Chapter 3

Long-term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L.

Co-authors: Jan Bogerd, Hans Komen, Jan G.D. Lambert, Henk J.Th. Goos

Biology of Reproduction 2001, 64(4): 1063-1071

Abstract

The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis. Stress has been shown to interfere with reproduction and the functioning of the BPG-axis. The response to chronic and severe stress may require much energy and force the organism to make adaptive choices. Energy that is normally available for processes like growth, immune response or reproduction will be channeled into restoration of the disturbed homeostasis. Cortisol plays a key role in the homeostatic adaptation during or after stress. In the present study, immature common carp were fed with cortisol containing food pellets covering the pubertal period. We showed that cortisol caused an inhibition of pubertal development, by affecting directly or indirectly all components of the brain-pituitary-gonad axis. The sGnRH content of the brain was decreased. LH and FSH encoding mRNA levels in the pituitary and LH plasma levels were diminished by long-term cortisol treatment, as was the testicular androgen secretion. Testicular development, reflected by gonadosomatic index and the first wave of spermatogenesis, was retarded.

Introduction

Adaptation to changing environmental conditions is essential for maintenance of physiological homeostasis. Stress can be defined as a disturbance of homeostasis by any kind of external or internal factor, referred to as the stressor. The consequence of the action of a stressor is that the homeostatic equilibrium is threatened. This will induce a coordinated set of behavioral and physiological responses that are compensatory and/or adaptive, enabling the organism to restore its homeostatic set points (Wendelaar Bonga, 1997). These adjustment reactions have been identified as the neuro-endocrine stress response (Selye, 1936). However, the response to prolonged stress may exceed the adaptive capacity. Energy, normally available for processes like growth, immune response or reproduction may then be channeled into restoration of the disturbed

homeostasis. This may result in maladaptation with adverse effects on reproduction, immune competence or growth.

Indeed, in several vertebrates, stress has been shown to interfere with reproduction and the functioning of the brain-pituitary-gonad (BPG) axis. In mammals, all levels of the BPG-axis are affected (Rivier & Rivest, 1991). For example, a decrease in plasma LH and hypothalamic LHRH in male rats after chronic restraint stress has been shown (López-Calderón *et al*., 1990) and similar results were found in rams and ewes (Tilbrook *et al*., 1999). Furthermore, adult rats submitted to immobilization stress from prepuberty showed decreased plasma LH and plasma testosterone (T) levels (Almeida *et al*., 1998), whereas Charpenet *et al*. (1981) demonstrated that chronic intermittent immobilization stress induced a strong decrease of plasma T levels and testicular T content in rats, without, however, detectable changes in plasma LH values. The precise mechanisms via which the stress response has its adverse effects on reproduction are still unknown.

Reports on the effects of stress on the reproductive capacity in fish are inconsistent since stimulatory as well as inhibitory effects of stress, or no effects at all, have been described. (reviewed by Wendelaar Bonga, 1997). In male brown trout, *Salmo trutta* L., acute and chronic stress suppressed the plasma levels of 11-ketotestosterone (11KT). However, plasma gonadotropin levels were elevated following 1 hour of handling stress (Pickering *et al*., 1987a). The variability of these results may depend on the nature and duration of the stressor, and the animal model that was used.

In all vertebrates, including fish, cortisol plays a key role in the restoration of homeostasis during or after stress. Furthermore, cortisol has frequently been indicated as the major factor mediating the suppressive effect of stress on reproduction. Carragher *et al*. (1989) showed that implantation of cortisol releasing pellets in the brown trout, *Salmo trutta* L. and in the rainbow trout, *Salmo gairdneri* Richardson, chronically elevated the plasma cortisol levels and affected a wide range of reproductive parameters. Cortisol-implanted maturing male brown trout had smaller gonads, lower plasma testosterone levels and their pituitaries had lower gonadotropin content.

The onset and regulation of puberty depends on the functional development of the BPG-axis. Several definitions for puberty exist, but for the purpose of this study, puberty will be considered as the period that spans the onset of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. Previous results have shown that in common carp (*Cyprinus carpio* L.) repeated temperature stress caused elevated plasma cortisol levels (Tanck *et al*., 2000) and a retardation of the first waves of spermatogenesis (chapter 2).

In the present experiments, stress-induced cortisol levels were mimicked in order to investigate (1) whether the effects of the temperature stress are due to elevated cortisol levels and, if so, (2) on which level the BPG-axis is affected by cortisol.

Temperature stress-induced cortisol levels (Tanck *et al.*, 2000) were mimicked by feeding the experimental animals with cortisol containing food pellets. As experimental animals we used isogenic male common carp, with highly uniform and predictable testicular development, with meiosis of spermatogonia starting around 90 days post hatching (dph) (Bongers *et al.*, 1999).

Material and Methods

Animals

Isogenic male common carp (*Cyprinus carpio* L., designated as strain E4xR3R8) were produced by crossing a homozygous gynogenetic E4 female (Komen *et al.*, 1991) with a YY-male of an unrelated homozygous androgenetic male R3R8 (Bongers *et al.*, 1997). Fry were produced and raised in the facilities of the Department for Fish Culture and Fisheries (Agricultural University, Wageningen, The Netherlands) and transported at 21 days post hatching (dph) to our department at the Utrecht University.

During the experiment, the fish were kept at 25°C in a flow-through system, exposed to a 12:12 hours light-dark regime and fed pelleted dry food daily (Provimi, 91 series, Rotterdam, The Netherlands) at a daily ration of 20 $g/kg^{-0.8}$. Fish were allowed to acclimatize till 63 dph after which the experiment started.

Experiment 1: Short-term steroid treatment

Cortisol (Steraloids Inc. Wilton, USA) containing food (100 mg/kg food) was prepared as described by Pickering *et al.* (1987b). In order to determine how to mimic the cortisol profile induced by temperature stress (Tanck *et al.*, 2000), 320 animals were equally divided over four groups. The first two groups received during one week, once daily, either control food or cortisol-treated food. In the same period the other two groups received either control food or cortisol-treated food daily over a 6 hours period, starting at 10:00 am (4 times, with intervals of 1.5 hours). Feeding the fish with cortisol containing pellets according to the latter regime mimicked the cortisol levels that were induced by the temperature stress (see Results). On the last day of the treatment, blood samples were taken for cortisol measurement by radioimmunoassay (RIA).

Experiment 2: Long-term steroid treatment

Two hundred animals, 63 dph, were divided over two groups. Group 1 served as controls and was fed with control food, while group 2 received four times daily the cortisol-containing food.

At the onset of the experiment, 63 dph, a start control group was sampled. At 90, 95, 101 and 106 dph, covering the pubertal development of this strain of common carp, 20 fish per group were sampled. The fish were caught and anaesthetized within one minute in TMS (Tricaine Methane Sulfonate, Crescent Research Chemicals, Phoenix AZ, USA). As shown by Weyts *et al*. (1997), a cortisol stress response due to handling is avoided in this procedure.

Body weight was determined and blood was collected by puncturing the caudal vasculature, using 1 ml syringes (needle: 26Gx½") rinsed with a solution of 7% sodium EDTA (pH 7.2). Plasma samples were stored at -20°C until use. After blood sampling, fish were immediately decapitated. Brains and pituitaries were collected, snap frozen in liquid nitrogen and stored at -80°C until use for hormone measurements, by means of RIA, or for mRNA quantification by RNase protection analysis (RPA). Testes were taken, weighed 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 spermatides; 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.

Steroid Radioimmunoassays (RIA)

Plasma levels of cortisol were determined by a RIA according to de Man *et al*. (1980) and Van Dijk *et al*. (1993). The plasma levels of the steroids 11KT, OA and T were measured in a RIA as described by Schulz (1985). In most male teleosts, 11KT is considered to be the most dominant androgen in the plasma (Borg *et al*., 1994). Also in the male common carp 11KT has been found to be the major androgen produced by the testes (Barry *et al*., 1990, Koldras *et al*., 1990). However, in immature common carp 11-ketoandrostenedione (OA) is the main androgen (Komen, personal communication). Testosterone was included since Cavaco *et al*. (1995) showed that this androgen is essential for gonadotroph development during puberty.

Plasma and pituitary LH

Luteinizing Hormone (LH) was quantified in the plasma and the pituitaries using a homologous RIA (slightly modified from Goos *et al*., 1986). Purified carp LHβ subunit (a gift from Dr. E. Burzawa-Gérard) was used for the

preparation of standards and for ^{125}I-labeling. Anti-LHβ (internal code #6.3) was used as a first antibody.

Ten pituitaries per treatment group were individually homogenized and assayed. Plasma LH levels were measured in all animals. In common carp, as in many species, the presence of a follicle-stimulating hormone (FSH) has also been demonstrated (Van Der Kraak *et al.*, 1992). However, a FSH specific assay is not available.

sGnRH content in the brain

Salmon GnRH, which is the native hypothalamic form for carp, was measured by RIA using a sGnRH specific antibody and iodinated sGnRH (Schulz *et al.*, 1993, Goos *et al.*, 1997). Ten brains per treatment group were individually homogenized in 2N acetic acid, heated at 90°C. for 10 minutes, snap frozen and sonicated. The homogenates were centrifuged, 3500 g at 4°C for 30 min. The supernatants were collected. The pellets were resuspended in 2N acetic and centrifuged. The second supernatants were added to the previous ones and stored at -70°C. Before assaying, the samples were lyophilized. The residues were reconstituted to a smaller volume with 2N acetic acid, sonicated and centrifuged at 3500 g and 4°C for 30 min. The supernatants were neutralized with 5N NaOH, centrifuged at 3500 g and 4°C for 5 min. and further diluted with the assay buffer.

RNase Protection Analysis (RPA)

To quantify the steady-state messenger RNA levels for glycoprotein hormone α-subunit (GPα), LHβ and FSHβ subunits, 10 pituitaries per treatment group were used for RPA, based on the method described by Rebers *et al.* (1997). However, the homogenization was performed in 50 µl lysis buffer to account for the low amounts of mRNA present in the pituitaries of immature common carp. For the quantification of the GPα, LHβ and FSHβ subunit mRNA levels, 45 µl of this homogenate was used. For the quantification of the 28S rRNA (internal standard) levels, 42.5 µl lysis buffer was added to 2.5 µl of the remaining homogenate.

The following oligodeoxynucleotide primers were used (Life Technologies, Breda, The Netherlands): GPα Fw, 5'-GAGGTCCAAGAAAACCATGCT-3'; GPα Rv, 5'-TTTAACTG*TAATACGACTCACTATA*GGGCAAAATCCGTAACACAAGCAAATCTTGAATGTC-3' (based on Huang *et al.*, 1992); LHβ Fw, 5'-TCCGACTGTACGATTGAAAGCC-3'; LHβ Rv, 5'-TTTAACTG*TAATACGACTCACTATA*GGGGTTGATATACTCTTCAGCTCAATATCCACGCC-3' (based on Chang *et al.*, 1992); FSHβ Fw, 5'-GGTCGACAGCGCTCACCAATATCTCCATTACCG-3'; FSHβ Rv, 5'-TTTACCTG*TAATACGACTCACTATA*GGGCCAAGAACGTGCATGTTATATTTATTGATGCTTGCA-3' (based on Kobayashi, unpublished, DDBJ

accession nr. AB003583); 28S rRNA Fw, 5'-CCATGCCTGGGTGAAAG CGGGGCCTCACGATCCT-3'; 28S rRNA Rv, 5'-GGTACC*TGTAATACGA CTCACTATA*GGGCCAGATTTGCCAGCTCACGTTCCCTATTAGTGG GT-3' (based on conserved sequences found in other 28S rRNA sequences). In all primers, the sequences in italics represent the T7 RNA polymerase promoter sequence used for cRNA probe synthesis (Rebers *et al.*, 1997). The underlined sequences are unable to hybridize to the mRNA to be detected and yield the difference in length between the cRNA probe and the protected fragment in the assay.

Statistics

All results are expressed as mean ± SEM. Plasma levels of the different steroids are given as ng per ml plasma. LH levels are given as ng per ml plasma or as ng per pituitary. sGnRH content is expressed in pg/brain. Messenger RNA levels for the GPα, LHβ and FSHβ subunits are corrected for 28S ribosomal RNA levels and expressed as percentage of the control. All results on the treatment effect of cortisol were processed for statistical analysis by Student's T-test ($p<0.05$). Differences between time-intervals were processed by one-way ANOVA, followed by Fisher's least significant difference test ($p<0.05$).

Results

Plasma cortisol

Plasma cortisol levels during and after a single or a 4 times daily cortisol food application are depicted in figure 1A and 1B, respectively. At the onset of the single treatment, plasma cortisol levels of the control group are elevated but decrease to basal within one hour. This profile reflects the normal stress reaction to the expectation of food. A single feeding with cortisol containing pellets caused a significant increase in plasma cortisol level, which reached a peak value

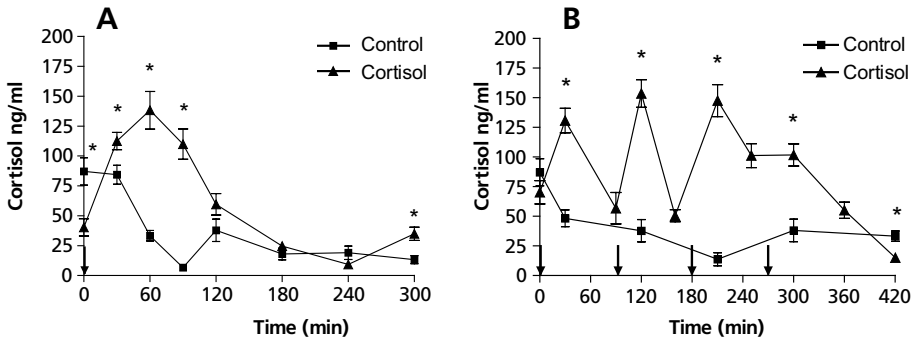

Figure 1. Plasma cortisol concentrations after a single (A) and a four times daily (B) cortisol food application (n=10). Arrows indicate the feeding times. * indicates a significant difference ($p<0.05$).

of 140 ng/ml plasma after one hour and was returned to basal level after 3 hours (Fig. 1A). Repeated application of the cortisol treated food, 4 times daily with intervals of 1.5 hours, induced an elevated cortisol profile over 7 hours. Cortisol peak values up to 150 ng/ml plasma were observed after each meal. In the control group, cortisol plasma values were all below 50 ng/ml plasma, except the first time point (Fig. 1B).

Growth, gonadosomatic index (GSI) and testicular histology

The growth curve (Fig. 2A) for the control group and cortisol treated group demonstrates that cortisol causes a slight retardation in growth which becomes significant from 101 dph onwards. The increase in GSI, observed in the control animals reflects the normal testicular development during puberty (Fig. 2B). In contrast, the cortisol treated animals show an impaired testicular development

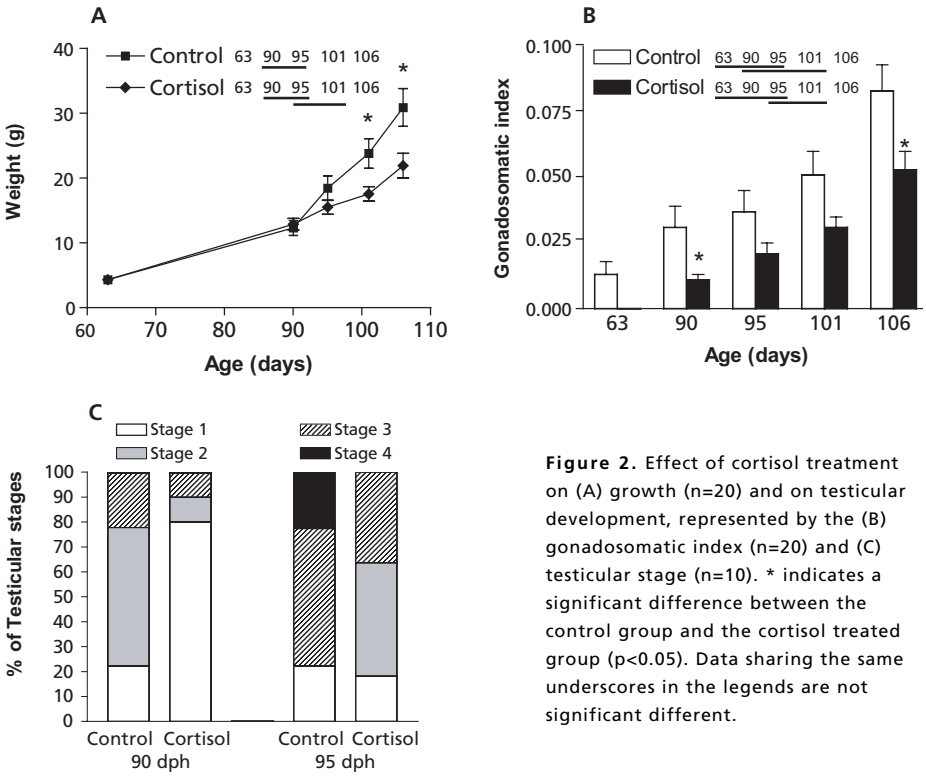

Figure 2. Effect of cortisol treatment on (A) growth (n=20) and on testicular development, represented by the (B) gonadosomatic index (n=20) and (C) testicular stage (n=10). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$). Data sharing the same underscores in the legends are not significant different.

as follows from the significantly lower GSI at 90 and 106 dph. This reflects the retardation in spermatogenesis as observed after histological analysis of the testis. Due to the some what higher variation in the control group, this difference is not statistically significant at 95 and 101 dph (Fig. 2C). At 90 dph most of the

control fish are in stage II whereas the cortisol treated fish remain in the first stage of spermatogenesis. When at 95 dph the control group is already in stage III-IV, all cortisol treated fish are still in stage II-III.

Plasma levels of sex steroids

Prolonged feeding with cortisol prevented the significant increase of 11KT plasma levels as observed in the control animals during the course of the experiment (Fig. 3A). The T levels were significantly lower at 106 dph only (Fig. 3C). Plasma OA levels are lower in cortisol treated animals. Statistical significance could not be calculated at 95 and 101 dph because most levels in cortisol treated animals were below the detection limit of the assay (8 out of 10 at 95 dph and 6 out 9 at 101 dph, respectively)(Fig. 3B).

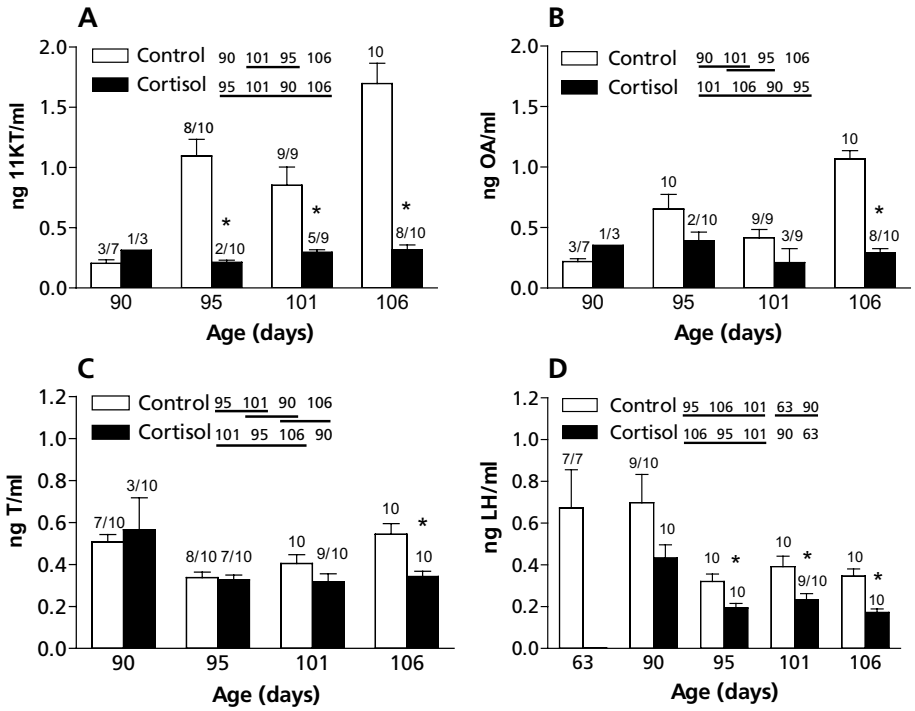

Figure 3. Effect of prolonged feeding with cortisol containing food pellets on plasma levels of (A) 11KT, (B) OA, (C) T and (D) LH (n=10). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$). Data sharing the same underscores in the legends are not significant different. Numbers above bars represent the number of values above the detection limit of the assay.

Plasma and pituitary LH

Plasma LH levels in both the control and cortisol treated fish gradually decreased during the experiment (Fig. 3D). However, the LH plasma levels for

the treated groups are significantly lower than the levels measured in the control group, except at day 90 dph, due to the larger variation in the control group.

The pituitary LH content shows a steady increase in the control group, which reflects the normal elevation of LH content during the pubertal development (Fig. 4A). This rise in LH content can also be observed in the cortisol treated group. However, cortisol treatment resulted in a slightly retarded elevation, which caused a significant difference at 101 dph.

sGnRH content in the brain

sGnRH content of the brain gradually decreased in the control animals from 90 to 101 dph, but were strongly increased at 106 dph. Prolonged feeding with cortisol resulted in lower brain contents of sGnRH (Fig. 4B) at 90, 95, 101 and 106 dph. Due to the somewhat larger variation in the cortisol group at 101 dph, there was no statistical difference between groups.

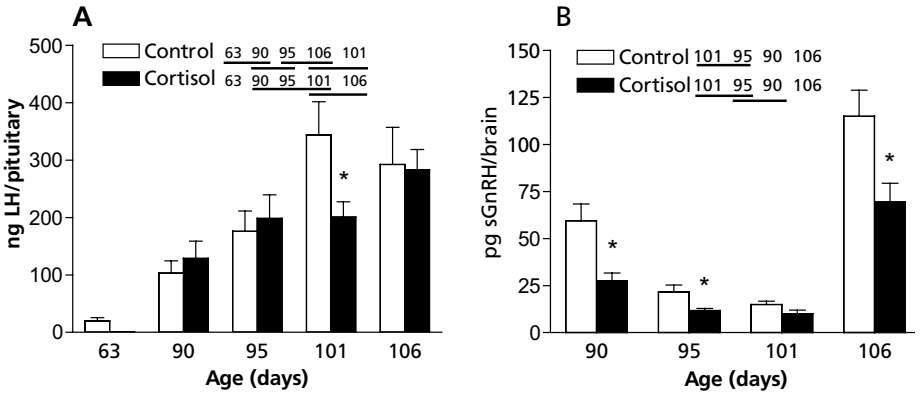

Figure 4. Effect of prolonged feeding with cortisol on (A) pituitary LH content, expressed in ng/pituitary and (B) sGnRH content in the brain, in pg/brain (n=10). * indicates a significant difference ($p<0.05$). Data sharing the same underscores in the legends are not significant different.

GPα, LHβ and FSHβ subunit steady-state mRNA levels

RNase protection analysis showed that cortisol treatment had different effects on the mRNA levels for GPα, LHβ and FSHβ depending on the age of the animals at time of the sampling (Fig. 5). At 90 dph, FSHβ subunit mRNA levels are significant lower compared to control values. Messenger RNA levels for GPα and LHβ tend to be lower in the cortisol treated group, but the differences are not significant. At 95 dph and 101 dph no differences are observed between the control and treated group. However at 106 dph GPα, FSHβ and LHβ subunit mRNA levels are significantly lower in the cortisol treated group.

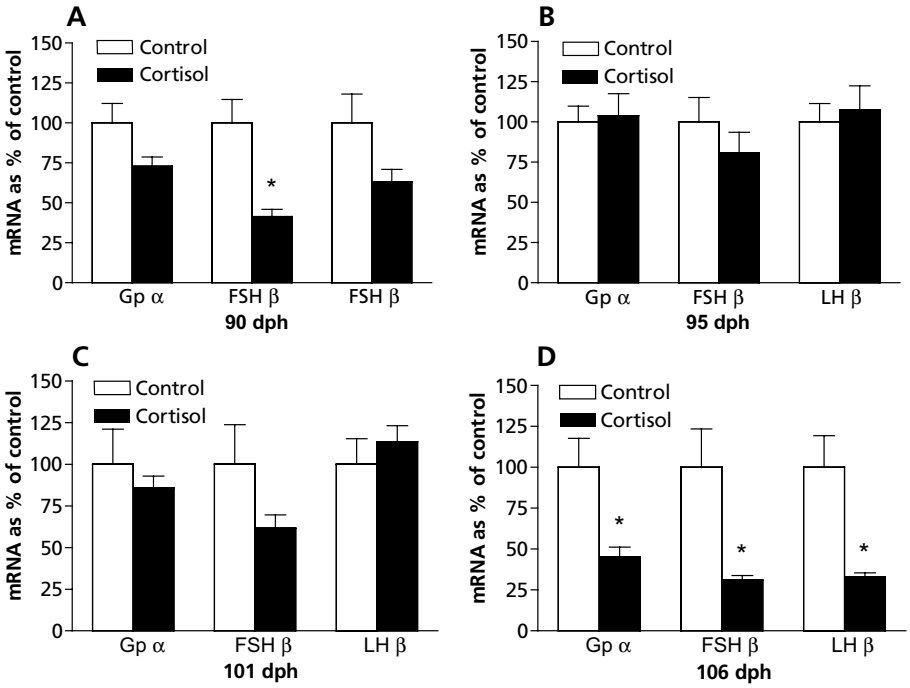

Figure 5. Effect of *in vivo* cortisol treatment on the mRNA levels for Gpα, FSHβ and LHβ subunit, respectively, at several ages during pubertal development (A) 90 dph, (B) 95 dph, (C) 101 dph and (D) 106 dph. Messenger RNA levels are expressed as a percentage of the control (n=10). * indicates a significant difference ($p<0.05$).

Discussion

In the present study we showed that long-term cortisol treatment caused an inhibition of pubertal development, affecting directly or indirectly all components of the BPG-axis. To our knowledge this is the first time that negative effects of cortisol on spermatogenesis during pubertal development have been described.

In this study we defined puberty as the period that spans the onset of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. In the isogenic common carp strain that was used, puberty occurs between 90 and 110 dph. Feeding with cortisol containing food pellets from 63 dph onwards caused retardation in weight gain between 90-100 dph. Similar results have been observed before for several species (reviewed in Van Weerd & Komen, 1998). Stress has been shown to have adverse effects on growth. In Atlantic salmon parr repeated stress once or twice daily over a period of 30 days reduced the growth rate significantly (McCormick *et al.*, 1998) and chronic crowding for a 9-month period in rainbow trout reduced the bodyweight as well (Pickering *et al.*, 1991).

Growth of the testis was affected by the cortisol treatment, reflected by lower GSIs at all sampling points. Due to the somewhat larger variation at 90 and 95 dph the difference was not statistically significant at these time points. Identical results have been obtained in other fish species. Hydrocortisone acetate treatment of male *Labeo gonius* caused a reduction in volume and length of the testes and in GSI (Joshi, 1982). Cortisol implanted maturing male brown trout had smaller gonads (Carragher *et al*., 1989). Female tilapia treated with cortisol showed reductions in GSI and oocyte size (Foo & Lam, 1993b). In mammals, reduction of testicular development was shown in stressed Siberian dwarf hamsters Castro & Matt, 1997). A causal relationship with declined testosterone (T) and estradiol (E2) levels has been suggested.

Reduction of plasma sex steroid levels due to stress or cortisol treatment has been reported for a variety of vertebrate species (mammals: Charpenet *et al*., 1981, Norman & Smith, 1992; reptiles: Moore *et al*., 1991, Mahmoud & Licht, 1997; amphibians: Coddington & Cree, 1995 and fish: Pickering *et al*., 1987a, Carragher *et al*., 1989, Foo & Lam, 1993a).

In African catfish, it has been shown by Cavaco *et al*. (1998b) that an important function of 11KT during sexual maturation is the stimulation of spermatogenesis. In Japanese eel, Miura *et al*. (1991) demonstrated that *in vitro* complete spermatogenesis could be induced by the application of 11KT to the culture medium. In the present study we show that cortisol caused a decline of 11KT levels, which is accompanied by an inhibition of the first wave of spermatogenesis, suggesting a causal relationship between the retardation of spermatogenesis and the decrease in 11KT secretion. Whether this is a direct effect of cortisol on the testicular androgen production, or an indirect action via the hypothalamic-pituitary gonadotropic system cannot be deduced from the present results. In mammals testosterone is secreted by the Leydig cells under LH stimulation, and testosterone may be considered to be the functional homologue of the fish androgen 11KT for promoting spermatogenesis (significance of testosterone for mammalian spermatogenesis reviewed by McLachlan *et al*. (1996) and Griswold (1998). Mammalian Leydig cells are known to express glucocorticoid receptors (GRs) (Schultz *et al*., 1993) and *in vitro* experiments suggest that stress or corticosteroids decrease the Leydig cell sensitivity to gonadotropins (Charpenet *et al*., 1981, Orr & Mann, 1992) either by reducing the LH receptor content (Bambino & Hsueh, 1981) or by inhibiting the 17α-hydroxylase and/or $C_{17,20}$-lyase activity (Fenske, 1997). In fish, the data on the direct effect of cortisol on steroidogenesis are less consistent compared to mammals. Carragher and Sumpter (1990) and Pankhurst *et al*. (1995a) found a reduction of 17β-estradiol and testosterone secretion by cultured ovarian follicles. In other species (goldfish, common carp and the sparid *Pagrus auratus*), however, Pankhurst *et al*. (1995b) found no evidence that the inhibitory effects

of stress on reproduction are mediated by the action of cortisol on ovarian steroidogenesis directly. From the present study, we do not have evidence for direct effects on the secretion of 11KT. However, in an earlier study (Consten *et al.*, 2000) we have shown that testes of cortisol treated common carp have a decreased OA and 11KT basal and LH-induced secretory capacity *in vitro*, indicating that a direct effect of cortisol on the Leydig cells may occur. Furthermore, in the same study we have shown that the addition of the non-metabolizable cortisol agonist, dexamethasone, to the incubation medium has similar effects.

In a successive study it will be investigated whether cortisol competitively inhibits the conversion of 11β-hydroxyandrostenedione (OHA) into OA or has an effect on the testicular steroid synthesizing capacity.

Cortisol may also have its effect on spermatogenesis via an action on Sertoli cells. In fish as in mammals, one of the functions of Sertoli cells is to mediate the action of androgens on spermatogenesis (Nagahama, 1994). Since glucocorticoid receptors (GRs) have been demonstrated in Sertoli cells in mammalian testes (Levy *et al.*, 1989) and these cells respond to glucocorticoids (Jenkins & Ellison, 1986, Lim *et al.*, 1996), an effect of cortisol on spermatogenesis via Sertoli cells can not be excluded. The presence of GR in the testis of fish has been confirmed by RT-PCR (Takeo *et al.*, 1996), but the exact localization is unknown yet.

Like in the African catfish (Schulz *et al.*, 1997), we observed an activation of the gonadotrophs in the pituitary during pubertal development, reflected by the increasing LH content. Schulz *et al.* (1997) suggested that a signal of testicular origin was responsible for the activation of the LH gene transcription and translation and LH storage. Indeed, several studies have shown that testosterone stimulates the maturation of gonadotrophs and the expression and storage of LH in various teleost species (Crim & Evans, 1979, Gielen & Goos, 1983, Magri *et al.*, 1985, Cavaco *et al.*, 1995, Rebers *et al.*, 2000). This is supported by studies of Cavaco *et al.* (1997), showing that testosterone is produced by the testis before sexual maturation. Moreover, castration slowed down gonadotroph maturation, a process that could be restored by testosterone replacement (Cavaco *et al.*, 1998c).

There is no evidence yet whether gonadotropin gene expression, storage and release are directly influenced by cortisol. The observed effect may be indirect via a reduced secretion of testosterone. Pituitary LH content was suppressed in the cortisol treated group only at 101 dph. There seems to be no relation with any of the other parameters, which limits the relevance of this observation. Gpα, FSHβ and LHβ mRNA steady state levels, however, were significantly decreased at 106 dph, which corresponds to the reduced T plasma content.

In the present experiments we found reduced plasma LH levels. Although a suppression of gonadotropin levels in fish by cortisol has been observed earlier (Zohar, 1980, Carragher *et al.*, 1989), the data are not always consistent. Some studies showed no effect (Pickering, 1981), others even an increase (Pickering, 1987a).

The reduced expression and release of gonadotropins after cortisol treatment may be related to the impaired testicular androgen secretion, but again, a direct effect of cortisol on the pituitary or via the hypothalamus cannot be excluded. In mammals, it has been demonstrated that corticosteroids inhibit the GnRH-induced LH release by inhibiting the responsiveness to GnRH (Padmanabhan *et al.*, 1983, Suter *et al.*, 1988). The inhibitory effects on the LH release may, however, also be caused by a suppression of the hypothalamic GnRH release (Rosen *et al.*, 1988). Both these pathways suppose the presence of GRs on GnRH neurons (or on neural elements controlling the GnRH neurons) or the gonadotrophs. Indeed, in fish GRs have been found in the hypothalamic GnRH neurons and in pituitary gonadotrophs of the rainbow trout (Teitsma *et al.*, 1999). Studies on the GnRH gene of several teleost species have shown that the GnRH promoter contains putative glucocorticoid responsive elements (GRE) (salmon GnRH: Klungland *et al.*, 1992, Higa *et al.*, 1997 and seabream GnRH and chicken GnRH-II: Chow *et al.*, 1998). *In vitro* experiments with immortalized GnRH-secreting cell lines, expressing a functional GR showed that dexamethasone repressed both the endogenous mouse GnRH gene by decreasing steady state levels of GnRH mRNA, and the transcriptional activity of transfected rat GnRH promoter-reporter gene constructs (Chandran *et al.*, 1994). The same author identified negative regulatory elements in the mouse GnRH encoding gene, which bind heteromeric complexes containing glucocorticoid receptor and mediate the repressive action of glucocorticoids (Chandran *et al.*, 1996). In addition, Attardi *et al.* (1997) showed that dexamethasone affected the GnRH secretion from GT1-7 cells as well.

In the present study, sGnRH content of the brain of control animals shows a gradual decrease from 90 to 101 dph and a sudden increase on 106 dph. We have no indication yet whether this profile reflects changes in synthesis, storage or release or a combination of these processes. However, our experiments demonstrate that prolonged cortisol treatment resulted in lower sGnRH levels in the brain, suggesting that the observed effects on the gonadotrophs may indeed be caused by a reduction of sGnRH secretion.

In conclusion, we show that cortisol inhibits pubertal development in common carp and that this inhibition is present at all levels of the BPG-axis. The present results are not sufficient to elucidate whether cortisol acts directly or indirectly in the different parts of the BPG-axis. Current investigations are designed to answer this question.