*Chapter 5*

# Corticosteroids affect the testicular androgen production in male common carp, *Cyprinus carpio* L.

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submitted

## Abstract

Our previous experiments to study the effect of cortisol on pubertal development in carp showed that repeated temperature stress, but especially prolonged feeding with cortisol containing food pellets caused a retardation of the first waves of spermatogenesis, and a decrease in 11-ketotestosterone (11KT) and LH plasma levels.

The objective of the present study was to investigate whether the decrease in plasma 11KT is caused by a direct effect of cortisol on the steroid producing capacity of the testis or by an indirect effect such as a decrease in plasma LH. Adolescent and pubertal isogenic male common carp were fed with either cortisol containing food pellets or control food pellets over a prolonged period. Our results indicate that cortisol has a direct inhibitory effect on the testicular androgen secretion, independent of the LH secretion. Furthermore, the pubertal period is critical to the influence of cortisol regarding testicular androgen secretion. The effect is no longer observed at adolescence.

## Introduction

In all teleost species, including the common carp, cortisol is the major corticosteroid produced by the interrenals under influence of stress (Barton & Iwama, 1991). Cortisol plays a key role in the restoration of homeostasis during or after stress and has frequently been indicated as the major factor mediating the suppressive effect of stress on reproduction. The developmental period during which the animal acquires the capacity to reproduce is defined as puberty. The basis of pubertal maturation is the development of the gonads and the endocrine system that regulates reproductive processes, the brain-pituitary-gonad (BPG) axis.

Our previous studies demonstrated that in common carp, repeated temperature-induced stress caused a retardation of the first waves of spermatogenesis (chapter 2). Furthermore, long-term cortisol treatment resulted in a similar

effect on spermatogenesis, accompanied by a decrease in plasma LH and plasma 11-ketotestosterone (11KT) (Consten *et al*., 2001a). Several studies indicate that 11KT has an important function of 11KT during sexual maturation. 11KT has been shown to stimulate spermatogenesis in African catfish (*Clarias gariepinus*) (Cavaco *et al*., 1998b), the common carp (*Cyprinus carpio*) (Komen, personal communication) and in Japanese eel (*Anguilla japonica*) (Miura *et al*., 1991).

The reduction of plasma sex steroids due to stress or cortisol has been demonstrated in a variety of vertebrate species (mammals: Norman and Smith, 1992, Charpenet *et al*., 1981; 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 mammals, the steroid producing cells of the testis, the Leydig cells, have been shown to contain glucocorticoid receptors (Schultz *et al*., 1993) and therefore corticosteroids may exert a direct effect on the steroidogenesis. 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 and 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 (*Carassius auratus*), 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.

The aim of this study was to investigate if the observed decrease in plasma 11KT levels is caused by a direct effect of cortisol on the steroid producing capacity of the testis or via a decreased LH secretion. Furthermore, we were interested if the negative effects of cortisol are correlated with age and development of the fish.

## Material and Methods

### *Animals*

Isogenic male common carp (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 the fish facilities 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 (Trouw, Putten, The Netherlands) at a daily ration of 20 $g/kg^{-0.8}$. Immature fish were allowed to acclimatize till 63 dph after which the experiment started or were kept until adolescence.

### *Experiment 1: maturing fish*

Cortisol (Steraloids Inc. Wilton, USA) treated food (100 mg/kg food) was prepared as described by Pickering *et al*. (1987). One hundred and twenty animals were equally divided over two groups. One group received control food, the other group the cortisol-treated food from 63 dph onwards as described previously (Consten *et al*., 2001).

Fish from both groups (n=20) were sampled at several time-intervals during the pubertal development, at 94 dph (early puberty), 100 dph (late puberty) and 120 dph (first wave of spermatogenesis completed). The fish were anaesthetized in TMS (Tricaine Methane Sulfonate, Crescent Research Chemicals, Phoenix AZ, USA). Body weight was determined and blood was collected by puncturing the caudal vasculature. After blood sampling fish were immediately decapitated and testes were removed for determining the gonadosomatic index (GSI) and *in vitro* incubation for determination of the steroid synthesizing capacity.

### *Experiment 2: adolescent fish*

Forty-eight adolescent fish were equally divided over two groups and were either fed, starting at 138 dph, control food or cortisol containing food, similar to the maturing fish. At 165, 183 and 197 dph fish were sampled (n=8) according to the same procedure as in experiment 1.

### *Androgen secretion in vitro*

The *in vitro* determination of the steroid secretory capacity of testicular tissue of maturing fish in experiment 1 was performed as described by Cavaco *et al*. (1998b). In short, the left and right testis of each male were divided in equal halves. Each half testis was weighed separately and transferred to a separate well of a 24-wells Costar plate, containing 0.5 ml HEPES buffered L-15 medium (15 mM HEPES, 100,000 U/l penicillin/streptomycin, pH 7.4). The testis halves were then cut into fragments of approximately 2 $mm^3$. The culture medium of the four halve testis was taken off and replaced by 0.5 ml medium, with or without dexamethasone (Sigma, St. Louis, USA) (150 ng/ml medium) and containing increasing amounts of LH (0, 10, 30 and 100 ng LH/ml medium). Carp pituitary extract, in which LH content determined by radioimmunoassay (RIA), was used as LH source. After incubation for 20 hours at 25°C, the medium was removed, heated for 1 hour at 80°C and centrifuged at 10,000 g for 30 min at

room temperature. The supernatant was stored at -20°C until steroid hormone measurement by RIAs.

Testicular tissue from each adolescent fish separately was prepared as described by Schulz *et al*. (1994). Then, for each fish five wells of a 24-wells Costar plate, containing 0.5 ml L-15 medium, were filled with 100 mg testicular tissue. The medium was taken off and replaced by 1 ml medium containing increasing amounts of LH (carp pituitary extract), respectively 0, 10, 30, 100 and 300 ng LH/ml medium, in the absence or presence of dexamethasone (Sigma, St. Louis, USA) (150 ng/ml medium). After an incubation of 20 hours at 25°C, the medium was treated as in experiment 1.

### *Pituitary extract and plasma LH*

Luteinizing Hormone (LH) was quantified in pituitary extract and in plasma 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 first antibody.

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.

### *Steroid Radioimmunoassays (RIA)*

The steroid levels in both plasma (11KT) and medium (11KT and 11-ketoandrostenedione, OA) were determined by RIA as described previously (Schulz, 1985). In most male teleosts 11KT is considered to be the most dominant androgen in the plasma (Borg, 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 OA is the main androgen produced by the testes (Komen, personal communication).

### *Statistics*

All results are expressed as mean ± SEM. Plasma levels of 11KT and LH are given as ng per ml plasma. *In vitro* data are given as ng of steroid secreted, corrected for total testis weight. All results on the treatment effect of cortisol were processed for statistical analysis by Student's T-test ($p<0.05$). *In vitro* data were processed by one-way ANOVA, followed by Fisher's least significant difference test ($p<0.05$).

## Results

### *Gonadosomatic index (GSI)*

In maturing control animals the gonadosomatic increases during the experimental period from 94 to 120 dph. This reflects the testicular growth that normally occurs during pubertal development. In contrast, prolonged treatment with cortisol containing food results in an impaired testicular development as

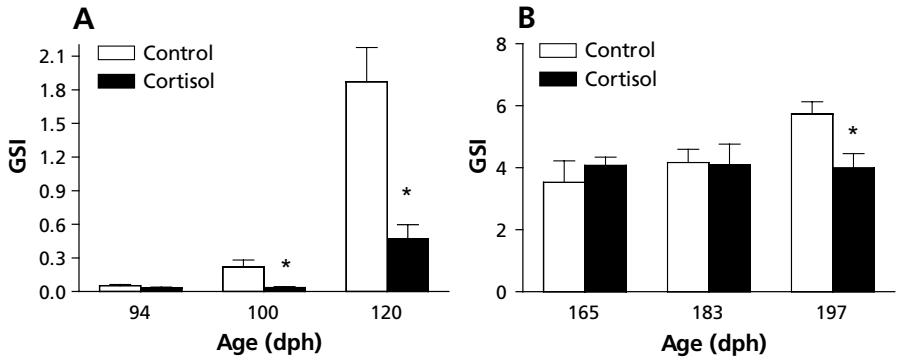


**Figure 1.** Effect of cortisol treatment on testicular development, represented by the gonadosomatic index in (A) pubertal fish (n=20) and (B) adolescent fish (n=8). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$).

follows from the significantly lower GSI at 100 and 120 dph (Fig. 1A). In adolescent fish, relative testicular growth has slowed down and only at 197 dph the GSI was significantly different from 165 dph. Consequently, the effect of cortisol treatment in adolescent fish is less pronounced compared to pubertal fish, but retardation in testicular growth could still be observed at 197 dph (Fig. 1B).

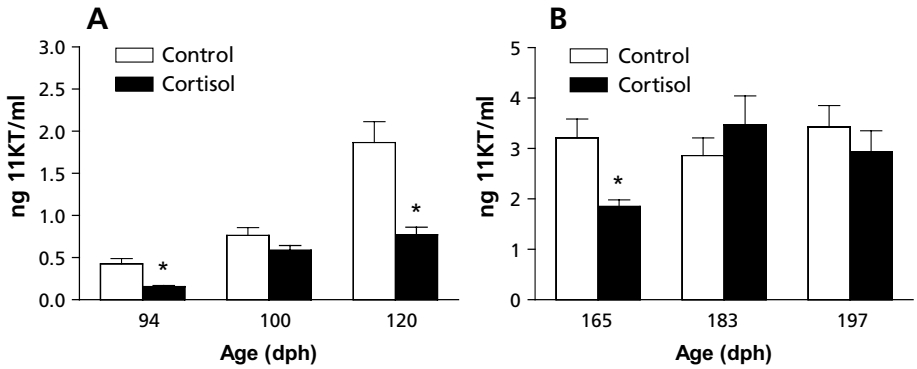


**Figure 2.** Effect of prolonged feeding with cortisol containing food pellets on plasma 11KT levels in (A) pubertal fish (n=20) and (B) adolescent fish (n=8). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$).

### *Plasma hormone levels*

Similar to the GSI in maturing control fish, plasma 11KT levels increased during pubertal development. Plasma 11KT levels of cortisol treated animals are significantly lower compared to control animals (at 100 dph, the difference is not significant) (Fig. 2A). In adolescent control fish, plasma 11KT levels have further increased and remain at the same level during the experimental period. In cortisol treated adolescent fish the 11KT levels at 165 dph are still behind the control values. However, during the experimental period the 11KT levels in cortisol treated fish become equal to the control values (Fig. 2B).

Prolonged feeding with cortisol results in maturing fish in lower plasma LH levels at 94 dph, equal at 100 dph and increased at 120 dph, compared to control fish (Fig. 3A). In adolescent fish, cortisol has no effect on plasma LH levels at 165 and 197 dph, but at 183 dph plasma LH levels are significantly higher in cortisol treated fish (Fig. 3B).

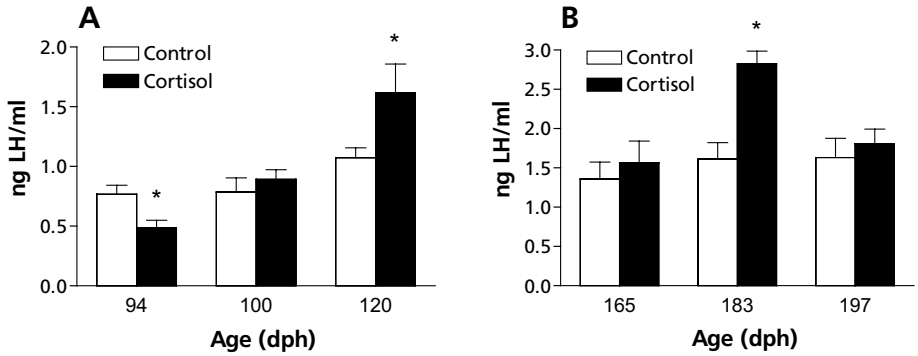


**Figure 3.** Effect of prolonged feeding with cortisol containing food pellets on plasma LH levels in (A) pubertal fish (n=20) and (B) adolescent fish (n=8). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$).

### *Androgen secretion in vitro*

Data on the *in vitro* androgen secretion are expressed as microgram steroid corrected by the total testes weight (µg per 2 testes). Schulz *et al*. (1996) have shown that differences are found in the steroid secretion per gram testes tissue, depending on the stage of testicular development during puberty. Since our previous results (Consten *et al*., 2001a) have shown that cortisol treatment leads to a retardation of the first cycle of spermatogenesis, we expressed our data as total secretion per 2 testes.

In maturing control fish, LH stimulated the *in vitro* steroid secretion dose dependently. Previous *in vivo* cortisol treatment from 63 dph on, reduced the LH-induced OA secretion significantly at 94 dph (Fig. 4A). Similar results were found on the 11KT secretion (Fig. 4B), although at 94 dph OA is the main pro-

duct produced by the testes. *In vitro* treatment with dexamethasone resulted in a reduction of the LH-induced androgen secretion as well (Fig. 4A&B).

At 100 dph, OA is still the main androgen produced by the testes. However, both OA and 11KT production have increased, compared to 94 dph (Fig. 4C&D). Cortisol treatment *in vivo* resulted in significantly lower LH-induced OA and 11KT secretion *in vitro*. The *in vitro* treatment with dexamethasone caused a reduction in the secretion of OA and 11KT, but this reduction is not significant due to the somewhat larger variation.

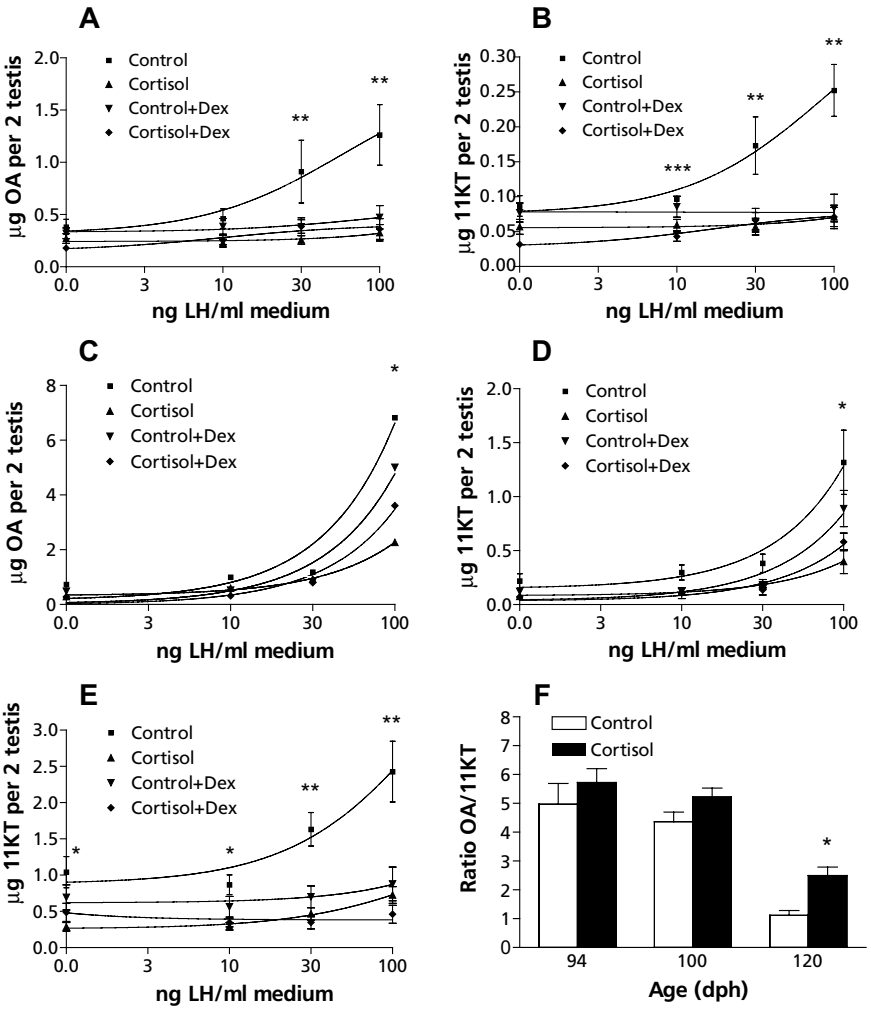


**Figure 4.** Effect of *in vivo* cortisol treatment and *in vitro* dexamethasone (Dex) treatment on the *in vitro* steroid secretion of pubertal fish, expressed as µg steroid per 2 testes (n=10). (A) OA secretion at 94 dph, (B) 11KT secretion at 94 dph, (C) OA secretion at 100 dph, (D) 11KT secretion at 100 dph, (E) 11KT secretion at 120 dph, (F) ratio between OA and 11KT secreted in vitro. * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$). ** indicates a significant difference of the control group with all other groups($p<0.05$). *** indicates a difference between the control group and the cortisol and dexamethasone treated group($p<0.05$).

At 120 dph, when in control animals 11KT is becoming the main steroid produced by the testes, both basal and LH-induced 11KT secretion are significantly reduced after prolonged *in vivo* cortisol treatment (Fig. 4E). The *in vitro* dexamethasone treatment has a comparable effect as both basal and LH-stimulated 11KT secretion are affected. Similar results were observed for the OA secretion (data not shown). The ratio OA/11KT shows that cortisol treatment

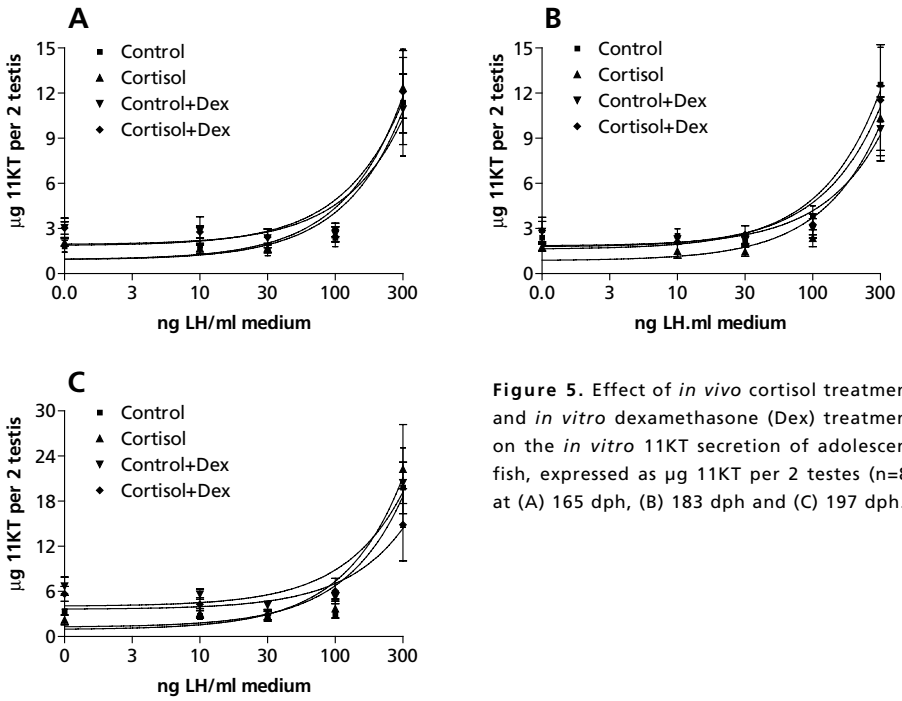


**Figure 5.** Effect of *in vivo* cortisol treatment and *in vitro* dexamethasone (Dex) treatment on the *in vitro* 11KT secretion of adolescent fish, expressed as µg 11KT per 2 testes (n=8) at (A) 165 dph, (B) 183 dph and (C) 197 dph.

not only affects the androgen production quantitatively, but also its pattern. In control animals there is a shift towards 11KT secretion at 120 dph, while cortisol treatment caused the relative high production of OA to be maintained at this age (Fig. 4F).

In contrast, in adolescent fish, there is no effect of either *in vivo* cortisol treatment or *in vitro* dexamethasone treatment on the *in vitro* basal and LH-induced androgen secretion throughout the experiment (Fig. 5A-C).

## Discussion

Previous work has shown that prolonged treatment with cortisol caused a retardation of the first waves of spermatogenesis, which are associated with the onset of puberty. This was accompanied by a decrease in plasma 11-ketotestosterone (11KT) (Consten *et al*., 2001a). In the present study we show that the observed decrease in plasma 11KT levels is caused by a direct effect of cortisol on the steroid producing capacity of the testis and is probably independent of the LH secretion.

As previously observed, cortisol treatment of maturing fish caused a retardation of pubertal testicular development as reflected by the lower GSI, the lower plasma 11KT levels, and the lower plasma LH levels at the onset of pubertal development, 94 dph. However, at 100 dph we observe plasma LH levels equal to the control group and at 120 dph the plasma levels in cortisol treated fish are even significantly elevated, but plasma 11KT levels are still lower than control fish. These results suggest that the decrease in plasma androgen levels is not caused by an effect of cortisol on LH levels. Pankhurst & Van Der Kraak (2000) also found evidence that the inhibitory effect of stress on plasma sex steroids is independent of the plasma LH levels.

In contrast, in adolescent fish we observe no effect of cortisol treatment on the testicular development at 165 and 183 dph. Only at 197 dph an inhibitory effect of cortisol treatment becomes apparent. At 165 dph, plasma 11KT levels is still lower in cortisol treated adolescent fish compared to controls, but during the experimental period they increase to same values as the controls. From these observations we conclude that fish become less sensitive to cortisol during sexual maturation. Apparently, cortisol sensitivity depends on the maturational status of the animal. Indeed, Pankhurst & Van Der Kraak (2000), demonstrated that in female rainbow trout the effect of cortisol on ovarian steroidogenesis depends on the stage of the reproductive cycle.

In mammals, cortisol may have a direct effect on the Leydig cells, since they have been shown to possess glucocorticoid receptors (Schultz *et al*., 1993). Studies by Charpenet *et al*. (1981) and by Orr & Mann (1992) demonstrate that stress decreases the sensitivity of the Leydig cell to gonadotropins. This may be caused by reducing the LH receptor content (Bambino and Hsueh, 1981) or by inhibiting the 17α-hydroxylase and/or $C_{17,20}$-lyase activity (Fenske, 1997).

Our results demonstrate that prolonged exposure to cortisol reduced the androgen secreting capacity of the testis. Both OA and 11KT secretion *in vitro* are significantly reduced and also the difference in the ratio OA/11KT shows once more that the testicular development in cortisol treated animals is retarded since the ratio still reflects a more immature pattern. Our results are not appropriate to reveal the precise mechanism via which cortisol affects the testicular androgen production. It is, however, unlikely that prolonged exposure to

cortisol causes a decrease in LH receptor content, since the sensitivity to LH is unchanged. At 100 dph and 120 dph the stimulation factor (data not shown) of LH is similar for control and cortisol treated animals. We therefore hypothesize that cortisol affects the enzyme activity involved in the androgen production. In a successive study we will investigate this hypothesis, as well as the possibility that cortisol competitively inhibits the conversion of 11β-hydroxyandrostenedione (OHA) into OA.

In contrast, *in vitro* treatment with dexamethasone does appear to affect LH sensitivity. At 94 and 120 dph, testes taken from control animals do not show an increase in the androgen production upon LH stimulation in the presence of dexamethasone. In several studies corticosteroids have been suggested (e.g. Pankhurst & Van Der Kraak, 2000; Valli *et al.*, 2000) and shown (reviewed by Borski, 2000) to mediate their inhibiting effect by interfering with signal transduction. In rat Leydig cells, chronic treatment with corticosterone diminished the production of testosterone, as well as the basal and LH-stimulated cyclic AMP production (Sankar *et al.*, 2000). Based on these results we hypothesize that the *in vitro* effect of dexamethasone in our experiments may be caused by an interference of corticosteroids with the LH signal transduction, thereby blocking the LH response and thus the LH-induced secretion of 11KT and OA.

In summary, we showed that cortisol has a direct inhibitory effect on the testicular androgen secretion, and not via plasma LH levels. The underlying mechanism may involve an inhibitory effect on expression of the steroid producing enzymes, substrate inhibition of enzymes that have a function in the conversion of cortisol as well as androgen precursors. Moreover, a direct interference with the LH signal transduction can not be excluded. Furthermore, our results demonstrate that cortisol sensitivity depends on the maturational status of the animal.