#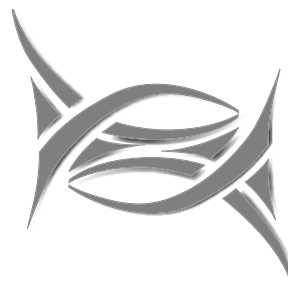 Cortisol effects on 11ß-hydroxysteroid dehydrogenase and the testicular androgen synthesizing capacity in common carp, *Cyprinus carpio* L.

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Abstract

Our previous studies on the effect of stress on pubertal development in carp have shown that repeated temperature changes caused an increase in cortisol levels and a retardation of the first waves of spermatogenesis. Identical effects, accompanied by a decrease in 11-ketotestosterone (11KT) plasma levels and the gonadosomatic index (GSI) were induced by cortisol administration via cortisol containing food pellets. The decrease in plasma 11KT is caused by a direct effect of cortisol on the steroid producing capacity of the testis, independent of LH levels. However, the precise mechanism via which cortisol interferes with testicular steroidogenesis is unknown. In the present study, we showed that *in vitro* physiological levels of cortisol can compete for the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), thereby inhibiting the conversion of 11β-hydroxyandrostenedione (OHA) into androstenetrione (OA), which is the precursor of 11KT. *In vivo*, cortisol could also interfere with this enzyme. However, our results demonstrate that an elevation of plasma cortisol levels during acute cortisol treatment did not result in lower plasma levels of OA and 11KT, but we did observe an accumulation of OHA. We suggest that the previously observed decrease in 11-oxygenated androgens, as an effect of long-term cortisol treatment, is caused by a retardation of the testicular development. This results in a lower steroid synthesizing capacity of the testis as a whole. Although the *in vitro* observed cortisol inhibition of the conversion of OHA into 11KT plays its role in the accumulation of OHA, it apparently has no effect on the final 11KT plasma concentration.

Introduction

Stress is a commonly used term and generally indicates a disturbing effect on the homeostatic state of the organism, caused by stressors (Chrousos & Gold, 1992). Prolonged and severe stress may have a deleterious effect on growth, immune competence and reproduction (Wendelaar Bonga, 1997).

Catecholamines (CA) and cortisol are frequently used as indicators of a stress response. Tanck *et al.* (2000) showed that in male common carp (*Cyprinus carpio*) temperature stress caused increased cortisol plasma levels. Previous results indicate that repeated temperature stress caused a retardation of the first waves of spermatogenesis (chapter 2) and this could be blocked by the cortisol antagonist RU486, indicating that the stress effects were mediated by cortisol. Furthermore, Consten *et al.* (2001a) demonstrated that cortisol administration mimicked effects of temperature stress on testicular development, since it resulted in both a decrease of the gonadosomatic index (GSI) and the androgen plasma levels. *In vitro* incubations showed that this inhibitory effect on the testicular androgen secretion is the result of a direct effect of cortisol (Consten *et al.*, 2000). There are at least two possibilities via which cortisol may cause a decrease of the androgen secretion. Cortisol could have an effect on the testicular steroidogenesis via a competition for enzymes, involved in the steroid synthesis. Secondly, cortisol may have an effect on the androgen production via the expression of steroid synthesizing enzymes, an effect that is probably mediated via the glucocorticoid receptor.

In contrast to mammals, in fish the main androgen is a derivative of T, namely 11-ketotestosterone (11KT) (reviewed by Borg, 1994). The main production route from pregnenolone (P_5) to 11KT is via progesterone (P_4), 17α–hydroxyprogesterone ($17\alpha P_4$), androstenedione (A_2), 11β–hydroxyandrostenedione (OHA) and 11–ketoandrostenedione (OA). OHA is converted to OA by the enzyme 11β–hydroxysteroid dehydrogenase (11β–HSD). The same enzyme converts cortisol into its inactive metabolite cortisone (Monder, 1991). This indicates a possible role for cortisol in the androgen synthesis of fish by competition for the enzyme 11β–HSD.

An effect of cortisol on the activity of enzymes, involved in steroid hormone production, via the glucocorticoid receptor is suggested for mammals (reviewed by Michael & Cooke, 1994), since the receptor was localized in the testis of rats (Schultz *et al.*, 1993). The presence of the glucocorticoid receptor in the fish testis, has been confirmed by RT-PCR in the rainbow trout (Takeo *et al*, 1996). Furthermore, it has been shown that cortisol can inhibit gonadal steroid hormone production independent of the action of gonadotrophic hormones (Pankhurst & Van der Kraak, 2000; Consten *et al.*, 2001b).

In the present study we investigated the conversion of OHA into OA, the precursor of 11KT, by testicular tissue and the possible interference by cortisol. *In vivo*, we examined the effect of cortisol on the androgen production by measuring the OHA, OA and 11KT plasma levels during cortisol feeding. Furthermore, we studied the testicular capacity for androgen production in 120 days old fish after prolonged treatment with cortisol in order to determine the long-term cortisol effects. This was performed by *in vitro* incubations with testicular fragments, using P_5 and A_2 as precursors. In the testis it are the Leydig

cells that are responsible for steroid production. The relative amount of these cells is one factor that determines the steroid synthesizing capacity of the testis per weight or volume unit. Therefore, the density of Leydig cells in the testis was determined by enzymecytochemical staining of the enzyme 3β–HSD and subsequent image analysis.

Materials 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 at the Fish and Fisheries of the Agricultural University of Wageningen, in the Netherlands. At 21 days post hatching (dph) the fish were transported to our department at the Utrecht University.

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. Before using, the fish were anaesthetized with TMS (Tricaine Methane Sulfonate, Crescent Research Chemicals, Phoenix AZ, USA), decapitated and the testes removed.

Experiment 1:
The effect of cortisol on the conversion of OHA by 11ß-HSD

Testicular homogenates were prepared of adult fish (approximately 5 months of age) to determine the conversion of OHA and cortisol into OA and cortisone respectively. Testes homogenates were prepared on ice with a glass-glass homogenizer in a sucrose phosphate buffer (w/v 1/10; 0.25 M sucrose; 0.1 M sodium phosphate buffer pH 7.4). To prepare a cell-free system the homogenate was centrifuged at 4°C for 10 minutes at 900 g. The supernatant was immediately used for incubations or frozen in liquid nitrogen and stored at –80°C until use.

Incubations with homogenates

The supernatant (16.67 mg/ml incubation mixture) was transferred to an incubation vial containing per ml endvolume 37 kBq [1,2-^{3}H]-OHA (19 nM) or [1,2-^{3}H]-cortisol (19 nM) dissolved in 100 μl propyleneglycol, the cofactor NAD$^+$ (1 mM) (Boehringer Mannheim) and sucrose phosphate buffer (0.25 M sucrose; 0.1 M sodium phosphate buffer pH 7.4). Higher substrate concentrations were obtained by adding non radio-labelled steroids. Incubations were performed at 25°C for 1 hour under continuous shaking in an air atmosphere. To

test the inhibitory effect of cortisol, the [1,2-^{3}H]-OHA incubation was carried out in the presence of different doses of non radio-labelled cortisol. Reactions were terminated by the addition of 5 ml dichloromethane and 2.5 ml water to the incubation.

Radioactive steroids and chemicals

[7-^{3}H]-Pregnenolone (sp. act. 925 GBq/mmol), [7-^{3}H]-androstenedione (sp. act. 907 GBq/mmol) and [1,2-^{3}H]-cortisol (sp. act. 1924 GBq/mmol) were purchased from NEN–Dupont life science. ^{3}H-labelled 11β–hydroxyandrostene-dione was chemically produced out of [1,2-^{3}H]-cortisol. Reference steroids were obtained from either Merck, Steraloids, Sigma, or Makor Chemical. The chemicals and solvents (Baker and Merck) were of analytical grade.

Extraction and separation

Before extraction steroids were added as carriers: 20 μg of OHA and OA to the incubations with [1,2-^{3}H]-OHA and 20 μg of cortisol and cortisone to incubations with [1,2-^{3}H]-cortisol. Steroids were extracted three times with dichloromethane (3 x 5 ml). The combined extracts were dried by evaporation. The steroid containing residue was dissolved in a few droplets of dichloromethane: methanol (9:1) and transferred to a thin layer chromatography (TLC)–plate.

TLC was carried out on precoated silica gel 60F$_{254}$ (Merck) plates in saturated tanks with the following systems: I) toluene-cyclohexane (1:1); II) chloroform–ethanol (95:5); III) toluene-ethylacetate (3:1). Each development was performed for 45 minutes and system I was used to separate steroids, remaining on the baseline, from apolar compounds. Carriers were identified by comparing them to the reference spots using a Universal-UV light (254nm and 366nm). Plates were analysed for radioactive areas by means of a chromatogram scanner, Berthold Automatic TLC Analyser (LB 2842). Moreover, from the radiochromatogram, it is possible to determine the percentage distribution of the tritiated compounds.

After a first treatment in system I, steroids were separated in system II.

Experiment 2:
The effect of short term elevation of plasma cortisol concentration on OHA, OA and 11KT plasma levels

If indeed the reduction in plasma OA and 11KT levels after long-term cortisol treatment, as observed in earlier studies, is caused by inhibition of the conversion of OHA, a short term elevation of cortisol plasma concentration may be expected to have the same effect.

Cortisol containing food (100 mg/kg food) was prepared as described by Pickering *et al*. (1987b). Fish received during one week, once daily, either con-

trol food or cortisol-treated food (Consten *et al.*, 2001a). On the last day of the treatment, blood samples were taken at regular time intervals following the last cortisol administration and stored at –20°C until steroid measurement by radioimmunoassay (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 OHA were measured in a RIA as described by Schulz (1985).

Experiment 3:
The effect of long-term cortisol treatment on testicular steroid synthesizing capacity. Incubations with ^{3}H-pregnenolone and ^{3}H-androstenedione

Fish were fed from 63 dph till 120 dph with cortisol containing food, 4 times daily with an interval of 1.5 hours, starting at 10 a.m. (Consten *et al.*, 2001a). Part of each of the testes was incubated for quantification of steroid production or for immunocytochemistry for 3β-HSD localization as marker for Leydig cells, respectively.

Incubations with testicular fragments

After determination of the body weight, fish were decapitated. Testes were weighed for determining the GSI (GSI= testis weight $\star$ 100/(Bodyweight-testis weight)) and a part of each testis was frozen into Tissue-Tek (Sakura finetek, USA) for 3β-HSD staining. The remaining parts of the testis were cut into fragments of approximately 2 mm^3. Each incubation mixture contained *ca*. 200 mg testis fragments in 1.9 ml HEPES buffered L15 medium (15 mM HEPES, 100.000 U/l penicillin/streptomycin; pH 7.4). The substrates, 37 kBq [7-^{3}H]-pregnenolone (40 nM) or [7-^{3}H]–androstenedione (41 nM), respectively, dissolved in 100 μl propyleneglycol, were added. Incubations were performed at 25°C for 0.5, 1.5 and 3 hours under continuous shaking in an air atmosphere. Reactions were terminated with 10 ml ethanol.

Extraction and separation

Before extraction, 40 μg of respectively, pregnenolone (P$_5$), 17α-hydroxypregnenolone (17αP$_5$) as well as 20 μg of respectively progesterone (P$_4$), 17α-hydroxyprogesterone (17αP$_4$), A$_2$, OHA and OA were added as carrier steroids to incubations with [7-^{3}H]-pregnenolone. For incubations with [7-^{3}H]-androstenedione, 20 μg A$_2$, OHA, OA, T, 11β-hydroxytestosterone (OHT) and 11KT, respectively, were added as carriers. Steroids were extracted and subjected to TLC as in experiment 1. Following treatment (three times) in system I, steroids were separated (three times) in system III.

3β-HSD staining and analysis

Cryo-sections of 10 μm were cut with a Jung Frigocut (cryostat 2800E, Leica), fixed to slides and stored at –20°C until use. Slides were air dried for 30 minutes, rinsed in ice-cold acetone during 2 minutes and air dried once again. Sections were incubated with freshly prepared medium containing 1 mg epiandrosterone (dissolved in 1ml dimethylformamide), 2 mg Nitro-blue tetrazolium (NBT) (BDH Chemicals), 5 mg NAD^+ (Boerhinger Mannheim), 0.2 mg EDTA and 10 ml 0.1 M sodium phosphate buffer (pH 8.3) for 1.5 hours at 37°C in a moist chamber. After the incubation, slides were rinsed shortly in distilled water, fixed in 5% formaldehyde for 5 minutes, rinsed in distilled water and embedded in glycerine/gelatine. The amount of Leydig cells was analysed with an image analysis system, equipped with the KS 400 software package (Carl Zeiss Vision, Germany). With this package, a special program was developed that measured the 3β-HSD stained areas in the testis. Testis sections were scanned with a black/white CCD camera (Sony XC-77CE) mounted on a Zeiss microscope. Objective 4.0 x was used in combination with Optovar 1.25. The total image size was 750 x 570 pixels; pixelsize 2.040 x 2.124 microns. On the basis of grey value the total tissue area was selected interactively. The automatic selection of stained parts in the tissue could be checked and corrected manually, if needed. Five control and cortisol treated fish, respectively, were analyzed. Seven representative slides per fish were measured, each slide in triplicate. To obtain the surface percentage that was occupied by the Leydig cells, the 3β-HSD stained area was corrected for the size of the testis section. Furthermore, the Leydig cell spot area and the number of spots per testis tissue area was measured.

Results

Experiment 1:
The effect of cortisol on the conversion of OHA
in testicular tissue

After the incubations with 17 mg of testis homogenates, 95-99 % of the total radioactivity was extracted from the medium.

Cortisol and OHA concentrations from 10 nM to 150 nM were converted into cortisone and OA for about 50% and 25%, respectively, within one hour. This indicates the presence of the enzyme 11β-HSD. The plot of the reaction velocity, as a function of the substrate concentration is still linear (Fig. 1A). At increasing substrate concentrations (from 1.5 to 60 μM) the conversion rates were decreased to 6% at the highest substrate concentration for cortisol and 2% for OHA (Fig. 1B). The decreasing slope indicates that the enzyme, 11ß-HSD, becomes saturated (Fig. 1B).
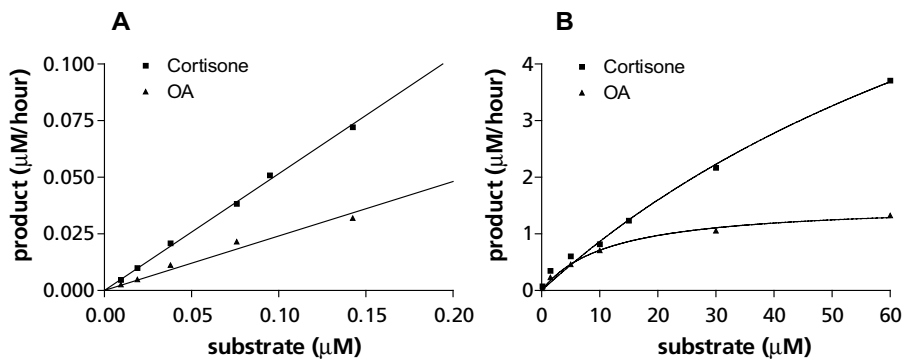
Figure 1. Conversion of cortisol and OHA into cortisone and OA, respectively, by testicular homogenates within one hour. (A) Linear regression for substrate concentrations in the range up to 150 nM, and (B) non-linear regression for substrate concentration in the range µM.

To test if the conversion of OHA into OA could be influenced by cortisol, OHA incubations were performed in the presence of cortisol concentrations that are physiological significant during stress adaptation, except for the highest concentration of 800 ng/ml. Analysis of the radiochromatogram showed that with an increasing concentration of cortisol, from 50 ng/ml up to 800 ng/ml, there was a linear decrease in the conversion of OHA to OA from about 55% to 10%, relative to the conversion where no cortisol was added (Fig. 2A). The concentration of 200 ng/ml cortisol, which is common during stress was further investigated in a one-hour time course. The obtained curve indicates a smaller OA production combined with a regression slope, that is only one-third of the incubation without cortisol (Fig. 2B).
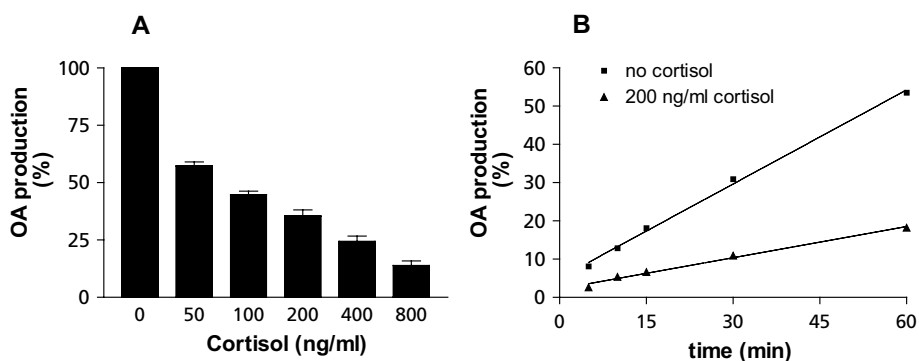

Figure 2. Effects of glucocorticoids on the conversion of OHA by 11β-HSD performed by incubations with testicular homogenates. conversion by 11β-HSD. (A) Relative production of OA after one hour, with increasing concentrations of cortisol. (B) conversion of OHA into OA, in a one hour time course in the absence or presence of cortisol (200 ng/ml).

Experiment 2:
The in vivo effect of short-term cortisol treatment
on OHA, OA and 11KT plasma levels

To investigate if cortisol also in vivo could affect the conversion of OHA into the 11keto–derivatives, steroid plasma levels were measured over a five–hour time–period, following cortisol treatment once daily for only one week. Blood samples were taken before and respectively, 30, 60, 90, 120, 180, 240 and 300 minutes after the last cortisol administration.

Cortisol plasma levels increased within one hour after feeding from 40 to 140 ng/ml, after which they returned to basal levels at 3 hrs (Fig. 3A). Cortisol administration had no inhibitory effect on the plasma levels of OA and 11KT (Fig. 3B and C). However, there was a clear effect on plasma OHA levels, which showed a 4 fold increase from 0.4 to 1.6 ng/ml within half an hour after corti–sol treatment and slowly decreased to control levels over de following 2.5 hours (Fig. 3D).
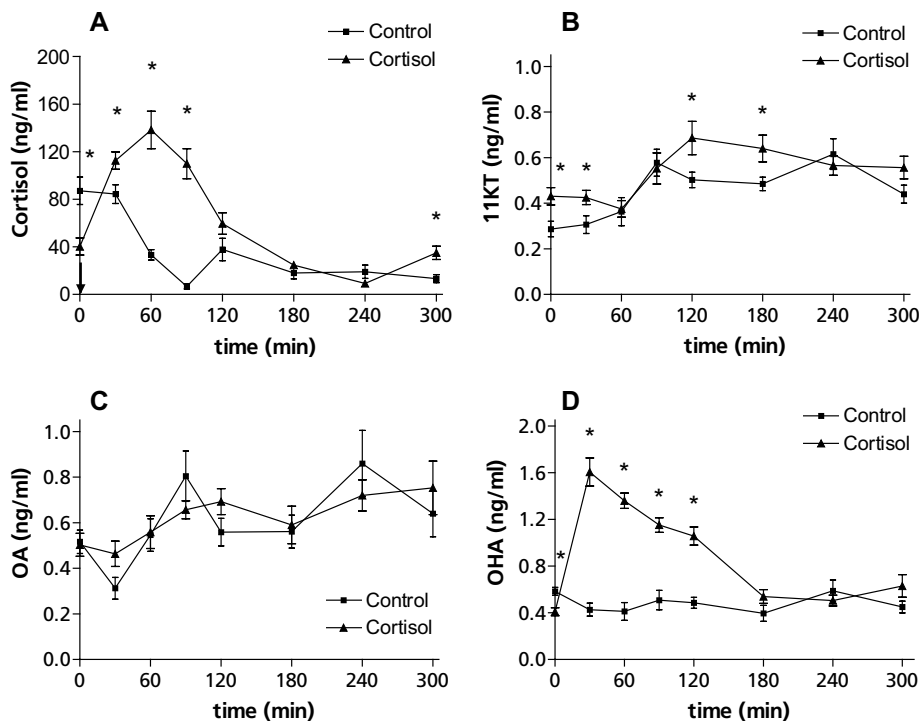

Figure 3. Plasma levels of (A) cortisol, (B) 11KT, (C) OA and (D) OHA over a 5 hour time period, after cortisol administration (n = 10). Arrow indicate the feeding time. * indicates a significant difference (p<0.05).

Triplicate incubations with testicular fragments from 120 days old control and cortisol treated fish were carried out for 0.5, 1.5 and 3 hours with ^{3}H–pregnenolone or ^{3}H–androstenedione as substrates. TLC–analysis showed that both P_5 (data not shown) and A_2 (data not shown) were converted to the same extent per gram testis tissue from control and cortisol treated fish, with the testis of the cortisol treated fish producing slightly more (not significant) OHA, OA and 11KT per gram testis.

Table 1. Leydig cell analysis in the testis of long-term cortisol treated fish versus control fish.

	Leydig cell area percentage	Leydig cell spot area (µm²)	Number of spots per testis area (mm²)
Control	0.721±0.060	100.04±2.97	71.60±5.34
Cortisol	1.129±0.124*	124.54±6.35*	93.19±10.51

(n = 5), mean ± SEM, * indicates significant difference (p<0.05)

Cryo–sections of control and cortisol treated fish of 120 days old, were subjected to the enzymecytochemical reaction for 3β–HSD. The relative area occupied by Leydig cells, the surface of concentrations of Leydig cells (spot area) and the number of spots were quantified by image analysis. The results, showed that Leydig cells occupy 1.57 times more of the surface of sections of the testis of cortisol treated fish. Furthermore, the area per spot is 1.25 fold bigger and there are 1.19 times more spots per area testis of cortisol treated fish (table 1).
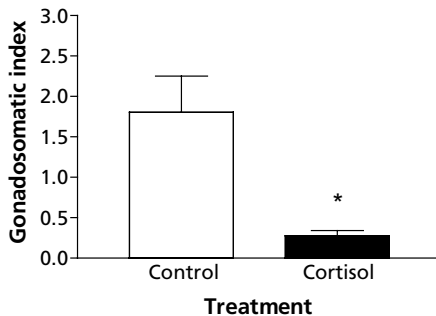

Figure 4. Effect of long-term cortisol treatment on the testicular development, represented by the gonadosomatic index at 120 dph (n = 15). * indicates a significant difference (p<0.05).

The effect of long-term cortisol treatment is shown by a 6.5 fold difference in the GSI (Fig. 4). Thus cortisol treatment inhibits testicular growth, as reflected by the GSI. Since P_5 and A_2 incubations (see above) showed no effect of long-term cortisol treatment and the relative amount of Ledig cells was not affected by cortisol treatment, the difference in testicular mass may be responsible for the observed reduced steroid production.

Discussion

The objective of the present study was to investigate how cortisol can influence androgen production in the testis of the common carp. Our data showed that both OHA and cortisol are converted in the testis to their 11keto-derivatives. The conversion of OHA into OA could be inhibited by the addition of physiological relevant cortisol levels comparable with levels during stress. However, a short-term elevation of the cortisol plasma levels did not result in a reduction of plasma OA and 11KT levels, as was previously observed during prolonged stress (chapter 2) or prolonged cortisol treatment (Consten *et al.*, 2001a). Our results indicate that the reduction of the plasma androgen levels is merely due to a reduction in the total androgen secretory capacity as a consequence of the retarded testicular development, observed under long-term cortisol treatment.

In vitro incubations with testicular homogenates showed that both OHA and cortisol were converted to OA and cortisone, respectively. This conversion is linear for both substrates in the range from 10 to 150 nM substrate. At concentrations of 1.5 μM and higher the enzyme becomes saturated. Cortisol was converted at a higher velocity than OHA, indicating that cortisol may be a strong competitor for the conversion of OHA into OA. Further results of *in vitro* incubations demonstrated that, indeed the conversion of OHA into OA was inhibited by cortisol, suggesting that cortisol can directly interfere with the OA and 11KT synthesis.

In vivo, long-term cortisol administration has been shown to result in decreased plasma OA and 11KT levels (Consten *et al.*, 2001a). This suggests that, in line with the *in vitro* OHA incubations, the direct effect of cortisol on the conversion from OHA to OA may also be responsible for the impaired OA and 11KT synthesis *in vivo*. However, the present results contradict this hypothesis. We observed no effect of short-term elevated cortisol concentrations on plasma OA and 11KT levels, but we do observe an increase in OHA. This suggests that *in vivo* 11β–HSD is, indeed, inhibited by cortisol, but that the plasma 11keto-androgens are somehow, kept constant. This may be caused by an effect of cortisol on the clearance of these steroids from the blood, but we have no proof for this hypothesis

Although our results indicate that there is a direct effect of cortisol on 11β-HSD, this does not explain the decreased plasma 11KT levels, observed by Consten *et al.* (2001a) in long-term cortisol treated fish. This suggests that cortisol affects the steroidogenic capacity via another mechanism. Therefore, we exposed fish to prolonged cortisol treatment and investigated at 120 dph the steroid producing capacity of the testis *in vitro* by incubation of testicular fragments with tritiated precursors. The production of androgens and the intermediate steroids were measured and corrected for the amount of testis tissue that was used. The results showed that per gram testis tissue the cortisol treated animals could produce slightly more (though not significantly) OHA, OA and 11KT. A higher production per gram testis may not be unexpected, since Consten *et al.* (2001a) showed that by prolonged cortisol treatment the spermatogenesis was inhibited. Control fish will have relatively more spermatogenetic elements, which leads to a "dilution" of the androgen producing Leydig cells per weight unit of testis. Similar results were found comparing early and later maturational stages during pubertal development of the African catfish (Schulz *et al.* 1996). For this reason we performed a 3β-HSD enzymecytochemical staining, to determine the relative amount of steroid producing cells, Leydig cells. Indeed, the testis of cortisol treated fish contained relatively more Leydig cells, which may explain the relative higher conversion of P_5 and A_2 per gram testis.

However, long-term cortisol treatment resulted in 6.5 fold less testis tissue, as reflected by the GSI. This more than compensates for the small difference in the steroid converting capacity, and as a consequence leads to a reduction of the steroidogenic capacity per pair of testis.

In summary, the present data suggest that in the common carp the secretion of 11-oxygenated androgens (OA and 11KT) may be influenced by cortisol, via the conversion of OHA into OA. This is supported by the *in vitro* inhibition of the conversion of OHA into OA in the presence of cortisol, and by the *in vivo* experiments in which we observed an accumulation of OHA in the plasma when cortisol plasma levels were elevated. However, we did not find subsequent changes in plasma OA and 11KT levels, indicating that the inhibition by cortisol of the conversion of OHA by 11β-HSD does not explain the previously observed decrease in plasma 11-oxygenated androgens.

The reduction in testicular size, resulting in a lower steroidogenic capacity, may be the most important factor for the limited testicular androgen secretion after long-term cortisol exposure.