Radioimmunoassay for Silver Carp Gonadotropin

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A radioimmunoassay for cyprinid gonadotropin (GtH) was developed using GtH purified from the silver carp *Hypophthalmichthys molitrix* and its specific antiserum. The minimum detectable quantity of silver carp GtH was 0.58 ng/ml (29 pg/tube). The intraassay and the interassay coefficients of variation were 6.8% (N=6) and 8.6% (N=5), respectively near 50% binding. This system was found to be suitable for the measurement of plasma GtH of goldfish *Carassius auratus* and carp *Cyprinus carpio* and pituitary GtH of some teleosts. The competition curves for goldfish and carp plasma were parallel to the standard curve with silver carp GtH. Pituitary extracts of goldfish, carp, honmoroko *Gnathopogon elongatus*, medaka *Oryzias latipes*, and chichibu goby *Tridentiger obscurus* showed competition curves parallel to the silver carp GtH standard, but plasma and pituitary samples of bitterlings (Cyprinidae) showed less steep curves than the standard. This system was physiologically validated by detecting the ovulatory GtH surge in individual goldfish. Plasma steroid hormone levels during ovulation were also measured and their role were discussed in relation to the GtH surge.

Radioimmunoassay (RIA) is one of the best methods to determine blood and tissue hormone levels. RIAs for teleost gonadotropin (GtH) have been developed and have contributed much information to the reproductive physiology of fish.¹⁻⁴⁾

Recently, the glycoprotein GtH was purified from the pituitary gland of the silver carp *Hypophthalmichthys molitrix*⁵⁾ and a highly specific antiserum to silver carp GtH was raised in our laboratory.⁶⁾ Using this antiserum, we developed a RIA for cyprinid GtH. In the present study, we examined the application of this system to the measurement of plasma and pituitary GtH in other cyprinid fishes such as goldfish *Carassius auratus*, carp *Cyprinus carpio*, rose bitterling *Rhodeus ocellatus ocellatus*, and kanehira bitterling *Acheilognathus rhombea*, all of which are commonly used as experimental fish. Although silver carp is abundant in Lake Kasumigaura in Japan, this fish is impractical for laboratory use because of its large size.

Since the occurrence of a GtH surge has been noted during ovulation in trout^{7,8)} and goldfish,⁹⁾ detecting the GtH surge would be a physiological validation of the GtH RIA. Previously changes in the plasma GtH levels during ovulation were presented by only as the mean value in a group of goldfish.⁹⁾ Therefore, we wanted to trace the changes in both the plasma GtH and

steroid hormone levels in individual fish during the process of final oocyte maturation and ovulation.

Materials and Methods

RIA for GtH

The silver carp GtH used in this study was purified by KOBAYASHI *et al.*⁵⁾ Antisera (No. 1-3) to the silver carp GtH were prepared in three male rabbits and their specificities were immunocytochemically validated by KANEKO *et al.*⁶⁾ The serum No. 1 which showed the most sensitive standard curve was used for the assay.

The silver carp GtH was iodinated by the lactoperoxidase method of THORELL and JOHANSSON¹⁰⁾ with some modifications. Silver carp GtH (2.5 μ g) dissolved in 25 μ l of 0.05 M phosphate buffer (pH 7.0) containing 0.14 M NaCl was reacted with 0.2 mCi ¹²⁵I (Amersham), 0.5 μ g of lactoperoxidase (Sigma) in 5 μ l of distilled water, and 5 μ l of H₂O₂ diluted 1:40,000 with 0.2 M phosphate buffer (pH 7.0). After three minute of reaction at room temperature, the mixture was applied to a Sephadex G-75 column (0.6 $\times$ 22 cm) and eluted with 0.05 M phosphate buffer (pH 7.0) containing 0.14 M NaCl to separate labeled hormone from free ¹²⁵I. The labeled GtH was stored at -20°C.

The standard hormone (purified silver carp GtH) was dissolved in Gel-PBS (0.01 M phosphate

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buffer, pH 7.5, containing 0.1% gelatin, 0.14 M NaCl and 0.1% sodium azide) at eight concentrations from 0.39 ng/ml (19.5 pg/tube) to 50 ng/ml (2.5 ng/tube). Dilutions of plasma and pituitary samples were made with Gel-PBS. The antiserum was diluted 10,000 fold with NRS-EDTA-PBS (0.01 M phosphate buffer, pH 7.5, containing 1% normal rabbit serum, 0.14 M NaCl, 0.05 M EDTA, and 0.1% sodium azide). Samples for competition curves and standards were assayed in duplicate.

The following double-antibody method was employed for RIA. First, 50 μ l of sample or standard hormone was incubated with 50 μ l of diluted antiserum, resulting in approximately 25% binding of labeled GtH in the absence of unlabeled GtH. After 24 h incubation at 4°C, 50 μ l of labeled hormone (approximately 20,000 cpm) were added to each tube, and incubation was continued for 24 h at 4°C. Then 100 μ l of second antibody (goat anti-rabbit gamma globulin serum) diluted 35-fold with EDTA-PBS (0.01 M phosphate buffer, pH 7.5, containing 0.14 M NaCl, 0.05 M EDTA and 0.1% sodium azide) were added and incubated for 20 h at 4°C. After adding 0.5 ml of Gel-PBS, the assay tubes were centrifuged at 1,500 g for 25 min. The supernatant was aspirated, and the radioactivity of the precipitate was counted in a gamma counter.

Pituitary glands were collected from maturing goldfish, carp, rose bitterling, and kanehira bitterling, honmoroko *Gnathopogon elongatus* (Cyprinidae), kokanee salmon *Oncorhynchus nerka* (Salmonidae), urohaze goby *Glossogobius olivaceus*, chichibu goby *Tridentiger obscurus* (Gobiidae), medaka *Oryzias latipes* (Cyprinodontidae), and shirogisu *Sillago japonicus* (Sillagonidae). Pituitary samples were dehydrated with cold acetone and homogenized with 1 ml of Gel-PBS. After a 24 h extraction at 4°C, the homogenates were centrifuged at 1,500 g, then, supernatants were used for the assay. Plasma samples were obtained from ovulated goldfish, carp, and kanehira bitterling. Plasma samples from six month old immature goldfish (including both sexes) weighing about 10 g were also taken and pooled for the assay.

Assay results were expressed as relative binding (B/Bo (%); radioactivity of sample tube $\times$ 100/total binding (radioactivity in the absence of unlabeled hormone)) and parallelism of the competition curves for plasma and pituitary samples to the standard curve was tested by analysis of variance.¹¹⁾ The minimum detectable level of the

GtH was calculated according to CAMPFIELD.¹²⁾

Induction of Ovulation

Sexually mature goldfish were purchased in May and were kept at 12°C under natural photoperiod until the experiment in July. Two days before the experiment, ten female goldfish (average body weight, 100 g) were transferred to four 50 liter tanks which were kept at 12°C and 15 h light-9 h dark (lights on at 04:00). Ovulation was induced by the method of YAMAMOTO *et al.*¹³⁾ through an increase in water temperature. The water temperature was raised to 24°C as illustrated in Fig. 3A. The experimental tanks were provided with sand filters and artificial water plants. Ten females were divided into two groups, from which 300 μ l of blood per fish were sampled alternately at 12:00 and 18:00 for five days. Ovulation was checked hourly from 23:00 to 04:00 by applying gentle pressure to the abdomen. In addition, blood was sampled at the time of ovulation and six hours after ovulation. Oxytetracycline (Sigma) was added to the water at a concentration of 10 ppm after the first blood sampling. Blood samples were taken from the caudal vasculature with a heparinized syringe and needle (21 gauge) after anesthetization with tricaine methanesulfonate (Crescent Res. Chem. Inc.). During the dark phase, fish were caught and anesthetized under a dim red light, and blood was taken in the light. After centrifugation, plasma samples were stored at -15°C.

RIA for Steroid Hormones

Plasma estradiol-17 β , 17 α -hydroxyprogesterone (17 α -OH-P), 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -diOH-P) and testosterone were measured by RIA according to the method of AIDA *et al.*¹⁴⁾ and LOU *et al.*¹⁵⁾ The antiserum to testosterone cross-reacted with both testosterone and 11-keto-testosterone (11-KT) at 100%. Plasma 11-KT was also measured by RIA using (1,2-³H) ketotestosterone (Amersham) and antibody raised against 11-ketotestosterone-3-CMO-BSA which was kindly provided by Dr. Y. NAGAHAMA (National Institute for Basic Biology, Okazaki). The antiserum to 11-KT cross-reacted with 11 β -hydroxytestosterone, testosterone, adrenosterone, 5 α -dihydrotestosterone, 11-ketoadrenosterone and androstenedione at 5.5, 1.9, 0.83, 0.49, 0.19 and 0.067% levels, respectively. The intraassay and the interassay coefficients of variation were 3.8% (N=6) and 7.8% (N=3), respectively, near 50% binding.

The sensitivity of steroid RIAs was 6 pg/tube. Since a small amount of plasma (20–50 μ l) was available for the assay in this study, minimum detectable limits (ng/ml plasma) were as follows: estradiol-17 β , 0.12 ng/ml; 17 α -OH-P, 0.3 ng/ml; 17 α ,20 β -diOH-P, 0.3 ng/ml; testosterone, 0.3 ng/ml; 11-KT, 0.12 ng/ml.

Results

RIA for GtH

A standard curve using the silver carp GtH is shown in Fig. 1. The minimum detectable quantity of silver carp GtH standard was 0.58 ng/ml (29 pg/tube). The intraassay and the interassay coefficients of variation were 6.8% (N=6) and 8.6% (N=5), respectively, near 50% binding.

Fig. 1. Dose-response competition curves for silver carp GtH and serial dilutions of plasma from carp, goldfish, and kanehira bitterling.

Competition curves for plasma from goldfish and carp were parallel to the standard curve, whereas the competition curve for plasma from kanehira bitterling was less steep in comparison with the standard curve as shown (Fig. 1). Plasma samples from immature goldfish showed no cross-reaction, and the GtH level was below the minimum detectable level.

In the cyprinid fishes tested, competition curves for pituitary extracts from carp, honmoroko, and goldfish showed parallelism to the standard, whereas those for two species of bitterling were less steep than the standard curve (Fig. 2). However, the competition curve for the pituitary extract from kanehira bitterling was parallel to that for plasma. The competition curve for goldfish pituitary extract was somewhat less steep when B/Bo was below 50%.

Pituitary extracts of the medaka and chichibu goby gave parallel competition curves to the standard (Fig. 2). Pituitary extracts of the kokanee salmon, shirogisu, and urohaze goby showed low cross-reaction to the antibody, and their competition curves were less steep than the standard curve (Fig. 2).

Ovulatory Surge of GtH

Ovulation occurred in 3 fish at 01:00 and in 1 fish at 04:00 on day 4. In these fish, a rapid increase in plasma GtH levels had already commenced at 18:00 before ovulation, and the highest levels were attained at the time of ovulation after midnight (Fig. 3A-D). Plasma GtH levels rapidly decreased after ovulation and reached the pre-surge levels in the morning. Plasma GtH levels in the fish which failed to ovulate were maintained

Fig. 2. Dose-response competition curves for silver carp GtH and serial dilutions of pituitary extracts of carp, goldfish, honmoroko, kanehira bitterling, rose bitterling, chichibu goby, urohaze goby, medaka, shirogisu, and kokanee salmon.

Fig. 3A-D. Changes in plasma hormone levels in individual ovulated goldfish. ov: ovulation.

Fig. 4A-F. Changes in plasma hormone levels in goldfish that failed to ovulate.

at low levels throughout the experiment (Fig. 4A-F).

Plasma Steroid Hormones

Plasma 17α -OH-P and 17α , 20β -diOH-P levels showed changes similar to those of GtH in ovulated fish as shown in Fig. 3A-D. Plasma 11-KT levels were usually below 0.12 ng/ml and the highest level was 1.0 ng/ml when the testosterone level (including 11-KT) was 22.7 ng/ml. Therefore, the main androgen in female goldfish is considered to be testosterone. An increase in testosterone before the GtH surge was clearly observed in three ovulated fish. Plasma estradiol- 17β levels changed asynchronously in each individual, and their changes were smaller than those of other steroids. In non-ovulated fish, plasma steroid hormone levels did not show distinct changes during the experiment (Fig. 4A-F).

Discussion

The silver carp GtH RIA established in this study was found to be suitable for the measurement of plasma GtH in goldfish and carp since the competition curves for plasma were parallel to the standard curve. The competition curve for the plasma of *kanehira* bitterling belonging to Cy-

prinidae was not parallel to the standard curve. Since the competition curve for the pituitary extract from this species showed a slope parallel to that for plasma from ovulated fish, this system seems to cross-react with GtH of *kanehira* bitterling, but further validation is required. Plasma samples from immature goldfish showed no cross-reaction in this RIA, indicating undetectable level of plasma GtH in immature goldfish and no competition of binding by plasma.

The pituitary GtH content can be measured in the carp and *honmoroko* with this system since their competition curves were completely parallel to the standard. The slope of the competition curve for the pituitary extract of goldfish was less steep than that of the silver carp GtH below 50% binding, whereas plasma samples showed a slope parallel to the standard. This suggests that goldfish pituitary may contain some other substances which cross-react with the antiserum. The pituitary extract from two species of bitterlings showed low cross-reactivity with the anti-silver carp GtH serum, and their competition curves were less steep than the standard curve. Species specificity of GtH in cyprinid fishes seems to be higher than that among salmonid fishes since a salmon GtH RIA was found to be applicable to

almost all salmonid fishes.* Pituitary extracts of medaka (*Cyprinodontidae*) and chichibu goby (*Gobiidae*) showed complete parallelism to the silver carp standard. This indicates that GtH content of the pituitary can be directly measured in these species using the silver carp GtH RIA, but the content appears to be underestimated as follows: 0.43 ng/one gland in medaka and 12 ng/one gland in chichibu goby. This may be due to the difference in the number of common antigenic determinants in GtH molecules. It is still unknown why competition curves for these fishes were parallel to the standard curve. The pituitary samples of other fishes examined in this study showed weak cross-reactivity to the antiserum raised against silver carp GtH, and the slopes of competition curves were gentle.

Since the assay sensitivity is high, only 25 to 50 μ l of plasma sample are required for the assay. Because only small volumes of plasma are needed for the assay, individual GtH changes in relatively small fishes can be traced. Undetectable levels of GtH in immature goldfish and distinct ovulatory GtH surges detected in this study indicate that this RIA system is physiologically valid in addition to the immunocytochemical validation shown by KANEKO *et al.*⁹⁾

GtH surges traced in individual fish showed a pattern similar to that demonstrated by STACEY *et al.*⁹⁾ and the synchronization of GtH surges was also observed. The timing of the GtH surge appears to be determined by photoperiodical cues in the goldfish since not only did abrupt changes in photoperiod shift the time of ovulation¹⁰⁾ but also the ovulatory GtH surge was observed in the dark phase under normal and reversed photoperiod (light phase: 04:00–19:00; 16:00–07:00, respectively) regardless of the time of raising water temperature.*

Plasma levels of 17α -OH-P and $17\alpha,20\beta$ -diOH-P increased almost at the same time as the GtH surge. These steroids are thought to be produced in ovarian follicles by the stimulation of GtH and to induce oocyte maturation as has been shown in some teleosts.^{17–19)} Maximum values of 17α -OH-P and $17\alpha,20\beta$ -diOH-P (4–75 ng/ml, 2–45 ng/ml respectively) were lower than those reported in salmonids^{7,8,15,20–23)} but considerably higher when compared with the values already reported in goldfish.²⁴⁾ Rapid changes in these hormone levels suggest short-time secretion and/or rapid plasma clearance of these hormones in goldfish.

There was no decrease in estradiol- 17β before ovulation in this study, conflicting with the suggestion in salmonids that decrease in estradiol- 17β level may trigger the hypothalamic-mediated GtH surge.⁷⁾ The changes in estradiol- 17β level were not synchronous; the physiological role of estradiol- 17β in ovulation remained unclear. On the other hand, following a rise in water temperature, plasma testosterone levels increased before the GtH surge in three ovulated fish. These elevations in testosterone levels might play some role in regulating the GtH surge as the result of positive feedback to the hypothalamus. Plasma 11-KT levels remained low in both ovulated and non-ovulated fish and this hormone may not be primarily involved in ovulation. These preliminary experiments revealed much about the hormone changes during ovulation in goldfish, but further investigation is required to elucidate the hormonal and environmental control of ovulation in teleosts.

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