bommelaer *et al.*, 1981).

Much attention has also been paid to the secretion of GTH and the regulation of this process in juvenile trout (Crim and Evans, 1979, 1980, 1983; Crim *et al.*, 1981a, b, 1982; Gielen *et al.*, 1982a–c; Gielen and Goos, 1983; Van den Hurk, 1982; Van den Hurk *et al.*, 1984; Fåhraeus-van Ree *et al.*, 1982, 1983). Treatment of male juvenile trout with GTH results in a precocious development of the gonads and an enhanced secretion of steroid hormones (Gielen *et al.*, 1982a, b; Magri *et al.*, 1982; Crim *et al.*, 1982). Subsequently, these steroids stimulate the synthesis and storage of glycoprotein gonadotrophin. Synthesis and storage of gonadotrophin, which can also be induced by exogenous steroids (Crim and Evans, 1979; Gielen *et al.*, 1982a, b; Van de Hurk, 1982; Van den Hurk *et al.*, 1984), are accompanied by signs of an accelerated development of the gonadotrophic cells, including enlargement of the cell and the nucleus, accumulation of secretory granules, development of the Golgi apparatus, and the appearance of large globules (Gielen *et al.*, 1982a, b). Moreover, *in vivo* and *in vitro* studies on cultured pituitaries demonstrate that steroids can stimulate the

[1] Present address: Intervet International B.V., P.O. Box 31, 5830 AA Boxmeer, The Netherlands.

[2] To whom correspondence should be sent.

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GTH cells directly (Gielen *et al.*, 1982c; Gielen and Goos, 1983; Fåhraeus-van Ree *et al.*, 1982, 1983). Although in these studies steroids were seen to stimulate the synthetic activity of the GTH cells, an increased release of their hormone was not observed. Possible explanations for the absence of a stimulated release of GTH could be that (1) the GTH cells have not yet fully developed a releasing mechanism responding to GnRH or (2) the circulating steroid has an inhibitory effect on the releasing process. The first explanation seems to be unlikely. A number of *in vivo* and *in vitro* studies have shown that pituitaries of juvenile rainbow trout, especially when pretreated with testosterone, are able to release GTH after application of luteinizing hormone-releasing hormone (LH-RH) or its analogs (Crim and Evans, 1980; Crim *et al.*, 1981a; Fåhraeus-van Ree *et al.*, 1983; Goos *et al.*, 1982).

With regard to the second explanation, results from castration experiments reported by Crim *et al.* (1982) and Gielen *et al.* (1982b) suggest that in juvenile trout no negative feedback is operative between the gonads and the GTH secretion. However, since the pituitary GTH content in juveniles is very low, not much of a release can be expected. Moreover, the levels of circulating steroids also are very low (cf. Gielen *et al.*, 1982a, b; Magri *et al.*, 1982; Crim *et al.*, 1982; Gielen and Goos, 1983) and, thus, might be of minor importance in inhibiting the release of GTH.

Therefore, in the present study the hypothesis of gonadal steroids inhibiting GTH release was checked by measuring the GTH content in the plasma of yearling rainbow trout, before and after removal of an implanted pellet containing testosterone. To study whether releasable GTH is still present in the pituitary after removal of the testosterone pellet the effect of a luteinizing hormone-releasing hormone analog (LH-RHa) on the plasma GTH level was investigated at different times after removal of the pellet.

MATERIALS AND METHODS

Animals

Juvenile rainbow trout, *S. gairdneri*, obtained from a Dutch hatchery, were kept in 200-liter aquaria of running copper-free tap water at 12 ± 1° under a simulated natural photoperiod. The fish were fed three times a week with Trouvit pellets (Trouw & Co., The Netherlands) in a quantity corresponding to 1–2 percent of their body weight. No food was supplied 2 days before and 3 days after operation. Animals of both sexes, aged 14 months in the first and 18 months in the second experiment, and with a body weight of 50–80 g were used. Before operation, blood sampling, injection, or decapitation the animals were anesthetised with 2-phenoxy-ethanol, 0.03%.

Experimental Design

Two experiments were carried out. The first experiment was performed in May 1982. Thirty animals were divided into three groups (Table 1). Fish of Groups 1 and 2 received cocoa butter pellets containing testosterone, while those of Group 3 received cocoa butter placebos.

To prepare the pellets containing testosterone, the steroid was dissolved in molten cocoa butter (30 μg/0.1 ml) at ~45°. Aliquots (0.1 ml) of the cocoa butter, each containing a silk thread, were allowed to solidify and were then stored at 4°.

A cocoa butter pellet, with or without testosterone, was implanted into the perivisceral cavity by making a small incision in the abdominal wall, and leaving the silk thread of the pellet outside the body. The wound was closed with a chrome-catgut thread.

Fourteen days after applying the cocoa butter pellets blood was sampled to measure the testosterone and the GTH levels. Subsequently, the pellets were removed from the animals of Group 2 by pulling the silk thread. After removal, the injury was stitched. Five days later a second blood sample was taken from the animals of Group 2, again to measure testosterone and GTH plasma levels.

Shortly after blood sampling of Groups 1 and 3 on Day 14 and of Group 2 on Day 19, five animals of each group were injected with LH-RHa and five with the LH-RHa vehicle. The LH-RHa used was des-Gly^{10} [D-Ala^{6}]LH-RH ethylamide (Sigma Chemical Co., St. Louis, Mo.). This LH-RHa has been demonstrated to be a potent GnRH in several teleosts (e.g., van der Kraak *et al.*, 1983). Animals were injected intraperitoneally with 2.5 μg LH-RHa, dissolved in 50 μl of a solution of 0.8% NaCl and 0.25% bovine serum albu-

TABLE 1
SCHEMATIC DESIGN OF THE FIRST EXPERIMENT (MAY)

	Treatment					
	Testosterone implantation			Injection of LH-RHa		
Group	Number of animals	Day 0	Day 14	Number of animals	Day 14	Day 19
1	10	+ T		5	+ LH-RHa	
				5	+ vehicle	
2	10	+ T	− T	5		+ LH-RHa
				5		+ vehicle
3	10	+ CB		5	+ LH-RHa	
				5	+ vehicle	

Note. Each animal received a pellet containing testosterone (T) or a cocoa butter placebo (CB). + T, − T = pellet containing testosterone administered and removed, respectively.

mine (BSA), or with 50 µl of the vehicle only. Two hours later blood samples were collected, and the animals were sacrificed by decapitation. The pituitaries were removed, quickly frozen over solid carbon dioxide and stored at −80°.

The experiment was repeated in September 1982. However, in this case the animals were divided into five groups (Table 2). Fish of Groups 1, 2, and 3 received pellets containing testosterone and those of Groups 4 and 5 were implanted with cocoa butter placebos. After fourteen days blood was sampled and the pellets were removed from animals of Groups 2, 3, and 5. Two days (Group 2) and 5 days (Groups 3 and 5) later another blood sample was taken. On Days 14, 16, and 19, the animals of each group received LH-RHa or vehicle as indicated in Table 2. Two hours after injection, blood was sampled and the animals were sacrificed. The blood samples were used for measuring the testosterone and GTH concentrations in the plasma.

Pilot studies carried out on both male and female juvenile trout showed that there was no difference in response to testosterone or LH-RHa between the two sexes (data not shown). For this reason in the present experiments no distinction has been made between males and females.

Hormone Assays

Blood was taken from the caudal vasculature using heparinized syringes. After centrifugation (10 min,

TABLE 2
SCHEMATIC DESIGN OF THE SECOND EXPERIMENT (SEPTEMBER)

	Treatment						
	Testosterone implantation			Injection of LH-RHa			
Group	Number of animals	Day 0	Day 14	Number of animals	Day 14	Day 16	Day 19
1	20	+ T		15	+ LH-RHa		
				5	+ vehicle		
2	5	+ T	− T	5		+ LH-RHa	
3	11	+ T	− T	6			+ LH-RHa
				5			+ vehicle
4	10	+ CB		5	+ LH-RHa		
				5	+ vehicle		
5	5	+ CB	− CB	5			+ LH-RHa

Note. Each animal received a pellet containing testosterone (T) or a cocoa butter placebo (CB). + T, − T = pellet containing testosterone administered and removed, respectively. + CB, − CB = cocoa butter pellet administered and removed, respectively.

3000*g*; 4°) the plasma was stored at −20° until assayed.

Pituitaries were homogenized in 400 μl phosphate-buffered saline (PBS, 0.01 *M*, pH 7.6) at 0°. Samples were then centrifuged for 20 min (5000*g*), supernatants were collected and frozen, the pellets were resuspended in 200 μl PBS, and stored overnight at 4°. The latter samples were then shaken well and recentrifuged, supernatants collected, and pooled with the initial ones. They were immunoassayed for GTH content.

The glycoprotein gonadotrophin was measured using the heterologous radioimmunoassay described by Gielen and Goos (1983). Salmon GTH (Con A II fraction, Dr. Idler, St. John's, Newfoundland, Canada) was used for labeling and as a standard preparation. As the primary antibody, an antiserum against SG-G100 salmon GTH was used in a final dilution of 0.5×10^{-5}. As a second antibody a donkey anti-rabbit serum (Wellcome Reagents Ltd., England) was used in a final dilution of 1:240. The limits of detection for the assay were as follows: for pituitary extracts the upper and the lower limits were 40 and 0.5 ng/pituitary, respectively; for the plasma the lower limit was 0.25 ng/ml. All plasma samples and all pituitaries were tested in one assay.

The radioimmunoassay for testosterone was based on the method described by van Landeghem *et al.* (1981), and can be summarized as follows. To 50-μl aliquots of plasma 50 μl 0.1 *M* sodium hydroxide was added. Following ether extraction the extracts were incubated with the antibody. After overnight incubation at 4°, separation of bound and unbound fraction was done by dextran-coated-charcoal treatment of the incubation mixture for 30 min. The bound fraction was measured by liquid scintillation counting. 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. The lower limit of detection was 0.1 pmol testosterone/ml plasma. All plasma samples were tested in the same assay.

Statistical Analysis

Bartlett's test for homogeneity of variances (Sokal and Rohlf, 1969) was performed. When the variances within the experimental groups were significantly heterogeneous an approximate test for differences between means recognizing this heterogeneity was used. Otherwise, the significance of differences between two means was assessed by Student's *t* test. Differences were considered to be significant if $P < 0.05$.

RESULTS

Effects of Testosterone (Table 3)

Both in May and September the plasma testosterone concentrations in yearling trout treated with cocoa butter pellets were very low, much lower than in animals treated with pellets containing testosterone. However, the increase in the plasma testosterone level due to the testosterone treatment was much more pronounced in May than in September. After removal of the source of exogenous testosterone in September the concentrations of testosterone in the plasma returned to base levels within 2 and 5 days. In May, 5 days after removal of the pellets containing testosterone, plasma testosterone had fallen below the level of detection.

In both experiments the pituitaries of placebo-treated animals had GTH contents that ranged from 0.7 to 1.0 ng. Treatment with testosterone led to levels exceeding the upper detectable limit. After removal of the source of exogenous steroid, the pituitary GTH content remained higher than 40 ng.

The plasma GTH levels in the placebo-treated fish were about the same in May and September. In September administering and subsequently withdrawing testosterone had no influence on the plasma GTH level. However, in May the presence of a testosterone-containing pellet caused a small but significant rise in GTH concentration in the plasma, which disappeared after removal of the pellet.

Effects of GnRH (Table 4)

Injection of LH-RHa into juvenile trout treated with testosterone resulted in an increased release of GTH. In both experiments, animals still carrying the testosterone pellets (and in consequence exhibiting high blood levels of testosterone at the time of LH-RHa-injections, showed more pronounced increases in the GTH concentrations in the plasma than did animals from which these pellets had been removed prior to LH-RHa administration (cf. Groups 1 and 2 in both experiments). However, the difference between the means of these

TABLE 3
PLASMA GONADOTROPHIN (GTH) AND TESTOSTERONE (T) LEVELS IN JUVENILE TROUT AFTER 14 DAYS OF TESTOSTERONE TREATMENT

Group	Treatment	N	Hormone levels (means ± SEM) Plasma GTH (ng/ml)	Plasma T (pmol/ml)	Pituitary GTH[a] (ng/pituitary)
			Experiment I (May)		
	T pretreatment				
1	Pellet not removed	10	0.61 ± 0.04**	23.06 ± 1.95*	>40
2	5 Days after removing the T pellet	10	0.33 ± 0.03	<0.1	>40
	CB pretreatment				
3	Pellet not removed	10	0.48 ± 0.04	0.92 ± 0.10	0.97 ± 0.04
			Experiment II (September)		
	T pretreatment				
1	Pellet not removed	20	0.41 ± 0.02	7.12 ± 0.76*	>40
2	2 Days after removing the T pellet	5	0.37 ± 0.02	0.60 ± 0.18	>40
3	5 Days after removing the T pellet	11	0.41 ± 0.03	0.53 ± 0.07	>40
	CB pretreatment				
4	Pellet not removed	10	0.40 ± 0.03	0.72 ± 0.08	0.83 ± 0.02
5	5 Days after removing the CB pellet	5	0.35 ± 0.05	0.43 ± 0.12	0.91 ± 0.03

Note. Each animal received a pellet containing testosterone (T) or a cocoa butter placebo (CB).
[a] GTH values were obtained after treatment of the animals with LH-RHa and vehicle; cf. Table 4.
* Significantly different from the other groups of both experiments ($P < 0.001$).
** Significantly different from Group 2 ($P < 0.001$) and Group 3 ($0.02 < P < 0.05$) of Experiment I.

Groups 1 and 2 was not significant, due to the strong individual variations within each group (variation in GTH increase/ml plasma: 0.8–10 ng in Groups 1 and 0.3–3.1 ng in Groups 2). On the other hand, the difference was observed twice, i.e., in May and in September.

Placebo-treated animals showed a significant response to LH-RHa in May, but not in September. However, the increased plasma GTH levels were significantly lower than those of animals pretreated with testosterone and having high pituitary GTH contents.

In animals which in May showed low plasma levels of testosterone, (i.e., the fish from which the testosterone pellets had been removed, and the placebo-treated fish) the testosterone concentration was increased significantly by the LH-RHa treatment. In contrast, in September injections of LH-RHa did not cause such differences in the plasma testosterone concentrations.

DISCUSSION

Treatment of juvenile rainbow trout with testosterone caused an increase in the amount of GTH present in the pituitary. This result is in accordance with earlier observations by Crim and Evans (1979), Gielen *et al.* (1982a–c) and Gielen and Goos (1983). Furthermore, these observations indicate that the accumulation of GTH in the pituitary is a result of stimulated synthesis of the hormone. This not only follows from the characteristic ultrastructural features of the GTH cells, (cf. Gielen *et al.*, 1982b), but also from an enhanced incorporation of radiolabeled amino acids into immunoprecipitable gonadotrophin from pituitaries of juvenile trout

TABLE 4

INCREASE IN PLASMA GONADOTROPHIN (GTH) CONTENT, ABSOLUTE PLASMA TESTOSTERONE (T) LEVEL AND THE AMOUNT OF GTH PER PITUITARY AFTER INJECTION OF LH-RHa INTO JUVENILE TROUT

Group	Treatment		N	Hormone levels (means ± SEM) Increase of plasma GTH (ng/ml)	Plasma T (pmol/ml)	Pituitary GTH (ng/pituitary)
			Experiment I (May)			
	T pretreatment					
1	Pellet not removed	+ LH-RHa	5	2.96 ± 1.14**[a]	22.22 ± 2.42*	>40
		+ vehicle	5	n.d.	23.45 ± 2.68	>40
2	5 Days after removing the T pellet	+ LH-RHa	5	1.17 ± 0.23***[b]	2.22 ± 0.24***	>40
		+ vehicle	5	n.d.[c]	<0.1	>40
	CB pretreatment					
3	Pellet not removed	+ LH-RHa	5	0.30 ± 0.09*	2.39 ± 0.20***	0.97 ± 0.07
		+ vehicle	5	n.d.	0.92 ± 0.11	0.95 ± 0.03
			Experiment II (September)			
	T pretreatment					
1	Pellet not removed	+ LH-RHa	15	2.79 ± 0.72***	7.06 ± 1.05*	>40
		+ vehicle	5	n.d.	5.28 ± 1.22	>40
2	2 Days after removing the T pellet	+ LH-RHa	5	1.23 ± 0.32	0.60 ± 0.16	>40
3	5 Days after removing the T pellet	+ LH-RHa	6	1.26 ± 0.48*	0.47 ± 0.12	>40
		+ vehicle		n.d.	0.60 ± 0.06	>40
	CB pretreatment			n.d.		
4	Pellet not removed	+ LH-RHa	5	n.d.	0.71 ± 0.14	0.86 ± 0.04
		+ vehicle	5	n.d.	0.73 ± 0.03	0.79 ± 0.02
5	5 Days after removing the CB pellet	+ LH-RHa	5		0.40 ± 0.12	0.91 ± 0.03

Note. Each animal was pretreated for 14 days with a pellet containing testosterone (T) or with a cocoa butter placebo (CB). Hormone levels were determined 2 hr after injection of 2.5 μg LH-RHa.

[a] Group 1 LH-RHa *vs* Group 3 LH-RHa:$0.01 < P < 0.02$.

[b] Group 2 LH-RHa *vs* Group 3 LH-RHa:$0.001 < P < 0.01$.

[c] n.d., increase in plasma GTH not detectable.

Asterisks indicate significant differences between experimental and control values.

* $0.02 < P < 0.05$.

** $0.01 < P < 0.02$.

*** $P < 0.001$.

treated with testosterone (Gielen and Goos, unpublished). This phenomenon is not limited to the rainbow trout but also occurs in other species of teleosts after treatment with steroid hormones (e.g., Olivereau and Olivereau, 1979). However, in none of the experiments mentioned above, was the storage of GTH followed by a full release of the hormone. It could be argued that testosterone has a negative effect on the release of gonadotrophin.

In juvenile trout, ablation of the gonads neither affects the hypophysial GTH content, nor the concentration of the hormone in the plasma (Gielen *et al.*, 1982a, b; Crim *et al.*, 1982). From these results the latter authors suggest the absence of a negative feedback relation between gonads and GTH release. Their conclusion, however, is based on experiments with juvenile, non-testosterone-pretreated rainbow trout in which not much GTH release may be expected. In the present experiments the GTH cells were stimulated to accumulate GTH but even then, after the testosterone implant had been removed and testosterone levels had dropped to control values, the pituitary failed to release GTH. Taking into account that even 5 days after removal of the testosterone implant there is a releasable pool of GTH present in the pituitary, these results point to the absence of an inhibitory effect of testosterone on the GTH release.

Instead of an inhibiting effect on the release of GTH one could even argue that a positive effect exists. It is not a low but a high testosterone concentration in the

plasma that leads to an enhanced GTH secretion. Namely, in the first experiment, carried out in May, high testosterone levels were accompanied by a limited but significant elevation of the GTH concentration in the plasma. Such an elevation was not observed in the second experiment, carried out in September. Different effects of exogenous testosterone on the plasma GTH level at different times of the year were also reported in a previous study (Gielen and Goos, 1983), in which exogenous testosterone was found to enhance GTH release in January–February, but not in November–December. Likewise, both in our previous study (Gielen and Goos, 1983) and in the present experiments, testosterone administration resulted in different concentrations of testosterone in the plasma depending on the time of year. There is no correlation between the higher and lower levels of circulating testosterone and the presence and absence, respectively, of an elevated GTH concentration in the plasma. Further experiments will have to elucidate the seasonal differences in uptake of exogenous testosterone and in the ensuing GTH release in juvenile trout.

The present results confirm and extend results from *in vitro* experiments. Crim *et al.*, (1981a) examined the *in vitro* GTH release of pituitaries from testosterone-pretreated juvenile rainbow trout and Fåhraeus-van Ree *et al.* (1983) cultivated pituitaries from 6-month-old trout in a medium containing 17α-methyltestosterone or oestradiol-17β. Under both circumstances GTH was accumulated but there was no full release. In addition, Gielen and Goos (1983) showed that a homo-grafted pituitary in a testosterone-treated juvenile trout accumulates GTH, but does not release the hormone. This means that the absence of GTH release after testosterone treatment is not caused by inhibition by the brain.

The results of the LH-RHa treatment allow some remarks. The results of these experiments make it clear that the GTH cells possess a releasable pool of GTH up to 5 days after removal of the testosterone pellet. All animals pretreated with testosterone, and having high levels of GTH in their pituitaries, showed prominent releases of GTH when stimulated with the GnRH. In May, but not in September, placebo-treated animals also responded slightly but significantly to GnRH. These differences in response to GnRH at different times of the year correspond to our previous observations (Goos *et al.*, 1982).

The response to GnRH is stronger in fish pretreated with testosterone and storing GTH in the pituitary than in animals not pretreated with the steroid and with small amounts of GTH in the GTH cells. At the same time, most of the animals accumulating GTH in their pituitaries and with high levels of circulating testosterone (pellet not removed) showed a much more pronounced release of GTH than the animals storing GTH and with a low plasma testosterone concentration (pellet removed). Such stimulatory effects of gonadal steroid on GTH responsiveness to GnRH resemble those observed in mammals (e.g., Kamel and Krey, 1982).

Injection of LH-RH into juvenile trout not only resulted in elevated levels of GTH in the plasma, but also in an increase of circulating testosterone. This effect could only be observed in experiments carried out in May. It seems likely that the enhanced secretion of testosterone is a result of the increased release of GTH. On the other hand, a direct effect of the analog on the gonads cannot be excluded, since in other vertebrates, such as mammals and birds, direct effects of GnRH on the steroidogenic activity of the gonads have been demonstrated (Hertelendy *et al.*, 1982; Hsueh and Jones, 1983; Sharpe and Harmer, 1983).

If the absence of GTH release after testosterone priming cannot be explained by a negative feedback action of the steroid nor by a hypothalamic inhibition, it might be that in juvenile trout a hypothalamic stimulation via GnRH is still missing. Recent

studies of Schreibman *et al.* (1983) describe an enhanced development of immunoreactive GnRH neurons in the hypothalamus of the platyfish after treatment of juvenile animals with androgens. Crim and Evans (1983) reported an elevation of plasma GTH and precocious sexual development in juvenile rainbow trout under the influence of a long-term and probably high dosage testosterone treatment. With regard to the ontogeny of the brain–pituitary–gonadal axis this might mean that steroid hormones, already being produced at very early stages (Van den Hurk *et al.*, 1982), not only are important for the development and synthetic activity of the GTH cells but also for the onset of GnRH secretion.

Studies to test whether the latter statement is applicable to juvenile trout are in progress.

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