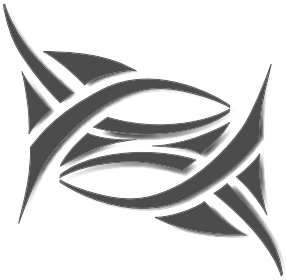
Chapter 8

Summarizing discussion



Summarizing Discussion

Every organism is subjected to stress in its day to day life. Environmental conditions, such as extreme temperatures, irregular food supply, heavy physical exercise, but also psycho-physical factors as being threatened by predators, crowding or domination by congeners may disturb the fine tuned internal balance, called homeostasis. Several definitions for stress exist (Barton & Iwama, 1991). In this study, we considered stress as any influence from the environment (the stressor) that disturbs homeostasis. All organisms have the capacity to respond to stress with physiological mechanisms in order to restore the disturbed homeostasis, during or after stress.

Several concepts on the physiological response to stress have been proposed as described in the general introduction but these concepts may be combined into one. In general, the stress response can be divided in three distinct phases. In the primary response, when an organism is suddenly confronted to a critical situation, the brain responds upon recognition of the stressor. This results in an activation of the hypothalamic-pituitary-interrenal (HPI) axis and as a consequence the release of stress hormones (catecholamines and corticosteroids). The secondary response is defined by the immediate actions and effects of these hormones. The organism tries to adapt to the altered conditions in order to meet the requirements of the new situation and to restore its homeostatic state. If the stress persists and the organism is not able to compensate, the final phase occurs, the phase of exhaustion or the tertiary response. This phase is mainly maladaptive and requires much energy, forcing the organism to make strategic choices in order to save its most vital functions. Energy that is normally available for processes like growth, immune response or reproduction may now be channeled into restoration of the disturbed homeostasis. Steroid hormones, cortisol being the most important one, play a key role in this homeostatic adaptation. In fish, as well as in all other vertebrates, stress has been shown to interfere with physiological processes such as growth, immune function and reproduction (Wendelaar Bonga, 1997). Especially in aquaculture, fish experience a number of different stressors from environmental or human origin, all affecting the well-being of the fish. This may result in suppression of the immune capacity, leading

to infectious diseases. This not only has its effect on the well-being of the fish, but also may lead to considerable economic losses.

Likewise, the reproductive performance is often affected by stress. There are numerous examples of animals in zoos, companion animals, but also from fish farming industry, showing that stress caused by captivity, overcrowding, false light regimes or any other factor has adverse effects on reproduction. The precise mechanisms via which the stress response affects reproduction are not known. The present study is an attempt to answer this intriguing question.

This project was part of a large, NWO-supported research program, directed towards questions as how stress activates the HPI-axis in fish, via which mechanisms the immune response is suppressed and whether in a given fish species there is genetic variation in the stress response. In all these projects, the same experimental animal was used: the isogenic male common carp, obtained by reproductive cloning. As stressor a temperature shock was used. At unexpected times, the fish were subjected to a sudden fall in water temperature of 11°C. In the present study, we focussed on the effect of stress on pubertal development of the male common carp. Juvenile, sexually immature male carp were exposed to repeated temperature stress. We could demonstrate an increase in cortisol secretion as part of the stress response. Long-term exposure to temperature shocks also caused an inhibition of testicular development. These results provided us with a model for studying the effects of cortisol on the pubertal development and to investigate which parts of the brain-pituitary-gonad (BPG) axis, the neuro-endocrine system of prevailing importance for reproduction, are affected by cortisol.

Stress, cortisol and the effects on pubertal development

(Chapter 2 and 3)

The developmental period during which the animal acquires the capacity to reproduce is defined as puberty. In our studies, we defined pubertal development as the time span that starts with the beginning of spermatogonial proliferation and ends when flagellated spermatozoa appear in the testis.

In a preparatory study (Tanck *et al.*, 2000), we showed that cold shock stress caused an elevation of the cortisol levels, indicating that, indeed, temperature stress elicits a stress response in the animal. In chapter 2, we showed that repeated temperature stress caused a chronic elevation of plasma cortisol levels. Furthermore, fish exposed to repeated temperature stress show a retardation of the testicular development during puberty. The growth of the testis is impaired as reflected by a lower gonadosomatic index. Histological analysis of the testis revealed that this is a consequence of an impaired spermatogenesis as indicated

by the presence of less advanced spermatogenetic stages in the testis of stressed fish. Based on these results we concluded that, indeed, temperature stress elicits a stress response and, if chronic, leads to a tertiary response, that affects the pubertal development of male common carp.

In order to investigate if indeed it is cortisol that mediates the suppressive effects of stress, we once more exposed fish to repeated temperature stress, but now treated them (by implantation) with the cortisol antagonist, RU486 (mifepristone). RU486 prevented the temperature stress-induced reduction in testicular growth. This indicated that cortisol is indeed responsible for the adverse effects of stress on pubertal testicular development.

Pubertal sexual maturation is associated with development to functional competence of the brain-pituitary-gonad (BPG) axis. This neuro-endocrine system, as well as the concept of puberty, has been described in the general introduction (chapter 1). In short, the brain integrates information from external and internal sources, leading to a coordinated synthesis and release of neurohormones. Probably gonadotropin-releasing hormone, GnRH and dopamine, (DA) are the key players. They control the synthesis and release of gonadotropic hormones (luteinizing hormone, LH and follicle-stimulating hormone, FSH) from the pituitary. These hormones reach the gonads via the circulation where, in general terms, LH stimulates the production and release of sex steroids and FSH controls gamete development. The sex steroids contribute to gamete development and control the development of secondary sexual characteristics and sexual behavior. Furthermore, the gonadal sexual steroids exert direct or indirect feedback effects on the pituitary and on the brain.

Stress effects have been reported to affect all levels of the BPG-axis. The aim of the subsequent studies was to investigate which level of the BPG-axis is affected and what mechanisms are involved.

During the chronic temperature stress experiments, the observed inhibition of testicular development was accompanied by lower plasma levels of the 11-oxygenated androgens: 11-ketoandrostenedione (OA) and 11-ketotestosterone (11KT). 11KT exerts an important function during sexual maturation and the stimulation of spermatogenesis (Miura *et al*., 1991; Cavaco *et al*., 1998b). In the goldfish, a close relative of the common carp, 11KT has been shown to induce spermatogenesis (Kobayashi *et al*., 1991) and also in the common carp, 11β-hydroxyandrostenedione, the precursor for 11KT has been shown to promote testicular development and spermatogenesis (Komen, personal communication). These observations suggest that the stress-induced suppression of the first wave of spermatogenesis may be a consequence of reduced plasma 11KT levels.

Since we showed that the temperature stress-induced response on testicular development is mediated by cortisol, we mimicked temperature stress by feeding the fish with cortisol containing food pellets (chapter 3). As expected, this resulted in a similar retardation of pubertal development as in the studies

described in chapter 2. These results provided us with an easy and much cheaper model to study the effects of stress on the different components of the BPG-axis. It appeared that all components of the brain-pituitary-gonad axis were affected by the cortisol treatment. On the hypothalamic level we noticed a reduction of the sGnRH content (salmon GnRH is the native GnRH for the common carp). On the pituitary level, the LH and FSH encoding mRNA levels and pituitary LH content were diminished. Plasma LH levels were slightly diminished. However, in subsequent experiments we showed that the plasma LH levels did not change consistently. Again, the androgen metabolism was influenced, reflected by reduced plasma levels. Once more, this suggested a causal relation between the observed retardation in testicular development and the depressed plasma levels of 11KT. Furthermore, in this chapter we also suggest that the observed decrease in brain sGnRH content and the effects on the LH and FSH encoding mRNA levels may be related to the impaired androgen secretion. However, we did not observe a consistent effect on plasma LH levels, which suggests that the effect of cortisol on the testis is probably not via LH. Cortisol certainly had an effect on the pituitary and hypothalamus, but the connection to testicular development is not yet clear.

Thus in summary, these two chapters demonstrate that repeated temperature stress leads to an impairment of the testicular development and this is mediated by cortisol. Furthermore, cortisol effects were observed at all levels of the BPG-axis. We were left, however, with the question whether cortisol acts directly or indirectly on the different parts of the BPG-axis.

Cortisol effects on pituitary and testis

(Chapter 4, 5 and 6)

In an attempt to solve that question, we designed experiments to study the direct effects of cortisol on the pituitary and the testis. Fish were again treated with cortisol and the pituitary and testis were incubated *in vitro* and tested for their response to GnRH or LH, respectively. Acute and direct effects of cortisol on testis and pituitary were investigated by performing incubations in the absence or presence of dexamethasone, a non-metabolizable cortisol agonist. The experiments on the pituitary, described in chapter 4, demonstrated that cortisol caused a decrease in pituitary LH content and consequently reduced the sGnRHa-stimulated LH secretion *in vitro*. Testosterone has been shown to induce development of pituitary gonadotrophs and a stimulation of the LH gene transcription, leading to an increase in LH content and GnRH-inducible LH release (Cavaco *et al*., 1995, 1998d; Rebers *et al*., 2000; Teves, personal communication). In combination with the observation that dexamethasone did not have any influence on the *in vitro* LH release, it was concluded that cortisol does not

directly influence the secretion of LH from the pituitary but that the decrease in testosterone secretion may be the reason for the impaired LH synthesis. However, we cannot exclude a direct effect of cortisol on the synthesis and storage of LH, since intracellular glucocorticoid receptors have been demonstrated in gonadotrophs of fish (Teitsma *et al.*, 1999).

In chapter 3, we suggested a causal relationship between the retardation of spermatogenesis and the reduced plasma 11KT levels under the influence of elevated cortisol levels. Whether the reduction in the 11KT production is the consequence of direct effect of cortisol on the testicular androgen production, or an indirect action via the hypothalamic-pituitary gonadotropic system could not be deduced from these results. Therefore, in chapter 5, we have investigated 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. Since plasma LH levels after prolonged cortisol treatment were not decreased, but even elevated at the end of the above described experiment, it is unlikely that LH is involved in the 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) demonstrated its presence in the common carp and revealed that carp LH and carp FSH share the same spectrum of biological activities, causing stimulation of steroidogenesis and inducing final oocyte maturation. However, a specific assay to quantify this hormone is not available and therefore we could not determine the effect of cortisol on the FSH secretion.

However, our results demonstrated that prolonged exposure to cortisol reduced the androgen secreting capacity of the total testes. Both OA and 11KT secretion *in vitro* were significantly reduced, but there is no apparent change in LH sensitivity. This indicates that corticosteroids cause directly an inhibition of the steroid producing capacity of the testis. This may be by a reduced synthesis of enzymes involved in the androgen production or because of a substrate competition for 11β-hydroxysteroid-dehydrogenase (11β-HSD), an enzyme involved both in the conversion of cortisol and OHA (see under). Based on these observations we concluded that cortisol acts directly on the testicular androgen secretion. The underlying mechanism may involve a long-term inhibitory effect on the steroid producing enzymes and/or substrate competition. Since dexamethasone could block the LH-induced increase in steroid secretion *in vitro* of testes taken from control animals, interference of cortisol with the LH signal transduction can not be excluded.

The possibility of substrate competition becomes apparent by taking a closer look to the steroidogenic pathway as described in figure 3 in the general introduction (chapter 1). Both 11β-hydroxyandrostenedione (OHA) and cortisol serve as a substrate for the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). In the fish testis, this enzyme converts OHA into OA, but the same

enzyme is involved in the inactivation of cortisol, by converting it into cortisone. This means that cortisol may inhibit the conversion of 11β-hydroxyandrostenedione (OHA) into OA and, in this way, may contribute to the inhibition of the testicular androgen secretion. Chapter 6 deals with this question. Our *in vitro* results demonstrated that, indeed, cortisol can compete dose dependently with OHA for the enzyme 11β-HSD, thereby reducing the conversion of OHA into OA. This supports the observations in the *in vivo* experiments where we observed an accumulation of OHA in the plasma. However, we do not find subsequent changes in plasma OA and 11KT levels during acute cortisol administration, indicating that the competition for 11β-HSD does not explain the previously observed decrease in plasma 11-oxygenated androgens.

In chapter 6, we showed by *in vitro* testis incubations that the testes of cortisol treated fish have the same potency to convert tritiated steroids per weight unit as untreated control fish. Furthermore, by enzymecytochemistry and subsequent image analysis, we demonstrate that the amount of steroidogenic tissue, reflected by the percentage of 3β-hydroxysteroid dehydrogenase (3β-HSD) active tissue per testicular volume, is equal in cortisol treated animals and untreated animals. However, the total amount testis tissue and thus of steroidogenic tissue, is up to 6.5 times larger in control animals, indicated by the GSI. Based on these data, we conclude that the diminished androgen secretion after long-term cortisol treatment is caused by a general retardation of testis growth, including the steroidogenic elements.

Cortisol-induced suppression of androgen secretion and retardation of pubertal development: correlation or causal relationship?

(Chapter 7)

Elevation of cortisol levels, either by temperature stress or by cortisol administration caused a suppression of plasma androgen levels and a retardation of testicular development. We suggested that androgens could mediate the cortisol effects, because of the numerous observations that androgens, especially 11KT stimulate testicular development and spermatogenesis. All experiments so far, however, did not provide a direct proof for this hypothesis. Therefore we designed an experiment, which is described in chapter 7, to elucidate the importance of the androgens. Cortisol treatment was combined with replacement of the testicular steroid hormones, testosterone or OA, which is converted to 11KT. Although this resulted in a restoration of plasma 11KT levels in the cortisol treated fish, the inhibitory effect of cortisol on testicular development could not be prevented: testicular growth and spermatogenesis were retarded to the

same extend as in cortisol-only treated fish. This suggests that cortisol acts more downstream than 11KT in the stimulatory cascade leading to spermatogenesis. We have no information about the site of action of cortisol, but an effect on developing germ cells may be possible, since we showed the presence of the glucocorticoid receptor mRNA in germ cells by *in situ* hybridization. Considering the effect of cortisol on the pituitary LH secretion, the combined testosterone and cortisol treatment resulted in restoration of the LH pituitary content and the basal and sGnRHa-stimulated LH secretion *in vitro*. In conclusion, cortisol has a direct inhibitory effect on the testis, affecting spermatogenesis downstream of the action of 11KT. The effect of cortisol on the LH secretion seems to be caused by an indirect effect, involving the reduced secretion of testosterone.

In summary

The main results of the present thesis can be integrated, describing how stress may interfere with the functioning of the BPG-axis and thus affect pubertal development. Exposure of immature fish to prolonged temperature stress results in increased plasma cortisol levels and leads to a suppression of testicular development. This inhibitory effect of stress could be blocked by the cortisol antagonist, RU486, indicating that the detrimental effects of stress on pubertal development indeed are mediated by cortisol.

Likewise, cortisol treatment resulted in a retardation of testicular development and gonadotroph maturation and caused a depression of the testicular androgen secretion. As mentioned before, androgens are involved in pubertal development as they model the BPG-axis into a functional, neuro-endocrine entity. Testosterone is implicated in gonadotroph maturation and development of the hypothalamic GnRH system, whereas 11-ketotestosterone serves an important role in the onset of spermatogenesis. Restoration of the reduced plasma 11KT levels, however, did not result in a restoration of the depressed testicular development. This suggests that cortisol acts more downstream than 11KT, possibly by an interaction with the Sertoli cell or even directly on the germ cells, as they have shown to possess the glucocorticoid receptor mRNA. In contrast, the pituitary gonadotrophs are indirectly affected by the cortisol treatment. Cortisol treatment inhibited the gonadotroph maturation and this effect could be restored by concomitant testosterone treatment. We have also observed a lower hypothalamic sGnRH content after cortisol treatment. We hypothesize that this may also be an indirect effect of cortisol, via the reduced testosterone secretion, but we have no data to support this.

Many of the observed effects of cortisol may have been realized via the second gonadotropic hormone, FSH. Since no specific assay was available, we

could not include this hormone in our studies. Moreover, in fish little is known about the role of FSH in spermatogenesis and we are unable to speculate about the importance of this factor in the stress-induced suppression of pubertal development.