

Manipulation of the hypothalamo-pituitary gonadal axis in the juvenile rainbow trout : influence of pituitary transplantation and LHRH analogue treatment

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Summary. The effect of implanting an extra pituitary containing large amounts of gonadotropic hormone (GtH), combined or not with a luteinizing hormone releasing-hormone analogue (LHRHa) treatment, on GtH levels and gonadal development was investigated in juvenile host fish. The extrapituitaries were collected from mature spermiating fish or from immature fish treated with testosterone. In recipient males and females circulating plasma GtH levels increased following transplantation of both types of pituitaries. Elevated GtH levels presumably triggered steroid synthesis by the immature male gonad since pituitary GtH content was observed to accumulate in recipient males and not in females. However, the potency of the two kinds of pituitaries seemed different since spermatogenesis was stimulated only in some recipient males bearing a mature adult pituitary. This divergence could be due to a different sensitivity to endogenous gonadotropin-releasing hormone (GnRH). Only mature extrapituitaries might be highly sensitive to GnRH, as suggested by results obtained in juvenile host fish after LHRHa treatment. At the end of the 6-week experimental period, this LHRHa treatment stimulated spermatogenesis and induced a significant increase in pituitary GtH content only in juvenile hosts transplanted with a mature pituitary. Such a result was not observed in juvenile hosts submitted to a LHRHa treatment combined or not with the transplantation of juvenile testosterone-treated pituitary. However, previous works have shown that pituitaries collected from immature testosterone-treated fish are sensitive to GnRH. In the present experiment, the amount of GnRH-induced GtH release might have been too low to initiate spermatogenesis during the 6-week experimental period.

Introduction.

Evidence is accumulating about the quiescent nature of the hypothalamo-pituitary gonadal axis (HPG) of the juvenile trout. Prior to puberty, gonadotropic hormone (GtH) in the pituitary and the circulation, as well as circulating sex steroid levels, are very low (see review in Crim and Evans, 1983).

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Although there is little evidence of the endocrine activity of the juvenile HPG even after removal of immature gonads (Crim *et al.*, 1982), the GtH responsiveness of juvenile trout gonads is easily demonstrated. GtH injections in males increase androgen levels (Ng and Idler, 1980; Magri *et al.*, 1982; Le Bail *et al.*, 1983) and stimulate gametogenesis (Magri *et al.*, 1981); pituitary GtH levels also begin to rise, presumably due to positive steroid hormone feedback (Crim *et al.*, 1982; Gielen *et al.*, 1982). In contrast to stimulation of the juvenile HPG by GtH treatment, attempts to induce the onset of sexual maturation with implants of an LHRH analogue (LHRHa) have not been successful (Crim and Evans, 1983).

Since dramatic accumulations of GtH in the pituitary gland are an early sign of sexual development in precocious Atlantic salmon parr (Crim and Evans, 1978), we decided to test the influence of grafting such sexually mature pituitaries upon the HPG of the juvenile trout. Since testosterone treatment produces a rapid increase in the pituitary GtH content of the juvenile trout, we also transplanted pituitaries obtained from testosterone-treated immature trout. Since *in vivo* pituitary from sexually mature male salmonids (Crim and Cluett, 1974; Weil *et al.*, 1978; Crim *et al.*, 1983) as well as pituitary from sexually immature, testosterone-treated fish (Crim and Evans, 1983; Gielen and Goos, 1984) are responsive to LHRH or LHRHa we wished to associate a LHRHa treatment with pituitary transplantation.

Material and methods.

A) *Host animals.* — We used a laboratory stock of 2-year old, sexually immature rainbow trout maintained in fresh water and exposed to a simulated natural photoperiod. Under these conditions, juvenile trout usually undergo their initial seasonal reproductive cycle at 3 years of age.

In experiment I, males and females weighing 20.93 ± 1.10 g ($\bar{x} \pm$ SEM) were used. In experiment II, 103 males weighing 24.80 ± 0.38 g were selected from a total of 177 animals after sex determination. Sex was determined by opening the abdominal cavity and examining the gonads with a fiber optic light source. The wound was closed with silk thread and healing and recovery were allowed for one month prior to pituitary transplantation. The survival rates after sex determination and surgery were both 98.1 %.

B) *Pituitary transplantation.*

1) *Extrapituitaries.*

Mature pituitaries. — The pituitaries were collected from a laboratory stock of spermiating landlocked salmon parr (*Salmo salar*). This stage of the reproductive cycle was chosen because pituitary GtH content reaches a peak at the onset of testis maturation (Crim and Evans, 1978). The pituitaries were implanted in the host fish of experiments I and II.

Juvenile pituitaries. — The pituitaries were obtained from the laboratory stock of juvenile rainbow trout which were implanted 3 months previously with 4.73 ± 0.77 mg of testosterone in silastic tubing in order to increase pituitary GtH content, as previously described (Crim and Evans, 1983). These pituitaries were implanted in the fish of experiment II.

2) *Surgical procedure*

The host fish were anesthetized with phenoxy-ethanol (0.8 ml/l). During surgery a lower concentration of the anesthetic (0.2 ml/l) was perfused through the gills. The skull of sham-operated and transplanted fish was cut and a 3-sided bone flap lifted, revealing the telencephalic lobes and the *saccus vasculosus*. The *saccus vasculosus* was pushed aside with forceps and the extrapituitary was put in its place between the telencephalic lobes and optic tectum with a microcapillary plugged with clay. In the sham operation, only the microcapillary was inserted into the brain. At the end of the operation, Cortland's saline solution (Wolf, 1963) was added to the skull cavity before closing the bone. The skull was held shut during healing by a ligature and glue (histoacryl blue).

C) Luteinizing hormone releasing hormone analogue (LHRHa) treatment. — At the end of the transplantation procedure, the host fish in experiment II were given either the LHRHa treatment or the control treatment. In the former, 25 μ g of (D-2 naphthylalanine)⁶ LHRH, contained in a cholesterol pellet, was surgically implanted into the perivisceral cavity as previously described (Crim *et al.*, 1983a). In the control treatment, surgery was carried out without inserting the pellet.

D) *Experimental design*

Experiment I. — The trial was conducted for a period of 9 weeks beginning on December 10, 1980. On week 0, some juvenile male and female host fish and some mature donor fish were killed to determine their initial pituitary and plasma GtH levels and their pituitary content, respectively. The remaining host fish underwent a sham transplantation operation or adult pituitary transplantation. Their pituitary and blood samples were collected at 2, 5 and 9 weeks following surgery to determine the GtH values. At the same time, the transplanted pituitaries were removed to determine their GtH content. On weeks 5 and 9, the intact controls were also observed.

Experiment II. — The experiment lasted 6 weeks from November 30, 1981 (week 0) to January 14, 1982 (week 6). The fish were treated with tetracyclin (1 g/10 l) twice a week between days 8 and 24. On week 0, samples of mature and juvenile donor fish were killed to determine the GtH content of the extrapituitaries before transplantation. On week 0, the gonadal stage and plasma and pituitary GtH levels of the host fish were determined by killing a sample of them. The remaining fish were distributed into three groups, one undergoing a sham transplantation operation, another juvenile pituitary transplantation, and

the third, adult pituitary transplantation. Six fish in each of the three groups received the LHRHa treatment, and the remaining fish were used as sham controls.

On week 2, samples of sham-transplanted host fish, juvenile pituitary-transplanted host fish and mature pituitary-transplanted host fish without LHRHa treatment were killed to determine plasma and pituitary GtH and gonadal development. The GtH content of the transplanted extrapituitaries was also determined.

On week 6, sham control and juvenile and mature pituitary-transplanted host fish, treated or not with LHRHa were killed. Samples of blood, pituitary and gonads were collected, including the transplanted pituitaries.

E) *Determination of plasma and pituitary GtH levels and gonad status.* — Plasma and pituitary GtH levels were determined using a salmon radioimmunoassay system previously validated for landlocked salmon and different species of trout (Crim *et al.*, 1975). Blood samples were taken from the caudal vasculature with an heparinized needle and syringe. The pituitaries were collected on dry ice and stored at -20°C until lyophilization and homogenization.

The state of gonadal development was determined from the weight of the gonads and an histological analysis conducted on $5\text{-}\mu\text{m}$ microscopic sections following fixation in Bouin's fluid and staining with Regaud's hematoxylin, Orange G and alinin blue.

F) *Statistical analysis.* — Non-parametric methods were used to compare the different treatments : the Kruskal-Wallis test for several groups and the Mann-Whitney U-test for two groups.

Results.

GtH profile of the extrapituitaries during the experiments (table 1). — At the time of transplantation, pituitary GtH levels were high in the donor groups of experiments I and II. In experiment II, the GtH values of mature landlocked salmon were lower than those observed in the pituitary of testosterone-treated fish ($P < 0.02$). By week 2 (compared to week 0), the pituitary GtH content decreased to about 50 % of the initial value for the mature pituitaries in experiments I and II and to about 85 % for the juvenile ones in experiment II. The values reached were identical in adult and juvenile pituitaries. Pituitary GtH content continued to decrease with time in both experiments.

Profile of pituitary and plasma GtH levels and the gonads of host fish during the experimental period.

Initial control, intact control and sham-transplanted fish. — On week 0, the immature male and female host fish of experiments I and II exhibited typically

TABLE 1
Profile of GtH content of transplanted pituitaries in experiments I and II (GtH content is expressed as μg per pituitary).

Origin of transplanted pituitary	Experiment I Week			Experiment II Week			
	0	2	5	9	0	2	
Mature landlocked salmon	3.24 ± 0.98 (5)	1.62 ± 0.16 (9)	0.98 ± 0.13 (10)	0.45 ± 0.10 (10)	4.33 ± 0.75 (6)	2.29 ± 0.26 (6)	0.76 ± 0.14 (12)
Juvenile testosterone- treated rainbow trout					10.37 ± 1.53 (10)	1.60 ± 0.64 (4)	0.84 ± 0.17 (11)

Values are $\bar{x} \pm \text{sem}$.
 () = number of observations.

low plasma and pituitary GtH levels (figs. 1, 2). Mean plasma GtH levels were 0.5 ± 0.02 ng/ml and 2.66 ± 0.26 ng/ml in experiments I and II, respectively. Mean GtH content was 0.31 ± 0.14 ng/pituitary for the females of experiment I. The mean pituitary GtH content of males was 1.50 ± 0.68 ng and 2.58 ± 0.89 ng for experiments I and II, respectively. In the second experiment, male gonad weight was low (8.6 ± 0.8 mg) and type A and B spermatogonia were the only stages present in the testes (fig. 2).

During the 9-week (experiment I) and 6-week (experiment II) experimental periods, sham and intact controls remained at this quiescent stage since the GtH values of plasma and pituitary samples were not different from those observed on week 0 ($P > 0.05$). Male gonadal stage did not change during the 6-week period either (fig. 2).

Pituitary-transplanted fish. — Two weeks after adult pituitary transplantation, male and female mean plasma GtH values in experiment I were elevated in comparison with the values of sham-operated fish ($P < 0.025$) (fig. 1). A significant increase in pituitary GtH content was detected only in males (fig. 1). These increases in plasma and pituitary GtH levels were also seen in the juvenile host males of experiment II bearing either a mature or an immature pituitary (fig. 2). At this time, GtH content was around 50-80 ng/pituitary. However, gonad weight was still low and similar to that of sham-operated fish; only type A and B spermatogonia were detected by histological examination (fig. 2).

By week 5 in experiment I or week 6 in experiment II, plasma GtH levels decreased in comparison with week 2.

By week 5, the pituitary GtH content of the males in experiment I decreased in comparison with week 2 ($P < 0.02$), although the levels were higher than those in the shams ($P < 0.02$); in females, mean pituitary GtH values were still low and similar to week-2 values (fig. 1). By week 6, the mean pituitary GtH levels of host males in experiment II were not different from those of week 2 and were identical in fish bearing a mature or juvenile pituitary (fig. 2). On the other hand, gonadal histology showed a difference. The gonad weight of all the fish with a juvenile pituitary was low, and microscopic examination showed that type A and B spermatogonia filled the testis. Two kinds of response to the adult transplantation treatment were found microscopically in the testes of the host fish. In 5 fish, the testes were still filled with type A and B spermatogonia. In the other three fish, the onset of spermatogenesis was evident. This onset was characterized by the presence of spermatocytes in one fish: meiosis was starting in all the lobules. In the other two, asynchronous spermatogenesis was beginning: some cysts were still immature (type A and B spermatogonia) while others were filled with spermatocytes in more or less advanced meiosis and with spermatozoa. However, there was no difference in plasma and pituitary GtH or gonad weight between fish which were starting spermatogenesis and those which were not.

Pituitary and sham-transplanted fish while LHRHa treatment (fig. 2). — In sham-transplanted fish and those bearing a juvenile pituitary, LHRHa had no

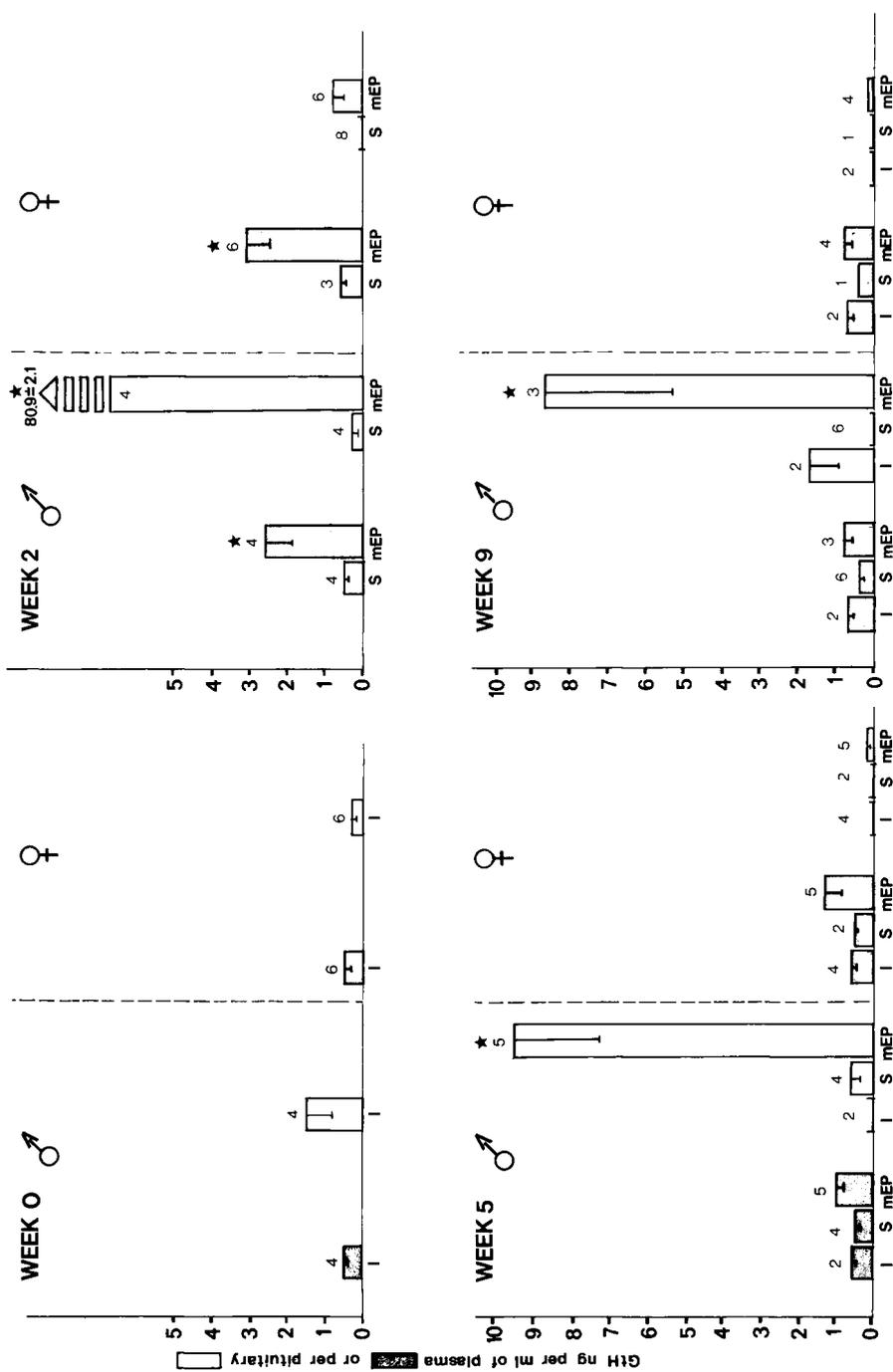


FIG. 1. — Pituitary and plasma GH values of intact (I), sham control (S) and pituitary-grafted (mEP) juvenile male and female rainbow trout during a 9-week experimental period. Extrapiuitaries were collected from adult spermating landlocked salmon.

Values are $\bar{X} \pm \text{SEM}$.

* : value significantly different from the corresponding sham value ($P < 0.025$).

The number of observations is shown above the appropriate column.

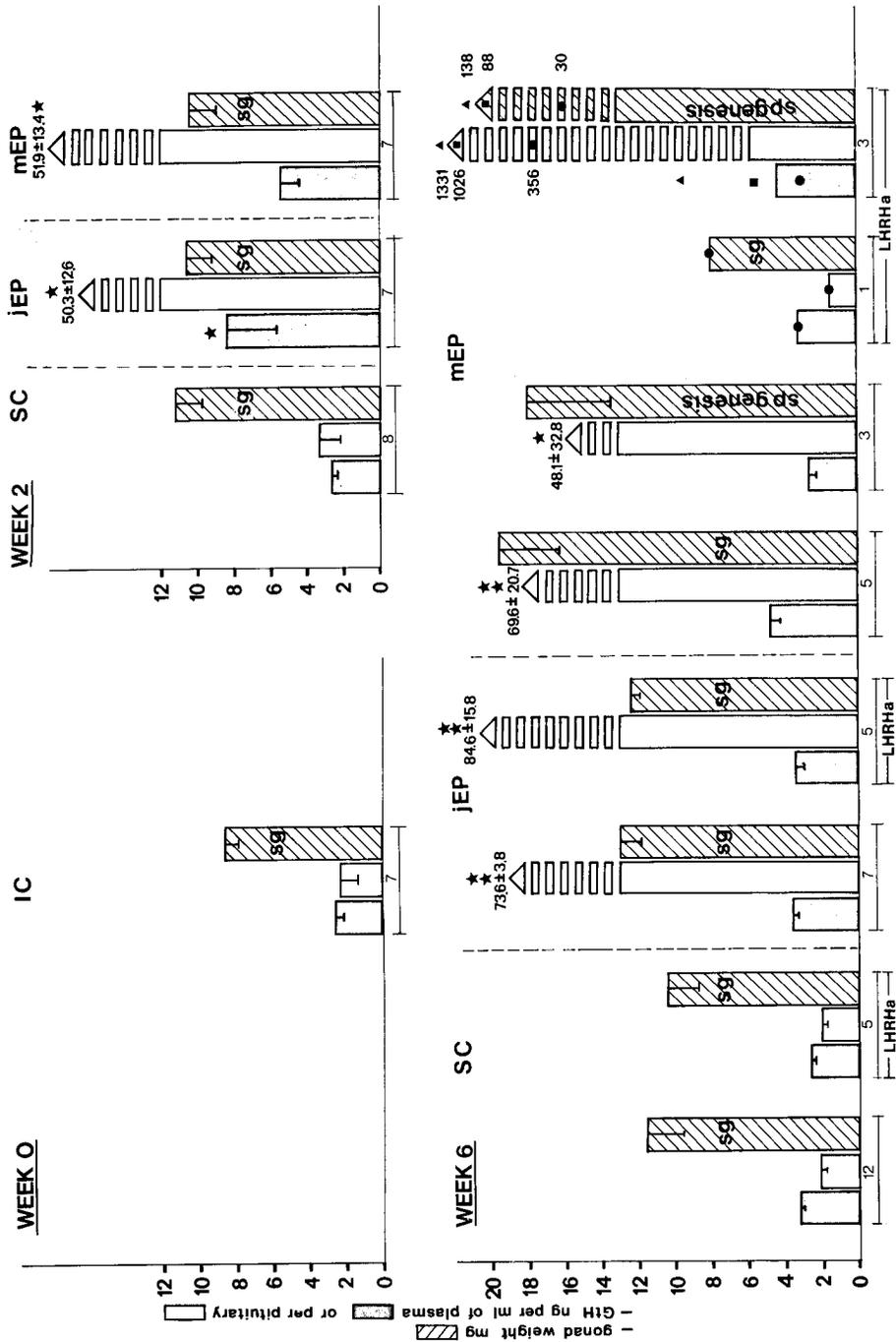


FIG. 2. — Profile of plasma and pituitary GH level and gonadal stage in juvenile male rainbow trout after extrapituitary transplantation with or without LHRHa treatment. Extrapituitaries were collected from adult spermating landlocked salmon or juvenile male and female rainbow trout previously treated with testosterone. IC : intact control ; SC : sham control ; JEP : juvenile extrapituitary ; mEP : mature extrapituitary. Values are $\bar{X} \pm \text{SEM}$ or individual values when heterogeneity was noted ; * , ** : value significantly different from the corresponding sham value (* $P < 0.02$; ** $P < 0.002$). Gonadal stage : sg : spermatogonia ; spgenésis : onset of spermatogenesis. For details see Results.

effect either on pituitary and plasma GtH values or on the gonads during the 6-week experimental period. These parameters were identical in LHRHa treated and untreated fish. On the other hand, LHRHa treatment was potent in 3 out of the 4 fish bearing a mature extrapituitary. In two, pituitary GtH content reached the μg -level and gonad weight was high. In the third fish, GtH content was 356 ng, a value higher than that observed in the corresponding fish not treated with LHRHa. Microscopic examination of these 3 fish showed the onset of typical (meiosis) or asynchronous spermatogenesis, as previously described in the testes of fish bearing a mature extrapituitary without LHRHa treatment. The plasma and pituitary GtH values and the gonads of the fourth fish were identical to those of the sham-operated fish.

Discussion.

A prolonged period of inactivity (minimal hormone secretion) characterizes the hypothalamo-pituitary gonadal axis of juvenile rainbow trout (for review see Crim and Evans, 1983). In many stocks of salmonid fish, the juvenile phase lasts for 3 years before seasonal reproductive cycles are initiated. However, such length in juvenile periods is not obligatory as evidenced by the presence of precocious males which may produce fertile sperm in their first year of life.

The present work was designed to examine some aspects of the chain of events regulating the onset of pubertal sexual development in trout. We decided to study the effect of pituitary and of LHRH. For this purpose we studied the influence of transplanting an extrapituitary, containing high amounts of gonadotropic hormone, on signs of sexual development. The donor pituitary gland, collected from either a sexually mature male or a testosterone-treated juvenile, was implanted into a rich vascular site near the *saccus vasculosus* between the telencephalic lobes and optic tectum. Releasing-hormone treatment consisting of the administration of a LHRH analogue contained in a cholesterol pellet was studied in addition to the effect of pituitary transplantation.

The data suggest that such transplanted pituitaries survive and can release GtH over experimental periods of 6 and 9 weeks. However, good cell survival was not assessed by microscopic examination. Such a study could not be carried out since we used the pituitaries to determine their GtH content. Macroscopically, they appeared to be undamaged, probably because they were placed in a rich vascular site. In both experiments, increases in plasma GtH in the juvenile host were reflected by week 2 by declining pituitary GtH content in the pituitary transplant, suggesting that the donor pituitary was the source of the increase in circulating GtH. Although the GtH content of the juvenile host male pituitary increased significantly, presumably in response to positive androgen feedback (Crim *et al.*, 1982 ; Gielen *et al.*, 1982 ; Magri *et al.*, 1985), it is unlikely that this small pool of accumulated GtH would produce detectable quantities in the plasma by week 2 (Crim and Evans, 1983). Furthermore, plasma GtH levels also increased in the females of experiment I after adult pituitary transplantation, although no increase in pituitary GtH content was noted. This fact supports the idea that this

increase in plasma GtH was due to adult and juvenile testosterone-treated transplanted pituitaries. It should be pointed out that the GtH content decreased more rapidly in juvenile transplanted pituitaries on week 2. The GtH release of both kinds of pituitaries does not seem different since circulating GtH levels were identical in juvenile host fish. On the other hand, the synthesis rate could be different. Indeed, pituitary GtH synthesis was stimulated in juvenile fish by high exogenous testosterone levels. Transferring these pituitaries into juvenile host fish not treated with testosterone could have decreased the synthesis rate.

As already mentioned, mature and juvenile testosterone-treated transplanted pituitaries induced pituitary GtH content to accumulate only in juvenile host males. In females, this lack of pituitary GtH augmentation was probably due to the incapacity of the juvenile ovary to respond to GtH stimulation by appropriate steroid synthesis (Crim *et al.*, 1981 ; Magri *et al.*, 1982) and, as a consequence, there would be no positive steroid feedback. For this reason we used only male fish in experiment II. However, in this experiment the potency of the two types of transplanted pituitaries seemed different since by week 6 spermatogenesis had begun in some host fish bearing a mature pituitary only. Mature pituitaries were collected from landlocked salmon and juvenile pituitaries from rainbow trout. Their different potency is probably not due to the fact that they were collected from different salmonid species, for spermatogenesis can be induced in juvenile trout by the injection of purified salmon GtH from mature animals (Magri *et al.*, 1982) as well as by endogenous GtH release after testosterone treatment (Crim and Evans, 1983 ; Magri *et al.*, 1985). This divergence could be due to their different sensitivity to endogenous gonadotropin-releasing hormone (GnRH), as suggested by their varied responsiveness to exogenous pelleted GnRH. Indeed, by week 6, pelleted LHRH analogue failed to induce spermatogenesis in sham fish as well as in fish grafted with a juvenile testosterone-treated pituitary. On the other hand, this GnRH treatment initiated spermatogenesis in some host fish after adult pituitary transplantation, suggesting that in a first step, only the adult transplanted pituitary is highly sensitive to GnRH. The lack of GnRH sensitivity of the pituitary gonadal axis of juvenile rainbow trout, previously described *in vitro* (Crim and Evans, 1980) and *in vivo* (Crim and Evans, 1983), is confirmed in the present work. On the other hand, pituitaries collected from testosterone-treated juvenile rainbow trout and containing about 50 ng of GtH have been shown to be sensitive to GnRH *in vitro* (Crim and Evans, 1980). This sensitivity was assessed by significant GtH release in 500 μ l of medium during 24 h of incubation. In addition, the combined treatment of pelleted GnRH and high doses of testosterone induced a high release (15-30 ng/ml) of plasma GtH *in vivo* one and two months after the beginning of the treatment. At that time, pituitary GtH content was high and the range of values was 5-10 μ g ; GSI stimulation reflected the onset of spermatogenesis in some fish (Crim and Evans, 1983). In the present experiment, the lack of sexual development in juvenile host males grafted with a juvenile testosterone-treated pituitary by week 6, does not necessarily mean that both host and donor pituitaries were insensitive to pelleted GnRH ; it must be remembered that they contained about 50 ng and 1 μ g, respectively, of GtH. Circulating plasma GtH levels following GnRH treatment were probably too low

to trigger spermatogenesis during a 6-week experimental period. This hypothesis could also be applied to juvenile host males grafted with a juvenile pituitary and submitted to only endogenous GnRH, although no data are available on circulating GnRH levels in salmonids. High circulating levels of a LHRH-like substance have been reported in another teleost, the immature eel (Dufour *et al.*, 1982). In both cases, if the experiments had lasted longer, a more permanent GtH release might have triggered the beginning of sexual development. Additional experiments are necessary to confirm or infirm this hypothesis.

We think that in a first step only the mature pituitaries are highly sensitive to endogenous and exogenous GnRH. As a consequence, by week 6, the highest plasma GtH levels were observed in the juvenile host fish beginning spermatogenesis and grafted with a mature pituitary associated with a GnRH treatment. These highest plasma GtH levels could have triggered intense androgen synthesis in the juvenile host gonad, leading to the maximal pituitary GtH levels observed by week 6. Indeed, juvenile host fish starting spermatogenesis after mature pituitary transplantation without GnRH treatment had lower pituitary GtH content. A positive relationship between pituitary GtH accumulation and the quantity of testosterone administered has already been shown (Crim and Evans, 1979).

The lack of response of the juvenile host immature pituitary gonadal axis to pelleted LHRHa requires further experimentation. As salmon GnRH is now available (Sherwood *et al.*, 1983), the role of the level and the type of GnRH used could be studied as well as the role of the profile of GnRH administration. However, the analogue used in the present experiment and administered at the same dose by a cholesterol pellet can advance the onset of spermiation in adult prespawning landlocked salmon (Weil and Crim, 1983).

Additional studies on the gonads are also needed to explain the heterogeneity reported in juvenile host male fish after adult pituitary transplantation. There is heterogeneity in the number of responsive fish as well as in gonad response : some testes present a synchronous onset of spermatogenesis and others an asynchronous one. This heterogeneity could be due to the GtH treatment or to the host immature gonad. In this work, GtH was not provided at a constant rate since during the experimental period we observed a decrease in the plasma GtH of the host fish associated with declining pituitary GtH content of the transplanted pituitary. On the other hand, the immature gonad might not have enough GtH receptors to allow a typical adult response.

In conclusion, further studies are needed to understand the chain of events regulating the onset of pubertal sexual development in rainbow trout.

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Résumé : *Manipulation de l'axe hypothalamus-hypophyse-gonade chez la truite arc-en-ciel juvénile : effet de l'implantation d'hypophyse et d'un traitement par un analogue du LHRH.*

Chez la Truite immature, on a étudié l'effet de l'implantation d'une extrahypophyse riche en hormone gonadotrope (GtH), en association ou non avec un analogue du LHRH (LHRHa), sur les teneurs en GtH circulante et hypophysaire et sur le développement des gonades. Les hypophyses implantées provenaient d'animaux matures spermiantes ou d'animaux immatures traités par la testostérone. Après transplantation des deux sortes d'hypophyses, on a observé chez les mâles et femelles receveurs une augmentation de la GtH circulante alors que l'augmentation de la GtH hypophysaire n'a été observée que chez les mâles. Chez ces derniers, la GtH circulante élevée a probablement entraîné une synthèse de stéroïdes par la gonade immature. Cependant, l'activité des deux sortes d'hypophyses transplantées semble différente puisqu'une stimulation de la spermatogenèse n'a été observée chez quelques animaux receveurs qu'après implantation d'une hypophyse adulte. Cette différence d'activité pourrait être due à une sensibilité différente au GnRH endogène. Seules les extrahypophyses matures seraient hautement sensibles au GnRH comme le suggère les résultats observés chez les animaux hôtes traités par le LHRHa. En effet, après un tel traitement pendant 6 semaines, un début de spermatogenèse et une augmentation de la charge hypophysaire ne sont observés que chez les animaux hôtes porteurs d'une extrahypophyse mature. Rien de tel n'a été observé chez les animaux hôtes, traités par le LHRHa, porteurs ou non d'une extrahypophyse juvénile prétraitée par la testostérone. Cependant des expériences antérieures ont montré que des hypophyses provenant d'animaux immatures ayant subi un traitement à la testostérone sont sensibles au GnRH. Dans la présente expérience, la quantité de GtH libérée par ces hypophyses n'a peut-être pas été suffisante pour induire en 6 semaines un début de spermatogenèse.

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