Chapter 4

Cortisol affects testicular development in male common carp, *Cyprinus carpio* L, but not via an effect on LH secretion

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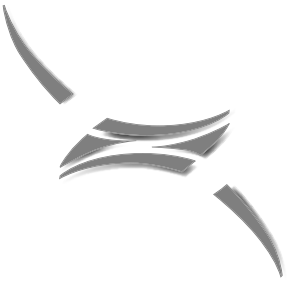

Abstract

Previous work showed that prolonged elevated cortisol levels, implicated in the stress adaptation, inhibits testicular pubertal development in male common carp, as well as an impairment of the synthesis of the 11-oxygenated androgens. This may be a direct effect of cortisol on the testis or via the gonadotropin secretion by the pituitary. The aim of the present study was to investigate whether cortisol has an effect on pituitary LH secretion. Juvenile common carp were fed with cortisol containing food pellets. Elevated cortisol levels blocked the increase in testosterone levels and pituitary LH content, but induced higher plasma LH levels at the end of puberty. The *in vitro* LH release capacity was correlated to the pituitary LH content. At the final stage of pubertal development, when a significant difference in pituitary LH content was observed, sGnRHa-induced LH release was also decreased. Testosterone has been shown to induce development of pituitary gonadotrophs, leading to an increase in LH content and GnRH-inducible LH release, but a decrease in plasma LH levels. We observed decreased plasma testosterone levels as a consequence of prolonged cortisol treatment. It is hypothesized that cortisol inhibits the testicular testosterone secretion and thereby prevents LH storage. *in vitro*, this leads to a reduced GnRH-inducible LH release, but *in vivo* to increased LH plasma levels. It is very unlikely that the impaired testicular development is due to an effect of cortisol on LH secretion.

Introduction

Adaptation to severe and chronic stress has been shown to interfere with processes such as growth, immune response or reproduction. In fish, the response to stress has many similarities to that of higher vertebrates, as it leads to an activation of the hypothalamic-pituitary-interrenal (HPI) axis, the equivalent of the mammalian hypothalamic-pituitary-adrenal (HPA) axis. In teleost fish, cortisol is the main glucocorticoid produced by the interrenals under stress adaptation.

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). Spermatogenesis was inhibited by the cortisol treatment and lower plasma 11-ketotestosterone (11KT) levels accompanied this.

In both mammals and fish, a decrease in plasma LH has been correlated to stress- and cortisol-induced adverse effects on reproduction (Carragher *et al*., 1989; Tilbrook *et al*., 1999). Indeed, cortisol may affect LH secretion directly, since glucocorticoid receptors (GRs) have been demonstrated in the pituitary, co-localized with the gonadotrophs (Teitsma *et al*., 1999). However, there is neither evidence for a direct effect of cortisol on gonadotropin, nor for an inhibition of testicular development under conditions of stress or cortisol treatment due to decreased LH secretion. In mammals it has been demonstrated that corticosteroids inhibit the GnRH-induced LH release by inhibiting the responsiveness to GnRH (Padmanabhan *et al*., 1983). But it has also been shown that the inhibitory effects on the LH release are caused by a suppression of the hypothalamic GnRH release (Rosen *et al*., 1988).

The aim of this study is to investigate if the inhibition of testicular development under elevated cortisol levels is mediated by an effect of cortisol on LH secretion.

Materials and Methods

Animals

Isogenic male common carp (*Cyprinus carpio* L.), 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 63 dph when the experiment started.

Steroid treatment

Cortisol (Steraloids Inc. Wilton, USA) containing food (100 mg/kg food) was prepared as described by Pickering *et al*. (1987b). One hundred and twenty animals, 63 dph, were equally divided over two groups. One group received control food, the other group the cortisol-containing food. 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 both groups (n=20) were sampled at several time-intervals during the pubertal development, at 94 (early puberty), 100 (late puberty) and 120 dph (pubertal development completed). 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 / (body-weight-testis weight)).

Pituitary incubations

Twenty pituitaries per group were collected individually. Ten pituitaries per group were pre-incubated for 18 h in L-15 medium (15 mM HEPES buffered, pH 7.4, 26 mM sodium bicarbonate, 100,000 U/l penicillin/streptomycin) containing 5% horse serum, whereas the remaining ten pituitaries were pre-incubated in the same medium containing dexamethasone (Sigma, St. Louis, USA) (150 ng/ml medium). The pituitaries were rinsed once and 0.5 ml fresh L-15 medium (without or with dexamethasone, respectively) 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 medium (without or with dexamethasone, respectively) containing 10 nM sGnRHa 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.

Plasma, medium and pituitary LH determination

Luteinizing Hormone (LH) was quantified in the plasma, incubation medium and the pituitaries using a homologous radioimmuno assay (RIA) (slightly modified from Goos *et al.*, 1986). Twenty pituitaries per treatment group were individually homogenized and assayed. Plasma LH levels were measured in all animals. For standards and iodine labeling, purified carp LHβ subunit (a gift from Dr. E. Burzawa-Gérard) was used and anti-LHβ (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 testosterone were measured in a RIA as described by Schulz (1985).

Statistics

All results are expressed as mean ± 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

Gonadosomatic index (GSI)

The increase in gonadosomatic index (GSI), observed in the control animals reflects the normal testicular development during puberty. In contrast, feeding pubertal fish with cortisol containing food pellets resulted in an impaired testicular development, reflected by a lower gonadosomatic index at 100 dph and 120 dph. (Fig. 1A)

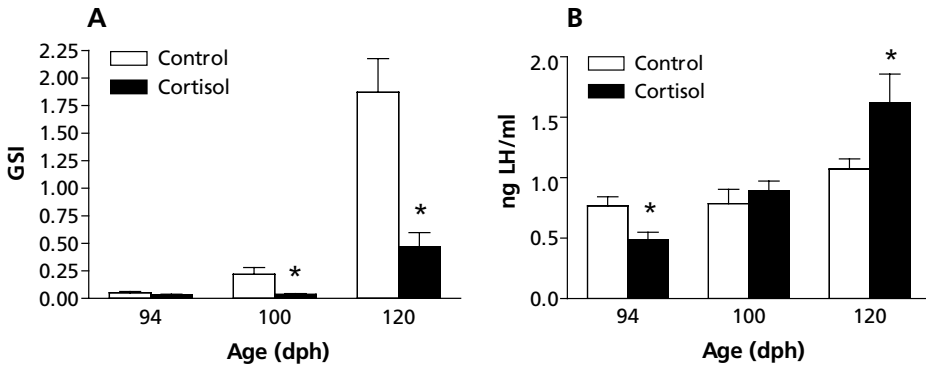

Figure 1. Effect of cortisol treatment on (A) gonadosomatic (GSI) index (n=20) and (B) plasma LH levels (n=20). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$, Student's T-test).

Pituitary LH content, sGnRHa-stimulated LH secretion in vitro and plasma LH levels

Pituitary LH content increased significantly during pubertal development. At 94 dph no significant difference was observed between control and cortisol treated fish. However, at 100 dph there is a slight difference (only significant at $p<0.1$) whereas at 120 dph the LH content of the control fish is significantly higher than in the cortisol treated fish (Fig. 3A).

In both control and cortisol treated animals the *in vitro* sGnRHa-induced LH release was stimulated by 10 nM sGnRHa (Fig. 2). Neither cortisol treatment, nor the addition of dexamethasone to the incubation medium had a con-

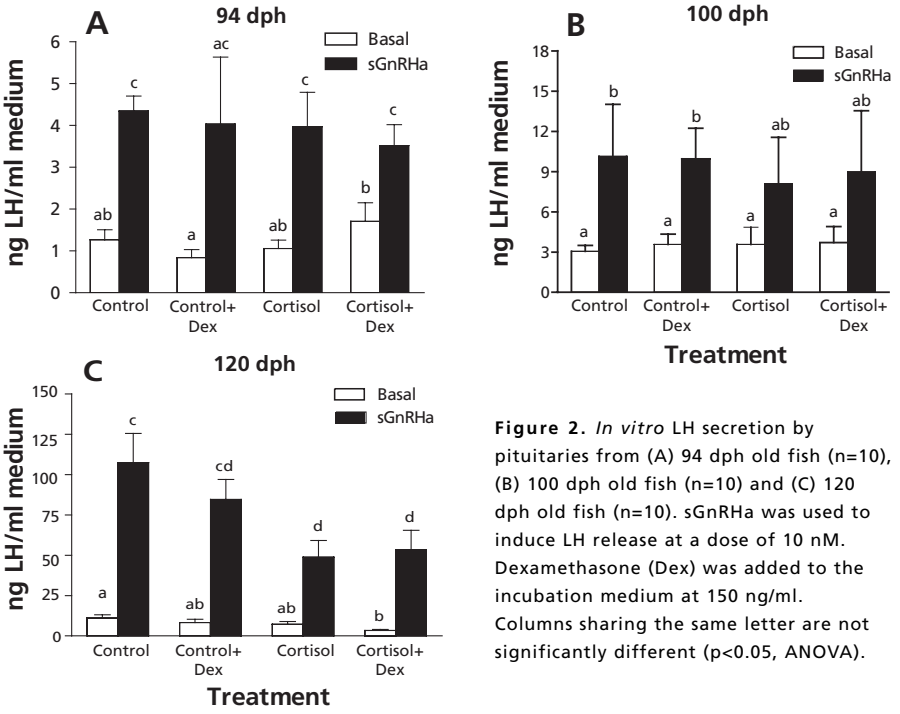

Figure 2. *In vitro* LH secretion by pituitaries from (A) 94 dph old fish (n=10), (B) 100 dph old fish (n=10) and (C) 120 dph old fish (n=10). sGnRHa was used to induce LH release at a dose of 10 nM. Dexamethasone (Dex) was added to the incubation medium at 150 ng/ml. Columns sharing the same letter are not significantly different ($p<0.05$, ANOVA).

sistent effect on basal LH release. Salmon GnRHa-stimulated LH release was unaffected by the *in vivo* cortisol treatment at 94 and 100 dph, but was significantly decreased at 120 dph. The addition of dexamethasone had no significant effect on the sGnRHa-stimulated release. It seems that the sGnRHa-stimulated secretion reflects the amount of LH in the pituitary.

Prolonged feeding with cortisol resulted in increased plasma LH levels at 120 dph (Fig. 1B).

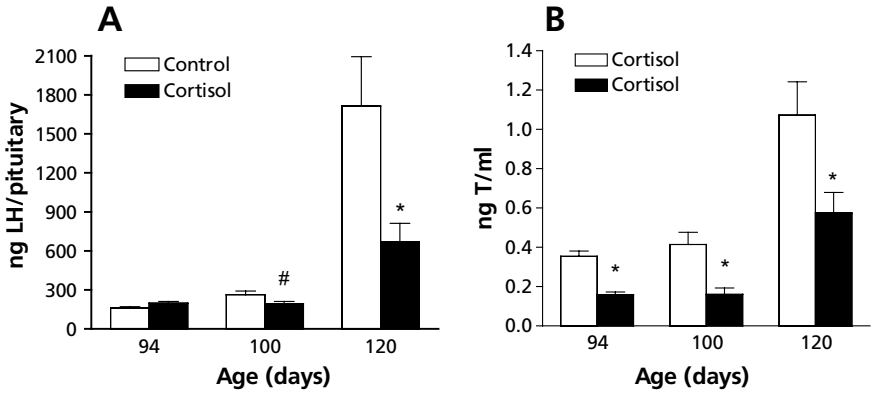

Figure 3. Effect of cortisol treatment on (A) pituitary LH content (n=20) and (B) plasma testosterone levels (n=20). * indicates a significant difference between the control group and the cortisol treated group ($p<0.05$, Student's T-test). # indicates a difference of $p<0.1$.

Plasma testosterone levels

Treatment of pubertal fish with cortisol resulted in significantly lower plasma levels of testosterone at all sampling days (Fig. 3B).

Discussion

The elevation of cortisol levels as a consequence of adaptation to stress is generally accepted as the main initial factor in the cascade of events that lead to disruption of processes like growth, immune capacity and reproduction. In earlier studies we have shown that testicular development in juvenile common carp was inhibited by chronic stress, induced by repeated changes in water temperature (chapter 2). Physiological adaptation to this stressor was accompanied by the elevation of cortisol levels (Tanck *et al*., 2000). In a following study, it was demonstrated that prolonged elevation of plasma cortisol levels in male juvenile common carp, indeed resulted in a retardation of testicular development, and a decrease of 11-oxygenated androgen plasma levels, assumed to be involved in the induction of spermatogenesis (Consten *et al*., 2001a).

The aim of the present investigation was to elucidate if the pituitary gonadotropin secretion mediates the effect of cortisol, and if so, whether the effect of cortisol on the pituitary is direct or indirect.

Cortisol treatment in the present study again caused a retardation of pubertal testicular development. Although cortisol treatment had an effect on pituitary LH, the inhibition of testicular development was unlikely to be caused by LH. Indeed, plasma LH levels were either not affected (at 100 dph), or even increased at 120 dph. The somewhat lower LH plasma levels at 94 dph should, however, not be neglected, in particular as they have been observed in other studies. Treatment of maturing male rainbow trout with cortisol significantly suppressed plasma gonadotropin levels (Carragher *et al*. 1989).

The pituitary incubations in the present study showed that at 120 dph the LH secretory capacity of the cortisol treated fish was lower compared to controls. Although basal LH secretion was unaffected, the sGnRHa-stimulated secretion was inhibited by the long-term cortisol treatment. Dexamethasone had only a small, non-significant, effect on both basal and sGnRHa-stimulated LH secretion, which may lead us to the suggestion that cortisol does not directly influence the secretion of LH from the pituitary of common carp.

In the present study, pituitary LH significantly increased during sexual maturation. Likewise, Schulz *et al*., 1997) observed the morphological and functional development of the gonadotrophs in the African catfish during puberty. In immature African catfish, treatment with testosterone increased the pituitary LH content (Cavaco *et al*., 1995) and Cavaco *et al*. (1997) showed that testo-

sterone is indeed produced by the premature testis. Moreover, castration slowed down gonadotroph maturation, a process that could be restored by testosterone replacement (Cavaco *et al*., 1998c). Earlier, Schulz *et al*. (1997) hypothesized that testosterone may be the testicular signal for gonadotroph maturation during pubertal development.

In the present study, we found lower pituitary LH contents in cortisol treated fish, which was first observed at 100 dph and became pronounced at 120 dph. Throughout the experiment, plasma testosterone levels were lower in cortisol treated fish. In several other studies it has also been shown that elevated cortisol levels lead to a decrease of plasma testosterone (e.g. Carragher *et al*., 1989) and our previous work showed that cortisol directly inhibits the secretion of androgens from the testis (Consten *et al*., 2000). Based on these observations, we hypothesize that cortisol resulted in a decrease in testosterone secretion, which may be the reason for an impaired gonadotroph maturation. However, we cannot exclude an effect of cortisol on the synthesis and storage of LH directly, since intracellular glucocorticoid receptors in gonadotrophs of fish have been demonstrated (Teitsma *et al*., 1999). The difference in plasma LH levels between control and cortisol treated animals was not apparent before 120 dph. Data on pituitary LH content and the *in vitro* LH release show that the LH releasable pool probably was not different in controls and cortisol treated animals before 120 dph.

Testosterone has been shown to potentiate the gonadotropin release response to GnRH in several fish species. Trudeau found that implantation of testosterone in goldfish (Trudeau *et al*., 1991a), common carp and Chinese loach (Trudeau *et al*., 1991b) increased the GnRH-stimulated gonadotropin secretion *in vivo*. In pubertal African catfish testosterone implantation resulted in an increase in pituitary LH content and reduced plasma LH levels, but a significant increase of the sGnRHa-stimulated LH secretion *in vitro*. This supports the concept that testosterone induces pubertal gonadotroph maturation, including LH expression and storage, and by consequence an increased GnRH-stimulated LH release. In the present study, elevated cortisol levels caused reduced testosterone levels and a decrease in pituitary LH content. This may correspond to a smaller LH releasable pool and consequently to a reduced sGnRHa-stimulated LH secretion *in vitro*. A direct effect of cortisol on gonadotroph sensitivity cannot be ruled out by the present data.

Our earlier studies have shown that cortisol not only affects testicular testosterone secretion, but also 11-ketotestosterone secretion, which is involved in the pubertal onset of spermatogenesis. Since plasma LH levels after prolonged cortisol treatment were not decreased, but even elevated at the end of the experiments, it is unlikely that LH is involved in the retardation of testicular development. Based on the present and earlier results we suggest that the reduced

steroid hormone secretion by the pre-pubertal testis not only had its effects on the maturation of pituitary gonadotrophs, but also on testicular development. Current experiments, combining cortisol treatment with a replacement of testicular steroid hormones (testosterone and 11-oxygenated androgens) may solve these questions.

Reduced testosterone levels may be the reason for the increase in plasma LH levels as observed at the end of this experiment. As we rule out LH to be involved in the observed retardation of testicular development, it is unfortunate that we were unable to collect information on the second gonadotropic hormone FSH. Van der Kraak *et al*. (1992) has demonstrated its presence in the common carp. However, its expression pattern and its specific functions are unknown. Moreover, a specific assay to quantify this hormone is not available.