Cortisol mediates the inhibitory effect of cold shock stress on pubertal development in male common carp, *Cyprinus carpio* L.

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Abstract

Prolonged stress has been shown to interfere with other processes like growth, immune response or reproduction. Stress leads to an activation of the hypothalamic-pituitary-interrenal (HPI) axis and this results in the end to an increase in cortisol secretion.

Cortisol plays a key role in the homeostatic adaptation during or after stress and is frequently indicated to be the major factor mediating the suppressive effect of stress on reproduction. The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis and stress has been shown to interfere with the functioning of the BPG-axis. In the present study we exposed immature common carp to repeated temperature stress. This stressor has been shown to cause elevated cortisol levels. Our results demonstrate that repeated temperature stress leads to a retardation of testicular development, reflected by the gonadosomatic index and the first wave of spermatogenesis, and a coinciding decrease of the 11-oxygenated androgen levels in the plasma. Furthermore, we could completely reverse the temperature stress-induced reduction in testicular growth by the concomitant treatment with RU486, a cortisol antagonist, indicating that the detrimental effects of stress on testicular development are mediated by cortisol.

Introduction

Stress is a ubiquitous feature of vertebrate life and may be defined as a disturbance of the homeostatic state of an organism by any kind of external or internal factor, referred to as the stressor. Prolonged stress has been shown to interfere with other processes like growth, immune response or reproduction. In fish, as in higher vertebrates, stress has been shown to cause an activation of the hypothalamic-pituitary-interrenal (HPI) axis, the equivalent of the mammalian hypothalamic-pituitary-adrenal (HPA) axis. This activation leads in the end to an increase in glucocorticoid secretion. In teleost fish, cortisol is the main glucocorticoid produced by the interrenals under influence of stress (Barton &

Iwama, 1991). Stressors such as handling and netting, common procedures in aquaculture, result in elevated levels of cortisol in turbot (Mugnier *et al.*, 1998), salmonids (Sumpter *et al.*, 1986; Sharpe *et al.*, 1998), and common carp (Weyts *et al.*, 1997). Rapid changes in water temperature are among the stressors with a high physiological impact on fish and previous results showed that cold shock stress caused an elevation of the cortisol levels in common carp (Tanck *et al.*, 2000). Cortisol plays a key role in the restoration of homeostasis and is frequently indicated to be the major factor mediating the suppressive effect of stress on reproduction. Treatment of brown trout, *Salmo trutta* L., and rainbow trout, *Salmo gairdneri* Richardson, with cortisol showed that a wide range of reproductive parameters were affected (Carragher *et al.* 1989).

Puberty is the period covering the transition from an immature, juvenile to a mature, adult state of the reproductive system. More precisely, it may be defined as the developmental period that spans the onset of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis. Stress has been shown to interfere at all levels of the BPG-axis (Rivier & Rivest, 1991) and also effects of cortisol can be observed throughout the BPG-axis.

The aim of the present experiments was (1) to demonstrate that repeated temperature stress affects pubertal development and (2) to investigate if the adverse effects of stress on reproduction are mediated by cortisol. As experimental animals we used isogenic male common carp.

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 at Department of Fish culture and Fisheries (Agricultural University, Wageningen, The Netherlands). At 21 days post hatching (dph) the fish were moved into the experimental aquaria and allowed to acclimatize till 60 dph after which the experiment started. The fish were kept at 25°C in a recirculation system, exposed to a 12:12 hours light-dark regime and fed daily pelleted dry food (Provimi, 91 series, Rotterdam, The Netherlands).

Cold shock stress

Stress was administered to the fish by lowering the water temperature from 25°C to 14°C as described by Tanck *et al.* (2000).

Experiment 1:
Repeated cold shock stress

Two hundred animals were divided over two groups. One group served as a control group whereas the other group received repeated cold shocks. Fish were submitted to cold shock stress 3 times a week, randomly divided over the week. At regular time intervals, covering the pubertal development of the common carp, 20 fish per group were sampled.

Experiment 2:
Repeated cold shock stress in the presence of RU486

Two hundred forty fish were divided over four groups. The first group served as a control. The second group received repeated cold shocks similar to Experiment 1. In order to investigate the role of cortisol during the cold shock stress the remaining two groups, of which one served as an control and the other was exposed to the repeated cold shocks, were implanted with cocoa butter containing a cortisol antagonist, RU486 (Sigma, St. Louis, USA). For this, Malaysia cocoa butter (a gift from drs. H. Kattenberg, ADM-Cocoa, Koog aan de Zaan, The Netherlands) was melted at a temperature not higher than 37.5°C so it will solidify within the bodycavity of the fish, even if the fish are kept at 25°C (van Malssen et al. 1996). The RU486 (50 mg/kg fish) was suspended in molten cocoa butter and fish were implanted with the cocoa butter by injecting 100 μl per 20 g fish with a 1 ml syringe (needle: 21Gx1½"). At regular time intervals, covering the pubertal development of the common carp, 20 fish per group were sampled.

Sampling

Fish were sampled at the onset of the experiment, 60 dph, and at several time-intervals during the period of pubertal development, 89, 94, 100 and 105 dph. Furthermore, at the onset of the experiment, 60 dph, a start control group was 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. Pituitaries were taken, snap frozen in liquid nitrogen and stored at –80°C until use for hormone measurements by means of radioimmunoassay. 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 spermatogenic stages, testis tissue of 10 fish per control and cortisol treated group, respectively, was processed for histology. Spermatogenesis was subdivided into four stages according to Cavaco *et al.* (1997). In short: stage I – spermatogonia only; stage II – spermatogonia and spermatocytes; stage III – spermatogonia, spermatocytes and spermatids; and finally stage IV – all stages of germ cells including spermatozoa.

Steroid Radioimmunoassays (RIA)

Plasma levels of cortisol were determined by radioimmunoassay according to de Man *et al.* (1980) and Van Dijk *et al.* (1993). The plasma levels of the steroids 11KT and OA 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, 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 produced by the testes (Komen, personal communication).

Plasma and pituitary LH

Luteinizing Hormone (LH) was quantified in the plasma and the pituitaries of common carp using a homologous RIA (slightly modified from Goos *et al.* (1986)). Ten pituitaries per treatment group were individually homogenized and assayed. Plasma LH levels were measured in all animals. As standards and label 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 also been demonstrated (Van Der Kraak *et al.*, 1992). However, a FSH specific assay is not available.

Statistics

All results are expressed as mean $\pm$ SEM. Plasma levels of the different steroids are given as ng per ml plasma. 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

Experiment 1: repeated cold shock stress

The increase in gonadosomatic index (GSI), observed in the control animals reflects the normal testicular development during puberty. Exposure of pubertal fish to repeated cold shock caused a retardation of the testicular development. This is shown by a lower gonadosomatic index of stressed fish at 94 dph (Fig. 1A). The histological analysis of the testes confirms that this is due to a retardation in spermatogenesis (Fig. 1B). At 100 dph and 105 dph a significant difference is no longer observed.
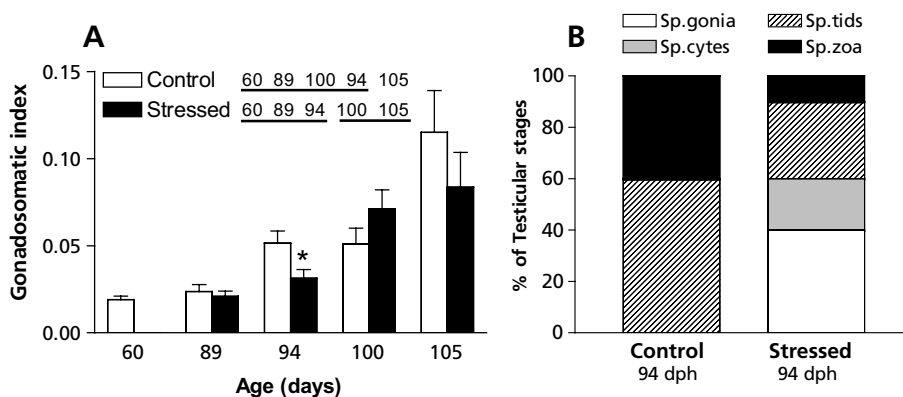

Figure 1. Effect of repeated temperature stress on testicular development, represented by (A) the gonadosomatic index (n=20) and (B) testicular stage (n=10). * indicates a significant difference between the control group and the stressed group (p<0.05). Data sharing the same underscores in the legends are not significant different.

Hormone measurements

Plasma cortisol measurements show that stressed fish have initially higher plasma cortisol levels and these level of as the experiment continues (Fig. 2A). No significant difference (p<0.05) is observed in plasma 11KT levels at 89, 100 and 105 dph (Fig. 2B). However, at 94 dph plasma 11KT levels are significantly lower in stressed fish. Similar results can be observed in plasma OA levels, where also at 94 dph stress leads to significantly lower plasma levels (Fig. 2C). Plasma LH levels and pituitary LH content were not different between stressed and control fish (data not shown)

Experiment 2: repeated cold shock stress in the presence of RU486

Similar to experiment 1 the GSI of control animals increased during pubertal development and the same is found for cold shock stressed fish (Fig. 3A).
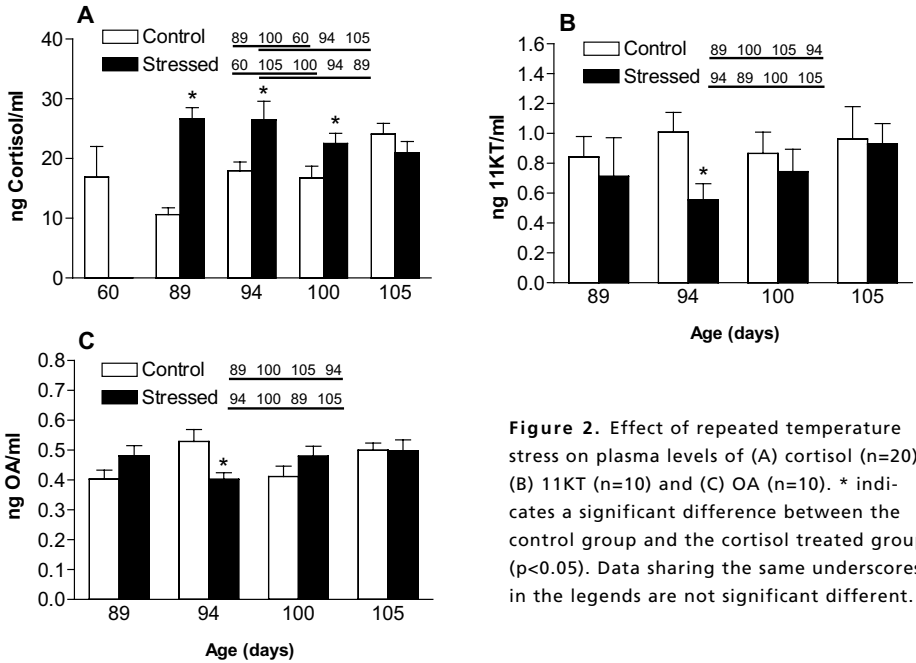
Figure 2. Effect of repeated temperature stress on plasma levels of (A) cortisol (n=20), (B) 11KT (n=10) and (C) OA (n=10). * indicates a significant difference between the control group and the cortisol treated group (p<0.05). Data sharing the same underscores in the legends are not significant different.

Again a retardation of testicular development is observed and this is significant at 95 dph. Prolonged treatment of stressed fish with the cortisol antagonist RU486 prevented the decrease in GSI at 95 dph. At 100 dph. the tendency is the same though the differences are not significant. Histological analysis of the testes show again that in stressed fish spermatogenesis is retarded, whereas in RU486–treated stressed fish show normal spermatogenesis (Fig. 3B).
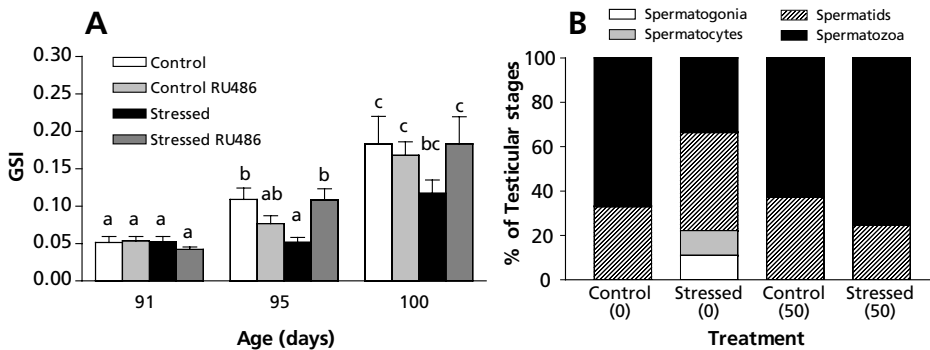

Figure 1. Effect of repeated temperature stress on testicular development, represented by (A) the gonadosomatic index (n=20) and (B) testicular stage (n=10). * indicates a significant difference between the control group and the stressed group (p<0.05). Data sharing the same underscores in the legends are not significant different.

Discussion

The aim of the present study was to demonstrate that chronic stress, induced by repeated temperature stress, affects pubertal development and that this effect is mediated by cortisol. Indeed, exposure to repeated cold shock stress had an inhibitory effect on pubertal testicular development. This effect could be prevented by administration of the cortisol antagonist RU486.

In males, the initiation of puberty is marked by the onset of spermatogenesis. Our results show that repeated exposure to temperature-induced stress adaptation leads to higher basal cortisol levels and a retardation of the testicular development during puberty, reflected by the lower GSI and the less advanced spermatogenic stages at 94 dph. However, from 100 dph onwards there is no longer a difference between control and stressed fish. Tanck *et al*. (2000) showed that in the same fish, at 120 dph habituation to the cold shocks occurred. In fish exposed to multiple cold shocks lower cortisol levels were found during exposure compared with a group that received at 120 dph the first temperature shock. Probably due to this phenomena we observe a recovery of the testicular growth from 100 dph onwards, although at 100 dph the basal cortisol levels are still slightly increased.

Adverse effects of stress on testicular development have been demonstrated in several studies, on a variety of experimental models. Siberian dwarf hamsters, submitted to separation stress had increased resting plasma cortisol levels and a decreased testis and seminal vesicle mass (Castro & Matt, 1997). A study, examining the annual cycle of testes weight of adult male rabbits of three populations in the sub-Antarctic Kerguelen archipelago, demonstrated that the population living under the most adverse environmental conditions had a deferred testis growth (Boussès & Chapuis, 1998). In rainbow trout (*Salmo gairdneri* Richardson), subjected to repeated acute stress, the quality of gametes produced by both sexes was reduced. Males had a significantly lower sperm count whereas females had a delayed ovulation and a reduced egg size. More importantly, the offspring from these stressed fish had significantly lower survival rates (Campbell *et al*., 1992).

The increased cortisol secretion during stress has frequently been associated with the subsequent decrease in reproductive capacity. Also in this experiment we observed chronically elevated cortisol levels. The role of cortisol on the reproductive capacity has been investigated in several studies. Treatment of male *Labeo gonius* with hydrocortisone acetate during the spawning season, inhibited maturation and spermiation. The volume of the testes and the gonadosomatic index were reduced (Joshi, 1982). In brown trout, *Salmo trutta* L. and rainbow trout, implantation of cortisol releasing pellets chronically elevated the plasma cortisol levels and affected a wide range of reproductive parameters.

Cortisol-implanted maturing male brown trout had smaller gonads and lower plasma testosterone levels (Carragher *et al*., 1989).

On plasma 11KT levels, we observed a clear effect of the temperature stress at 94 dph, whereas at 89, 100 and 105 dph the levels are not significantly lower. Similar results are obtained from the plasma OA levels. A reduction in plasma sex steroids, due to stress or cortisol, has been reported for a variety of vertebrate species (mammals: Norman & 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 African catfish, it has been shown by Cavaco *et al*. (1998b) that 11KT has an important stimulatory effect on spermatogenesis during sexual matura-tion. Furthermore, in Japanese eel spermatogenesis can be completed *in vitro* by 11KT administration to the medium (Miura *et al*., 1991). Also in the common carp, 11-oxygenated androgens have been shown to promote spermatogenesis during puberty (Komen, personal communication). If the lower 11KT levels observed in the present experiment contribute to the retardation of the testicu-lar development during puberty remains to be investigated. A more direct effect of corticosteroids is also a possibility, since in rat the glucocorticoid receptor has been localized on different types of germ cells, indicating that corticosteroids may directly inhibit spermatogenesis Schultz *et al*., 1993; Weber *et al*., 2000).

In order to elucidate the role of cortisol as a mediator of the adverse effects of stress on reproduction, we implanted fish that were exposed to repeated tem-perature stress with the cortisol antagonist, RU486 (mifepristone). RU486 has been proven to bind with high affinity to the glucocorticoid receptor (reviewed by Cadepond *et al*., 1997). In mammals, several studies have shown that RU486 can be used to attenuate stress-induced effects. For example, exposure of male rats to acute and chronic immobilization stress leads to a reduction of the pitui-tary LH release. Systemic treatment with RU486 significantly attenuated this decline of circulating LH (Briski *et al*., 1995). This effect is in mammals medi-ated via the type II glucocorticoid receptor (Briski *et al*., 1994). Furthermore, Orr & Mann (1992) investigated the role of glucocorticoids in the stress-induced inhibition of testicular steroidogenesis in male rats by examining the effect of *in vivo* treatment with RU486. Immobilization reduced plasma testo-sterone (T) levels without affecting the LH levels. This reduction could partial-ly be reversed by *in vivo* injections of RU486, prior to the stress. Similar results were observed *in vitro*, both corticosterone and dexamethasone inhibited the hCG-stimulated T production and co-incubations with RU486 reversed this glucocorticoid-induced suppression.

In fish, RU486 has also been used as a potent antagonist of glucocorticoid action. In brook charr, *Salvelinus fontinalis*, RU486 treatment increased the liver glycogen content and prevented the handling stressor-related elevation of

plasma glucose levels (Vijayan & Leatherland, 1992). In rainbow trout, cortisol enhanced the metabolic potential of hepatocytes. RU486 treatment blocked the cortisol–induced increases in alanine gluconeogenesis and glycogen utilization for endogenous use (Vijayan *et al*., 1994). Also in the common carp, the species used in our study, RU486 has been proven to be a potent anti–glucocorticoid. Weyts *et al*. (1998) used RU486 to block the cortisol–induced apoptosis on carp peripheral blood leukocytes *in vitro*. These studies provide validation for the use of RU486 as a potent glucocorticoid antagonist in fish. In our experiment, implantation of RU486 completely reversed the stress–induced reduction in testicular growth at 95 dph, indicating that, indeed, cortisol mediates the adverse effects of stress on reproduction. Similar to experiment 1, we found no longer a difference in testicular development at 100 dph, indicating that the effect of habituation, as observed by Tanck *et al*. (2000), is consistent.

In summary, our experiments demonstrate that exposure to repeated temperature stress causes a delay in pubertal development and this inhibitory effect of stress is mediated via cortisol. The present results are not sufficient to elucidate whether cortisol acts directly or indirectly on spermatogenesis. Current investigations are designed to answer this question.