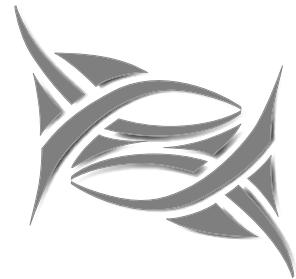


**Sex steroids and their
involvement in the cortisol-
induced inhibition of pubertal
development in male common
carp, *Cyprinus carpio* L**

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in preparation



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, exert an important function in the onset of puberty. Stress has been shown to interfere 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. Previous work showed that prolonged elevated cortisol levels, implicated in the stress adaptation, inhibit pubertal development in male common carp. Cortisol treatment caused a retardation of pubertal testis development and reduced the luteinizing hormone (LH) content and the salmon GnRH α -stimulated LH secretion *in vitro*. Furthermore, a reduced synthesis of androgens was observed. This suggests that the cortisol-induced inhibition of the testicular development, as well as the maturation of pituitary gonadotrophs is 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, in order to investigate the role of these steroids in the cortisol-induced suppression of pubertal development. Our results indicate that the effect of cortisol on spermatogenesis is independent of 11KT, whereas the effect on the pituitary is an indirect one, involving the testicular secretion of testosterone.

Introduction

In juvenile fish, the importance of sex steroids in the onset of puberty has been shown in several studies. Sex steroids have been demonstrated to stimulate the development of all levels of the BPG axis. In the African catfish, *Clarias gariepinus*, treatment with 11-oxygenated androgens stimulated testicular growth and spermatogenesis as well as the development of secondary sexual characteristics (Cavaco *et al.*, 1998b). Stimulation of spermatogenesis by 11-oxygenated androgens has also been observed in pre-pubertal common carp, *Cyprinus carpio* L. (Komen, personal communication).

Treatment with testosterone activated the gonadotroph maturation (Cavaco *et al.*, 1995) and accelerated the development of the hypothalamic GnRH system (Dubois *et al.*, 1998).

The adaptation to severe and chronic stress has been shown to interfere with processes such as growth, immune response or reproduction. Cortisol has frequently been indicated as a major factor mediating the suppressive effect of stress on reproduction. Our previous work showed that prolonged cortisol treatment inhibits pubertal development in male common carp (Consten *et al.*, 2001a). Elevated cortisol levels resulted in an impairment of spermatogenesis, as well as a reduction of the synthesis of the 11-oxygenated androgens. We showed that these effects were not mediated by an effect of cortisol on LH secretion (Consten *et al.*, 2001b). However, cortisol does affect the LH secretion at the level of the pituitary as we observed a smaller LH releasable pool and a reduced salmon GnRH α -stimulated LH secretion *in vitro*. Testosterone has been shown to induce development of pituitary gonadotrophs, leading to an increase in LH content and GnRH-inducible LH release maturation (Cavaco *et al.*, 1995). 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 not only had its effects on the maturation of pituitary gonadotrophs, but also on the testicular development (Consten *et al.*, 2001a, 2001b).

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. Therefore we combined cortisol treatment with a replacement of the testicular steroid hormones, testosterone and 11-oxygenated androgens.

Material and Methods

Animals

Isogenic male common carp, designated as strain E4xR3R8, were produced and raised as described by Tanck *et al.* (2000) at the Department of Fish Culture and Fisheries, Agricultural University, Wageningen, The Netherlands. After transportation at 21 days post hatching (dph) to the fish facilities in Utrecht, the fish were kept under similar conditions and were allowed to acclimatize till 61 dph when the experiment started.

Steroid treatment

Three hundred and twenty animals were equally divided over six groups. At 61 dph, two days before the onset of cortisol treatment, three control groups (designated C (control), CK (control + OA) and CT (control + T)) and three

future cortisol groups (designated F (cortisol), FK (cortisol + OA), FT (cortisol + T)) were implanted with cocoa butter containing either no steroid, 11-ketoandrostenedione (OA) or testosterone (T) at a dose of 5 mg/kg bodyweight (dose determined by pilot studies). For this, Malaysia cocoa butter (a gift from drs. H. Kattenberg, ADM-Cocoa, Koog aan de Zaan, The Netherlands) was melted at a temperature not higher than 37.5°C. So it solidified within the body-cavity of the fish, even if the fish are kept at 25°C (van Malssen *et al.* 1996). The steroids, T and OA (5 mg/kg fish) were suspended in molten cocoa butter and fish were implanted with the cocoa butter by injecting 100 µl per 20 g body weight with a 1 ml syringe (needle: 21Gx1¹/₂"). OA has been shown to be rapidly converted into 11-ketotestosterone (11KT). Pilot studies revealed that steroid levels peaked one day post-injection and were back to control levels ten days post-injection. Therefore all groups were re-implanted every ten days and also one day before the onset of sampling at 89 dph, in order to measure the effectiveness of the implantation. At several time intervals, covering the pubertal development of the common carp, 15 fish per group were sampled.

Cortisol (Steraloids Inc. Wilton, USA) containing food (100 mg/kg food) was prepared as described by Pickering *et al.* (1987b). Starting at 63 dph, all control groups received control food (C), the other groups the cortisol-containing food (F). Fish were fed daily over a 6 hours period, starting at 10:00 am (4 times, with intervals of 1.5 hours). This treatment induced an elevation of plasma cortisol levels up to 150 ng/ml over a period of 6 hours daily (Consten *et al.*, 2001a).

Sampling

Fish from all groups (n=15) were sampled at several time-intervals during the pubertal development at 89, 95, 100 and 120 dph. The fish were anaesthetized in TMS (Tricaine Methane Sulfonate, Crescent Research Chemicals, Phoenix AZ, USA). Body weight was determined. After blood sampling, the fish were immediately decapitated. Pituitaries were collected individually and immediately transferred to L-15 medium for determining the LH secretion *in vitro*. Testes were taken for determining the gonadosomatic index (GSI = testes weight * 100 / (bodyweight-testis weight)) and fixed for histological determination of the testicular development.

Testicular histology

For determination of the spermatogenetic stages, testis tissue of 10 fish per control and cortisol treated group, respectively, was processed for histology.

Spermatogenesis was subdivided into four stages according to Cavaco *et al.* (1997). In short: stage I - spermatogonia only; stage II - spermatogonia and spermatocytes; stage III - spermatogonia, spermatocytes and spermatids; and finally stage IV - all germ cells including spermatozoa. The number of animals

per group with the same stage of testicular development are counted and expressed as a percentage of the total group.

Pituitary incubations

Ten pituitaries per group were collected individually and pre-incubated for 18 h in L-15 medium (15mM HEPES buffered, pH 7.4, 26mM sodium bicarbonate, 100,000 U/l penicillin/streptomycin) containing 5% horse serum. The pituitaries were rinsed once and 0.5 ml fresh L-15 medium was added and the incubation was continued for 3 h, after which the medium was collected for determination of the basal LH secretion. The pituitaries were rinsed once more and 0.5 ml of fresh containing 10 nM salmon GnRHa was added for another 3 h incubation. Thereafter, the medium was collected for determination of the sGnRHa-stimulated LH release. The pituitaries were collected, snap frozen in liquid nitrogen and stored at -80°C LH measurements.

Incubation medium and pituitary LH determination

LH was quantified in the incubation medium and the pituitaries using a homologous radioimmunoassay (RIA) (slightly modified from Goos *et al.*, 1986). Ten pituitaries per treatment group were individually homogenized and assayed. For standards and iodine labeling, purified carp LH β subunit (a gift from Dr. E. Burzawa-Gérard) was used and anti-LH β (internal code #6.3) as first antibody. In common carp, as in many species, the presence of a follicle-stimulating hormone (FSH) has been demonstrated. However, a FSH specific assay is not available.

Plasma testosterone measurement

The plasma levels of the steroids 11-ketotestosterone (11KT), 11-ketoandrostenedione (OA) and testosterone (T) were measured in a RIA as described by Schulz (1985).

Cloning of a carp glucocorticoid receptor partial cDNA

Total RNA was isolated from common carp brains by the method of Chirgwin *et al.* (1979). 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 glucocorticoid receptor (GR) cDNA of the carp was PCR amplified using degenerate primers based on the rainbow trout (Ducouret *et al.*, 1995), tilapia (Tagawa *et al.*, 1997) and Japanese flounder (Tokuda, unpublished results, acc.no. AB013444) GR sequences. The following oligodeoxynucleotide primers were used (Life Technologies, Breda, The Netherlands): carp GR-Fw, 5'-CTGCAGT-GCTCCTGGCTITTYCTIATG-3' and carp GR-Rv, 5'-GTIAGCTGATAGAAICKCTGCCARTTYTG-3'. The amplified fragment was subcloned into

pGEM-T vector (Promega) and transformed into *Escherichia coli* competent cells. The sequence of the clone was checked by nucleotide sequence analysis.

In situ hybridization

In order to investigate the presence of the GR mRNA in the testis, a non-radioactive *in situ* hybridization, as described by Braat *et al.* (1999), was performed on sections of testis taken from 95 dph old carp, fixed in 4% paraformaldehyde, 5% acetic acid in phosphate buffered saline (PBS). Digoxigenin-rUTP-labeled anti-sense and sense probes were synthesized after linearization of the GR cDNA fragment by performing PCR on the pGEM-T vector with vector-based primers PBS-A and PBS-E, followed by *in vitro* transcription with T7- and SP6 RNA polymerase, respectively.

Statistics

All results are expressed as mean \pm SEM. Results on the effect of cortisol were processed for statistical analysis by Student's T-test ($p < 0.05$) or by one-way ANOVA, followed by Fisher's least significant difference test ($p < 0.05$), as indicated in the legends.

Results

Steroid treatment

Implantation of cocoa butter containing OA leads to a significant elevation of the plasma levels for 11KT at 89 dph, one day post-implantation (Fig. 1A). A slight, not significant increase in plasma OA (data not shown) was observed indicating that the conversion of OA to 11KT is nearly 100%. Implantation of T also leads to elevated plasma T levels at 89 dph (Fig. 1B).

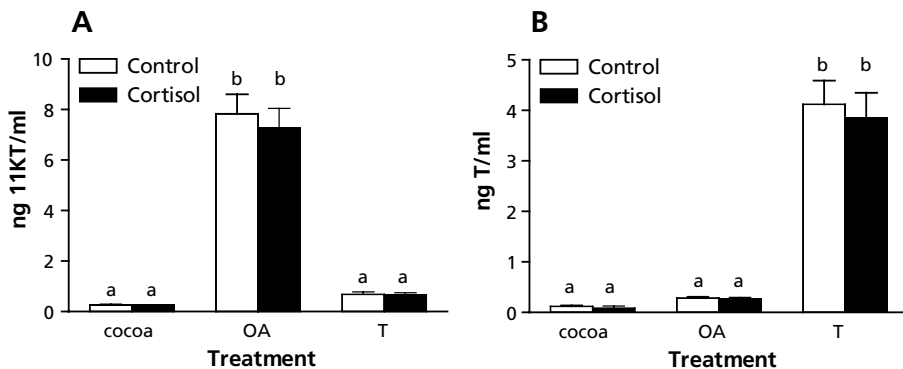


Figure 1. Plasma levels for (A) 11KT and (B) T one day after implantation (89 dph) with cocoa butter alone, OA or T, respectively (n=10). Data sharing the same letter are not significantly different ($p < 0.05$).

Gonadosomatic index (GSI)

The increase in GSI, as indicated by the increase in the values on the Y-axis, reflects normal testicular development of the control fish (C) during puberty (Fig. 2). In contrast, the cortisol treated fish (F) show an impaired testicular development as follows from the significantly lower GSI at 95, 100 and 120 dph 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 fed animals (CK) at all days. Cortisol treated animals, implanted with OA (FK), also showed an impaired spermatogenesis, similar to the cortisol treated fish (F). However, only at 120 dph, concomitant cortisol and OA treatment leads to an increase in GSI compared to cortisol treatment alone. Due to the somewhat higher variation in this group, the difference, however, is not statistically significant

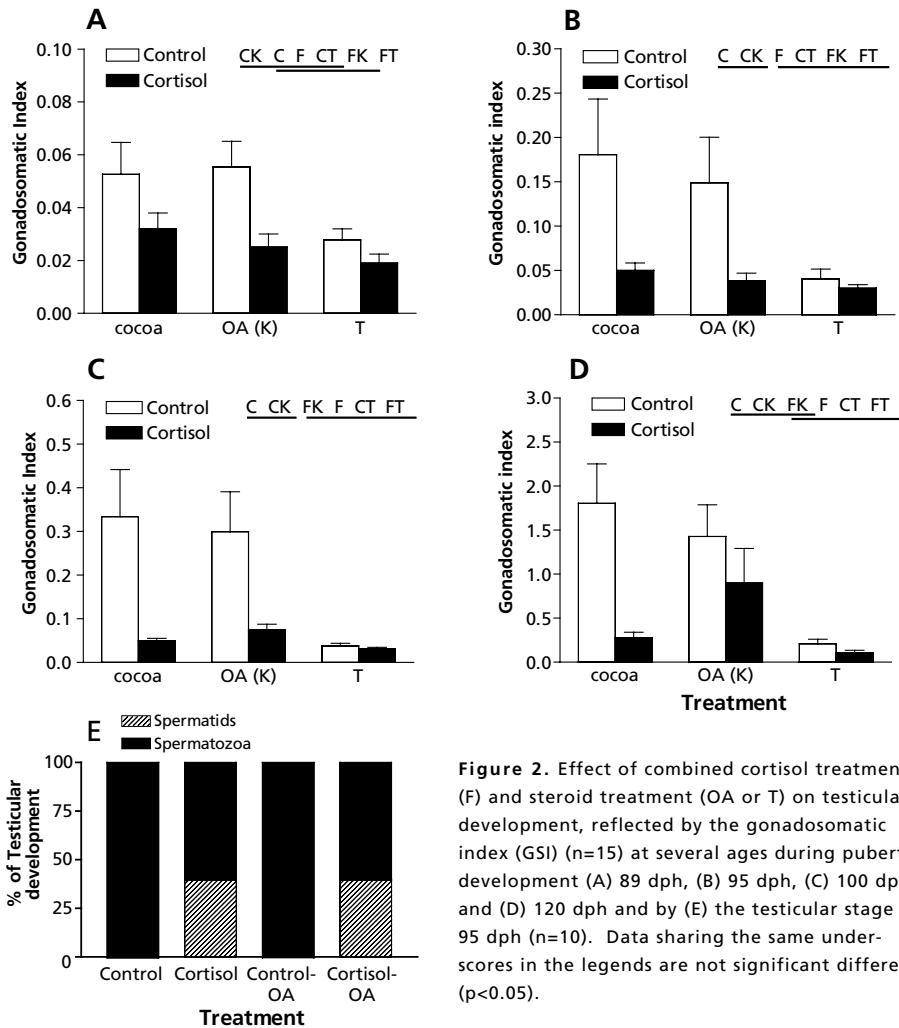


Figure 2. Effect of combined cortisol treatment (F) and steroid treatment (OA or T) on testicular development, reflected by the gonadosomatic index (GSI) (n=15) at several ages during pubertal development (A) 89 dph, (B) 95 dph, (C) 100 dph and (D) 120 dph and by (E) the testicular stage at 95 dph (n=10). Data sharing the same under-scores in the legends are not significant different (p<0.05).

(Fig. 2D). Treatment with T had a similar effect on testicular development as cortisol treatment. At all days sampled both the control (CT) and cortisol (FT) treated group, implantation with T caused a significant inhibition of the testicular development (Fig. 2).

Pituitary content

Pituitary LH content increased significantly during pubertal development (Fig. 3). At 89 and 95 dph no significant difference was observed between control and cortisol treated fish (Fig. 3A, B). However, at 100 dph there is a slight, not significant, difference (Fig. 3C), whereas at 120 dph the LH content of the control fish is significantly higher than in the cortisol treated fish (Fig. 3D). Concomitant treatment with OA resulted in somewhat lower pituitary LH levels at 95 and 100 dph (Fig. 3B, C). However, at 120 dph the inhibitory effect of OA becomes apparent, both OA-treated groups (CK and FK) are significant-

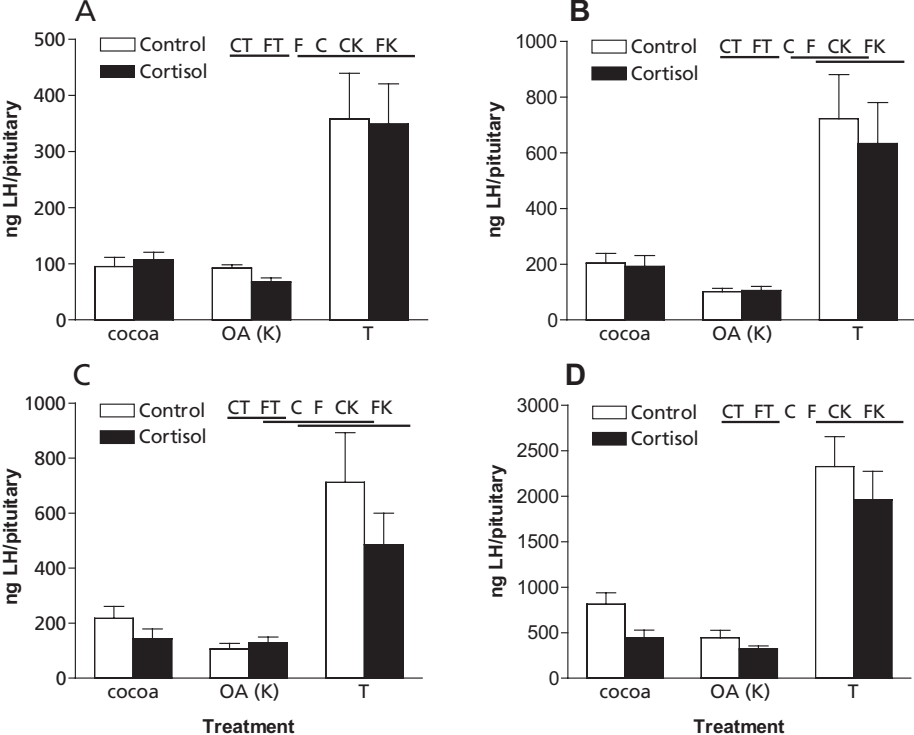


Figure 3. Effect of combined cortisol treatment (F) and steroid treatment (OA or T) on pituitary LH content (n=10) at several ages during pubertal development (A) 89 dph, (B) 95 dph, (C) 100 dph and (D) 120 dph. Data sharing the same underscores in the legends are not significant different (pK0.05).

ly 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 caused to some extent an inhibition of the T-induced increase in the pituitary LH content (Fig. 3C).

Salmon GnRH α -stimulated LH secretion *in vitro*

In all groups the *in vitro* LH release was significantly stimulated by 10 nM sGnRH α (Fig. 4). Cortisol treatment alone had no effect on both basal and sGnRH α -stimulated LH release at 94dph (Fig. 4A). At 100 dph basal secretion was unaffected by the cortisol treatment, but the sGnRH α -stimulated release was significantly decreased (Fig. 4B), whereas at 120 dph both basal and sGnRH α -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 sGnRH α -stimulated LH release at 95 dph (Fig. 4A). This reduction is significant at 100 and 120 dph; both OA-treated groups (CK and FK) are significantly different from the control group (C) (Fig. 4B, C).

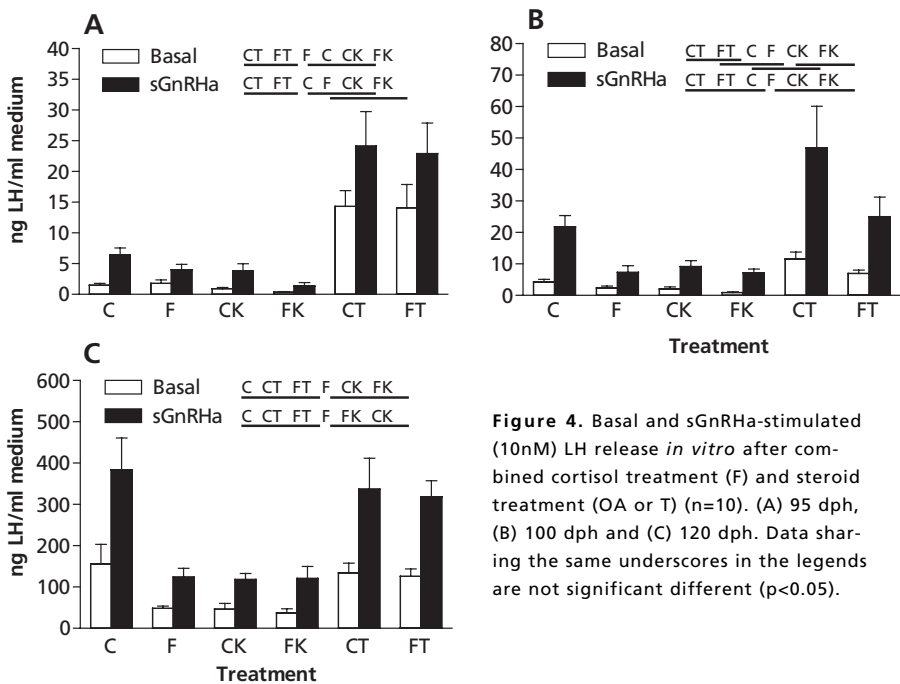


Figure 4. Basal and sGnRH α -stimulated (10nM) LH release *in vitro* after combined cortisol treatment (OA or T) (n=10). (A) 95 dph, (B) 100 dph and (C) 120 dph. Data sharing the same underscores in the legends are not significant different ($p < 0.05$).

Treatment with T caused a significant increase in the basal as well as the sGnRH α stimulated LH release *in vitro* at 95 and 100 dph (Fig. 4A, B). At 120 dph both T treated groups (CT and FT) are, however, no longer significantly different from the control group (C) (Fig. 4C). Concomitant treatment with cor-

tisol has no significant effect on the basal and sGnRHa-stimulated LH release *in vitro*, although at 100 dph slightly lower levels are found in the combined cortisol and T treated group (FT) compared to the T treated control group (CT) (Fig. 4B)

Carp glucocorticoid receptor

Part of the glucocorticoid receptor was amplified from common carp brain cDNA, using the carp GR-Fw and carp GR-Rv primers. This yielded 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.

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          S   F   G   L   G   W   R   S   Y   Q   Q   C   N   G
Carp GR-Fw- TCC'TTC'GGC'TTG'GGC'TGG'AGA'TCC'TAC'CAG'CAG'TGT'AAC'GGA'
          G   M   L   C   F   A   P   D   L   V   I   N   E   E   R   M   K
GGC'ATG'CTG'TGT'TTC'GCT'CCA'GAC'CTG'GTC'ATC'AAT'GAG'GAG'AGG'ATG'AAA'
          L   P   Y   M   N   D   Q   C   S   Q   M   L   K   I   T   S   E
CTA'CCC'TAC'ATG'AAT'GAC'CAG'TGT'AGC'CAG'ATG'CTG'AAG'ATC'ACC'AGT'GAC'
          L   V   R   L   Q   V   S   Y   D   E   Y   L   C   M   K   V   L
CTG'GTC'AGG'CTG'CAG'GTG'TCC'TAC'GAC'GAG'TAT'CTC'TGC'ATG'AAA'GTC'CTC'
          P   L   L   S   T   V   P   K   D   G   L   K   S   Q   A   V   F
CCG'CTC'CTC'AGC'ACA'GTA'CCA'AAG'GAC'GGC'CTT'AAA'AGC'CAA'GCT'GTG'TTT'
          D   E   I   R   M   S   Y   I   K   E   L   G   K   A   V   V   K
GAT'GAA'ATC'CGC'ATG'TCG'TAC'ATC'AAG'GAG'CTG'GGC'AAA'GCC'GTT'GTT'AAA'
          R   E   E   N   S   S
CGA'GAG'GAG'AAC'TCC'AGC'-carp GR-Rv

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Figure 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.

In situ hybridization

The *in situ* hybridization on testicular tissue of common carp revealed that the glucocorticoid receptor mRNA is present in several types of germ cells. Spermatogonial stem cells and early spermatogonia showed no staining, whereas late spermatogonia show specific staining for the GR. The most intense staining is found in spermatocytes. In spermatids and spermatozoa the messenger for the GR was not detected. Sections incubated with the cRNA sense probe yielded no signal (Fig. 6).

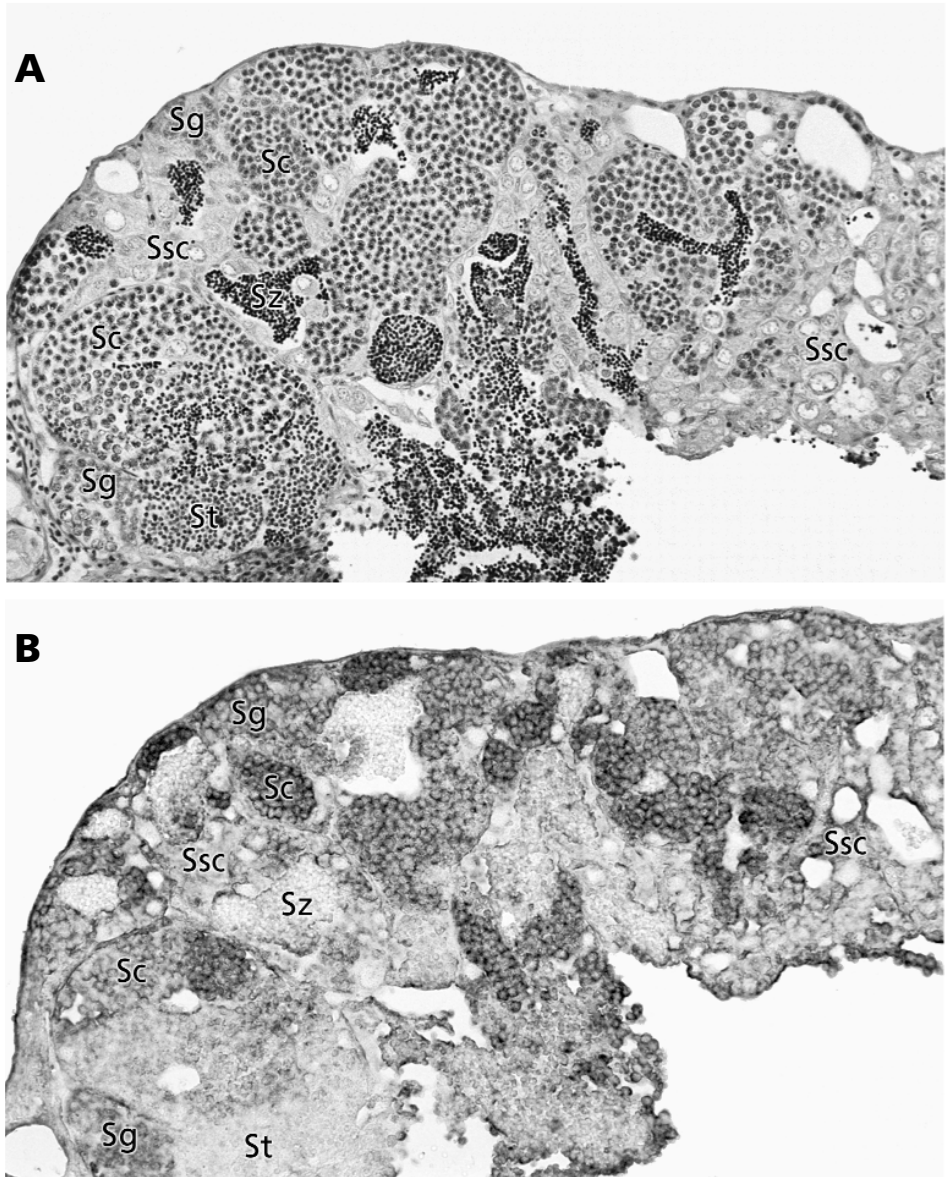


Figure 6. *In situ* hybridization of common carp testis with the GR cRNA probe. (A) Haemalun eosin staining; (B) *in situ* hybridization. Staining is found in late spermatogonia (Sg) and spermatocytes (Sc), whereas spermatogonial stem cells (Ssc), spermatids (St) and spermatozoa (Sz) show no signal (magn: 240x).

Discussion

In an earlier study (Consten *et al.*, 2001a) we observed that the inhibitory action of cortisol on pubertal development of the testis in common carp was accompanied by a suppressed testicular androgen secretion. 11-Oxygenated androgens have been shown to stimulate spermatogenesis in the Japanese eel, *Anguilla japonica* (Miura *et al.*, 1991), in the African catfish (Cavaco *et al.*, 1998b) and in the common carp (Komen, personal communication). Also in the goldfish, *Carassius auratus*, a close relative of the common carp, 11KT has been shown to induce spermatogenesis (Kobayashi *et al.*, 1991). The inhibitory effect of cortisol on spermatogenesis could be a direct one, or its effect may be 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. Therefore, in this study we combined cortisol treatment with the replacement of 11KT, by implanting the fish with OA, which is readily converted to 11KT.

In this study, cortisol treatment caused again a retardation of pubertal development, reflected by significantly lower GSIs from 95 dph onwards and the less advanced spermatogenetic stages at 95 dph. However, restoration of the 11KT levels in cortisol treated animals did not result in a testicular development similar to the control animals. Testosterone treatment caused a clear suppression of the testicular growth.

These results indicate that, even when the 11KT levels are restored in the cortisol treated fish, the inhibitory effect of cortisol on testicular development cannot be prevented. Thus, cortisol interferes with spermatogenesis at a lower level of the stimulatory cascade than 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 (Nagahama, 1994). Possibly, cortisol acts on the Sertoli cell, interfering with the production and secretion of activin B. In mammalian testes, the presence of glucocorticoid receptors (GRs) in Sertoli cells has been demonstrated (Levy *et al.*, 1989, Weber *et al.*, 2000) and these cells respond to glucocorticoids (Jenkins *et al.*, 1986; Lim *et al.*, 1996). Therefore, an effect of cortisol on spermatogenesis via Sertoli cells can not be excluded. However, in rat testes, the GRs have also been shown to be localized on spermatogenetic elements (Schultz *et al.*, 1993; Weber *et al.*, 2000). The labeling of germ cells indicates that a more direct inhibitory effect of glucocorticoids on spermatogenesis may occur. This 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 (Rogatsky *et al.*, 1997; Samuelsson

et al., 1999; Smith *et al.*, 2000). We partially cloned the GR of the common carp to investigate the localization of the GR mRNA in the testis by means of *in situ* hybridization. GR mRNA appeared to be present in late spermatogonia and spermatocytes. This indicates that also in fish, cortisol may act directly on the germ cells, interfering with the cell cycle proteins and thereby hampering spermatogenesis.

Similar to the testicular development, also the pituitary gonadotrophs were affected by the cortisol treatment. Cortisol treated fish had lower pituitary LH contents, which was first observed at 100 dph and became pronounced at 120 dph. Furthermore, the *in vitro* studies showed that from 100 dph on the sGnRHa-stimulated LH secretory capacity of the pituitaries of cortisol treated fish was lower compared to controls. From 120 dph on, both basal and sGnRHa-stimulated LH secretion are significantly depressed in cortisol treated fish. These results are comparable to our earlier study (Consten *et al.*, 2001b), in which we suggested that cortisol inhibits the testicular testosterone secretion and thereby prevents LH storage, leading to a reduced GnRH-inducible LH release *in vitro*. Also in other species testosterone has been shown to induce development of pituitary gonadotrophs, leading to an increase in LH content and GnRH-inducible LH release (Cavaco *et al.*, 1995, 1998d). In the present study, we combined cortisol treatment with the replacement of T. T replacement was able to overcome the cortisol-induced inhibition in gonadotroph maturation. In contrast, 11KT treatment had an inhibitory effect on the pituitary development, which is significant on 120 dph. Studies in the African catfish, also indicate an inhibitory effect of 11KT on the pituitary LH levels (Cavaco *et al.*, 1995; Rebers *et al.*, 2000).

In conclusion, we show that cortisol has a direct inhibitory effect spermatogenesis, which is independent of 11KT. The effect of cortisol on the hypophysial LH secretion is, however, is caused by an indirect effect, involving the testicular secretion of testosterone.