Sex Steroids and Their Involvement in the Cortisol-Induced Inhibition of Pubertal Development in Male Common Carp, *Cyprinus carpio* L.  


Graduate School for Developmental Biology, Research Group for Comparative Endocrinology, Utrecht University, 3584 CH Utrecht, The Netherlands  
Fish Culture and Fisheries Group, Wageningen University, 6700 AH Wageningen, The Netherlands

**ABSTRACT**

The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis. Sex steroids produced in the gonads play an important role in the onset of puberty. Stress interferes with reproduction and the functioning of the BPG axis, and cortisol has frequently been indicated as a major factor mediating the suppressive effect of stress on reproduction. Prolonged elevated cortisol levels, implicated in stress adaptation, inhibited pubertal development in male common carp (*Cyprinus carpio*). Cortisol treatment caused a retardation of pubertal testis development and reduced the LH pituitary content and the salmon GnRHa-stimulated LH secretion in vitro. A reduced synthesis of androgens was also observed. These findings suggest that the cortisol-induced inhibition of testicular development and the maturation of pituitary gonadotrophs are mediated by an effect on testicular androgen secretion. In this study, we combined cortisol treatment with a replacement of the testicular steroid hormones (testosterone and 11-oxygenated androgens) to investigate the role of these steroids in the cortisol-induced suppression of pubertal development. The effect of cortisol on spermatogenesis was independent of 11-ketotestosterone, whereas the effect on the pituitary was an indirect one, involving the testicular secretion of testosterone.

cortisol, glucocorticoid receptor, pituitary, spermatogenesis, testosterone

**INTRODUCTION**

In juvenile fish, the importance of sex steroids for the onset of puberty has been shown in several studies. Sex steroids stimulate the development of all levels of the brain-pituitary-gonad (BPG) axis. In the African catfish (*Clarias gariepinus*), treatment with 11-oxygenated androgens stimulated testicular growth and spermatogenesis and the development of secondary sexual characteristics [1]. Treatment with testosterone activated the gonadotroph maturation [2] and accelerated the development of the hypothalamic GnRH system [3].

Adaptation to severe and chronic stress interferes with processes such as growth, immune response, and reproduction. Cortisol has frequently been implicated as a major factor mediating the suppressive effect of stress on reproduction. Prolonged cortisol treatment inhibited pubertal development in male common carp (*Cyprinus carpio*) [4], and elevated cortisol levels resulted in an impairment of spermatogenesis and a reduction of the synthesis of the 11-oxygenated androgens. These effects were not mediated by an effect of cortisol on LH secretion [5]. However, cortisol does affect LH secretion at the level of the pituitary, resulting in a smaller LH releasable pool and reduced salmon GnRHa (sGnRHa)-stimulated LH secretion in vitro. Testosterone induces development of pituitary gonadotrophs, leading to an increase in LH content and GnRH-inducible LH release [2]. We hypothesized that cortisol inhibits the testicular testosterone secretion and thereby prevents LH storage, which leads to a reduced GnRH-inducible LH release in vitro. Furthermore, we suggested that the reduced steroid hormone secretion would effect the maturation of pituitary gonadotrophs and testicular development [4, 5].

The aim of the present study was to investigate whether cortisol has a direct effect on the development of pituitary and testis or an indirect effect via the reduced androgen secretion. We combined cortisol treatment with a replacement of the testicular steroid hormones testosterone and 11-oxygenated androgens.

**MATERIALS AND METHODS**

**Animals**

Isogenic male common carp (strain E4×R3R8) were produced and raised as described previously [6] at the Department of Fish Culture and Fisheries, Agricultural University, Wageningen, The Netherlands. After transportation at 21 days posthatching (dph) to the fish facilities in Utrecht, the fish were kept under similar conditions and were allowed to acclimate until 61 dph, when the experiment started.

**Steroid Treatment**

Three hundred twenty fish were equally divided into six groups. At 61 dph, 2 days before the onset of cortisol treatment, three control groups designated C (control), COA (control + 11-ketoandrostenedione [OA]), and CT (control + testosterone [T]) and three future cortisol groups designated F (cortisol), FOA (cortisol + OA), and FT (cortisol + T) were implanted with cocoa butter containing no steroid, OA, or T at a dose of 5 mg/kg body weight (dose determined by pilot studies). To produce the implantation material, Malaysia cocoa butter (a gift from Dr. H. Kattenberg, ADM-Cocoa, Koog aan de Zaan, The Netherlands) was melted at a temperature of 37.5°C to solidify and stay solid within the body cavity of the fish, even if the fish were kept at 25°C [7].

Three hundred twenty fish were equally divided into six groups. At 61 dph, 2 days before the onset of cortisol treatment, three control groups designated C (control), COA (control + 11-ketoandrostenedione [OA]), and CT (control + testosterone [T]) and three future cortisol groups designated F (cortisol), FOA (cortisol + OA), and FT (cortisol + T) were implanted with cocoa butter containing no steroid, OA, or T at a dose of 5 mg/kg body weight (dose determined by pilot studies). To produce the implantation material, Malaysia cocoa butter (a gift from Dr. H. Kattenberg, ADM-Cocoa, Koog aan de Zaan, The Netherlands) was melted at a temperature of 37.5°C to solidify and stay solid within the body cavity of the fish, even if the fish were kept at 25°C [7].

The steroids T and OA (5 mg/kg fish) were suspended in molten cocoa butter, and fish were implanted with this mixture by injection of 100 μl/20 g body weight with a 1-ml syringe and a 21-gauge 1.5-inch needle. OA rapidly converts into 11-ketotestosterone (11KT). Pilot studies revealed that steroid levels peaked 1 day postinjection and were back to control levels at 10 days postinjection. Therefore, all groups were reimplanted every 10 days and also 1 day before the onset of sampling at 89 dph to measure the effectiveness of the implantation. At several intervals...
FIG. 1. Plasma levels for 11KT and T, respectively, at 89 dph (A and B), 95 dph (C and D), 100 dph (E and F), and 123 dph (G and H) after implantation with cocoa butter vehicle alone, vehicle plus OA, or vehicle plus T (n = 10). Values sharing the same letter are not significantly different (P < 0.05).

(89, 95, 100, and 123 dph) covering the pubertal development of the common carp, 15 fish/group were sampled.

Cortisol (Steraloids, Newport, RI) in food (100 mg/kg food) was prepared as previously described [8]. Starting at 63 dph, all control groups (C groups) received control food, and the other groups (F groups) received the cortisol-containing food. Fish were fed daily over a 6-h period, starting at 1000 h (four times, at 1.5-h intervals). This treatment induces a significant elevation of plasma cortisol levels over a period of 6 h daily, with peak levels of up to 150 ng/ml [5].

Sampling

 Fifteen fish from each group were sampled at 89, 95, 100, and 123 dph during pubertal development. The fish were anaesthetized in tricaine methane sulfonate (Crescent Research Chemicals, Phoenix, AZ). Body weight was determined, blood was obtained, and the fish were immediately decapitated. Pituitaries were collected individually and immediately transferred to L-15 medium for determination of LH secretion in vitro. Testes were weighed for determination of the gonadosomatic index, GSI = testes weight \times 100/(body weight - testis weight), and then fixed for histological determination of the testicular development.

Testicular Histology

For determination of the spermatogenetic stages, testis tissue from 10 fish from each control and cortisol-treated group was processed for histology. Spermatogenesis was subdivided into four stages according to Cavaco et al. [9]: stage I = spermatogonia only; stage II = spermatogonia and spermatocytes; stage III = spermatogonia, spermatocytes, and spermatids; stage IV = all germ cells including spermatozoa. The number of fish per group with the same stage of testicular development was expressed as a percentage of the total group.
Pituitary Incubations

Pituitaries were collected from 10 fish per group on 95, 100, and 123 dph and were preincubated for 18 h in L-15 medium (15 mM HEPES buffer, pH 7.4, 26 mM sodium bicarbonate, 100 000 U/L penicillin/streptomycin) containing 5% horse serum. Pituitaries were then rinsed once and placed in 0.5 ml fresh L-15 medium. After 3 h, the medium was collected for determination of basal LH secretion. The pituitaries were rinsed again and placed in 0.5 ml of fresh medium containing 10 nM sGnRHa. After 3 h, this medium was collected for determination of the sGnRHa-stimulated LH release. The pituitaries were then snap frozen in liquid nitrogen and stored at \(-80^\circ C\) until processed for LH measurements.

LH Determination in Incubation Medium and Pituitaries

LH was quantified in the incubation medium and the pituitaries using a homologous RIA [10, 11]. Ten pituitaries per treatment group were individually homogenized and assayed. For standards and iodine labeling, purified carp LHβ subunit (a gift from Dr. E. Burzawa-Gérard) was used, with anti-LHβ (internal code 6.3) as the first antibody. In common carp, as in many species, the presence of FSH has been demonstrated [12]. However, an FSH-specific assay is not available.

Plasma T Measurement

The plasma levels of the steroids 11KT, OA, and T were measured in an RIA as described by Schulz [13].

Cloning of a Carp Glucocorticoid Receptor Partial cDNA

Total RNA was isolated from common carp brains by the method of Chirgwin et al. [14]. Oligo dT-primed cDNA was synthesized using Superscript II RNaseH– reverse transcriptase (Life Technologies, Breda, The Netherlands) according to the manufacturer’s instructions. A partial 372-bp base pair (bp) glucocorticoid receptor (GR) cDNA of the carp was amplified by polymerase chain reaction (PCR) using degenerate primers based on GR sequences from the rainbow trout [15], tilapia [16], and Japanese flounder (Tokuda, unpublished results, accession AB013444). The following oligodeoxynucleotide primers were used (Life Technologies): carp GR-Fw, 5’-CTGCAGTGCTCCTGGCTITTYCTIATG-3’ and carp GR-Rv, 5’-GTIAGCTGATAGAAICKCTGCCARTTYTG-3’. The amplified fragment was subcloned into pGEM-T vector (Promega, BE-NELUX, Leiden, The Netherlands) and transformed into Escherichia coli competent cells. The sequence of the clone was checked by nucleotide sequence analysis.

In Situ Hybridization

To investigate the presence of the GR mRNA in the testis, nonradioactive in situ hybridization [17] was performed on sections of testis taken from 95-dph carp. Sections were fixed in 4% paraformaldehyde and 5% acetic acid in PBS. Digoxygenin-dUTP-labeled anti-sense and sense probes were synthesized after linearization of the GR cDNA fragment using PCR on the pGEM-T vector with vector-based primers PBS-A and PBS-E fol-
followed by in vitro transcription with T7- and SP6 RNA polymerase, respectively.

Statistics

All results are expressed as mean ± SEM. Results of cortisol treatment were analyzed using the Student t-test or a one-way ANOVA, followed by the Fisher least significant difference test, as indicated. Differences were considered significant at P < 0.05.

RESULTS

Steroid Treatment

Implantation of cocoa butter containing OA or T resulted in a significant elevation of the plasma levels of 11KT (Fig. 1, A, C, E, and G) or T (Fig. 1, B, D, F, and H), respectively, at all sampling points. A slight, but not significant increase in plasma OA (data not shown) was observed, indicating that the conversion of OA to 11KT is nearly 100%.

Gonadosomatic Index

The increase in GSI, as indicated by the increase in the values on the y-axis in Figure 2, reflects normal testicular development of the control fish (C) during puberty. In contrast, the cortisol-treated fish (F) show an impaired testicular development as indicated by the significantly lower GSI at 95, 100, and 123 dph (Fig. 2, A–D) and the histological analysis of the testis at 95 dph (Fig. 2E). Implantation of OA had no significant effect on the gonadal development in the control group (COA) at all sampling days. However, at 123 dph the inhibitory effect of OA became apparent, both OA-treated groups (COA and FOA) were significantly different from the control group (C) (Fig. 2D). However, this difference in GSI was not significant. The effect of T treatment on testicular development was similar to that observed with cortisol treatment. Implantation with T caused a significant inhibition of testicular development at all days sampled in both the control (CT) and cortisol-treated (FT) group (GSI: Fig. 2, A–D; histological analysis on 95 dph: Fig. 2E).

Pituitary LH Content

Pituitary LH content increased significantly during pubertal development (Fig. 3, A–D). At 89 and 95 dph, no significant difference was observed between control and cortisol-treated fish (Fig. 3, A and B). At 100 dph, there was a slight but nonsignificant difference (Fig. 3C), whereas at 123 dph the LH content of the control fish is significantly higher than that of the cortisol-treated fish (Fig. 3D). Concomitant treatment with OA resulted in somewhat lower pituitary LH levels at 95 and 100 dph (Fig. 3, B and C). However, at 123 dph the inhibitory effect of OA became apparent, both OA-treated groups (COA and FOA) were significantly different from the control group (C) (Fig. 3D). Treatment with T strongly elevated the pituitary LH content in both control and cortisol-treated groups (CT and FT) at all sampled days. At 100 dph, concomitant treatment with cortisol never caused a significant inhibition of the T-induced increase in pituitary LH content (Fig. 3C).

Salmon GnRHa-Stimulated LH Secretion In Vitro

In all groups, the in vitro LH release was significantly stimulated by 10 nM sGnRHa (Fig. 4). Cortisol treatment alone had no effect on both basal and sGnRHa-stimulated LH release at 95 dph (Fig. 4A). At 100 dph, basal secretion was unaffected by the cortisol treatment, but the sGnRHa-stimulated release was significantly decreased (Fig. 4B).
FIG. 4. Basal and sGnRHa-stimulated (10 nM) LH release in vitro after combined cortisol treatment (F) and steroid treatment (OA or T) (n = 10) at 95 dph (A), 100 dph (B), and 123 dph (C). Values sharing the same underscores are not significantly different (P < 0.05).

whereas at 123 dph both basal and sGnRHa-stimulated release were significantly depressed (Fig. 4C). Similar to the pituitary LH content, treatment with OA resulted in a slight reduction of the basal and sGnRHa-stimulated LH release at 95 dph (Fig. 4A). This reduction was significant at 100 and 123 dph, when both OA-treated groups (COA and FOA) were significantly different from the control group (C) (Fig. 4, B and C).

Treatment with T caused a significant increase in the basal and sGnRHa-stimulated LH release in vitro at 95 and 100 dph (Fig. 4, A and B). However, at 123 dph both T-treated groups (CT and FT) were no longer significantly different from the control group (C) (Fig. 4C). Concomitant treatment with cortisol had no significant effect on the basal and sGnRHa-stimulated LH release in vitro, although at 100 dph slightly lower levels were found in the combined cortisol- and T-treated group (FT) compared with the T-treated control group (CT) (Fig. 4B).

Carp Glucocorticoid Receptor

Part of the GR was amplified from common carp brain cDNA using the carp GR-Fw and carp GR-Rv primers, yielding a PCR product of approximately 370 bp. The amplified fragment was subcloned and identified by DNA sequence analysis. The sequence showed highest homology with the rainbow trout GR. The nucleotide sequence and the deduced amino acid sequence are shown in Figure 5.

In Situ Hybridization

The in situ hybridization of testicular tissue of common carp revealed that GR mRNA is present in several types of germ cells. Spermatogonial stem cells and early spermatogonia showed no staining, whereas late spermatogonia showed specific staining for GR. The most intense staining was found in spermatocytes, but no staining was detected in spermatids and spermatozoa. Sections incubated with the cRNA sense probe yielded no signal (Fig. 6).

DISCUSSION

The 11-oxygenated androgens stimulate spermatogenesis in several teleost species, such as the Japanese eel [18], the African catfish [1], and the goldfish [19]. In an earlier study in common carp [4], we observed an inhibitory action of cortisol on pubertal development of the testis that was accompanied by a suppressed testicular androgen secretion.

FIG. 5. Nucleotide sequence of the partial GR cDNA (372 bp) and the deduced amino acid sequence. The depicted sequence is the sequence between the degenerate primers carp Gr-Fw and carp GR-Rv.

FIG. 6. Sections incubated with the cRNA sense probe yielded no signal.
The inhibitory effect of cortisol on spermatogenesis could be direct or could be indirect via the reduced androgen production.

The aim of the present study was to elucidate the role of the androgens 11KT and T in the cortisol-induced suppression of testicular development in the male common carp. We combined cortisol treatment with the replacement of the main androgens 11KT and T by implanting the fish with OA (which is readily converted to 11KT) and T, respectively.

Cortisol treatment caused a retardation of pubertal development, reflected by significantly lower GSIs from 95 dph onwards and less advanced spermatogenetic stages at 95 dph. This retardation was accompanied by lower 11KT and T plasma levels due to a decrease of the total steroid production capacity of the testis [20]. Restoration of the androgen levels in cortisol-treated animals did not result in testicular development similar to that of the control animals.

Cortisol appears to interfere with spermatogenesis at a lower level of the stimulatory cascade than does 11KT. In the endocrine regulation of spermatogenesis, 11KT acts on the Sertoli cells, in which it triggers the production of activin B. Activin B then acts on the spermatogonia to induce mitosis, leading to the formation of spermatocytes [21]. Cortisol may also act on the Sertoli cell, possibly interfering with the production and secretion of activin B. In mammalian testes, the presence of GRs in Sertoli cells has been demonstrated [22, 23], and these cells respond to glucocorticoids [24, 25]. Therefore, the possibility of an effect of cortisol on spermatogenesis via Sertoli cells cannot be excluded. However, in rat testes, the GRs have also been localized on spermatogenetic elements [23, 26]. The labeling of germ cells indicates that a more direct inhibitory effect of glucocorticoids on spermatogenesis may occur. This hypothesis is supported by the observation that in several tissues glucocorticoids have been shown to interfere with cell cycle proteins, thereby inhibiting the cell cycle progression [27–29]. We partially cloned the GR of the common carp to investigate the localization of the GR mRNA in the testis by in situ hybridization. GR mRNA appeared to be present in late spermatogonia and spermatocytes. This finding indicates that in fish, cortisol may act directly on the germ cells, interfering with the cell cycle.
proteins and thereby hampering spermatogenesis. Because of the relatively low resolution of in situ hybridization, it was not possible to determine whether Sertoli cells also express GRs.

Testosterone treatment caused a clear suppression of testicular growth and spermatogenesis. Identical results were obtained by Cavaco et al. [30] in the African catfish. Testosterone inhibited 11KT synthesis by a still unknown mechanism, resulting in an impairment of pubertal testicular development. OA treatment had no effect on the GSI or spermatogenesis, although positive effects have been observed in other species. A possible explanation for this discrepancy may be that the strain of carp we used for the present study displays a very rapid testicular development that cannot be stimulated any further.

Similar to the testicular development, the pituitary gonadotrophs also were affected by the cortisol treatment. Cortisol-treated fish had lower pituitary LH, which was first observed at 100 dph and became pronounced at 123 dph. Furthermore, in the in vitro studies showed that from 100 dph onward the sGnRH-stimulated LH secretory capacity of the pituitaries of cortisol-treated fish was lower compared to that of controls. From 123 dph onward, both basal and sGnRH-stimulated LH secretion were significantly depressed in cortisol-treated fish. These results are comparable to those of our earlier study [5], in which we suggested that cortisol inhibits testicular T secretion and thereby prevents LH storage, leading to a reduced GnRH-inducible LH release in vitro. In other species, T has been shown to induce development of pituitary gonadotropins, leading to an increase in LH content and GnRH-inducible LH release [2, 31]. In the present study, we combined cortisol treatment with the replacement of T, which was able to overcome the cortisol-induced inhibition in gonadotroph maturation. A direct effect of cortisol on pituitary LH content could be ruled out because of an earlier study in which we tested the effect of cortisol on in vitro LH secretion [5].

Increased 11KT levels had an inhibitory effect on pituitary development; this effect was significant at 123 dph. Studies in the African catfish also indicated an inhibitory effect of 11KT on the pituitary LH levels [2, 32] that could be compensated for by additional T treatment [30]. From these studies, we concluded that the inhibitory effect of 11KT on pituitary LH levels was indirect via a decreased secretion of T.

Cortisol has an inhibitory effect spermatogenesis, and this effect is independent of 11KT. The effect of cortisol on the hypophysial LH secretion, however, is caused by an indirect effect, involving the testicular secretion of T.

REFERENCES