Growth hormone receptors in ovary and liver during gametogenesis in female rainbow trout (*Oncorhynchus mykiss*)

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Changes of growth hormone receptivity in the ovary during the reproductive cycle were studied in rainbow trout (*Oncorhynchus mykiss*). A method for characterizing growth hormone receptors in crude ovary homogenate was required for this. Binding of radiolabelled recombinant rainbow trout growth hormone (^{125}I -labelled rtGH) to crude ovary preparation was dependent on ovarian tissue concentration. The sites were specific to growth hormone, with no affinity for prolactins and gonadotrophins. Similar high affinities for ^{125}I -labelled rtGH were obtained with crude ovary ($4.2 \times 10^9 \pm 0.3 \text{ mol l}^{-1}$) and crude liver preparations ($4.9 \times 10^9 \pm 0.1 \text{ mol l}^{-1}$) at all stages of ovogenesis, and with ovarian membrane preparations ($8.2 \times 10^9 \text{ mol l}^{-1}$) tested at the beginning of vitellogenesis. Ovarian growth hormone receptor concentration was highest during the early phases of follicular development (endogenous vitellogenesis: $315\text{--}310 \text{ fmol g}^{-1}$ ovary) and decreased regularly during oocyte and follicular growth (exogenous vitellogenesis) to reach a minimal value at oocyte maturation (42 fmol g^{-1} ovary). In postovulated fish, binding was at a similar level (297 fmol g^{-1} ovary) to that found in endogenous vitellogenesis. Conversely, the absolute binding capacity of the whole ovary was low from immaturity to early exogenous vitellogenesis ($0.1\text{--}0.6 \text{ pmol}$ per pair of gonads), increased slowly during vitellogenesis and more markedly during rapid oocyte growth and at the time of final maturation (10.8 pmol per pair of gonads). In postovulated fish, the absolute binding capacity decreased partially (4.4 pmol per pair of gonads). Mean hepatic growth hormone receptor concentration did not vary with the reproductive stage for most of the cycle ($3.0\text{--}4.5 \text{ pmol g}^{-1}$ liver) except in endogenous vitellogenesis where significantly higher concentrations were observed (6.7 pmol g^{-1} liver). Individual ovarian growth hormone receptor concentrations were correlated with hepatic growth hormone receptor concentrations, indicating that they are regulated in a similar way. We conclude that growth hormone receptors are present in the ovary during the entire ovarian cycle in rainbow trout, probably mainly in somatic cells as indicated by the same concentration of binding sites in immature and in postovulated fish. Growth hormone is potentially important during oocyte recruitment in vitellogenesis and initiation of growth and during final follicular maturation.

Introduction

Although gonadotrophins are the major regulators of ovarian function in vertebrates, accumulating evidence indicates a role for growth hormone (GH) in the control of the female reproductive process (for review, see Adashi *et al.*, 1992; Katz *et al.*, 1993; Le Gac *et al.*, 1993). Delayed pubertal development in human and animal isolated GH deficiency can be restored by GH treatment (Sheikhislam and Stempfel, 1972; Ramaley and Phares, 1980; Advis *et al.*, 1981; Ovesen *et al.*, 1992), and GH therapy has been used with success as an adjuvant to gonadotrophin for ovulation induction in assisted human reproduction (Volpe *et al.*, 1989;

Homburg *et al.*, 1990; Burger *et al.*, 1991; Jacobs *et al.*, 1991). Direct effects of GH on the ovary were proposed after the first *in vitro* studies had demonstrated that GH stimulates granulosa cell differentiation of murine (Jia *et al.*, 1986; Hutchinson *et al.*, 1988), pig (Hsu and Hammond, 1987) and human (Mason *et al.*, 1990) ovarian follicles. A large body of evidence indicates that GH effects may be mediated, in part, through an intra-ovarian insulin-like growth factor (IGF) system (for review, see Adashi *et al.*, 1992; Guidice, 1992). The detection of low GH receptor and/or binding protein (GH-R/BP) mRNA in rat (Tiong and Herington, 1991; Bingham *et al.*, 1994), rabbit (Ymer and Herington, 1992) and human (Mercado *et al.*, 1994) ovaries also indicated a direct action for GH. Attempts to localize ovarian cells carrying GH-R have revealed some discrepancies. Expression of mRNA encoding GH-R/BP and GH-R/BP immunoreactivity

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were widespread in the rat ovary (Lobie *et al.*, 1990), mainly in granulosa and theca cells (human: Mertani *et al.*, 1995) and localized in the granulosa cells of preantral and antral follicles (rat: Carlsson *et al.*, 1993; sheep: Eckery *et al.*, 1997), in the granulosa cells of dominant follicles and luteal cells (humans: Sharara and Nieman, 1994; Tamura *et al.*, 1994) or in luteal cells (cattle and pigs: Lucy *et al.*, 1993; Yuan and Lucy, 1996). Finally, expression of GH-R/BP mRNA was low in ovine and bovine oocytes (Eckery *et al.*, 1997; Izadyar *et al.*, 1997). Binding studies reported the possible occurrence of functional GH receptors in human and rabbit ovary (Carlsson *et al.*, 1992; Ando *et al.*, 1994), and both experiments used human GH which reacts with the lactogenic receptor as well as (or even better than) the somatogenic receptor. In fact, most attempts have been unsuccessful in demonstrating and quantifying GH receptors in mammalian ovarian tissues by binding assays (Webb *et al.*, 1994; Eckery *et al.*, 1997), due to high non-specific binding.

In teleost fish, the presence of binding sites for homologous GH has been detected in ovary (Yao *et al.*, 1991; Gray *et al.*, 1990), and Mourot *et al.* (1992) have described GH-specific binding with general characteristics of GH-R in rainbow trout ovarian membrane preparations. These GH-R appear functional in teleost fish, as GH treatments are able to modify the gonadal production of sexual steroids *in vivo* and *in vitro* (Singh *et al.*, 1988; Van Der Kraak *et al.*, 1990; Le Gac *et al.*, 1992; Singh and Thomas, 1993).

In rainbow trout, the ovogenesis and ovulation of thousands of gametes occur synchronously in the entire ovary, providing a particularly convenient model in which to study physiological changes during successive stages of folliculogenesis and oocyte maturation.

In the present study, changes in gonadal GH-R concentration during the initial ovarian cycle in rainbow trout were studied to investigate the role of GH in gonadal development during puberty, follicular and oocyte growth, and final maturation. First, it was necessary to characterize the specific binding of GH to crude ovarian preparation and to validate this method to allow quantitative studies in individual and small gonads. Changes in ovarian GH-R concentration during the reproductive cycle were analysed in relation to plasma GH concentrations and in comparison with hepatic GH-R contents during the same period.

Materials and Methods

Animals

One-year-old female rainbow trout (*Oncorhynchus mykiss*) of the Cornec Autumnal strain (fall spawning) reared at the INRA experimental fish farm (Sizun, Finistère, France) were used. During the entire experimental period (January 1995–November 1995), fish were kept under natural temperature conditions (February: 8.5°C; August: 20°C) and photoperiod (48°N) in circulating fresh water tanks (capacity 1800 l), and fed once per day for 6 days per week (except for 2 days before sampling) with commercial pellets (Aqualife number 17, Aqualim SA, St Estephe, France) at the rate recommended by the manufacturer.

Experimental design

This study was designed to investigate the changes in ovarian GH-R during one ovogenetic cycle. Approximately once per month, 35–45 females (sexually immature, 546 ± 19 g body weight in January; sexually mature, 2200 ± 170 g body weight in November) randomly caught were killed ($n = 11$ samplings). At each sampling, after rapid anaesthesia (3–4 min) in phenoxy-2-ethanol (0.5 ml l⁻¹) the body weight (± 1 g) and length (± 0.1 cm) of each female were measured. Blood samples were collected rapidly from the caudal vasculature in heparinized syringes. The samples were then centrifuged (4°C) at 3200 g for 20 min, and the plasma was stored in aliquots at -20°C until assayed. Ovary and liver were dissected out, weighed to determine gonadosomatic index (GSI = ovarian mass $\times$ 100/body weight) and hepatosomatic index (HSI = liver mass $\times$ 100/body weight), and then frozen individually in liquid nitrogen, and stored at -70°C until used.

Transverse sections from the middle part of the ovaries were fixed in Bouin's solution for histological examination. The ovarian stage of each fish was determined by histological examination for early stages (oocytes with diameter < 1 mm) and by GSI measurements as described by Breton *et al.* (1983), and by macroscopic observation for oocyte maturation (Jalabert, 1976). The stages were defined as: stage 1: previtellogenic ovary containing oogonia and primary oocytes; stages 2 and 3: early and advanced endogenous or type I vitellogenesis; stages 4.1 to 5: subclasses of exogenous or type II vitellogenesis based on the increasing proportion of oocytes containing vitellus deposit in yolk globules and platelets (4.1 and 4.2) and on increasing GSI which was found directly proportional to the oocyte diameter in stages 4.2 to 5 (in this particular cohort, vitellogenesis continued until close to final maturation); stage 5: preovulatory stage with macroscopic signs of oocyte final maturation; stage 6: post-ovulatory stage. 'Previtellogenic' oocytes remained observable in the gonad, although in decreasing number, at least until stage 4.2 (Table 1)

Hormones

Recombinant rainbow trout GH (rtGH) and recombinant tilapia GH (rtiGH; Rentier-Delrue *et al.*, 1989) were generously provided by J. Smal (Eurogentec, Liège) and F. Rentier-Delrue, respectively. The mammalian hormone preparations were pituitary-extracted bovine GH (batch bGH-B-1, NIDDK, NIH, Bethesda, MD) and pituitary-extracted ovine prolactin (batch oPRL-19, NIDDK, NIH). Trout gonadotrophins (tGTH I and tGTH II; Govoroun *et al.*, 1997) and salmon prolactin (sPRL; Prunet and Houdebine, 1984) were purified in our laboratory.

Five micrograms rtGH was radiolabelled with 0.5 mCi Na¹²⁵I (IMS 30, Amersham, Les Ulis, France) by the chloramine-T method (Greenwood *et al.*, 1963), with the modification introduced by Martal (1972). The specific activity of ¹²⁵I-labelled rtGH, measured by self displacement on hepatic membrane preparation (5 mg pellet per tube) was 120 μ Ci μ g⁻¹ for studies on the ovary and 46 μ Ci μ g⁻¹ for

Table 1. Ovarian stages of rainbow trout defined on the basis of oocyte histological and macroscopic characteristics and on gonadosomatic index (GSI)

Stages	Characteristics	GSI
1. Immature (previtellogenesis)	Previtellogenic oocytes + rare oocytes with cortical alveoli	0.1 ± 0.004
2. Early endogenous vitellogenesis	Previtellogenic + 25% oocytes with cortical alveoli	0.1 ± 0.01
3. Endogenous vitellogenesis	Tissue consists mainly of oocytes with cortical alveoli and lipidic globules	0.2 ± 0.02
4.1. Early exogenous vitellogenesis	Scarce oocytes with yolk globules	0.4 ± 0.02
4.2. Mid-exogenous vitellogenesis	All maturing oocytes with lipidic globules	0.7 ± 0.04
4.3. Advanced exogenous vitellogenesis	Plus increasing number of yolk globules	4.3 ± 0.4
5. Final maturation (pre-ovulation)	From germinal vesicle migration to oocyte periphery to germinal vesicle breakdown	13.1 ± 0.5
6. Postovulation	1 day to 4 weeks after ovulation	0.7 ± 0.05

Exogenous vitellogenesis occurs in stages 4.1–5 and corresponds to the accumulation of vitellus and rapid growth of the oocyte and of the follicular layers; stages 4.1–4.3 are subclasses based on oocyte diameter, which is directly proportional to the GSI; at stage 5 macroscopic signs of final oocyte maturation are also detected.

studies on the liver. The maximum binding activity (MBA) of ^{125}I -labelled rtGH, estimated with an excess amount of liver membrane preparation, ranged from 60 to 65% of total added hormone. The labelled hormone was stable for about 3 weeks when stored in glycerol (1/1, v/v) at -20°C . Radioactivity was measured in a γ -counter (Packard Instrument Co., Meriden, CT) with a counting efficiency of 75%.

Tissue preparations

Crude ovarian fractions were obtained at $0\text{--}4^{\circ}\text{C}$ using chilled buffers according to the following method. Ovaries were minced and homogenized with a Polytron homogenizer ($2 \times 15\text{ s}$, 8000 r.p.m.) in ice-cold homogenization buffer (1:5 w/v) (20 mmol Tris-HCl l^{-1} pH 7.5, 5 mmol MgCl_2 l^{-1} , 5 mmol CaCl_2 l^{-1} , 0.1% (w/v) NaN_3), complemented with para-aminobenzamidine (0.25 mg ml^{-1}), 4-(2-aminoethyl)-benzenesulfonyl fluoride (0.1 mg ml^{-1}) and soya bean trypsin inhibitors (0.05 mg ml^{-1}). Gonads were crushed and washed twice in homogenization buffer before being processed to eliminate yolk in vitellogenic and mature ovaries. The preparation was further homogenized by passing through a Dounce homogenizer and centrifuged at 3200 g for 30 min. The pellet was then washed in 5 volumes of buffer and centrifuged at 3200 g for 30 min. The supernatant was removed and the final pellet, which contained membrane fractions of all ovarian cell types (stages 1–5) or no more oocyte membranes (stage 6), was weighed and resuspended in ice-cold incubation buffer (homogenization buffer containing soya bean trypsin inhibitors 0.05 mg ml^{-1} and 0.5% (w/v) BSA).

Ovarian membrane preparations were obtained as described by Mourot *et al.* (1992). Briefly, ovaries were homogenized in 5 mmol Tris-HCl l^{-1} pH 7.2, 100 mmol NaCl l^{-1} , 5 mmol CaCl_2 l^{-1} , 100 mmol sucrose l^{-1} containing inhibitors of proteolytic enzymes (1/5, w/v), and centrifuged at 600 g for 20 min. The supernatant was recentrifuged at

30 000 g for 45 min and the resulting pellet was resuspended as described above. Crude hepatic preparations were obtained according to the method of Yao *et al.* (1991). All preparations were used immediately in the binding assay.

Binding assay

Three hundred microlitres of crude ovary homogenate corresponding to 20 or 30 mg pellet per tube (approximately 2 or 3 mg of protein per tube, depending on the stage of ovogenesis) were added to $12 \times 75\text{ mm}$ polystyrene tubes containing 100 μl ^{125}I -labelled rtGH (saturation studies: increasing amounts ranged from 30 000 to 1 200 000 c.p.m. per tube, equivalent to 13–520 pmol l^{-1} ; single point binding studies: 300 000 c.p.m. per tube, equivalent to 130 pmol l^{-1}), with (non-specific binding, NSB) or without (total binding, TB) unlabelled rtGH (500 ng per tube) in a final volume of 500 μl . Incubation was carried out at 12°C for 20 h, under gentle agitation (120 strokes min^{-1}) and was terminated by the addition of 3 ml ice-cold incubation buffer followed by centrifugation at 3 200 g for 30 min. The supernatants were discarded and the radioactivity in the pellet was counted. The binding assay with other preparations was performed as described above with 50 mg pellet per tube for ovary membrane preparations, and 5 mg pellet per tube for crude hepatic preparation. Specific binding per mg of pellet or mg of protein was calculated by subtracting NSB from the TB. Since GH-specific binding to crude ovary preparations were low, all binding measurements were performed in quadruplicate.

Other assays

The protein concentration in the final (ovary and liver) preparations was determined in duplicate by the bicinchoninic acid method (BCA protein assay reagent,

Pierce, Rockford, IL), with bovine serum albumin (BSA) as a standard. The amount of plasma GH was determined using a homologous radioimmunoassay developed in our laboratory (Le Bail *et al.*, 1991). The sensitivity of the assay (ED₉₀) was 0.2 ng ml⁻¹ for 50 µl assayed plasma and the ED₅₀ value was 1.0 ng ml⁻¹.

Data analysis

For each ovogenetic stage, affinity constants (K_a) and binding capacities (B_{max}) were calculated according to the method of Scatchard (1949). Scatchard plot analyses were performed with free (U) hormone values corrected for MBA. Scatchard plots were compared using covariance analysis. The statistical difference among groups was analysed by one-way analysis of variance (ANOVA) followed by multiple range test (Kruskal-Wallis test; differences were considered significant when $P < 0.05$). Linear regression analysis was used to detect the relationships among variables. All results are expressed as mean $\pm$ SEM.

Ovary and liver size change with sexual development and also with body growth. The total GH receptor capacity was expressed in pmol per organ and compared with the same parameter normalized for body weight (pmoles per liver kg⁻¹ body weight) with a view to analysing changes specifically linked to the reproductive stage.

Results

Characterization of ovarian growth hormone binding sites

The specific binding of ¹²⁵I-labelled rtGH to increasing amounts of crude ovarian preparation obtained at the beginning of exogenous vitellogenesis (Fig. 1) and in the immature stage (data not shown) was dependent on a membrane concentration in the range of 10 to 50 mg pellet per tube (approximately 1–5 mg of protein per tube). In the following experiments 20 mg pellet per tube was used for quantitative studies (single point binding), and 20 or 30 mg pellet per tube was used for saturation experiments (depending on the stage of ovogenesis).

¹²⁵I-labelled rtGH-specific binding appeared saturable when increasing concentrations of ¹²⁵I-labelled rtGH were incubated with a fixed amount of ovarian preparation (Fig. 2a). Scatchard plot analyses were linear, indicating the presence of a single population of binding sites with high affinity ($K_a = 4.2 \pm 0.3 \times 10^9$ mol l⁻¹, $n = 17$) and a limited number of sites. The affinity constants in crude ovary preparations were of the same order of magnitude as those observed both in ovary membrane preparations (Fig. 2b) and in crude liver preparations (Fig. 2c).

The specificity of GH binding sites to crude ovarian preparation was tested in competition experiments (Fig. 3). Unlabelled rtGH at concentrations of 0.1–250 ng per tube progressively inhibited the specific binding of the tracer. Bovine GH and rtiGH competed with ¹²⁵I-labelled rtGH in a dose-dependent manner but appeared to be 30 and 100 times less effective (calculated at 50% displacement) than

Fig. 1. Specific binding (---) and non-specific binding (—) of ¹²⁵I-labelled recombinant trout GH to increasing amounts of crude ovarian preparation obtained from a pool of gonads at stage 4.2 of ovogenesis. B, fraction of hormone bound; T, total added hormone in the incubate.

unlabelled rtGH, respectively. Salmon prolactin, ovine prolactin and trout gonadotrophins (GTH I, GTH II) did not significantly compete with ¹²⁵I-labelled rtGH for the binding sites at the tested concentrations.

Changes of gonadosomatic index and hepatosomatic index during ovogenesis

In these autumnal spawning rainbow trout, after 1 year of prepubertal immaturity, vitellogenesis developed more or less synchronously in the whole gonad from March–April, as indicated by the presence of vitellogenic follicles (stage 2) to September–October (ovulation). The definition and characteristics of the ovarian stages used are presented (Table 1). In this population, the gonadosomatic index remained low in the early stages until July (early endogenous vitellogenesis through advanced exogenous vitellogenesis) and increased markedly at the end of exogenous vitellogenesis to reach maximum values during final maturation (Fig. 4a). The hepatosomatic index also increased at the end of vitellogenesis and appeared significantly higher ($P < 0.001$) before ovulation (Fig. 4b). In the present experiment, the whole experimental trout population matured.

Changes in ovarian growth hormone receptors during ovogenesis

Saturation experiments were conducted at each stage of ovogenesis (1–4 experiments per stage) on pools of ovaries at the same histological stage (Table 2). Scatchard plots revealed that affinity constants were of the same order of magnitude (covariance analysis) during the entire cycle ($4.2 \times 10^9 \pm 0.3$ mol l⁻¹, $n = 17$), while binding capacities (B_{max}) showed significant changes.

Fig. 2. Effect of increasing concentrations of ^{125}I -labelled recombinant trout GH (rtGH) (30 000–1 200 000 c.p.m. per tube) on specific binding to (a) crude ovarian preparation (20 or 30 mg pellet per tube; $K_d = 4.9 \times 10^9 \text{ mol l}^{-1}$; $B_{\text{max}} = 31 \text{ pmol l}^{-1}$), (b) ovary membrane preparation (50 mg pellet per tube; $K_d = 8.2 \times 10^6 \text{ mol l}^{-1}$; $B_{\text{max}} = 89 \text{ pmol l}^{-1}$), (c) crude liver preparation (5 mg pellet per tube; $K_d = 4.7 \times 10^9 \text{ mol l}^{-1}$; $B_{\text{max}} = 115 \text{ pmol l}^{-1}$). The insets represent the

Fig. 3. Competition curves for specific binding of ^{125}I -labelled recombinant trout GH (rtGH) (30 000 c.p.m. per tube) to crude ovarian preparation (40 mg pellet per tube, stage 3 of ovogenesis) with increasing amounts of unlabelled hormone preparations. Binding is expressed as a percentage of ^{125}I -labelled rtGH specifically bound in the absence of competitor. rtiGH, recombinant tilapia GH; bGH, bovine GH; sPRL, salmon prolactin; oPRL, ovine prolactin; tGTH I, tGTH II, trout gonadotrophins.

Since the equilibrium association constant at each stage of ovogenesis was found to be similar, binding studies with only 50% of the saturating concentration of ^{125}I -labelled rtGH were carried out to work with the small amounts of tissue and to estimate changes of GH receptors in a large number of individual ovaries. Specific binding of ^{125}I -labelled rtGH was measured on 40 individual ovaries at different stages of ovogenesis (except for immature stage ovaries and those with oocytes at the final stage of maturation, where pools of gonads were used) (Fig. 5a). The results show that binding was highest during endogenous vitellogenesis (315–310 fmol g^{-1} ovary) and decreased regularly ($P < 0.001$) during the entire exogenous vitellogenesis to reach a minimal value during oocyte maturation (42 fmol g^{-1} ovary). In postovulated fish, binding was similar (297 fmol g^{-1} ovary) to that of fish in endogenous vitellogenesis. The binding tended to change similarly when the results were expressed in fmol mg^{-1} protein: the highest amount of binding occurring during endogenous vitellogenesis (230–235 fmol mg^{-1} protein) and in postovulated fish (231 fmol mg^{-1} protein), and the lowest amount (60 fmol mg^{-1} protein) occurring at oocyte maturation. These results were in good agreement with data obtained in the saturation experiments (Table 1). A different pattern was observed when the data were expressed in fmol g^{-1} pellet (Fig. 5b). During the first stages, the binding was high and increased significantly from stages 1 to 3 (690–1110 fmol g^{-1} pellet), decreased ($P < 0.001$) in stage 4.1 to stay

derived Scatchard plots. Scatchard plot analyses were performed with values for free (U) hormone corrected for maximum binding activity of the tracer. B, fraction of hormone bound.

Fig. 4. Changes in (a) gonadosomatic index (GSI) and (b) hepatosomatic index (HSI) in rainbow trout during ovogenesis. Different letters above histograms represent significant differences (Kruskal–Wallis test). ANOVA, $P < 0.001$. Results are expressed as the means $\pm$ SEM for 22–83 fish, except for stage 6 of ovogenesis from which 14 fish were used. Imm, immature.

unchanged during the rest of the cycle. The absolute binding capacity of the whole gonad (Fig. 6) expressed in pmol per pair of gonads (or in pmol per pair of gonads kg^{-1} body weight, that is, normalized for body size; data not shown) was low from the immature stage to early exogenous vitellogenesis (0.1–0.6 pmol per pair of gonads), increased significantly during exogenous vitellogenesis (stage 4.1–4.3) and more markedly in the rapidly growing ovary and before ovulation (stage 5: 10.8 pmol per pair of gonads). In postovulated fish, the absolute binding capacity was decreased (4.4 pmoles per pair of gonads).

Changes of hepatic growth hormone receptors and plasma growth hormone during ovogenesis

The characteristics of ^{125}I -labelled rtGH binding to crude hepatic preparations obtained from rainbow trout at different stages were also determined (Table 1). Scatchard plot analyses of the data indicate the presence of a single

population of binding sites during the entire ovogenetic cycle with unchanged high affinity ($4.9 \times 10^9 \pm 0.1 \text{ mol l}^{-1}$, $n = 16$) for ^{125}I -labelled rtGH and significant changes of binding capacity. Specific ^{125}I -labelled rtGH binding to individual livers corresponding to the ovaries studied above was measured in single point binding studies (Fig. 7). Binding was constant during most of the cycle except during endogenous vitellogenesis (6.7 pmoles g^{-1} liver) where there was an increase. The GH-R concentration in liver, expressed in fmol g^{-1} tissue, appeared to be 20–80-fold higher, according to the sexual stage (calculated with binding data), than GH-R concentration in ovary. The total binding capacity of the liver, expressed in fmol per liver kg^{-1} body weight (corrected for general body growth), showed similar changes to the hepatic GH-R concentration (data not shown).

The plasma concentrations of GH were low ($< 1 \text{ ng ml}^{-1}$) and tended to decrease (non-significantly) during the entire reproductive cycle. The relationships among plasma GH, GH binding in liver and GH binding in ovary were assessed by linear regression analysis of individual values. A significant correlation was found between ovarian GH-R concentration and hepatic GH-R concentration (expressed in fmol g^{-1} ovary: $r = 0.5$, $P = 0.01$ or expressed in fmol g^{-1} pellet: $r = 0.6$, $P = 0.01$), while no relation was found between plasma GH concentration and GH-R concentration (ovarian GH-R concentration:plasma GH concentration: $r = -0.2$; hepatic GH-R concentration:plasma GH concentration: $r = -0.2$).

Discussion

This study describes a method that allows the measurement of GH receptors in individual ovaries at all stages of ovogenesis in rainbow trout. As has been described for crude testicular preparations (Gomez *et al.*, 1998), crude ovarian preparations were chosen because they gave a less variable and a higher recovery yield of receptors than protocols using enriched membrane preparations. In protocols using enriched membrane preparations, changes in ovary composition during the reproductive cycle mainly due to vitellogenin incorporation influence the yield of membrane recovery and create technical variability among stages (data not shown). Furthermore, in mammals (Hocquette *et al.*, 1989; Fraser and Harvey, 1992) and teleost fish (Yao, 1993), GH-R is localized preferentially in membranes associated with intracellular structures as well as plasma membranes of GH target cells, and a crude membrane preparation could be more representative of the tissue receptivity potential than purified plasma membrane preparations. Finally, this method allows the quantification of GH-R on small amounts of tissue and, therefore, on individual gonads at most stages of the ovarian cycle. Desaturation by MgCl_2 treatment (Kelly *et al.*, 1979) of the binding sites possibly occupied by endogenous GH was not applied to crude ovary preparation. However, the low concentration of plasma GH found at all stages of oogenesis indicates that the number of free binding sites estimated in this study was a good assessment of total number of binding sites.

The results of the present study revealed that the affinity and specificity of GH binding to crude ovarian homogenates

Table 2. K_a and B_{max} values for 125 I-labelled rtGH binding in ovary and liver during the first ovogenetic cycle in rainbow trout

	Stages							
	1	2	3	4.1	4.2	4.3	5	6
Ovary								
$K_a \times 10^9$ (mol $^{-1}$)	2.8 $\pm$ 0.4	3.2	4.3 $\pm$ 0.4	4.7 $\pm$ 1.6	3.0	6.7	5.2 $\pm$ 0.7	4.0 $\pm$ 0.7
B_{max} (fmol g $^{-1}$ ovary)	152 $\pm$ 38	183	200 $\pm$ 14	267 $\pm$ 71	43	54	35 $\pm$ 1	390 $\pm$ 126
<i>n</i>	2	1	4	2	1	1	2	4
Liver								
$K_a \times 10^9$ (mol $^{-1}$)	4.7 $\pm$ 0.1	4.8 $\pm$ 0.4	4.9 $\pm$ 0.4	4.9 $\pm$ 0.4	5.4 $\pm$ 0.5	4.8 $\pm$ 0.2	5.1 $\pm$ 0.1	5.1 $\pm$ 0.2
B_{max} (pmol g $^{-1}$ liver)	6.3 $\pm$ 0.5	4.6 $\pm$ 0.2	5.2 $\pm$ 0.5	3.5 $\pm$ 0.2	3.2 $\pm$ 0.1	1.9 $\pm$ 0.3	7.1 $\pm$ 0.3	5.3 $\pm$ 2.8
<i>n</i>	2	2	2	2	2	2	2	2

Pools of ovaries at the same ovogenetic stage (pools of 12–18 pairs of gonads from the immature stage to early endogenous vitellogenesis; pools of 3–12 pairs of gonads from endogenous vitellogenesis to ovulated fish) and livers corresponding to the same animals were used.

Results are expressed as mean $\pm$ SEM; *n*, number of saturation experiments.

Fig. 5. Changes of GH receptor concentrations expressed in (a) fmol g $^{-1}$ ovary or (b) fmol g $^{-1}$ ovarian pellet in rainbow trout during ovogenesis. 125 I-labelled recombinant trout GH (rtGH)-specific binding (300 000 c.p.m. per tube) was performed with 20 mg pellet per tube. Specific 125 I-labelled rtGH binding was measured on individual gonads chosen at each stage of ovogenesis (except for

were similar to those described in rainbow trout with crude hepatic preparations (Sakamoto and Hirano 1991; Yao *et al.*, 1991), and with ovarian or hepatic membrane preparations (Le Gac *et al.*, 1992; Mourot *et al.*, 1992). The apparent K_a obtained in the present study was higher than those described in partially purified ovarian membrane (B. Mourot and A. Fostier, unpublished), owing probably to differences in tracer preparation and apparent specific activity. Binding was specific to GH with little or no affinity for other hormones tested. Previous studies in teleosts also found that prolactin does not bind to GH-R, indicating that GH and prolactin have distinct binding sites in fish (Yao *et al.*, 1991; Auferin *et al.*, 1994). K_a values were in the same range as those described in the testis (Gomez *et al.*, 1998), central nervous system (Perez-Sanchez *et al.*, 1991), gill (Sakamoto and Hirano, 1991) of the same species, and in the liver of other fish (Hirano, 1991; Mori *et al.*, 1992). The apparent dissociation constant of the GH-R (0.2 nmol l $^{-1}$) may be considered high compared with the plasma concentration measured in the present study (0.03 nmol l $^{-1}$). However, plasma GH concentration in rainbow trout shows episodic fluctuations (Gomez *et al.*, 1996) that can lead to transient high concentrations of GH, which could bind efficiently to GH-R. Moreover, the expression of GH mRNA has been detected by PCR in testis (Untergasser *et al.*, 1997), indicating that the local production of small amounts of GH is not impossible. However, it was not possible to show the expression of GH mRNA in rainbow trout testis by northern blot analysis (F. Le Gac, unpublished). The present study is in agreement with results in mammals revealing GH-R mRNA and immunoreactivity in the ovary.

In rainbow trout, ovarian GH-R concentration was highest during the first stages of maturation, and decreased regularly throughout exogenous vitellogenesis to reach a minimal value during the pre-ovulatory period. In contrast, in postovulated fish, GH-R concentration had returned to

immature stage (Imm) and final oocyte maturation from which pools of 3–6 pairs of gonads were used). Different letters above histograms represent significant differences (Kruskal-Wallis test). ANOVA, $P < 0.001$. Results are expressed as means $\pm$ SEM for 4–7 values (*n* given inside the bars in (a)).

Fig. 6. Changes of GH receptor capacity (expressed in pmol per ovary) in rainbow trout ovary during ovogenesis. ^{125}I -labelled recombinant trout GH (rtGH)-specific binding (300 000 c.p.m. per tube) was performed with 20 mg pellet per tube at all stages of ovogenesis. Specific ^{125}I -labelled rtGH binding was measured on individual gonads chosen at each stage of ovogenesis (except for immature stage (Imm) and final oocyte maturation from which pools of 3–6 pairs of gonads were used). Different letters above histograms represent significant differences (Kruskal–Wallis test). ANOVA, $P < 0.001$. Results are expressed as the means + SEM for 4–7 values.

concentrations similar to those observed in endogenous vitellogenesis. These changes in GH binding result from a progressive change in the GH-R capacity, with no alteration of their affinity. Attempts to quantify binding sites for GH in the ovary were unsuccessful in several vertebrates owing to the low ratio of specific:non-specific binding (Eckery *et al.*, 1997). Only one developmental study showed that ovarian GH-R/BP mRNA expression decreases between 1 and 5 weeks of age in rats, and does not change during the oestrous cycle (Carlsson *et al.*, 1993). The 6–7-fold decrease in GH-R concentration (pmol g⁻¹ ovary) during the second part of the oogenetic cycle reflects the increase of oocyte size and yolk accumulation during vitellogenesis, which results in a ‘dilution’ of the cells or tissues bearing the receptors. In fact, when the same data were expressed in fmol g⁻¹ crude pellet (that is mainly exempt of yolk), GH binding was remarkably stable during vitellogenesis (stages 4.1–5). In trout, a large number of oocytes develop and ovulate synchronously in the entire gonad and, after ovulation, the ovarian tissue is mainly constituted of oogonia, primary oocytes, granulosa, luteal and theca cells as well as fibroblasts and blood vessels. The similar concentration of GH-R (pmol g⁻¹ ovary) during endogenous vitellogenesis and in postovulatory stages indicates that GH receptors are localized primarily in ovarian somatic cell membranes rather than in maturing oocyte membranes. This finding is in accordance with the detection in mammals of GH-R/BP mRNA and immunoreactivity on granulosa and, sometimes, in theca cells. However, the possibility cannot be excluded that there is a low concentration of GH-R in oocyte membranes because studies have detected GH-R/BP mRNA in sheep and cow oocytes (Eckery *et al.*, 1997; Izadyar *et al.*, 1997).

Fig. 7. Changes of GH receptor concentrations (expressed in pmol g⁻¹ of liver) in rainbow trout during ovogenesis. ^{125}I -labelled recombinant trout GH (rtGH)-specific binding (300 000 c.p.m. per tube) was performed with 5 mg pellet per tube. Specific ^{125}I -labelled rtGH binding was measured on individual livers chosen at each stage of ovogenesis. Different letters above histograms represent significant differences (Kruskal–Wallis test). ANOVA, $P < 0.001$. Results are expressed as means + SEM for 5–8 values.

Although the roles and mechanisms of GH action in the ovary have not been elucidated, information has been obtained, particularly in domestic mammals. In brief, regulatory effects of GH on follicular growth and the number of follicles developing during an oestrous cycle have been documented in cows and ewes and GH enhances the response to superovulation treatments in women and domestic animals (Gong *et al.*, 1991, 1993; Eckery *et al.*, 1997). GH stimulates the development of early (preantral) follicles in vitro (Liu *et al.*, 1998), it affects the maturation of cumulus-enclosed bovine oocytes and may promote early embryonic development (Izadyar *et al.*, 1996). Numerous GH effects on ovarian cells have been described, including the stimulation of granulosa cell proliferation, differentiation and steroidogenic activities, the increased production of insulin-like growth factors (IGFs) and modulation of IGF-binding proteins (Wathes *et al.*, 1995; Sirotkin, 1996).

In the present study in trout, the highest GH-R concentrations (expressed in fmol g⁻¹ ovary, fmol g⁻¹ pellet or in fmol mg⁻¹ protein) were observed in stages 2–3 of ovarian development, when the oocytes are recruited to the vitellogenic process and start growing. This finding may indicate that the ovary is a target tissue for GH during this period. A hypothetical role of GH could be to adjust the number of growing oocytes depending on the general metabolic status of the animal. In fact, the influence of growth on fish fecundity has been suggested previously. GH could also enhance follicular growth and development in these early stages. In fish, GH may act by enhancing the gonadotrophic stimulation of ovarian steroidogenesis (Van Der Kraak *et al.*, 1990) or could be effective alone on testosterone and oestradiol production as found using ovarian tissues in vitro or hypophysectomized female fish (Singh *et al.*, 1988; Singh and Thomas, 1993). In another

respect the highest total binding capacity for GH in trout ovary was found during the final stage of the cycle and in ovulated gonads, and a potential role for GH in final maturation and ovulation in fish must be considered. The increase in plasma GH concentrations at the end of the reproductive cycle was described in several species (for review, see Le Gac *et al.*, 1993) and studies have shown that GnRH peptides (and sex steroids) play an important role in the regulation of growth hormone secretion in several teleosts, especially when undergoing gonadal development or in sexually mature fish (Peter and Marchant, 1995). Several authors have emphasized the possible 'gonadotrophic' function of GH. GH action may occur through the local production of IGF-I, as has been suggested in fish testis (Le Gac *et al.*, 1996). In fact, IGF-I mRNA and IGF-I receptors are expressed in fish ovary (Gutierrez *et al.*, 1992; Duan *et al.*, 1993) and IGF-I was found to act on final oocyte maturation (Kagawa *et al.*, 1994) and to increase the ovarian production of oestradiol and of $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (the meiosis inducing steroids in trout) (Maestro *et al.*, 1995, 1997; Fostier *et al.*, 1994). However, to our knowledge, no effect of GH on ovarian IGF production has been demonstrated (Duguay *et al.*, 1994).

During the reproductive cycle, the hepatic GH-R concentration g^{-1} tissue increased significantly at the initiation of the ovarian cycle during endogenous vitellogenesis, and then decreased and remained at the same concentration for the rest of the oogenetic cycle. These changes differ from those observed in rodents, in which large and continuous increases of hepatic GH binding or GH-R mRNA have been described during sexual maturation (Maes *et al.*, 1983; Mathews *et al.*, 1989). However, these changes may be related to the rapid growth and to the metabolic status of the animals during puberty, rather than directly to their sexual status. The lower hepatic GH-R concentration observed in rainbow trout in stages 4.2 and 4.3 might be accounted for by temperature and nutritional effects since, at the time these fish were sampled, they had just been submitted to the increase in water temperature that occurs during summer, and to a reduced food ration at the end of summer administered to compensate for the higher temperature. Indeed, decreased liver GH-R has been shown to occur in the case of food restriction in teleost fish (Gray *et al.*, 1992; Perez-Sanchez *et al.*, 1994). The liver, which is the source of vitellogenin, a major component in yolk production, increases in size during rapid vitellogenesis. The total amount of binding sites per liver was found to be increased in fish killed at the end of this period. These fish presented morphological signs of final oocyte maturation (stage 5). Whether this increase is related to external factors, or is somehow related to vitellogenesis or final sexual maturation remains unclear.

The changes of ovarian GH-R concentration in comparison with hepatic GH-R and plasma GH concentrations were analysed. Changes in hepatic and ovarian GH-R concentration during the reproductive cycle showed similar general patterns and significant positive individual correlation. Such correlation indicates that GH-R in these two tissues may be subject to common regulatory factors. Such a hypothesis is not in agreement with the

demonstration that the regulation of GH-R expression may be controlled by a tissue-specific mechanism (Frick *et al.*, 1990; Ymer and Herington, 1992). In mammals, one speculation is that an alternative promoter controls GH-R expression in reproductive organs that are target tissues for placental somatotrophin or lactogen hormones (see Heap *et al.*, 1996), but such a mechanism would not be relevant to the ovarian cycle in teleosts. On the other hand no correlation was found in male rainbow trout between liver and testis GH-R concentration during spermatogenesis (Gomez *et al.*, 1998). Hepatic GH receptors are either up- or downregulated by plasma GH in mammals (for review, see Gluckman *et al.*, 1989). In teleost fish, regulation of hepatic GH-R by high plasma GH has also been reported (Gray *et al.*, 1990; Hirano, 1991; Mori *et al.*, 1992). In the present study, variations in ovarian or hepatic GH receptors did not appear to be significantly correlated with changes in plasma GH concentration (in particular, the decrease in ovarian GH-R concentration observed during vitellogenesis cannot be attributed to downregulation or to binding sites occupied by endogenous GH, as the amount of this hormone tended to decrease at this time). However, in the strain of rainbow trout observed in the present study, particularly low GH concentrations and small fluctuations of this hormone were probably not the best conditions for such an analysis. Luteinizing hormone (Juengel *et al.*, 1997) and gonadal steroids (Baumbach and Bingham, 1995) were also proposed as powerful regulators of hepatic GH-R messenger. A possible relationship between oestradiol or gonadotrophin concentrations and GH-R expression is under investigation.

Finally, factors such as water temperature and nutrition may affect GH-R in gonads as well as in the liver.

In conclusion, a method for the measurement of GH-specific binding in crude ovarian preparations has been validated in rainbow trout. The presence of GH receptors has been observed at all stages of ovogenesis, including in immature fish, but the highest GH-R concentration was observed during the initial step of ovarian growth and the total receptor capacity was maximal during final follicular maturation. The marked decrease in ovarian GH-R concentration during ovogenesis was found to be related to similar changes of hepatic GH-R but not to plasma GH concentration. The present data support the occurrence of GH-R mainly in somatic ovarian cells. In view of these results and of other data obtained in fish and mammals, it is suggested that GH has a gonadotrophic function during final maturation, but also that this hormone is potentially important for gonadal functions during the first steps of ovogenesis (recruitment in vitellogenesis and initiation of oocytes growth). It will be necessary to identify the cell types that express the GH receptor and to develop *in vivo* or *in vitro* experimental models to test the effects of GH treatments on oocyte recruitment, follicular growth or final maturation.

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