Growth hormone, sexual maturity and steroids in male carp
(Cyprinus carpio)

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Abstract

Samples of pituitary, blood plasma and gonad were taken from male carp. The growth hormones (GH) in the pituitary and plasma were measured in fish of various body weights (BW) and degrees of gonad development, and compared with the levels of 17β-estriol (E2), testosterone (T), 17α-hydroxyprogesterone (17-P), 11-ketotestosterone (11-KT) and progesterone (P) in the testes and plasma. The gonadosomatic index increased rapidly with BW from 100 to 600 g, and then decreased at 900 g. The pituitary GH did not change with BW, but plasma GH was higher in fish weighing 300±50 and 600±50 g, than in fish weighing 900±50 g. In fish weighing 150±50 to 300±50 g, the level of T rose significantly in the testes (2.27 ng g⁻¹) and plasma (1.3 ng g⁻¹); E2 was very low in both testes (0.06 pg g⁻¹) and plasma (11.28 pg ml⁻¹), increasing as BW rose from 150 to 600 g. The level of P rose mainly at BW of 300±50 and 600±50 g, from 0 to 25 ng g⁻¹ in the testes and from 0 to 17 ng ml⁻¹ in the plasma. The level 17-P rose from 2.5 to 20 ng g⁻¹ in the testes at 600±50 g BW, but no significant change was recorded in the plasma. The level of 11-KT rose significantly in the testes of fish at 300, 600 and 900 g (0.5–6 ng g⁻¹). The application of different steroids (E2, T and 17-P) on a primary culture of pituitary cells led to the release of GH. Release was significantly higher (P < 0.05) after 4 h at steroid concentrations of 10⁻⁸ M. © 1998 Elsevier Science Inc. All rights reserved.

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1. Introduction

Growth hormones (GH) have been isolated from the pituitary glands in a number of fish species: Cyprinus carpio [4]; Oncorhynchus mykiss [4,42]; Anguilla japonica [43]; Seriola quinquemaculata [19]; Katsuwonus pelamis [29]; Gadus morhua [32]; Oreochromis mossambicus [43].

The biological activities of GH have been examined for many of these species [33]. During recent years, several GHs have been successfully cloned and expressed in Escherichia coli [10,13,33,35]. GH plays a role in the growth and development of teleost fishes [7,8,22,36,37,40]. Recombinant salmon GH increases and mediates steroid production in vitro in rainbow trout, Oncorhynchus mykiss and sea trout, Cynodon nebulosus [37]. High affinity, low capacity GH binding sites have been detected in rainbow trout testes [36]. The two gonadotropin-releasing hormones, cGnRH-I and cGnRH-II, stimulate the release of GH from the pituitary of goldfish, Carassius auratus [31].

Little information has been published on the GH level in the plasma of teleosts during growth and maturation, but existing data support the hypothesis that there is a correlation between GH and gonad development or steroid levels. Bjornsson et al. [3] found that the GH level is higher in sexually mature Atlantic salmon, Salmo salar, than in immature fish. In the white sucker, Catostomus commersoni, where there is no
obvious relationship between males and the reproductive status of the female, the GH level in the latter rises during ovulation and remains high after spawning [38]. In chum salmon, *Oncorhynchus keta*, caught in the wild, an increase in GH level during sexual maturation has been related to a changed osmoregulatory function [15]. Sampter and colleagues [39] showed that the GH level increases during ovulation in rainbow trout, and Le Gac and colleagues [22], observed an increase in the GH level in male rainbow trout at the beginning of sperm production, concomitant with an increase in the level of 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P). The changes in gonadotropin (GTH) and ovarian steroids associated with oogenesis have been described in detail in carp [9,20,23–25]. The spermatogenesis and spermatiation of carp are controlled by GTH and steroids [27,28]. Although the level of 11-ketotestosterone (11-KT) in salmonids drops during spermatiation, the level of GTH remains high, while levels of progesterone (P) and 17,20-P rise [34]. The GH level in the serum of goldfish, *Carassius auratus*, was found to correlate with seasonal change, with the highest level in July and the lowest level in February and March [26]. The GH level in mature female carp gonads was higher than in undeveloped fish. Altogether, other steroid levels correlated with carp GH [6]. However, the relationship between GH, steroid levels and gonadal development in carp during growth and gonadal development has not previously been reported.

The aims of the present study were to measure the level of GH in the pituitary and plasma of male common carp of different and body weight (BW) and stages of gonadal maturation, and to relate these findings to the levels of sex steroids in the plasma and testes.

2. Materials and methods

2.1. Fish

Male carp, weighing 100–900 g and aged 0.5–2 years, were collected from fish ponds at Kfar Giladi, in northern Israel, in April 1994. The fish were brought to the laboratories of the Galilee Technological Center (MIGAL) and were divided into four groups of ten fish each, on the basis of size: 150, 300, 600 and 900 g (all ±50 g) and gonad development (1% GSI, 1.6% GSI and 3.2% GSI). Pituitaries for cell culture were taken from the smallest fish (150 g), in which the gonads were still undeveloped.

2.2. Blood and pituitary samples

Blood samples (0.1–0.5 ml) were extracted from the hearts of the specimens by heparinized syringe, after anesthesia [5]. The plasma was separated by centrifugation and stored at −20°C until assayed.

The specimens were then killed and a sample of 0.5 g was taken from the testes of each, to establish the state of maturity. These samples were immediately fixed with Bouin solution, for histological observation. A further 0.5 g of the testes was taken and stored at −20°C for later analysis of the steroid content, which was performed according to Ando [1], as improved by Venkatesh [41].

The pituitary was removed from each specimen and stored at −20°C, until measurement of GH. The pituitary was homogenized with phosphate buffer, pH 7.4, and the supernatant was separated by centrifugation.

2.3. Extraction of steroids

Samples of fish testis (0.5 g) were homogenized with 1 ml water for 3–4 min, in an automatic homogenizer (Kinematica). Dichloromethane (DCM; 5 ml) was added to the mixture in tubes, which were then placed in storage at 0°C for 10 min. Two phases appeared: an upper frozen aqueous phase and a lower phase containing the DCM and the steroids. The lower phase was transferred to new tubes. When the DCM had evaporated completely, hexane acetone was added (7:1) and the mixture was stored overnight at 0°C. The tubes were then centrifuged again (5 min, 8000 × g) and the upper phase was again transferred to a new tube. All the hexane acetone was allowed to evaporate.

2.4. Hormone assay

Plasma GH was assayed in three replicates (two 50–μl samples) by double antibody radioimmunoassay (RIA), according to Fine [10] in 1.5-ml centrifuge tubes, each containing 250 μl RIA buffer (50 mM TRIZMA Base, 0.5% NaNO₃, 0.1% Triton X-100, 1% BSA; pH 7.8; 100 μl of a known or unknown concentration of cGH; 100 μl carp GH (cGH) antiserum (1:10000); 50 μl 125I-cGH (40000–50000 cpm per tube). After incubation overnight at 4°C, 0.1 ml anti-rabbit IgG (containing 2–3 μg protein) was added, and the tubes were incubated for an additional 3 h. Next, 0.1 ml bovine gamma globulin (1% w/v) and 0.5% polyethylene glycol (25% w/v) were added. The tubes were thoroughly mixed and centrifuged at 9000 × g. The supernatant was aspirated, and the precipitates were counted with 65% efficiency. The quantity of cGH in each tube was calculated automatically, using the log-logit program. In the absence of unlabelled antigen, 15–20% 125I-cGH was specifically bound. The standard curve covered the range 0.03–200 ng cGH per tube.

The homogenized pituitary sample was centrifuged and the protein concentration measured with a biuret protein assay kit (BioRAD, Jerusalem).

The steroid concentrations in the plasma and testes were measured by means of an RIA kit [125I-cGH] from.
Count-A-Count (California). The steroids thus measured, using a γ-counter (version 2, Eurogenetics, Belgium), were 17β-estradiol (E2) (range: 8–3600 ng ml⁻¹; coefficient of variation: r = 0.99); 17α-hydroxyprogesterone (17-P) (0.25–20 ng ml⁻¹; r = 0.99); P (0.1–20 ng ml⁻¹; r = 0.98); testosterone (T) (0.1–30 ng ml⁻¹; r = 0.98); and 11-ketotestosterone (11KT) (0.1–30 ng ml⁻¹; r = 0.99). These steroids were selected for measurement because of their importance in the reproductive cycle [44]. The RIA kits have highly specific antisera. The cross reactivity among the various steroids was never higher than 0.02%, except for the antiserum against 17-P, which showed a cross reactivity of 3.5% with progesterone. The intra-assay coefficients were calculated for each of four samples from the results of 20 pairs of tubes in a single assay; they ranged from 4.0 to 7.0%. The interassay coefficients were calculated for each of five samples from the results of pairs of tubes in 20 different assays; they ranged from 4.2 to 8.1%. The results of the measurements by RIA were cross checked against high performance liquid chromatography (HPLC) [6].

2.5. Specificity of HPLC assay

In addition to the samples, standard steroids (Sigma) were injected into the HPLC: 17-P, 17-20-P, P and T at 244 nm, and E₂ at 210 nm, each at a concentration of 0.1 mg ml⁻¹. The standard steroids (mixed in two groups, according to wavelength) underwent the same extraction treatment as the samples, in order to determine their recovery rates. In all cases, recovery was 80%, except for 17-P (58%) and P (100%).

Injection was into an RP 18 column (Merck Lichrospher 250-4), using two solvents: 100% D2O (A) and 100% ACN for UV (Lab-Scan). The following gradients were used, at a flow-rate of 1.0 ml min⁻¹:

<table>
<thead>
<tr>
<th>Time</th>
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<td>15</td>
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2.6. Pituitary cell culture

A total of 20 fish were killed, and the pituitaries removed and immediately placed in a solution of medium M-199, plus 0.5% antibiotic (10000 units ml⁻¹ penicillin⁻¹, 10 mg ml streptomycin⁻¹, 1250 units ml nystatin⁻¹), 3% BSA, 10 mM Hepes, in which they were washed three times before they were cut into small pieces, and 0.25% (w/v) trypsin, 1% glucose and EDTA were added. The suspension was incubated for 1 h, the reaction stopped by adding fetal calf serum, and the suspension was transferred to conical tubes. The bigger pieces were allowed to sink and the total number of cells was estimated from the supernatant. The cells were counted by means of a hemocytometer. A total number of 9.9 × 10⁶ cells was counted.

The cell suspension was then again diluted with M-199, plus 10% fetal calf serum, 10 mM Hepes and 0.5% antibiotic, and divided into 96 plate wells (200 µl solution containing 55000 cells per well), where the cells adhered to the bottom. The percentage of live cells after dissociation was 91%. They were incubated for 3 days (28°C, 95% humidity, 5% CO₂), then washed twice and incubated for 15 min with the same solution. Next, 180 µl M-199, 1% antibiotic, 0.1% BSA and 1% Hepes were added, and 20 µl of the steroid was diluted in alcohol. Each dose of every steroid was assayed in six replicates.

The plates were incubated again (4 h), and aliquots from each well were taken for GH to be determined by RIA assay.

2.7. Histological examination

The pieces of testes were embedded in paraffin plastic medium. Histological sections of 2–5 µm were obtained by the use of a Reichert Jung (Austria) microtome. The trichrome of Mallory was used to stain the sections [12,17].

2.8. Stages of testes development

The stages of testes development were determined by calculating the gonadosomatic index (% GSI) and by histological observation of stained sections.

2.9. Statistical analysis

The differences between means of hormone concentrations (GH and steroids) among fish groups were analyzed and compared by student’s t-test [30] with the level of significance in different groups set at P < 0.05. Correlations between the various hormones and GSI and BW were determined by linear regression, and the correlation coefficient was calculated. Differences in percentage GSIs were examined by the proportional d-test [30].

3. Results

Fish weighing 150 g were immature, with 46% reaching maturity at 300 g; and all the fish were mature above that weight. Changes in the GSI, the ratio of testis to BW, of carp at various weights, are presented in Fig. 1. The GSI rose significantly (P < 0.01) as BW
rose to 600 ± 50 g, and decreased slightly at 900 ± 50 g. Fig. 2 illustrates the relation between the gonadal development and GSI. A and B: histological section of fish weighing 150 g, with GSI = 1 ± 0.5%; the mature spermatozoa (S) occupy a small area of the gonad. C and D: histological section of fish weighing 300 ± 50 g with GSI = 1.6 ± 0.8%; E and F: histological section of fish weighing 600 ± 50 g with GSI of 3.2 ± 0.8%; the mature spermatozoa completely fill the lobule and occupy a big volume of the gonad.

The pituitary GH per protein unit is significantly higher in fish weighing 200 ± 50 g than in fish at 150 ± 50 g, but showed no further significant difference when compared to bigger fish (500 ± 50 and 900 ± 50 g) (Fig. 3). However, the plasma GH showed significant difference (P < 0.01) between fish weighing 150 ± 50 and fish of 600 ± 50 g. Fish weighing 900 ± 50 g showed plasma GH levels similar to small fish (150 ± 50 g) (Fig. 3).

The level of T in the testes was very low in small fish (0–0.15 ng g⁻¹). It was higher in both testes and plasma in fish weighing 300 ± 50 and 600 ± 50 g, and lower in fish weighing 900 ± 50 g (Fig. 4). Differences in levels of T between the groups were significant (P < 0.05) in the testes, while in the plasma there was a significant difference in T between immature fish (up to 150 g) and other groups.

The concentrations of E2 in the testes were also very low. Significant differences (P < 0.05) in the levels of E2 were found in the testes, between fish weighing 150 ± 50 and 900 ± 50 g, and fish weighing 300 ± 50 and 600 ± 50 g; and in the plasma between the two lower weight groups and the two higher weight groups (Fig. 5).

The levels of P in the testes and plasma were higher at fish weights of 300 ± 50 and 600 ± 50 g than at 150 ± 50 and 900 ± 50 g (Fig. 6).

The steroid 17-P was very low in both plasma and testes. There is a positive correlation between the level of hormone in testes and weight in fish weighing from 100 ± 50 to 650 ± 50 g, while in fish weighing 900 ± 50 g there was a significant decrease in the hormone level (Fig. 7).

The level of 11-KT in the testes correlated directly to fish weight (Fig. 8).

The short-term effects of E2, T, 17,20-P and 17-P on GH secretion from primary culture pituitary cells in vitro are shown in Table 1. The release of GH from the cells affected by various steroids was significantly higher (P < 0.05) at a concentration of 10⁻⁷ M after 4 h (Table 1). The level of GH dropped under lower dose treatments (10⁻⁸ M) of T and 17-P. Only with 17,20-P was there a significant rise in GH secretion with a decrease in steroid levels.
Fig. 3. Growth hormone (GH) levels in pituitary gland and plasma of male carp of various sizes, gonadosomatic index and gonadal development. 150 g: 1% GSI; 300 g: 1.6% GSI; 600 g: 3.2% GSI; 900 g: 1.8% GSI. Lower case letters indicate statistical comparison (t-test). Distinct letters indicate significant differences (P < 0.05).

Table 2 shows the correlation among GSI, BW and the various hormones. High correlation was found between GH in the pituitary and the plasma (r² > 0.99), and between GH in the pituitary and the steroids T (plasma) (r² > 0.98), 17P (plasma) and 11-KT (testes) (r² > 0.83). The GH in the plasma also correlated highly with E₂ and T (r² > 0.99) and P (r² > 0.96) in the testes (Table 2).

Fig. 4. Levels of testosterone (T) in testes and plasma of male carp of various sizes, gonadosomatic index and gonadal development. 150 g: 1% GSI; 300 g: 1.6% GSI; 600 g: 3.2% GSI; 900 g: 1.8% GSI. Lower case letters indicate statistical comparison (t-test). Distinct letters indicate significant differences (P < 0.05).

Fig. 5. Levels of 11-estradiol (E₂) in testes and plasma of male carp of various sizes, gonadosomatic index and gonadal development. 150 g: 1% GSI; 300 g: 1.6% GSI; 600 g: 3.2% GSI; 900 g: 1.8% GSI. Lower case letters indicate statistical comparison (t-test). Distinct letters indicate significant differences (P < 0.05).

4. Discussion

The results of this study show that the GSI of the male C. carpio is related to BW and gonadal development, reaching a maximum when the fish attained a size of 600 ± 50 g. There is no evident correlation between plasma GH and BW, but a significant correlation between plasma GH and GSI. The GH level in the pituitary remained almost the same in fish at 300 ± 30 and 900 ± 50 g; in the plasma it is higher in fish weighing 300 ± 50 and 600 ± 50 g than in fish of 900 ± 50 g. We know of no study that shows a similar correlation between GH and weight. A number of parameters can affect GH secretion into the plasma, including season, temperature and water quality [25]. There is strong evidence that GH is an important
regulator of gonadal growth and development in teleosts [11, 14, 45]. Van der Kraak [40] studied the effect of GH on the stimulation of GTH and secretion of steroids, and suggested that GH has a direct modulatory effect on GTH-stimulated steroid production, and also that it may be an important regulator of follicular development in the goldfish. In killifish, injection of recombinant salmon GH stimulated the growth of the gonads and raised the level of sex steroids circulating in the blood [36]. Similarly, injection of recombinant rainbow trout GH raised the E2 level in immature trout and killifish. Levavi-Zeremsky and Yaron [23] found that E2 is the hormone that controls vitellogenesis, while 17β-estradiol controls oocyte maturation; and found the same pattern with T in the plasma, which was very high when oocytes reached vitellogenesis. Marchant and Peter [26] found that daily levels of GH in the serum correlated closely with seasonal changes in the length of the day. Billard et al. [2] studied the hormonal changes in testis, pituitary and plasma during growth in Cyprinus carpio. Their results showed that plasma GTH was in the same range in mature and immature (6 months) males, in the pituitary, GTH content was even lower in mature than in immature males. In older fish (19–37 months) no significant changes in plasma GTH were detected during gonadal development, but an acute rise in the plasma level of GTH was detected around the spawning period. In the pituitary, the GTH showed a general tendency to rise with the age of fish. In the present study it was found that pituitary GH levels did not change. However, GH plasma levels in fish weighing 600 ± 50 g were significantly higher than in 300 ± 50 and 900 ± 50 g fish. The dynamic of production and secretion of GH and GTH is apparently distinct.

The composition of the sertoli and interstitial cells of the testis changes as it matures. This may affect the rate of secretion of steroids, such as was found in the present work.

Growth in fish can be stimulated by the administration of GH [7, 8]. Several research groups have succeeded in cloning GHs from carp [10, 21].

In the present study it was found that at a high concentration (10−6 M) the steroids E2, T and 17β-P increase the release of the GH from the carp pituitary in vitro. Similar results were obtained by Hugard and Habibi [16]. In an in vitro study in which an increased mRNA level in pituitary was found, after treatment with 20 ng ml−1 T. Comparing the in vivo and in vitro results, these authors suggested that T had a direct stimulatory effect on the GH mRNA level.

The relationship between GH in plasma, body weight and sexual maturation suggests that, before maturation, GH affects mainly growth, whereas during maturation in males, its function appears to be related to another biological event, for example gonadal development. During the maturation period there is not a positive relation between weight and GH levels in the plasma. This has also been found in other fish species. During the growth period and at the beginning of maturation, when the gonads develop and the levels of steroids rise,
the secretion of GH into the plasma accelerates growth. At maturity, however, steroid and GH levels correlate with reproductive development and not with BW. This proposed model of the relationship between growth, steroids and secretion of GH is supported by a number of other studies, which have shown that GH is involved in the reproductive cycle [3].

References