Stress response and pubertal development in the male common carp, *Cyprinus carpio* L.

Dimitri Consten

Stress response and pubertal development in the male common carp, *Cyprinus carpio* L.

Stressreaktion og pubertetsudvikling i den mandlige karpe, *Cyprinus carpio* L.

Stress respons en puberteitsontwikkeling in de mannelijke karper, *Cyprinus carpio* L.

(With a summary in English)

(Med en forkortet udgave i Dansk)

(Met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht,
op gezag van de Rector Magnificus, Prof. Dr. W. H. Gispen
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
maandag 23 april 2001 des namiddags te 14.30

door

Dimitri Consten

Geboren op 22 februari 1973, te Utrecht

Give a man a fish and you feed him for a day
Teach him how to fish and you feed him for life.
Confucius

The more experience and insight I obtain into human nature,
the more convinced do I become that
the greater portion of a man is purely animal.
Henry Morton Stanley

Domplein, Utrecht

Contents

Summary for electronic version

Every organism experiences the effects of stress in its day to day life. Stress can be defined as any disturbance of the organism's homeostasis. The internal or external stimulus that causes stress is called the stressor. Usually, the organism is well equipped to adapt to the stress. It has the mechanisms, generally referred to as the stress response, to restore its disturbed homeostasis. However, severe and chronic stress may exceed the ability to adapt and this may then lead to suppression of growth, the immune response or the reproductive performance. In fish, as in higher vertebrates, stress adaptation has been shown to be related to an activation of the hypothalamic-pituitary-interrenal (HPI) axis. In teleost fish, cortisol is the main glucocorticoid produced by the interrenals under influence of stress. 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 the reproductive performance.

This thesis addresses the effect of stress adaptation on the pubertal development. Puberty is the developmental process by which the animal acquires the capacity to reproduce. In males, the period of pubertal development may be defined as the time span that starts with the beginning 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 effects have been reported to affect all levels of the BPG-axis. However, the precise mechanisms via which the stress response has its adverse effects on reproduction are still unknown.

The present study is an attempt to find more answers to solve this intriguing question. Firstly, we investigated the effects of repeated temperature stress on the pubertal development (Chapter 2). Since cortisol has been shown to play a key role in the homeostatic adaptation during or after stress, we focussed in chapter 3 on the effects of cortisol on the pubertal development. In the same chapter we also paid attention to the question at which level the BPG-axis is affected by cortisol. Since our results indicate that all levels of the BPG-axis were affected by cortisol, we studied the cortisol effects on the pituitary and

the testis in more detail (chapter 4 and in chapter 5) and tried to answer the question whether cortisol has its effects, directly or indirectly on the different components of the BPG-axis. Based on these studies we hypothesized that the cortisol-induced suppression of pubertal development is mediated by effects on the androgen production. In the following chapter, chapter 6, we therefore intensified our research on the testis, focussing on the steroid synthesis. Finally, in chapter 7, we investigated the role of the androgens, which possibly play a key role in the cortisol-induced suppression of pubertal development, by combined cortisol treatment and steroid replacement therapy.

Keywords: Stress, cortisol, reproduction, puberty, pituitary, testis, spermatogenesis, steroidogenesis, testosterone, feedback

General introduction

General introduction

Fish appeared more than 500 million years ago and probably all vertebrate life has evolved from them. Nowadays, fish are the most abundant of the vertebrates in terms of both species and individuals. Approximately 60% of all vertebrates are fishes and over 20,000 different species have been described.

Since the beginning of mankind, fish have been used as a food source. The remains of hominids, long before the advent of *Homo sapiens*, have been found together with prehistoric fishbone and pebbles used for the killing of the fish. The history of fishing is thus older than agriculture. Originally, the only interest was to catch sufficient fish for the daily needs, but at some point in the history of mankind it became possible to trade fish and this increased the necessity for more catch. Nowadays, fish are of high commercial interest and fishing has developed into a large-scale industry. However, this has resulted in a depletion of some of the main fish stocks in the world and more and more efforts are made to introduce commercially important fish species such as cod, halibut, sea bass and sea bream, in aquaculture. However, captivity stress is unavoidable and will influence processes like growth, reproduction and immune response. For successful rearing fish and to be able to prevent the adverse effects of stress, knowledge the biology of stress and reproduction is essential.

1.1 Stress

Successful existence and survival of a species depends on the capacity of the organism to cope with its environment and the ability to reproduce. Stress may be defined as a condition in which the dynamic equilibrium of an organism, referred to as homeostasis, is threatened or disturbed as a result of the actions of internal or external stimuli. Such stimuli are commonly characterized as stressors (Wendelaar Bonga, 1997).

Stress, and the physiological response to stress by the organism has been described as early as 1936 by Selye (Selye, 1936). According to Selye's concept, a stressed organism passes through three distinct phases. The first phase is the so-

called alarm reaction, which occurs when the organism is suddenly confronted to a critical situation. This is followed by the second phase, the phase of resistance. The organism tries to adapt to the altered conditions in order 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, which in the end may lead to a combination of pathologies, in Selye's concept referred to as the General Adaptation Syndrome (GAS).

A variation on this has been proposed by Moberg (1985), reviewed by Barton & Iwama, 1991). In this model the stress response is divided into three categories: a) the recognition of a threat to homeostasis, b) the stress response itself and c) the consequences of stress.

More focussed on the stress response in fish, still another concept has been introduced (Pickering, 1981, reviewed by Wendelaar Bonga, 1997). This concept

Stressor

Stress

Stress Response

Adaptive

↑ Plasma cortisol
↑ Plasma catecholamines
↑ Branchial blood flow
↑ Plasma glucose
↑ Muscular activity

Maladaptive

↑ Ion / water fluxes
↑ Metabolic acidosis
↓ Immunocompetence
↓ Growth capacity
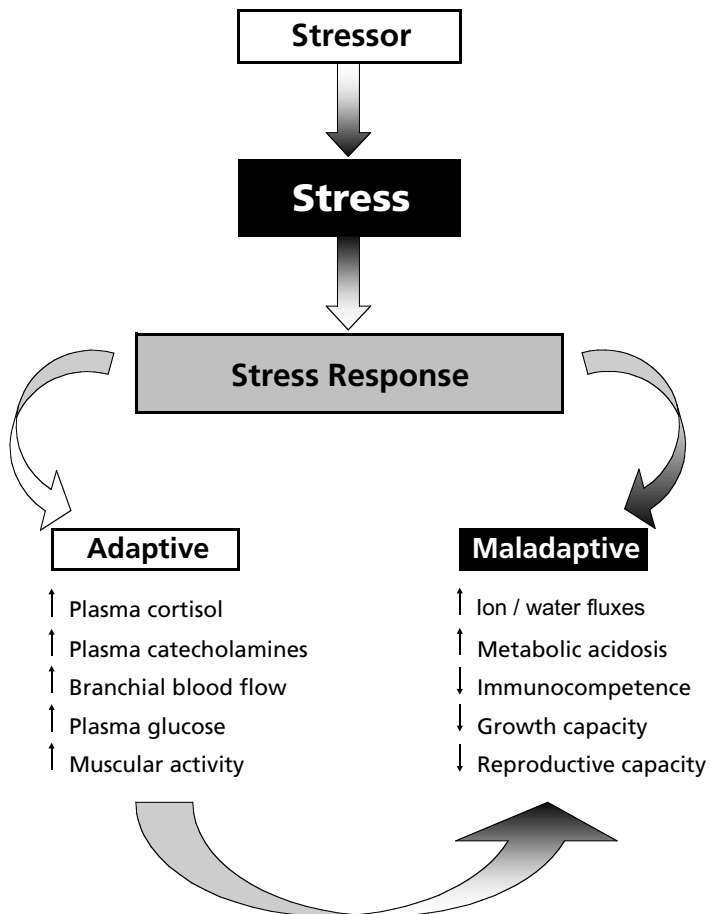↓ Reproductive capacity

Figure 1. Simplified scheme showing the concept of stress (adapted from Barton & Iwama, 1991).

makes a distinction between primary, secondary and tertiary responses. In the primary response the brain responds upon recognition of the stressor, resulting in activation of the hypothalamic-pituitary-interrenal (HPI) axis and as a consequence the release of stress hormones (catecholamines and corticosteroids). The secondary responses are defined as the immediate actions and effects of these hormones, mainly upon metabolism and the cardiovascular system. These two responses are essentially adaptive, enabling the organism to regain its original homeostatic state. However, in contrast, the tertiary response is mainly maladaptive and only occurs when the response to prolonged stress exceeds the adaptive capacity. Then, energy that is normally available for processes like growth, immune response or reproduction will be channeled into restoration of the disturbed homeostasis. All these concepts can easily be combined, which leads to the simplified representation as depicted in figure 1 (adapted from Barton & Iwama, 1991).

1.2 The Hypothalamic-Pituitary-Interrenal axis

In aquacultural practice, stress to the fish cannot be avoided. Fish experience handling, netting and transport, common procedures that can hardly be avoided, as stressors. In general, all forms of aquaculture-related stressors have been shown to cause an elevation of the plasma cortisol levels (reviewed in Barton & Iwama, 1991). Cortisol is the main product of the fish interrenal and is the end product of the neuro-endocrine system that is referred to as the stress axis or the hypothalamic–pituitary–interrenal (HPI) axis. This axis is the equivalent of the mammalian hypothalamic–pituitary–adrenal (HPA) axis.

1.2.1 Brain hormones

Upon recognition of the stressor, neuro-endocrine cells of the hypothalamus release peptides such as corticotropin-releasing hormone (CRH), a 41-amino acid peptide. This neuro-peptide belongs to a family of peptides that are all structurally related and include peptides as frogskin sauvagine and urotensin I (Turnbull & Rivier, 1997). CRH is generally considered to be the principal factor that stimulates the release of adrenocorticotropin (ACTH) from the anterior pituitary gland (Vale *et al*., 1981). However, other factors of hypothalamic origin, like urotensin I, thyrotropin releasing hormone (TRH) and arginine vasotocin (AVT) were also proven to possess corticotropin-releasing activity (reviewed by Wendelaar Bonga, 1997).

1.2.2. Pituitary hormones

The pituitary is an important endocrine gland that secretes a variety of polypeptides essential for growth, reproduction, metabolism and adaptation. The

basic structure of the pituitary is similar among vertebrates. The fish pituitary can be divided into a neuro–intermediate lobe (NIL) and a distal lobe. In the NIL small of groups of melanotropic (MSH) cells and somatolactin (SLT) cells can be found. Furthermore, th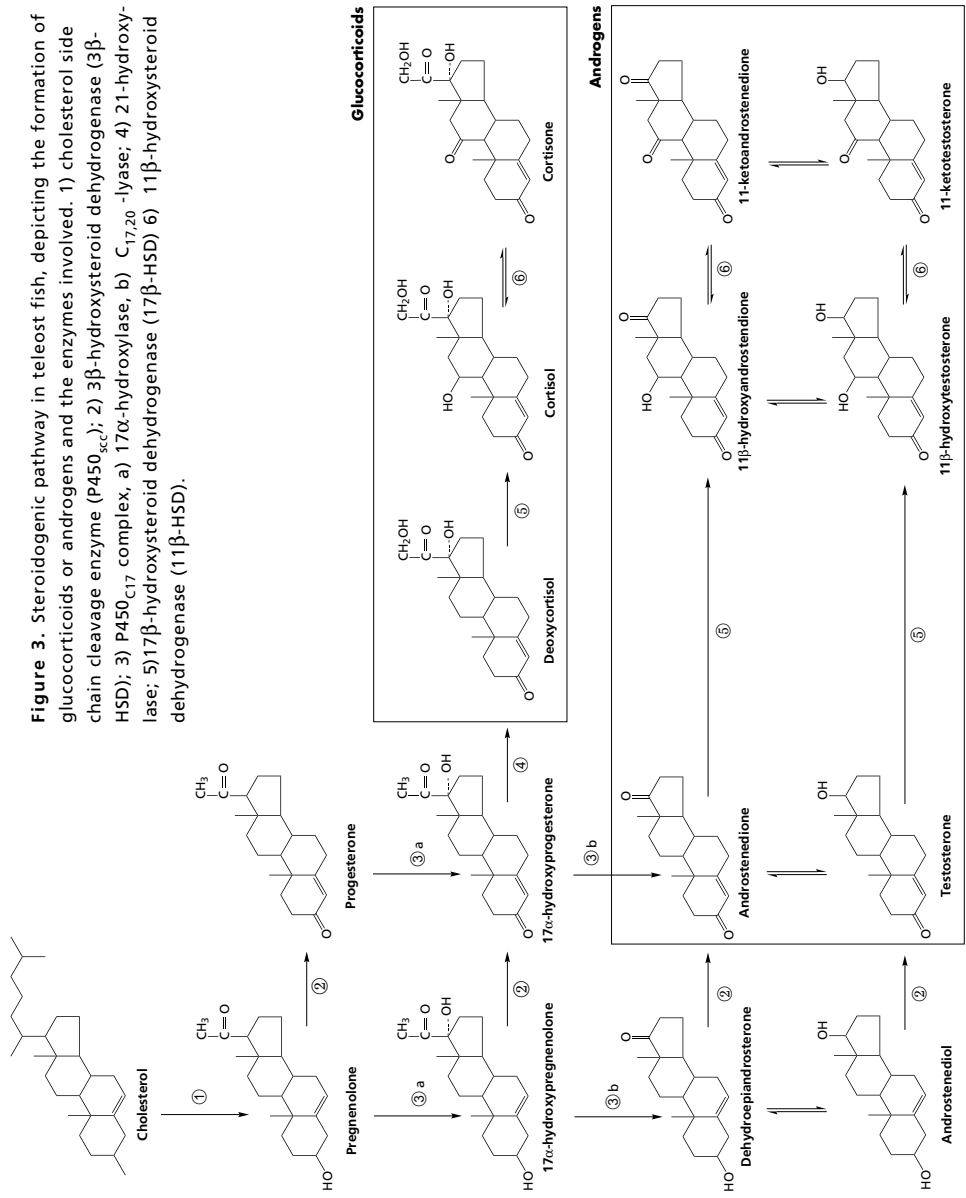e NIL contains neuro–secretory nerve endings from the diencephalic preoptic nucleus. The distal lobe or pars distalis can be subdivided into two parts: the rostral pars distalis (RPD) and the proximal pars distalis (PPD) (Van Oordt & Peute, 1983). The pars distalis contains several hormone–secreting cells. The distribution of the various endocrine cells is regionalized and summarized in figure 2. In the PPD, the gonadotrophs, thyrotrophs and somatotrophs are found, whereas the RPD mainly contains prolactin cells. The corticotrophs are mostly located at the border between the rostral and proximal pars distalis.

Figure 2. Schematic representation of a fish pituitary. The fish pituitary can be divided into a neuro-intermediate lobe (NIL) and a distal lobe. The NIL contains melanotrophs (MSH), somatolactin cells (SLT) and neurosecretory nerve endings. The distal lobe or pars distalis can be subdivided into two parts: the rostral pars distalis (RPD), containing prolactin cells (PRL) and the proximal pars distalis (PPD), which contains the gonadotrophs (LH/FSH), thyrotrophs (TSH) and somatotrophs (GH). The corticotrophs (ACTH) are mostly located at the border between the rostral and proximal pars distalis (adapted from Cavaco, 1998a).
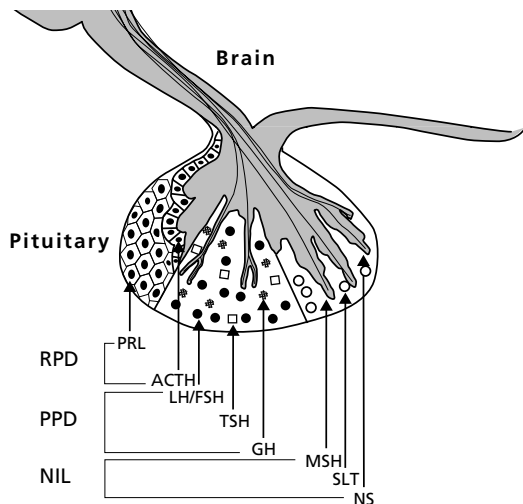
The corticotrope cells produce adrenocorticotropin (ACTH) derived from the hormone precursor proopiomelanocortin (POMC). From the same precursor, POMC, the melanotrope cells synthesize melanocyte–stimulating hormone (α–MSH). Both ACTH and α–MSH are the main pituitary products of the HPI–axis. Elevated ACTH and α–MSH levels in the circulation have been associated with stress in salmonids (Sumpter *et al.*, 1985, 1986). The role of ACTH in controlling the secretion of cortisol has been well established in fish (reviewed by Donaldson, 1981). However, α–MSH has also been shown to possess corticotropic activity. For example, chronic exposure of Mozambique tilapia to acidified water leads to an activation of the α–MSH cells. In addition, α–MSH stimulated the release of cortisol from interrenal tissue, incubated *in vitro* with diacetyl α–MSH (Lamers *et al.*, 1992). This activity may even be potentiated by β–endorphin; also a product derived from POMC (Balm *et al.*, 1995).

Figure 3. Steroidogenic pathway in teleost fish, depicting the formation of glucocorticoids or androgens and the enzymes involved. 1) cholesterol side chain cleavage enzyme (P450scc); 2) 3β-hydroxysteroid dehydrogenase (3β-HSD); 3) P450C17 complex, a) 17α-hydroxylase, b) $C_{17,20}$-lyase; 4) 21-hydroxylase; 5)17β-hydroxysteroid dehydrogenase (17β-HSD) 6) 11β-hydroxysteroid dehydrogenase (11β-HSD).

1.2.3 Cortisol

In teleost fish, cortisol is the end product of the HPI-axis. Cortisol is secreted in the interrenals upon stimulation by ACTH or α-MSH. The interrenals are the functional equivalent of the mammalian adrenal, although they differ in their structural organization. In mammals the adrenal consists of two parts; the medulla, producing catecholamines (adrenaline and nor-adrenaline), and the cortex. The cortex itself can be divided into three layers, based on morphological characteristics as well as steroid producing properties. The outer zone, the zona glomerulosa, produces mineralocorticoids. The zona fasciculata and the innermost zone, the zona reticularis, produce glucocorticoids and androgens. In fish the organization is different, in the sense that in fish interrenals no distinction in zones can be made. The interrenals, or also referred to as the headkidneys, share endocrine, haemopoeitic and lymphoid tissue in one organ. The endocrine tissue can be divided in two cell types; the chromaffin cells, which are the catecholamine producing cells and are thus the equivalent of the adrenal medulla of mammals, and the interrenal tissue, that produces the glucocorticoids.

In fish, cortisol is the main corticosteroid secreted by the interrenal cells. As all steroid hormones, cortisol is synthesized from the precursor cholesterol. The steroidogenic pathway and the enzymes that catalyze the cascade of conversions are summarized in figure 3.

The importance of cortisol in the stress response is indicated by its early presence in development. In carp, cortisol has been shown to be present at the time of hatching and the entire HPI-axis is already fully functional at that stage (Stouthart *et al.*, 1998). In fish, cortisol has a broad spectrum of activities. As the end product of the HPI-axis, it has both glucocorticoid and mineralocorticoid activities (Wendelaar Bonga, 1997). Furthermore, 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 growth, immune functions and reproduction.

1.3 Puberty

Reproduction is one of the most important events in the life of any organism, since it guaranties the continuation of the species. The period during which the animal develops the capacity to reproduce is defined as puberty. The basis of pubertal maturation is the development of the gonads and the neuroendocrine system that regulates reproductive processes, the brain–pituitary–gonad (BPG) axis (Fig. 4). Typically, in males the initiation of puberty is marked by the onset of spermatogenesis. However, the question how the transition from a juvenile, quiescent state to an adult, active state of the reproductive system is achieved has not yet been fully elucidated (Schulz & Goos, 1999). In this con-
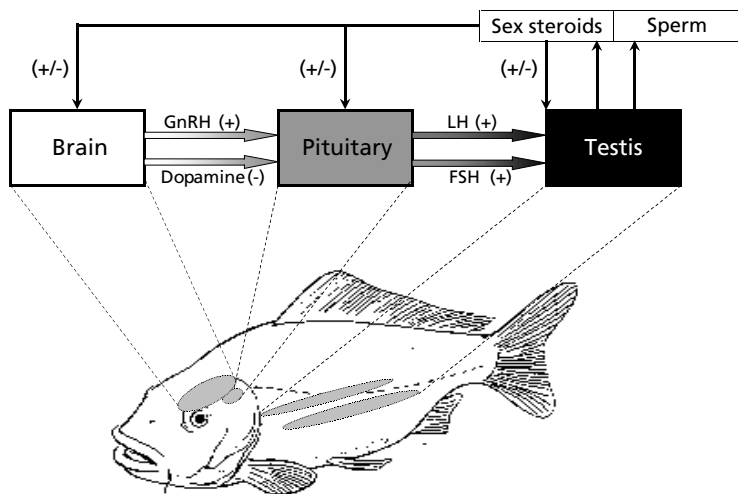
Figure 4. Schematic representation of the Brain-Pituitary-Gonad (BPG) axis. For an explanation, see paragraph 1.4.

text, two main concepts were postulated, the gonadostat concept and the "missing link" concept (reviewed by Goos, 1993). Although both concepts are presented in literature, the difference between the two is mainly semantic. The gonadostat concept is based on mammalian research. It states that the onset of puberty is a result of the disappearance of the negative feedback of sex steroids on the hypothalamic–hypophysial system. This leads to an increase of the secretion of gonadotropic hormone-releasing hormone (GnRH) by an augmentation of both the release pulse frequency and the GnRH pulse amplitude. On the other hand, the missing link concept assumes that one or more components of the BPG axis are non-functional before puberty. In that sense the gonadostat concept can also be referred to as a missing link concept, since the disappearance of the negative feedback can be considered a missing link.

In fish, several studies demonstrate that sex steroids stimulate the development of the BPG axis on all levels (e.g. Cavaco *et al.*, 1995, 1998b, Dubois *et al.*, 1998). Therefore it was suggested that in juvenile teleost fish, the production of sex steroids and/or the expression of their cognate receptors are (part of) the missing link for the initiation of puberty (Schulz & Goos, 1999).

1.4 The Brain-Pituitary-Gonad axis in fish

The brain–pituitary–gonad axis (Fig. 4) is the predominating neuro-endocrine system that regulates reproductive processes, including pubertal deve-

lopment. The brain integrates information from external and internal sources and as a consequence neuro–secretory cells, mostly located in the hypothalamus, release a variety of hormones, such as GnRH and dopamine (DA) that control the synthesis and secretion of gonadotropic hormones (luteinizing hormone, LH and follicle-stimulating hormone, FSH) from the pituitary. These gonadotropic hormones reach, via the circulation, the gonads where, in general terms, LH stimulates the production and release of sex steroids and FSH stimulates gamete development. Sex steroids also contribute to gamete development and are responsible for the development of secondary sexual characteristics and sexual behavior. Furthermore, the sex steroids together with other gonadal factors exert direct or indirect feedback effects on the pituitary and on the brain (reviewed by Nagahama, 1994; Peter & Yu, 1997).

1.4.1. Gonadotropin-releasing hormone

Gonadotropin–releasing hormones have been identified in the brains of all vertebrates. The first GnRH was characterized in mammals and was named mammalian luteinizing hormone-releasing hormone (mLHRH) (Matsuo *et al.*, 1971; Amoss *et al.*, 1971). Since it was found that GnRH not only stimulated the release of luteinizing hormone from the pituitary, but also the release of follicle stimulating hormone, the name LHRH has been substituted by the term gonadotropin-releasing hormone (GnRH). Later detected GnRHs were named after the species from which they were first isolated. However, their distribution among species is not restricted to the namesake species. In all vertebrate species, two or more different molecular forms of GnRH can be distinguished. Recent phylogenetic analysis shows that their corresponding GnRH genes can be divi - ded into three distinct branches, each of which share a similar molecular signature and a characteristic site of expression in the brain. GnRH1 is the hypo-thalamic form and the predominant GnRH in the regulation of the pituitary gonadotropins. GnRH2 is found in the midbrain tegmentum and is named the mesencepahlic form. Its function remains unclear although a function as neuro-modulator has been suggested. The last GnRH class, GnRH3 or the telen-cephalic form, is found in the forebrain and its function also remains to be cla-rified (Fernald & White, 1999).

To date, 15 different forms of GnRHs have been found in different species (Table 1). Comparison of the structure of GnRHs shows that it is a highly con-served peptide that consists of ten amino acids. All GnRHs analyzed so far show homology in amino acid residues 1, 4, 9 and 10, whereas the highest diversity is found among residues 5 and 8 (Table 1).

GnRH is produced in neurons located in the brain and transported to the pituitary. In mammals, the GnRH neurons deliver their GnRH via the axons to the median eminence, a system of portal vessels. This portal system connects to the pituitary and the GnRH reaches the gonadotrophs to execute their function,

Table 1. Amino acid sequence of the 15 molecular forms of gonadotropin-releasing hormone (GnRH) identified to date. The amino acids are indicated by their three-letter abbreviations. Residues that differ from the mammalian GnRH are indicated in bold.

GnRH	1	2	3	4	5	6	7	8	9	10	Reference
Mammalian	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH$_2$	Matsuo et al., 1971; Amoss et al., 1971
Guinea pig	pGlu	**Tyr**	Trp	Ser	Tyr	Gly	**Val**	Arg	Pro	Gly-NH$_2$	Jimenez-Linan et al.,1997
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Gln**	Pro	Gly-NH$_2$	King and Millar, 1982
Chicken II	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Tyr**	Pro	Gly-NH$_2$	Miyamoto et al., 1984
Rana	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Trp**	Pro	Gly-NH$_2$	Yoo et al., 2000
Catfish	pGlu	His	Trp	Ser	**His**	Gly	Leu	**Asn**	Pro	Gly-NH$_2$	Bogerd et al., 1994
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	**Trp**	**Leu**	Pro	Gly-NH$_2$	Sherwood et al., 1983
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Ser**	Pro	Gly-NH$_2$	Powell et al., 1994
Medaka	pGlu	His	Trp	Ser	**Phe**	Gly	Leu	**Ser**	Pro	Gly-NH$_2$	Okubo et al., 2000
Herring	pGlu	His	Trp	Ser	**His**	Gly	Leu	**Ser**	Pro	Gly-NH$_2$	Carolsfeld et al., 2000
Dogfish	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Leu**	Pro	Gly-NH$_2$	Lovejoy et al., 1992
Lamprey I	pGlu	His	**Tyr**	Ser	**Leu**	**Glu**	**Trp**	**Lys**	Pro	Gly-NH$_2$	herwood et al., 1986; Sower et al., 1993
Lamprey III	pGlu	His	Trp	Ser	**His**	**Asp**	**Trp**	**Lys**	Pro	Gly-NH$_2$	Sherwood et al., 1986; Sower et al., 1993
Tunicate I	pGlu	His	Trp	Ser	**Asp**	**Tyr**	**Phe**	**Lys**	Pro	Gly-NH$_2$	Powell et al., 1996
Tunicate II	pGlu	His	Trp	Ser	**Leu**	**Cys**	**His**	**Ala**	Pro	Gly-NH$_2$	Powell et al., 1996

which is stimulating the release of gonadotropins. In fish, the pituitary gonadotrophs are directly innervated by GnRH nerve fibers originating in the hypothalamus (Dubois *et al.*, 2000).

In carp brain, the presence of two GnRH forms has been demonstrated (Amano *et al.*, 1992). Salmon GnRH (sGnRH) as the hypothalamic GnRH (GnRH1) and chicken GnRH-II (cGnRH-II) as the form found in the midbrain tegmentum (GnRH2).

1.4.2. Gonadotropic hormones

Gonadotropic hormones are produced and secreted by the gonadotrophs in the pituitary. The gonadotropic cells are located in the pars distalis of the pituitary. In most vertebrate species two distinct gonadotropins are found, luteinizing hormone, LH and follicle-stimulating hormone, FSH. Both LH and FSH, together with thyroid stimulating hormone (TSH) and chorionic gonadotropin, belong to the family of the glycoprotein hormones (Pierce & Parsons, 1981). These hormones are heterodimers, constituted of two different subunits, a α–subunit and a β–subunit. Within a species, all members of this family share the same common glycoprotein α–subunit (GPα), whereas the β–subunit is specific and mediates the biological specificity. In general, the main function of LH is the regulation of steroidogenesis and that of FSH is the control of gametogenesis and gonadal growth.

In contrast, in teleost fish it has originally been assumed that gonadal functioning was controlled by only one gonadotropin, combining all functions that normally are fulfilled by FSH and LH (Burzawa-Gérard & Fontaine, 1972). Nowadays, it is well established that also in most teleost fish two chemically distinct gonadotropins are present (e.g. Itoh *et al.*, 1998; Van Der Kraak *et al.*, 1992). However, in fish, the functional difference between the two gonadotropins is not as distinct as in mammals. Indeed, in common carp, the two gonadotropins can be separated based on their chemical and physicochemical characteristics, but it proved to be impossible to distinguish the two hormones on a functional base. Both gonadotropins were found to stimulate steroidogenesis as well as gametogenesis, similarly (e.g. Van Der Kraak *et al.*, 1992).

1.4.3 Spermatogenesis and steroidogenesis

In the BPG-axis the testis has a dual function, the production of spermatozoa and the production of sex steroids. In teleosts, the testis is an elongated paired organ, located in the dorsal part of the body cavity. The testicular structure can be divided into two compartments, the interstitial compartment and the lobular or tubular (depending on the species) compartment. The lobular compartment, which is typical for most teleosts including the common carp (Yaron, 1995), consists of a central lumen formed by the surrounding cysts. In the cysts spermatogenesis takes place and the produced spermatozoa are released into the
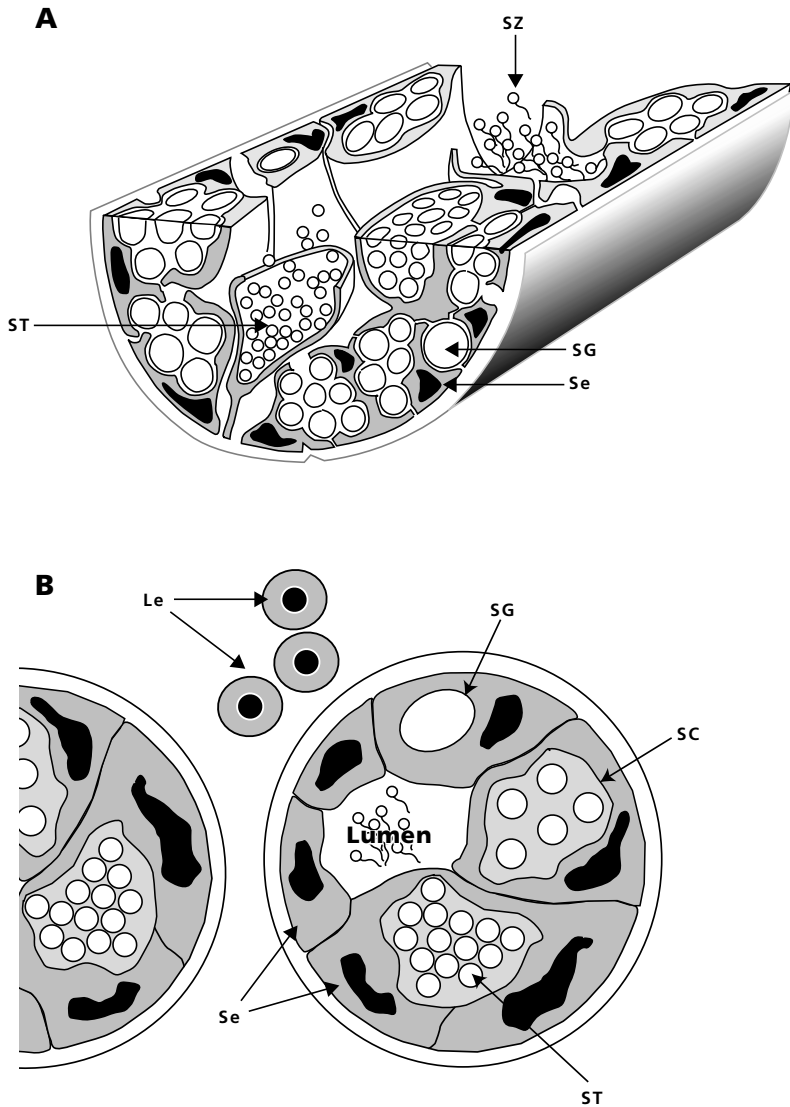
Figure 5. Schematic representation of the anatomical structure of a fish testis. (a) A testicular lobule; (b) a cross section of two lobules. Inside the lobules, Sertoli cells (Se) support the developing germ cells in different stages of development; spermatogonia (SG), spermatocytes (SC), spermatids (ST) or spermatozoa (SZ). In the interstitial compartment, between adjacent lobules, the Leydig cells (Le) are found (adapted from Cavaco, 1998a)

lumen. The tubular type, found in some cyprinodontiforms, such as the guppy, has no lumen. The cysts migrate to the end of the testis during the process of spermatogenesis (Grier & Harry, 1981, Billard *et al*., 1982).

The lobules of the testis contain two distinct cell types, the germ cells and somatic cells, the Sertoli cells (Fig. 5). The Sertoli cells have a central role in spermatogenesis. These cells form the lining of the cyst in which the germ cells

are enclosed. In this microenvironment, the germ cells proceed through spermatogenesis, supported and nurtured by the Sertoli cells. The regulation of spermatogenesis by hormones such as FSH or sex steroids is mediated by the Sertoli cell (Griswold, 1998).

During spermatogenesis the germ cells differentiate from a precursor cell (spermatogonial stem cell) into mature spermatozoa via a sequence of cytological events. Three stages can be distinguished: a) mitotic proliferation, leading to new stem cells and differentiated spermatogonia; b) meiosis, to reduce the number of chromosomes in each germ cell and leading to the haploid spermatids and c) spermiogenesis, in which spermatids transform to flagellated spermatozoa. In teleost fish, other than in mammals, germ cell development occurs synchronously in each separate cyst (Billard *et al.*, 1982).
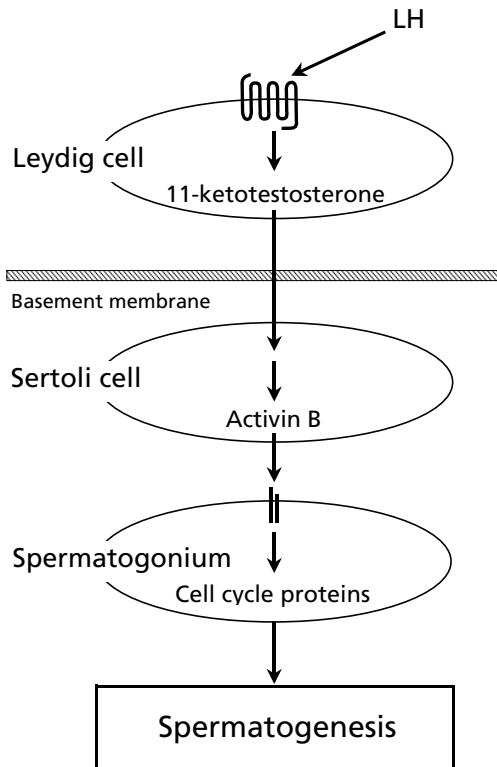
According to Cavaco *et al.* (1997), we divided spermatogenesis in the carp into four stages, based on the presence of the developmental stages of the germ cells in the testis. In stage I only spermatogonial stem cells and spermatogonia are present. This is the mitotic phase, in which spermatogonia undergo cell proliferation. In stage II, also spermatocytes are present, representing the meiotic phase. Stage III contains also spermatids, while stage IV is characterized by the additional presence of mature spermatozoa.

The interstitial spaces between the lobules consist of blood and lymph vessels, fibroblasts and interstitial cells, the latter known as the Leydig cells. These Leydig cells form clusters, located in the connective tissue surrounding the lobules. Their primary function is to produce sex steroids. In mammals and other tetrapods the testes mainly produce testosterone. Although the teleost testes produce also testosterone, 11KT is considered to be the most dominant androgen in the plasma (reviewed by Borg, 1994). Indeed, in male common carp 11KT has been found to be the major androgen in the circulation (Barry *et al.*, 1990; Koldras *et al.*,1990). However, this was not the case in immature common carp, where 11-ketoandrostenedione (OA) is the main androgen (Komen, personal communication). During pubertal development a shift from OA to 11KT occurs.

Cholesterol is the precursor molecule for al natural occurring steroid hormones. It is converted by a cascade of enzyme driven steps into bioactive hormone molecules. All naturally occurring androgens are so-called C_{19}–steroids. The steroidogenic pathway and the respective enzymes are summarized in figure 3.

The importance of sex steroids in the onset of puberty has been shown in several studies. In the African catfish, treatment with 11-oxygenated androgens stimulated testicular growth and spermatogenesis, as well as the development of secondary sexual characteristics (Cavaco *et al.*, 1998b) of sexually immature animals. Even more striking, in Japanese eel, Miura *et al.* (1991) demonstrated that complete spermatogenesis could be induced *in vitro* by the application of 11KT

LH

Leydig cell

11-ketotestosterone

Basement membrane

Sertoli cell

Activin B

Spermatogonium

Cell cycle proteins

Spermatogenesis

Figure 6. Hormonal regulation of spermatogenesis in fish (adapted from Nagahama, 1994)

to the culture medium. Also in the common carp, 11β-hydroxyandrostenedione, the precursor to 11KT has been shown to promote testicular development and spermatogenesis (Komen, personal communication). The mechanism of action of 11KT on spermatogenesis was proposed by Nagahama (1994). In short, LH stimulates the release of 11KT from the Leydig cells, which triggers the Sertoli cells to stimulate spermatogenesis via the secretion of activin-B (Fig. 6).

Besides the effects of steroid hormones on spermatogenesis, they also affect the pituitary and brain during pubertal development. For example, in immature African catfish it was shown that testosterone could activate the maturation of gonadotrophs (Cavaco *et al*., 1995) and accelerate the development of the hypo-thalamic GnRH system in the brain (Dubois *et al*., 1998).

1.5 Stress and reproduction

In 1936, Hans Selye was the first to propose a concept on stress. In this early paper in Nature, he already assessed the impact of stress on several physiological

processes such as reproduction (Selye, 1936). Later on this has been shown in numerous studies on animals, but also in humans there are many examples for stress having serious consequences on reproduction. In a recent study performed by Tahirovic (1998), the impact of "stress of war" on the pubertal development of deported girls in besieged Srebrenica was assessed. This study showed that the Srebrenica girls had a significantly higher mean menarchal age (approximately 1.4 years later) compared to a control group, living mainly in peaceful communities in the unoccupied territory of Bosnia. These results suggest that menarche is a very sensitive pubertal event which is strongly subjected to environmental and emotional factors. Also the disappearance of menstrual cycles in women under severe psychological or physical stress is a well-known phenomenon, which may occur for example under heavy sports training.

1.5.1 Stress and reproduction in mammals

In several mammals, stress has been shown to interfere with reproduction and the functioning of the brain-pituitary-gonad (BPG) axis. In male Siberian dwarf hamsters, separation stress decreased the seminal vesicle mass and the testicular mass (Castro & Matt, 1997). In lactating dairy cows, high summer temperature has been shown to be a major stressor contributing to low fertility (reviewed in Wolfenson *et al.*, 2000).

In mammals, all levels of the BPG-axis have been shown to be affected by stress (Rivier & Rivest, 1991, Wolfenson *et al.*, 2000). For example, a decrease in plasma LH and hypothalamic GnRH in male rats after chronic restraint stress has been observed (López-Calderón *et al.*, 1989) and similar results were found in rams and ewes (Tilbrook *et al.*, 1999). Furthermore, adult rats, submitted to immobilization stress from pre-puberty onwards, showed decreased plasma LH and plasma testosterone (T) levels, as well as a decrease in the amount of spermatids in the testis and lower sperm counts, together with a reduction in the seminal vesicle weight (Almeida *et al.*, 1998). Charpenet *et al.* (1981) demonstrated that chronic intermittent immobilization stress induced a drastic fall in plasma and testicular testosterone concentration of rats, without detectable changes in plasma LH. The precise mechanisms via which the stress response has its adverse effects on reproduction are still largely unknown.

1.5.2 Stress and reproduction in fish

In fish, as in mammals, it has been recognized that stress has detrimental effects on growth, immune functions and reproduction (Wendelaar Bonga, 1997). In rainbow trout, exposure to repeated acute stress resulted in delayed ovulation and reduced egg size. In stressed males, significantly lower sperm counts were observed and more important, the progeny from stressed fish had significantly lower survival (Campbell *et al.*, 1992). In another study, female tilapia even failed to spawn in crowded holding tanks but the same fish spawned

soon after transfer to individual aquaria. Furthermore, as the period of confinement lasted, oogenesis was affected and an increase in atresia was observed, coinciding with reduced plasma 17β-estradiol and testosterone levels. The authors suggested that the reduced levels of 17β-estradiol and testosterone during crowding are insufficient to allow completion of vitellogenic growth (Coward *et al.*, 1998).

Reported effects of stress on the BPG-axis in fish are inconsistent since stimulatory as well as inhibitory effects of stress, or no effects at all, have been described. (reviewed by Wendelaar Bonga, 1997). In male brown trout, *Salmo trutta* L. acute and chronic stress suppressed the plasma levels of 11-ketotestosterone (11KT). However, plasma gonadotropin levels were elevated following 1 hour of handling stress (Pickering *et al.*, 1987). The variability of these results may depend on the nature and duration of the stressor.

In all vertebrates, including fish, cortisol plays a key role in the restoration of homeostasis during or after stress. Cortisol has frequently been indicated as a major factor mediating the suppressive effect of stress on reproduction. Carragher *et al.* (1989) showed that chronically elevated plasma cortisol levels by the implantation of cortisol releasing pellets affected a wide range of reproductive parameters in the brown trout and in the rainbow trout, *Salmo gairdneri* Richardson. Cortisol–implanted maturing male brown trout had smaller gonads, lower plasma testosterone levels and their pituitaries had lower gonadotropin content.

In general, it can be concluded that stress has an inhibitory effect on reproduction in fish. However, the precise mechanisms via which the stress response affects reproduction are also in fish to be elucidated.

1.6 Models used in this thesis

1.6.1. Temperature stress

Rapid changes in water temperature are among the stressors with a high physiological impact on fish. Changes in the water temperature have immediate effects on fish, because of the high rate of heat exchange between the animal and the surrounding water. However, for each species there is a range of temperatures to which the species can acclimate, as well as a more narrow optimal temperature range in which the efficiency for most physiological processes is maximal. The fish can easily adapt to gradual changes within the tolerance zone but rapid changes will disturb the internal homeostasis and thus elicit a stress response (Elliot, 1981). In figure 7 (based on Elliot, 1981) the thermal requirements for the common carp are summarized.

In this study our experimental model, the common carp, was submitted to rapid temperature decreases of 11°C (standard rearing temperature is 25°C) as

described by Tanck *et al.* (2000). Previous results showed that this kind of cold shock stress caused an elevation of the cortisol levels in common carp.
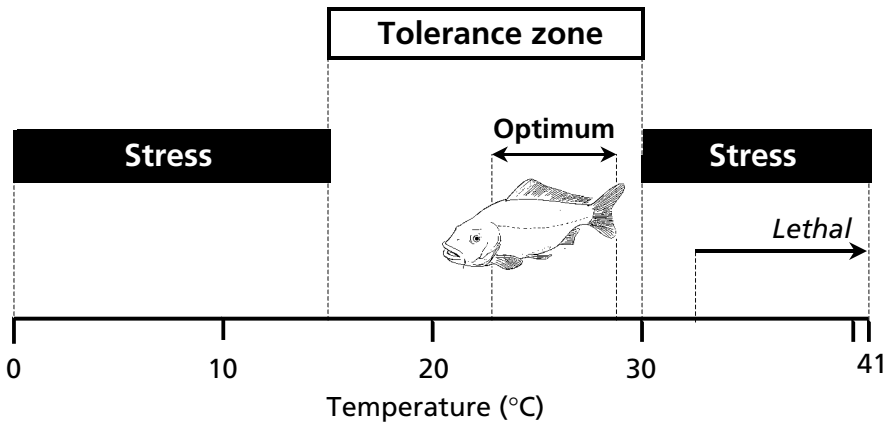

Figure 7. Thermal requirements for the common carp (adapted from Elliot, 1981).

1.6.2. An all-male carp population

As experimental animal we used an isogenic, all–male population of the common carp, *Cyprinus carpio* L., produced and raised in the facilities of the Department for Fish Culture and Fisheries from the Agricultural University of Wageningen. This population was obtained by the crossing of a homozygous gynogenetic E4 female (Komen *et al.*, 1991) with a so-called "super male" of an unrelated homozygous androgenetic strain R3R8 (Bongers *et al.*, 1997). This super male is homogametic, which means that these males possess two Y-chromosomes, and hence the resulting offspring of this crossing is an all–male population.

1.6.3. Puberty in carp

Pubertal development in this strain of isogenic male common carp (E4xR3R8) is highly uniform and predictable, summarized in figure 8. Before
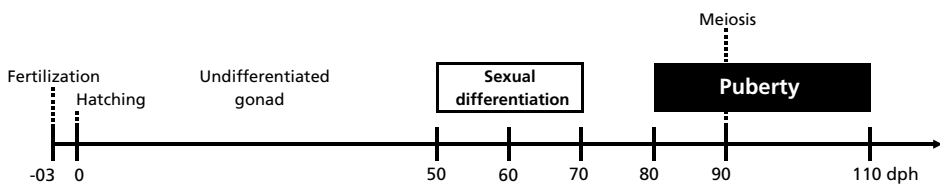

Figure 8. Life span of the common carp strain used in this study (E4xR3R8). The period of sexual differentiation and pubertal development is indicated.

50 days post–hatching (dph), the gonad is still undifferentiated and contains only few primordial germ cells. At this stage the difference between a testis and an ovary is indistinguishable. From 50 dph onwards in females the appearance of an ovarian cavity can be observed and sexual differentiation commences. At the same time the primordial germ cells start to proliferate. The organization of the testis cords and the appearance of the first testis tubules occurs at around 80 dph as a consequence of the spermatogonial multiplication. At around 90 dph the spermatogonia enter meiosis and spermatogenesis continues until the appearance of the first flagellated spermatozoa between 100 and 110 dph.

1.7 This thesis

In mammals, as in fish, it is well recognized that stress has adverse effects on the reproductive process. However, the precise mechanisms via which the stress response has its adverse effects on reproduction are still unknown. The present study is an attempt to find more answers to solve this intriguing question. Firstly, we investigated the effects of repeated temperature stress on the pubertal development (chapter 2). Since cortisol has been shown to play a key role in the homeostatic adaptation during or after stress and is frequently indicated as a major factor mediating the suppressive effect of stress on reproduction, we focussed in chapter 3 on the effects of cortisol on pubertal development. In the same chapter we also paid attention to the question, at which level the BPG–axis is affected by cortisol. Since our results indicate that all levels of the BPG–axis were affected by cortisol, we studied the cortisol effects on the pituitary and the testis in more detail (chapter 4 and in chapter 5) and tried to answer the question whether cortisol has its effects, directly or indirectly on the different components of the BPG–axis. Based on these studies we hypothesized that the cortisol–induced suppression of pubertal development is mediated by effects on the androgen production. In the following chapter, chapter 6, we therefore intensified our research on the testis, focussing on the steroid synthesis. Finally, in chapter 7, we investigated the role of the androgens, which possibly play a key role in the cortisol–induced suppression of pubertal development, by combined cortisol treatment and steroid replacement therapy. In the summarizing discussion, we present a model that integrates our findings and describes a possible mechanism via which stress adaptation affects reproduction.

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

Co-authors: Edward Kuijer, Tom W.L. Groeneveld, Marc Y. Engelsma, Michael W.T. Tanck, Menno ter Veld, Hans Komen, Jan G.D. Lambert, Henk J.Th. Goos

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).

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: 21Gx1$\frac{1}{2}$"). 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: 26Gx$\frac{1}{2}$") 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 ± 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.
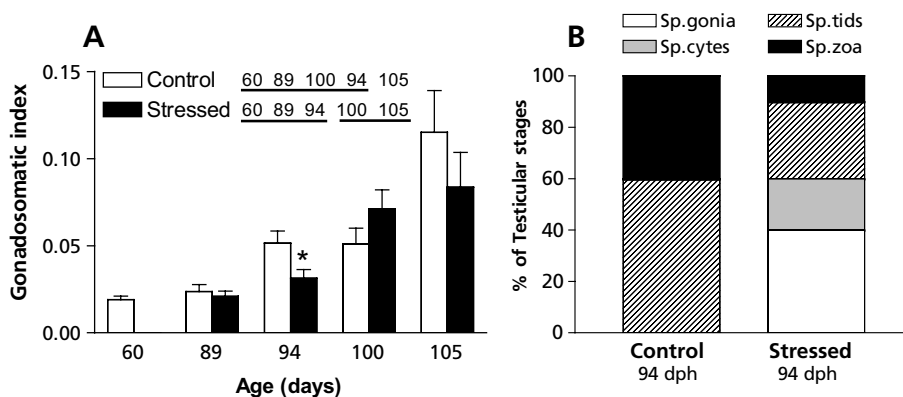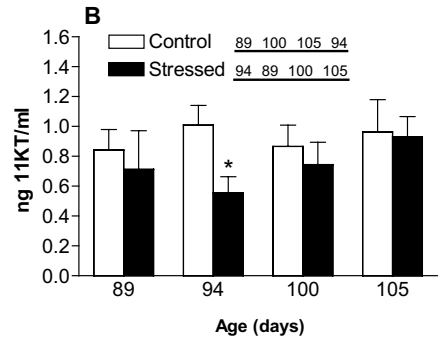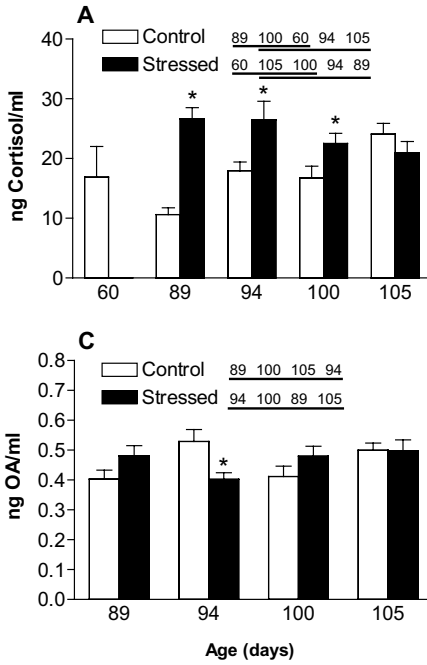

Figure 1. Effect of repeated temperature stress on testicular development, represented by (A) the gonadosomatic index (n=20) and (B) testicular stage (n=10). * indicates a significant difference between the control group and the stressed group (p<0.05). Data sharing the same underscores in the legends 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).
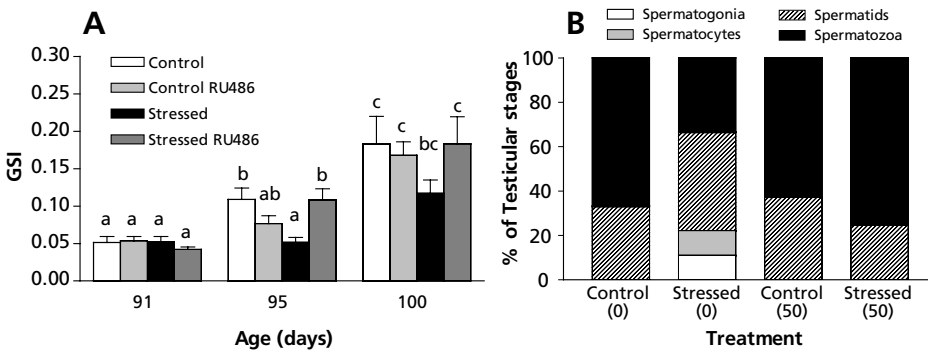
Figure 1. Effect of repeated temperature stress on testicular development, represented by (A) the gonadosomatic index (n=20) and (B) testicular stage (n=10). * indicates a significant difference between the control group and the stressed group (p<0.05). Data sharing the same underscores in the legends 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 maturation. 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 testicular 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 temperature 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 pituitary LH release. Systemic treatment with RU486 significantly attenuated this decline of circulating LH (Briski *et al.*, 1995). This effect is in mammals mediated 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 testosterone (T) levels without affecting the LH levels. This reduction could partially 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.

Long-term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L.

Co-authors: Jan Bogerd, Hans Komen, Jan G.D. Lambert, Henk J.Th. Goos

Abstract

The onset and regulation of puberty is determined by functional development of the brain–pituitary-gonad (BPG) axis. Stress has been shown to interfere with reproduction and the functioning of the BPG-axis. The response to chronic and severe stress may require much energy and force the organism to make adaptive choices. Energy that is normally available for processes like growth, immune response or reproduction will be channeled into restoration of the disturbed homeostasis. Cortisol plays a key role in the homeostatic adaptation during or after stress. In the present study, immature common carp were fed with cortisol containing food pellets covering the pubertal period. We showed that cortisol caused an inhibition of pubertal development, by affecting directly or indirectly all components of the brain-pituitary-gonad axis. The sGnRH content of the brain was decreased. LH and FSH encoding mRNA levels in the pituitary and LH plasma levels were diminished by long-term cortisol treatment, as was the testicular androgen secretion. Testicular development, reflected by gonadosomatic index and the first wave of spermatogenesis, was retarded.

Introduction

Adaptation to changing environmental conditions is essential for maintenance of physiological homeostasis. Stress can be defined as a disturbance of homeostasis by any kind of external or internal factor, referred to as the stressor. The consequence of the action of a stressor is that the homeostatic equilibrium is threatened. This will induce a coordinated set of behavioral and physiological responses that are compensatory and/or adaptive, enabling the organism to restore its homeostatic set points (Wendelaar Bonga, 1997). These adjustment reactions have been identified as the neuro-endocrine stress response (Selye, 1936). However, the response to prolonged stress may exceed the adaptive capacity. Energy, normally available for processes like growth, immune response or reproduction may then be channeled into restoration of the disturbed

homeostasis. This may result in maladaptation with adverse effects on reproduction, immune competence or growth.

Indeed, in several vertebrates, stress has been shown to interfere with reproduction and the functioning of the brain–pituitary–gonad (BPG) axis. In mammals, all levels of the BPG–axis are affected (Rivier & Rivest, 1991). For example, a decrease in plasma LH and hypothalamic LHRH in male rats after chronic restraint stress has been shown (López-Calderón *et al.*, 1990) and similar results were found in rams and ewes (Tilbrook *et al.*, 1999). Furthermore, adult rats submitted to immobilization stress from prepuberty showed decreased plasma LH and plasma testosterone (T) levels (Almeida *et al.*, 1998), whereas Charpenet *et al.* (1981) demonstrated that chronic intermittent immobilization stress indu-ced a strong decrease of plasma T levels and testicular T content in rats, without, however, detectable changes in plasma LH values. The precise mechanisms via which the stress response has its adverse effects on reproduction are still unknown.

Reports on the effects of stress on the reproductive capacity in fish are inconsistent since stimulatory as well as inhibitory effects of stress, or no effects at all, have been described. (reviewed by Wendelaar Bonga, 1997). In male brown trout, *Salmo trutta* L., acute and chronic stress suppressed the plasma levels of 11-ketotestosterone (11KT). However, plasma gonadotropin levels were elevated following 1 hour of handling stress (Pickering *et al.*, 1987a). The variability of these results may depend on the nature and duration of the stressor, and the animal model that was used.

In all vertebrates, including fish, cortisol plays a key role in the restoration of homeostasis during or after stress. Furthermore, cortisol has frequently been indicated as the major factor mediating the suppressive effect of stress on reproduction. Carragher *et al.* (1989) showed that implantation of cortisol releasing pellets in the brown trout, *Salmo trutta* L. and in the rainbow trout, *Salmo gairdneri* Richardson, chronically elevated the plasma cortisol levels and affected a wide range of reproductive parameters. Cortisol–implanted maturing male brown trout had smaller gonads, lower plasma testosterone levels and their pituitaries had lower gonadotropin content.

The onset and regulation of puberty depends on the functional development of the BPG–axis. Several definitions for puberty exist, but for the purpose of this study, puberty will be considered as the period that spans the onset of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. Previous results have shown that in common carp (*Cyprinus carpio* L.) repeated temperature stress caused elevated plasma cortisol levels (Tanck *et al.*, 2000) and a retardation of the first waves of spermatogenesis (chapter 2).

In the present experiments, stress–induced cortisol levels were mimicked in order to investigate (1) whether the effects of the temperature stress are due to elevated cortisol levels and, if so, (2) on which level the BPG–axis is affected by cortisol.

Temperature stress–induced cortisol levels (Tanck *et al.*, 2000) were mimicked by feeding the experimental animals with cortisol containing food pellets. As experimental animals we used isogenic male common carp, with highly uniform and predictable testicular development, with meiosis of spermatogonia starting around 90 days post hatching (dph) (Bongers *et al.*, 1999).

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 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 our department 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 daily (Provimi, 91 series, Rotterdam, The Netherlands) at a daily ration of 20 g/kg$^{-0.8}$. Fish were allowed to acclimatize till 63 dph after which the experiment started.

Experiment 1:
Short-term steroid treatment

Cortisol (Steraloids Inc. Wilton, USA) containing food (100 mg/kg food) was prepared as described by Pickering *et al.* (1987b). In order to determine how to mimic the cortisol profile induced by temperature stress (Tanck *et al.*, 2000), 320 animals were equally divided over four groups. The first two groups received during one week, once daily, either control food or cortisol–treated food. In the same period the other two groups received either control food or cortisol–treated food daily over a 6 hours period, starting at 10:00 am (4 times, with intervals of 1.5 hours). Feeding the fish with cortisol containing pellets according to the latter regime mimicked the cortisol levels that were induced by the temperature stress (see Results). On the last day of the treatment, blood samples were taken for cortisol measurement by radioimmunoassay (RIA).

Experiment 2:
Long-term steroid treatment

Two hundred animals, 63 dph, were divided over two groups. Group 1 served as controls and was fed with control food, while group 2 received four times daily the cortisol–containing food.

At the onset of the experiment, 63 dph, a start control group was sampled. At 90, 95, 101 and 106 dph, covering the pubertal development of this strain of common carp, 20 fish per group were 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: 26Gx$\frac{1}{2}$") 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. Brains and pituitaries were collected, snap frozen in liquid nitrogen and stored at –80°C until use for hormone measurements, by means of RIA, or for mRNA quantification by RNase protection analysis (RPA). Testes were taken, weighed for determining the gonadosomatic index (GSI = testes weight $\star$ 100 / (bodyweight-testis weight)) and fixed for histological determination of the testicular development.

Testicular histology

For determination of the spermatogenetic 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 spermatides; and finally stage IV – all germ cells including spermatozoa. The number of animals per group with the same stage of testicular development are counted and expressed as a percentage of the total group.

Steroid Radioimmunoassays (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 T 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 *et al.*, 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 (Komen, personal communication). Testosterone was included since Cavaco *et al.* (1995) showed that this androgen is essential for gonadotroph development during puberty.

Plasma and pituitary LH

Luteinizing Hormone (LH) was quantified in the plasma and the pituitaries 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 a first antibody.

Ten pituitaries per treatment group were individually homogenized and assayed. 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.

sGnRH content in the brain

Salmon GnRH, which is the native hypothalamic form for carp, was measured by RIA using a sGnRH specific antibody and iodinated sGnRH (Schulz *et al.*, 1993, Goos *et al.*, 1997). Ten brains per treatment group were individually homogenized in 2N acetic acid, heated at 90°C. for 10 minutes, snap frozen and sonicated. The homogenates were centrifuged, 3500 g at 4°C for 30 min. The supernatants were collected. The pellets were resuspended in 2N acetic and centrifuged. The second supernatants were added to the previous ones and stored at –70°C. Before assaying, the samples were lyophilized. The residues were reconstituted to a smaller volume with 2N acetic acid, sonicated and centrifuged at 3500 g and 4°C for 30 min. The supernatants were neutralized with 5N NaOH, centrifuged at 3500 g and 4°C for 5 min. and further diluted with the assay buffer.

RNase Protection Analysis (RPA)

To quantify the steady-state messenger RNA levels for glycoprotein hormone α-subunit (GPα), LHβ and FSHβ subunits, 10 pituitaries per treatment group were used for RPA, based on the method described by Rebers *et al.* (1997). However, the homogenization was performed in 50 µl lysis buffer to account for the low amounts of mRNA present in the pituitaries of immature common carp. For the quantification of the GPα, LHβ and FSHβ subunit mRNA levels, 45 µl of this homogenate was used. For the quantification of the 28S rRNA (internal standard) levels, 42.5 µl lysis buffer was added to 2.5 µl of the remaining homogenate.

The following oligodeoxynucleotide primers were used (Life Technologies, Breda, The Netherlands): GPα Fw, 5'-GAGGTCCAAGAAAACCATGCT-3'; GPα Rv, 5'-TTTAACTG*TAATACGACTCACTATA*GGGCAAAATCC GTAACACAAGCAAATCTTGAATGTC-3' (based on Huang *et al.*, 1992); LHβ Fw, 5'-TCCGACTGTACGATTGAAAGCC-3'; LHβ Rv, 5'-TTTAACTG*TAATACGACTCACTATA*GGGGTTGATATACTCTTCAG CTCAATATCCACGCC-3' (based on Chang *et al.*, 1992); FSHβ Fw, 5'-GGTCGACAGCGCTCACCAATATCTCCATTACCG-3'; FSHβ Rv, 5'-TTTACCTG*TAATACGACTCACTATA*GGGCCAAGAACGTGCATGTT ATATTTATTGATGCTTGCA-3' (based on Kobayashi, unpublished, DDBJ

accession nr. AB003583); 28S rRNA Fw, 5'-<u>CCATGCCTGGG</u>TGAAAG CGGGGCCTCACGATCCT-3'; 28S rRNA Rv, 5'-GGTACC*TGTAATACGA CTCACTATA*<u>GGGC</u>CAGATTTGCCAGCTCACGTTCCCTATTAGTGG GT-3' (based on conserved sequences found in other 28S rRNA sequences). In all primers, the sequences in italics represent the T7 RNA polymerase promoter sequence used for cRNA probe synthesis (Rebers *et al.*, 1997). The underlined sequences are unable to hybridize to the mRNA to be detected and yield the difference in length between the cRNA probe and the protected fragment in the assay.

Statistics

All results are expressed as mean ± SEM. Plasma levels of the different steroids are given as ng per ml plasma. LH levels are given as ng per ml plasma or as ng per pituitary. sGnRH content is expressed in pg/brain. Messenger RNA levels for the GPα, LHβ and FSHβ subunits are corrected for 28S ribosomal RNA levels and expressed as percentage of the control. 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

Plasma cortisol

Plasma cortisol levels during and after a single or a 4 times daily cortisol food application are depicted in figure 1A and 1B, respectively. At the onset of the single treatment, plasma cortisol levels of the control group are elevated but decrease to basal within one hour. This profile reflects the normal stress reaction to the expectation of food. A single feeding with cortisol containing pellets caused a significant increase in plasma cortisol level, which reached a peak value
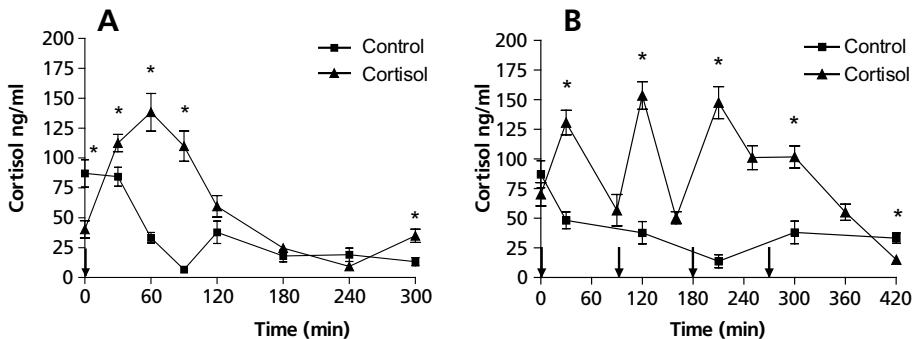

Figure 1. Plasma cortisol concentrations after a single (A) and a four times daily (B) cortisol food application (n=10). Arrows indicate the feeding times. * indicates a significant difference (p<0.05).

of 140 ng/ml plasma after one hour and was returned to basal level after 3 hours (Fig. 1A). Repeated application of the cortisol treated food, 4 times daily with intervals of 1.5 hours, induced an elevated cortisol profile over 7 hours. Cortisol peak values up to 150 ng/ml plasma were observed after each meal. In the control group, cortisol plasma values were all below 50 ng/ml plasma, except the first time point (Fig. 1B).

Growth, gonadosomatic index (GSI) and testicular histology

The growth curve (Fig. 2A) for the control group and cortisol treated group demonstrates that cortisol causes a slight retardation in growth which becomes significant from 101 dph onwards. The increase in GSI, observed in the control animals reflects the normal testicular development during puberty (Fig. 2B). In contrast, the cortisol treated animals show an impaired testicular development
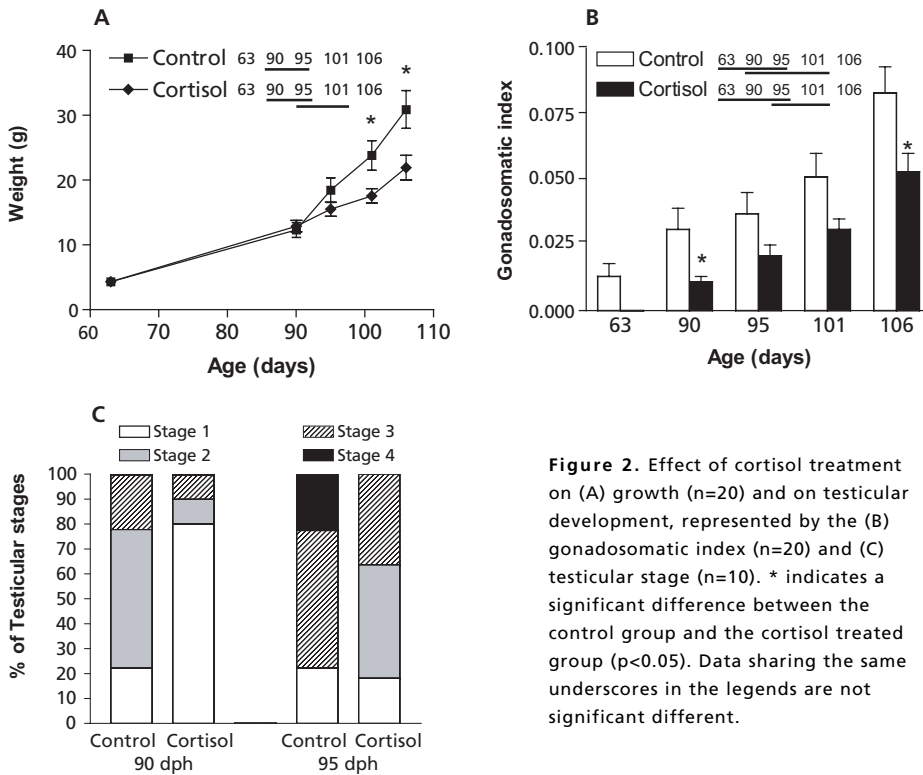
Figure 2. Effect of cortisol treatment on (A) growth (n=20) and on testicular development, represented by the (B) gonadosomatic index (n=20) and (C) testicular stage (n=10). * indicates a significant difference between the control group and the cortisol treated group (p<0.05). Data sharing the same underscores in the legends are not significant different.

as follows from the significantly lower GSI at 90 and 106 dph. This reflects the retardation in spermatogenesis as observed after histological analysis of the testis. Due to the some what higher variation in the control group, this difference is not statistically significant at 95 and 101 dph (Fig. 2C). At 90 dph most of the

control fish are in stage II whereas the cortisol treated fish remain in the first stage of spermatogenesis. When at 95 dph the control group is already in stage III–IV, all cortisol treated fish are still in stage II–III.

Plasma levels of sex steroids

Prolonged feeding with cortisol prevented the significant increase of 11KT plasma levels as observed in the control animals during the course of the experiment (Fig. 3A). The T levels were significantly lower at 106 dph only (Fig. 3C). Plasma OA levels are lower in cortisol treated animals. Statistical significance could not be calculated at 95 and 101 dph because most levels in cortisol treated animals were below the detection limit of the assay (8 out of 10 at 95 dph and 6 out 9 at 101 dph, respectively)(Fig. 3B).
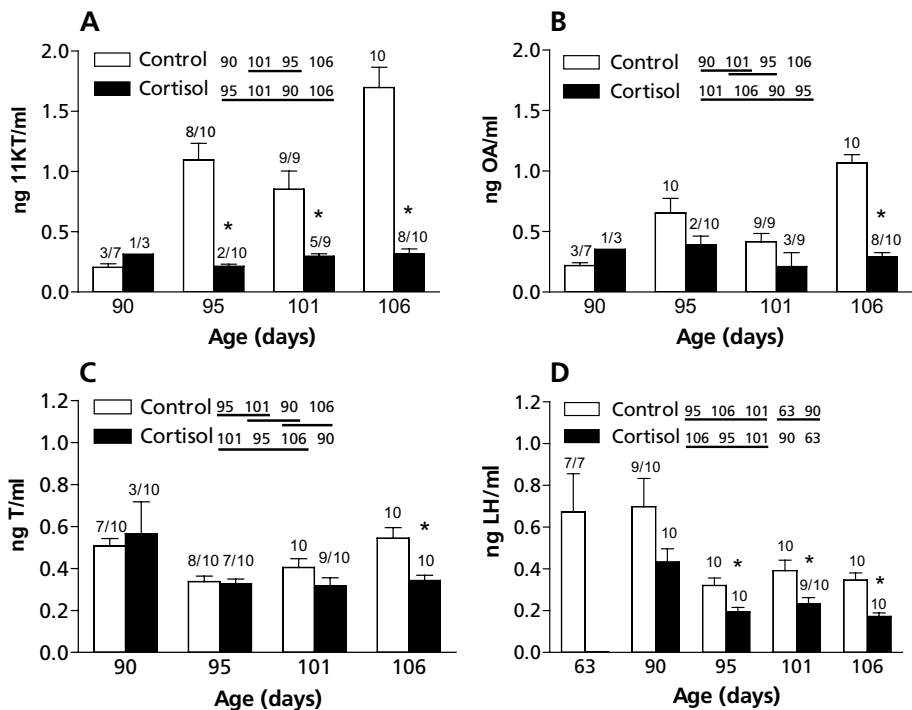
Figure 3. Effect of prolonged feeding with cortisol containing food pellets on plasma levels of (A) 11KT, (B) OA, (C) T and (D) LH (n=10). * indicates a significant difference between the control group and the cortisol treated group (p<0.05). Data sharing the same underscores in the legends are not significant different. Numbers above bars represent the number of values above the detection limit of the assay.

Plasma and pituitary LH

Plasma LH levels in both the control and cortisol treated fish gradually decreased during the experiment (Fig. 3D). However, the LH plasma levels for

the treated groups are significantly lower than the levels measured in the control group, except at day 90 dph, due to the larger variation in the control group.

The pituitary LH content shows a steady increase in the control group, which reflects the normal elevation of LH content during the pubertal development (Fig. 4A). This rise in LH content can also be observed in the cortisol treated group. However, cortisol treatment resulted in a slightly retarded elevation, which caused a significant difference at 101 dph.

sGnRH content in the brain

sGnRH content of the brain gradually decreased in the control animals from 90 to 101 dph, but were strongly increased at 106 dph. Prolonged feeding with cortisol resulted in lower brain contents of sGnRH (Fig. 4B) at 90, 95, 101 and 106 dph. Due to the somewhat larger variation in the cortisol group at 101 dph, there was no statistical difference between groups.
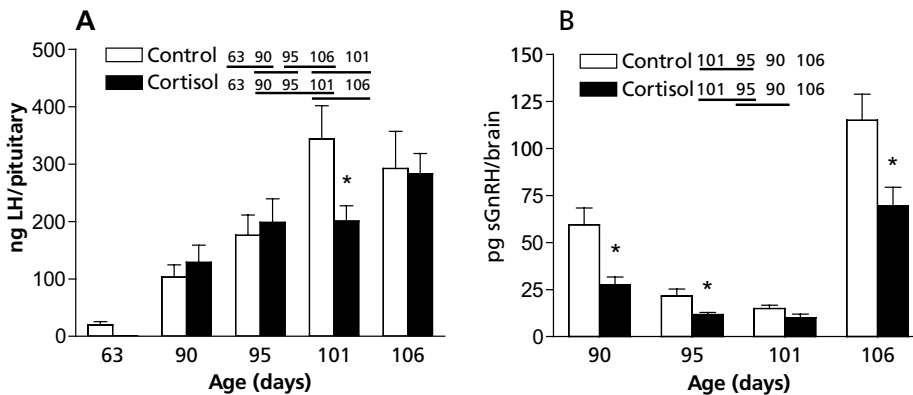
Figure 4. Effect of prolonged feeding with cortisol on (A) pituitary LH content, expressed in ng/pituitary and (B) sGnRH content in the brain, in pg/brain (n=10). * indicates a significant difference (p<0.05). Data sharing the same underscores in the legends are not significant different.

GPα, LHβ and FSHβ subunit steady-state mRNA levels

RNase protection analysis showed that cortisol treatment had different effects on the mRNA levels for GPα, LHβ and FSHβ depending on the age of the animals at time of the sampling (Fig. 5). At 90 dph, FSHβ subunit mRNA levels are significant lower compared to control values. Messenger RNA levels for GPα and LHβ tend to be lower in the cortisol treated group, but the differences are not significant. At 95 dph and 101 dph no differences are observed between the control and treated group. However at 106 dph GPα, FSHβ and LHβ subunit mRNA levels are significantly lower in the cortisol treated group.
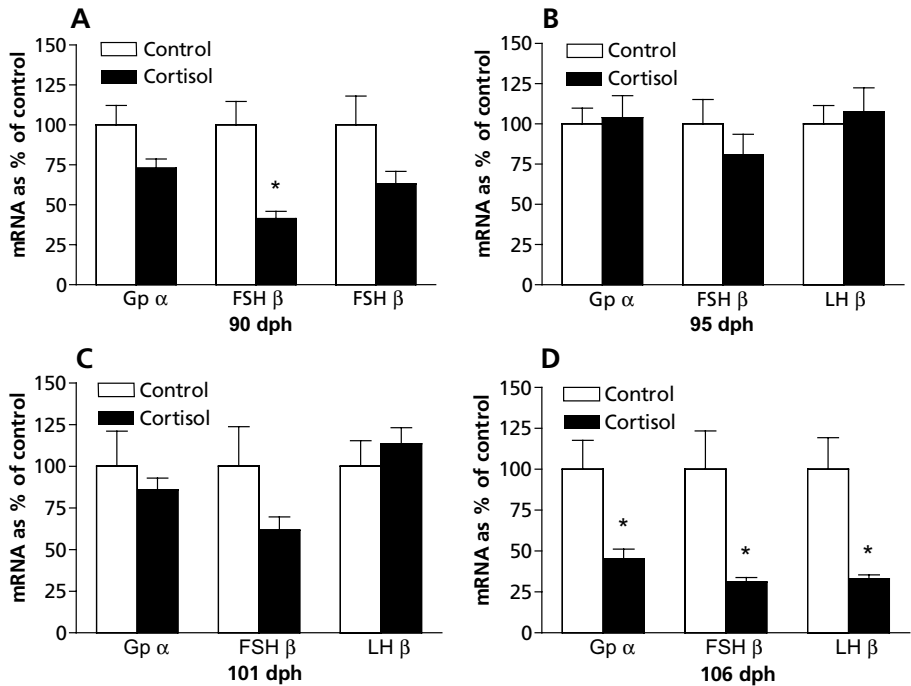
Figure 5. Effect of *in vivo* cortisol treatment on the mRNA levels for Gpα, FSHβ and LHβ subunit, respectively, at several ages during pubertal development (A) 90 dph, (B) 95 dph, (C) 101 dph and (D) 106 dph. Messenger RNA levels are expressed as a percentage of the control (n=10). * indicates a significant difference (p<0.05).

Discussion

In the present study we showed that long-term cortisol treatment caused an inhibition of pubertal development, affecting directly or indirectly all components of the BPG-axis. To our knowledge this is the first time that negative effects of cortisol on spermatogenesis during pubertal development have been described.

In this study we defined puberty as the period that spans the onset of spermatogonial multiplication until the appearance of the first flagellated spermatozoa. In the isogenic common carp strain that was used, puberty occurs between 90 and 110 dph. Feeding with cortisol containing food pellets from 63 dph onwards caused retardation in weight gain between 90-100 dph. Similar results have been observed before for several species (reviewed in Van Weerd & Komen, 1998). Stress has been shown to have adverse effects on growth. In Atlantic salmon parr repeated stress once or twice daily over a period of 30 days reduced the growth rate significantly (McCormick *et al.*, 1998) and chronic crowding for a 9-month period in rainbow trout reduced the bodyweight as well (Pickering *et al.*, 1991).

Growth of the testis was affected by the cortisol treatment, reflected by lower GSIs at all sampling points. Due to the somewhat larger variation at 90 and 95 dph the difference was not statistically significant at these time points. Identical results have been obtained in other fish species. Hydrocortisone acetate treatment of male *Labeo gonius* caused a reduction in volume and length of the testes and in GSI (Joshi, 1982). Cortisol implanted maturing male brown trout had smaller gonads (Carragher *et al.*, 1989). Female tilapia treated with cortisol showed reductions in GSI and oocyte size (Foo & Lam, 1993b). In mammals, reduction of testicular development was shown in stressed Siberian dwarf hamsters Castro & Matt, 1997). A causal relationship with declined testosterone (T) and estradiol (E2) levels has been suggested.

Reduction of plasma sex steroid levels due to stress or cortisol treatment has been reported for a variety of vertebrate species (mammals: Charpenet *et al.*, 1981, Norman & Smith, 1992; 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 an important function of 11KT during sexual maturation is the stimulation of spermatogenesis. In Japanese eel, Miura *et al.* (1991) demonstrated that *in vitro* complete spermatogenesis could be induced by the application of 11KT to the culture medium. In the present study we show that cortisol caused a decline of 11KT levels, which is accompanied by an inhibition of the first wave of spermatogenesis, suggesting a causal relationship between the retardation of spermatogenesis and the decrease in 11KT secretion. Whether this is a direct effect of cortisol on the testicular androgen production, or an indirect action via the hypothalamic–pituitary gonadotropic system cannot be deduced from the present results. In mammals testosterone is secreted by the Leydig cells under LH stimulation, and testosterone may be considered to be the functional homologue of the fish androgen 11KT for promoting spermatogenesis (significance of testosterone for mammalian spermatogenesis reviewed by McLachlan *et al.* (1996) and Griswold (1998). Mammalian Leydig cells are known to express glucocorticoid receptors (GRs) (Schultz *et al.*, 1993) and *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 & 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, 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. From the present study, we do not have evidence for direct effects on the secretion of 11KT. However, in an earlier study (Consten *et al.*, 2000) we have shown that testes of cortisol treated common carp have a decreased OA and 11KT basal and LH–induced secretory capacity *in vitro*, indicating that a direct effect of cortisol on the Leydig cells may occur. Furthermore, in the same study we have shown that the addition of the non–metabolizable cortisol agonist, dexamethasone, to the incubation medium has similar effects.

In a successive study it will be investigated whether cortisol competitively inhibits the conversion of 11β–hydroxyandrostenedione (OHA) into OA or has an effect on the testicular steroid synthesizing capacity.

Cortisol may also have its effect on spermatogenesis via an action on Sertoli cells. In fish as in mammals, one of the functions of Sertoli cells is to mediate the action of androgens on spermatogenesis (Nagahama, 1994). Since glucocorticoid receptors (GRs) have been demonstrated in Sertoli cells in mammalian testes (Levy *et al.*, 1989) and these cells respond to glucocorticoids (Jenkins & Ellison, 1986, Lim *et al.*, 1996), an effect of cortisol on spermatogenesis via Sertoli cells can not be excluded. The presence of GR in the testis of fish has been confirmed by RT–PCR (Takeo *et al.*, 1996), but the exact localization is unknown yet.

Like in the African catfish (Schulz *et al.*, 1997), we observed an activation of the gonadotrophs in the pituitary during pubertal development, reflected by the increasing LH content. Schulz *et al.* (1997) suggested that a signal of testicular origin was responsible for the activation of the LH gene transcription and translation and LH storage. Indeed, several studies have shown that testosterone stimulates the maturation of gonadotrophs and the expression and storage of LH in various teleost species (Crim & Evans, 1979, Gielen & Goos, 1983, Magri *et al.*, 1985, Cavaco *et al.*, 1995, Rebers *et al.*, 2000). This is supported by studies of Cavaco *et al.* (1997), showing that testosterone is produced by the testis before sexual maturation. Moreover, castration slowed down gonadotroph maturation, a process that could be restored by testosterone replacement (Cavaco *et al.*, 1998c).

There is no evidence yet whether gonadotropin gene expression, storage and release are directly influenced by cortisol. The observed effect may be indirect via a reduced secretion of testosterone. Pituitary LH content was suppressed in the cortisol treated group only at 101 dph. There seems to be no relation with any of the other parameters, which limits the relevance of this observation. Gpα, FSHβ and LHβ mRNA steady state levels, however, were significantly decreased at 106 dph, which corresponds to the reduced T plasma content.

In the present experiments we found reduced plasma LH levels. Although a suppression of gonadotropin levels in fish by cortisol has been observed earlier (Zohar, 1980, Carragher *et al.*, 1989), the data are not always consistent. Some studies showed no effect (Pickering, 1981), others even an increase (Pickering, 1987a).

The reduced expression and release of gonadotropins after cortisol treatment may be related to the impaired testicular androgen secretion, but again, a direct effect of cortisol on the pituitary or via the hypothalamus cannot be excluded. In mammals, it has been demonstrated that corticosteroids inhibit the GnRH-induced LH release by inhibiting the responsiveness to GnRH (Padmanabhan *et al.*, 1983, Suter *et al.*, 1988). The inhibitory effects on the LH release may, however, also be caused by a suppression of the hypothalamic GnRH release (Rosen *et al.*, 1988). Both these pathways suppose the presence of GRs on GnRH neurons (or on neural elements controlling the GnRH neurons) or the gonadotrophs. Indeed, in fish GRs have been found in the hypothalamic GnRH neurons and in pituitary gonadotrophs of the rainbow trout (Teitsma *et al.*, 1999). Studies on the GnRH gene of several teleost species have shown that the GnRH promoter contains putative glucocorticoid responsive elements (GRE) (salmon GnRH: Klungland *et al.*, 1992, Higa *et al.*, 1997 and seabream GnRH and chicken GnRH-II: Chow *et al.*, 1998). *In vitro* experiments with immortalized GnRH-secreting cell lines, expressing a functional GR showed that dexamethasone repressed both the endogenous mouse GnRH gene by decreasing steady state levels of GnRH mRNA, and the transcriptional activity of transfected rat GnRH promoter-reporter gene constructs (Chandran *et al.*, 1994). The same author identified negative regulatory elements in the mouse GnRH encoding gene, which bind heteromeric complexes containing glucocorticoid receptor and mediate the repressive action of glucocorticoids (Chandran *et al.*, 1996). In addition, Attardi *et al.* (1997) showed that dexamethasone affected the GnRH secretion from GT1-7 cells as well.

In the present study, sGnRH content of the brain of control animals shows a gradual decrease from 90 to 101 dph and a sudden increase on 106 dph. We have no indication yet whether this profile reflects changes in synthesis, storage or release or a combination of these processes. However, our experiments demonstrate that prolonged cortisol treatment resulted in lower sGnRH levels in the brain, suggesting that the observed effects on the gonadotrophs may indeed be caused by a reduction of sGnRH secretion.

In conclusion, we show that cortisol inhibits pubertal development in common carp and that this inhibition is present at all levels of the BPG-axis. The present results are not sufficient to elucidate whether cortisol acts directly or indirectly in the different parts of the BPG-axis. Current investigations are designed to answer this question.

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

Co-authors: Jan G.D. Lambert, Henk J.Th. Goos
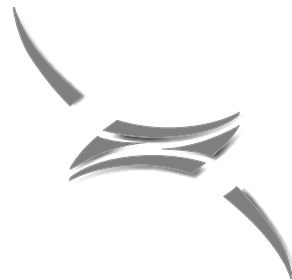
Abstract

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).

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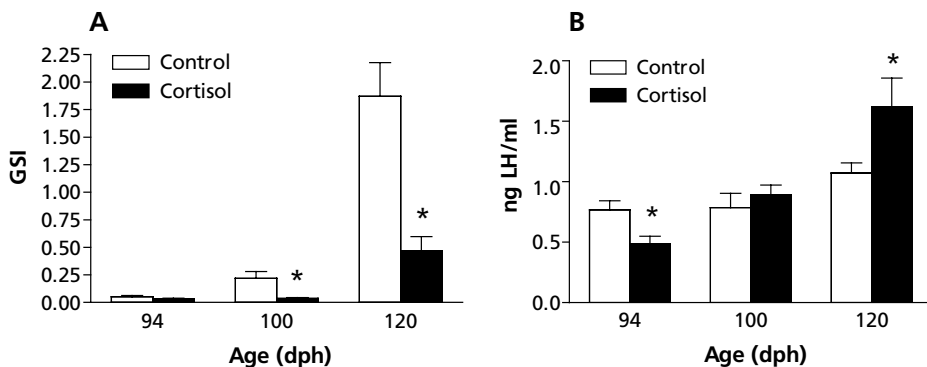
Figure 1. Effect of cortisol treatment on (A) gonadosomatic (GSI) index (n=20) and (B) plasma LH levels (n=20). * indicates a significant difference between the control group and the cortisol treated group (p<0.05, Student's T-test).

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-
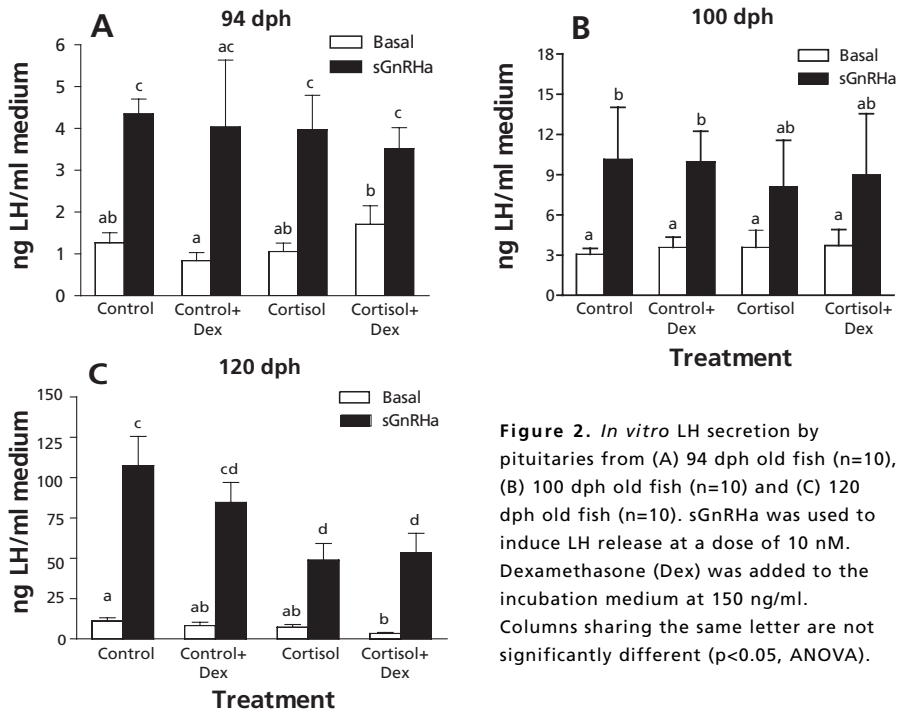
Figure 2. *In vitro* LH secretion by pituitaries from (A) 94 dph old fish (n=10), (B) 100 dph old fish (n=10) and (C) 120 dph old fish (n=10). sGnRHa was used to induce LH release at a dose of 10 nM. Dexamethasone (Dex) was added to the incubation medium at 150 ng/ml. Columns sharing the same letter 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).
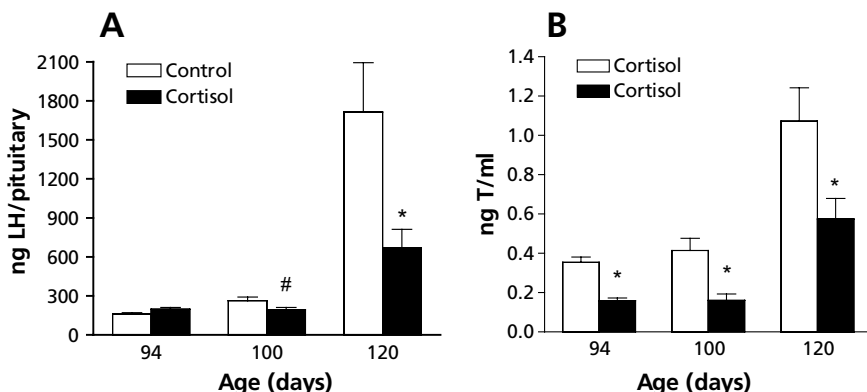
Figure 3. Effect of cortisol treatment on (A) pituitary LH content (n=20) and (B) plasma testosterone levels (n=20). ∗ indicates a significant difference between the control group and the cortisol treated group (p<0.05, Student's T-test). # indicates a difference of p<0.1.

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.

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

Co-authors: Jan G.D. Lambert, Henk J.Th. Goos

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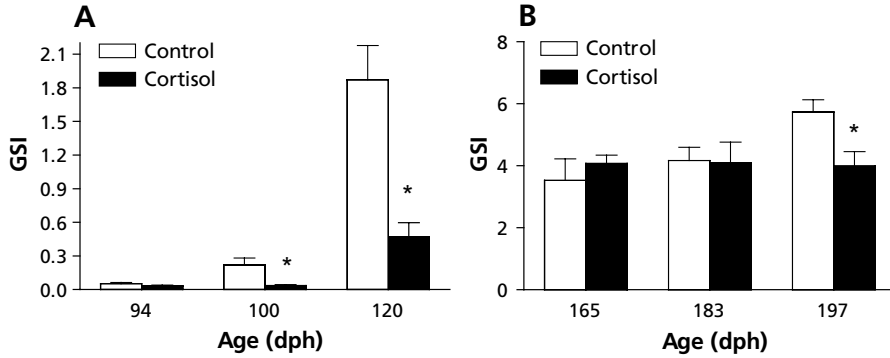
Figure 1. Effect of cortisol treatment on testicular development, represented by the gonadosomatic index in (A) pubertal fish (n=20) and (B) adolescent fish (n=8). * indicates a significant difference between the control group and the cortisol treated group (p<0.05).

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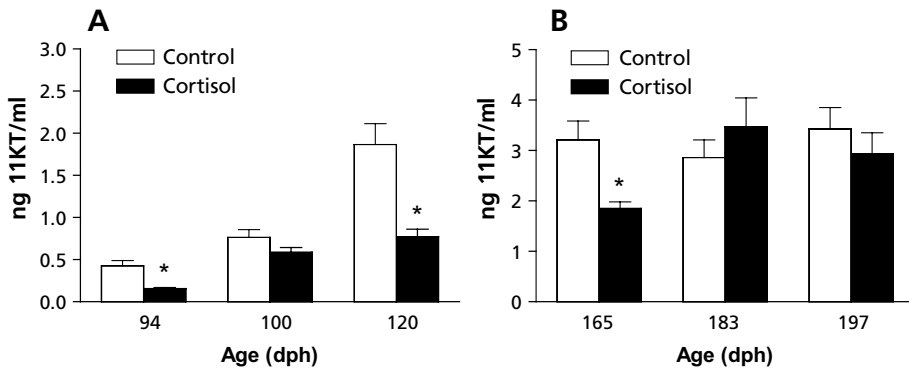
Figure 2. Effect of prolonged feeding with cortisol containing food pellets on plasma 11KT levels in (A) pubertal fish (n=20) and (B) adolescent fish (n=8). * indicates a significant difference between the control group and the cortisol treated group (p<0.05).

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).
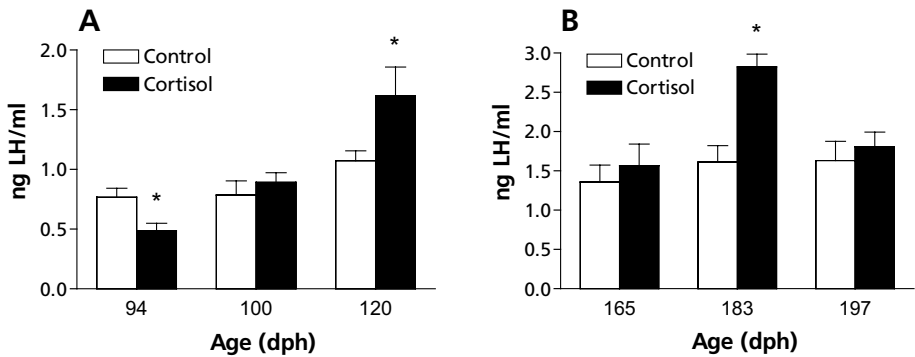

Figure 3. Effect of prolonged feeding with cortisol containing food pellets on plasma LH levels in (A) pubertal fish (n=20) and (B) adolescent fish (n=8). * indicates a significant difference between the control group and the cortisol treated group ($p < 0.05$).

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.
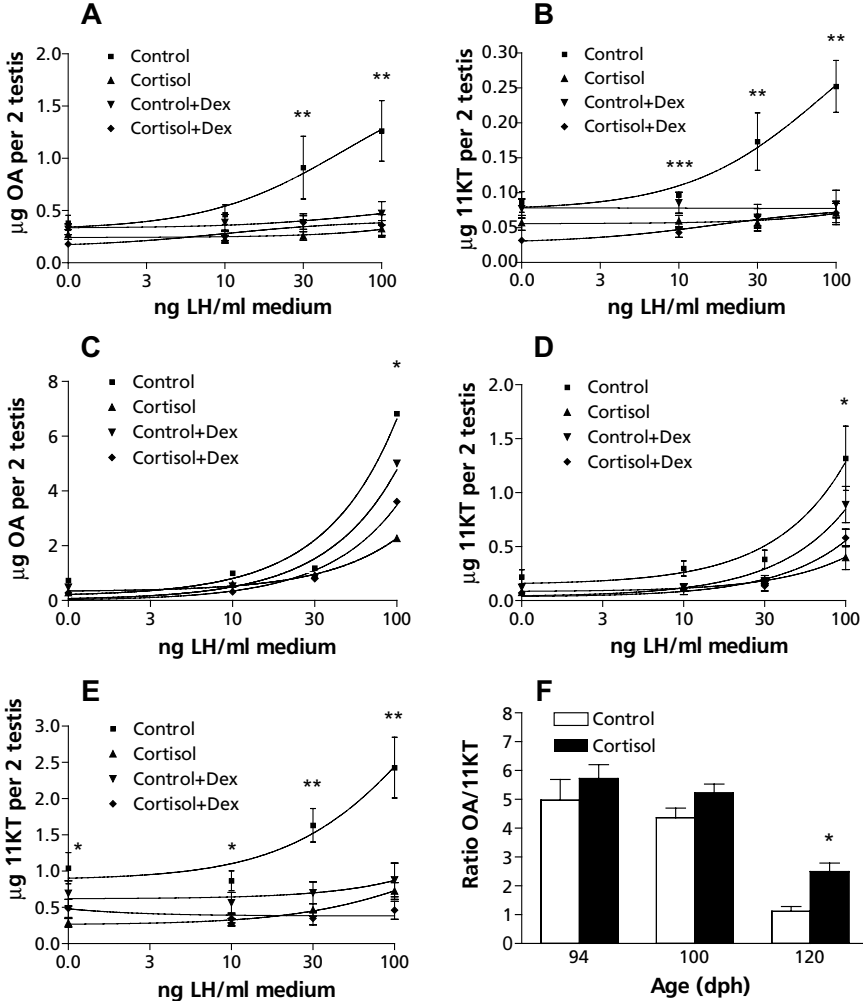
Figure 4. Effect of *in vivo* cortisol treatment and *in vitro* dexamethasone (Dex) treatment on the *in vitro* steroid secretion of pubertal fish, expressed as µg steroid per 2 testes (n=10). (A) OA secretion at 94 dph, (B) 11KT secretion at 94 dph, (C) OA secretion at 100 dph, (D) 11KT secretion at 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
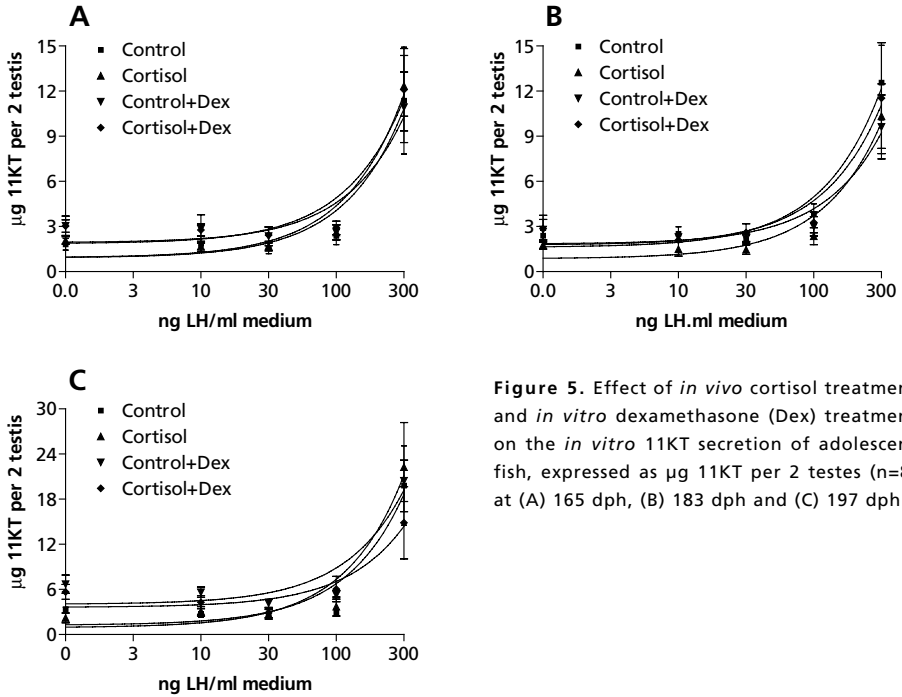
Figure 5. Effect of *in vivo* cortisol treatment and *in vitro* dexamethasone (Dex) treatment on the *in vitro* 11KT secretion of adolescent fish, expressed as µg 11KT per 2 testes (n=8) at (A) 165 dph, (B) 183 dph and (C) 197 dph.

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–hydroxyandrostene-dione (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 trans-duction. 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 pro-ducing enzymes, substrate inhibition of enzymes that have a function in the con-version 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.

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

Co-authors: Eelco D. Keuning, Maarten Terlou, Jan G.D. Lambert, Henk J.Th. Goos
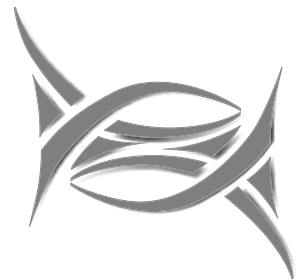
Abstract

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 $[1,2\text{-}^3\text{H}]$-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
$[7\text{-}^3\text{H}]$-Pregnenolone (sp. act. 925 GBq/mmol), $[7\text{-}^3\text{H}]$-androstenedione (sp. act. 907 GBq/mmol) and $[1,2\text{-}^3\text{H}]$-cortisol (sp. act. 1924 GBq/mmol) were purchased from NEN–Dupont life science. ^3H-labelled 11β–hydroxyandrostene-dione was chemically produced out of $[1,2\text{-}^3\text{H}]$-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 $[1,2\text{-}^3\text{H}]$-OHA and 20 μg of cortisol and cortisone to incubations with $[1,2\text{-}^3\text{H}]$-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 ⋆ 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 tetra-zolium (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 micro-scope. 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 selec-tion 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).
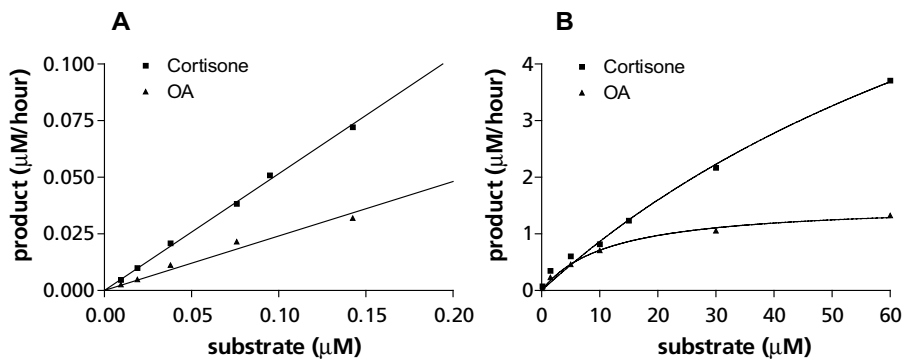
Figure 1. Conversion of cortisol and OHA into cortisone and OA, respectively, by testicular homogenates within one hour. (A) Linear regression for substrate concentrations in the range up to 150 nM, and (B) non-linear regression for substrate concentration in the range μM.

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).
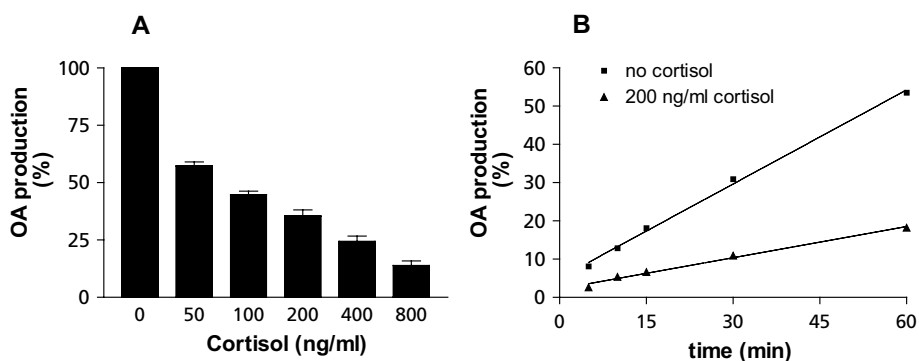
Figure 2. Effects of glucocorticoids on the conversion of OHA by 11β-HSD performed by incubations with testicular homogenates. conversion by 11β-HSD. (A) Relative production of OA after one hour, with increasing concentrations of cortisol. (B) conversion of OHA into OA, in a one hour time course in the 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 cortisol treatment and slowly decreased to control levels over de following 2.5 hours (Fig. 3D).
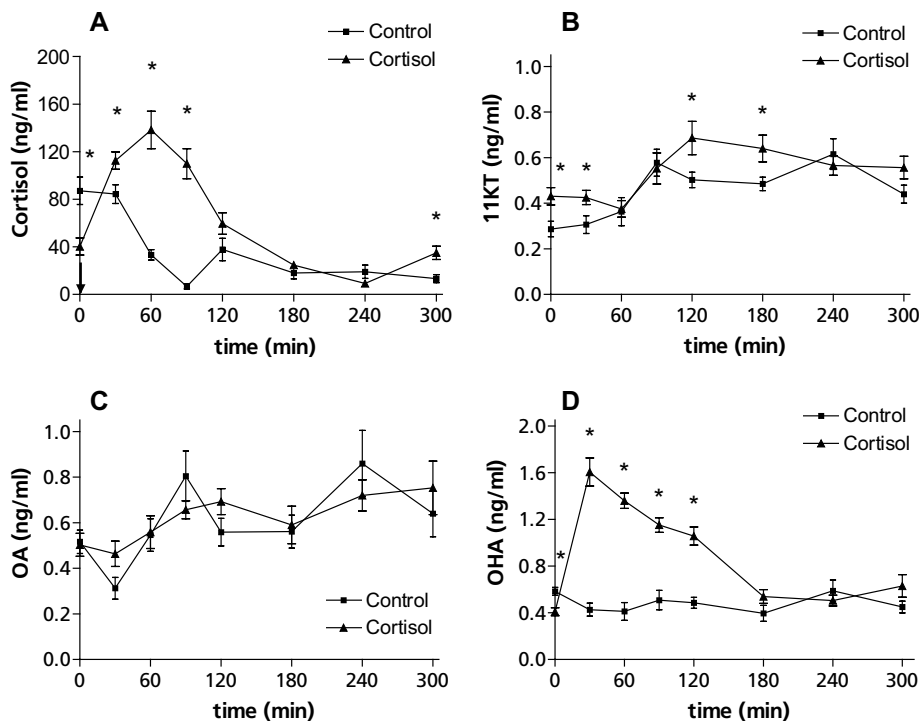
Figure 3. Plasma levels of (A) cortisol, (B) 11KT, (C) OA and (D) OHA over a 5 hour time period, after cortisol administration (n = 10). Arrow indicate the feeding time. * indicates a significant difference (p<0.05).

Experiment 3:

The effect of long-term cortisol treatment

on testicular steroid production capacity and

determination of the relative amount of Leydig cells.

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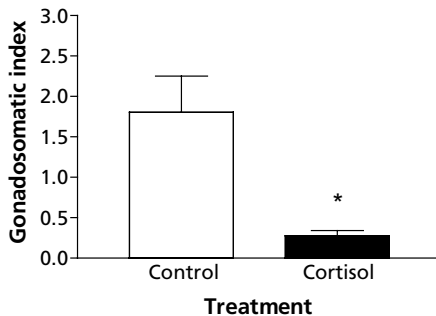
Figure 4. Effect of long-term cortisol treatment on the testicular development, represented by the gonadosomatic index at 120 dph (n = 15). * indicates a significant difference (p<0.05).

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.

Sex steroids and their involvement in the cortisol-induced inhibition of pubertal development in male common carp, *Cyprinus carpio* L

Co-authors: Eelco D. Keuning, Jan Bogerd, Thijs A. Zandbergen, Jan G.D. Lambert, Henk J.Th. Goos
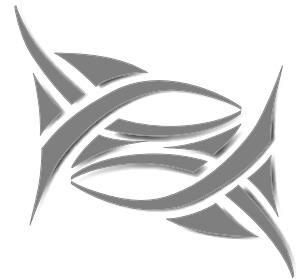
Abstract

The onset and regulation of puberty is determined by functional development of the brain-pituitary-gonad (BPG) axis. Sex steroids, produced in the gonads, exert an important function in the onset of puberty. Stress has been shown to interfere with reproduction and the functioning of the BPG-axis and cortisol has frequently been indicated as a major factor mediating the suppressive effect of stress on reproduction. Previous work showed that prolonged elevated cortisol levels, implicated in the stress adaptation, inhibit pubertal development in male common carp. Cortisol treatment caused a retardation of pubertal testis development and reduced the luteinizing hormone (LH) content and the salmon GnRHa-stimulated LH secretion *in vitro*. Furthermore, a reduced synthesis of androgens was observed. This suggests that the cortisol-induced inhibition of the testicular development, as well as the maturation of pituitary gonadotrophs is mediated by an effect on testicular androgen secretion. In this study we combined cortisol treatment with a replacement of the testicular steroid hormones, testosterone and 11-oxygenated androgens, in order to investigate the role of these steroids in the cortisol-induced suppression of pubertal development. Our results indicate that the effect of cortisol on spermatogenesis is independent of 11KT, whereas the effect on the pituitary is an indirect one, involving the testicular secretion of testosterone.

Introduction

In juvenile fish, the importance of sex steroids in the onset of puberty has been shown in several studies. Sex steroids have been demonstrated to stimulate the development of all levels of the BPG axis. In the African catfish, *Clarias gariepinus*, treatment with 11-oxygenated androgens stimulated testicular growth and spermatogenesis as well as the development of secondary sexual characteristics (Cavaco *et al.*, 1998b). Stimulation of spermatogenesis by 11-oxygenated androgens has also been observed in pre-pubertal common carp, *Cyprinus carpio* L. (Komen, personal communication).

Treatment with testosterone activated the gonadotroph maturation (Cavaco *et al.*, 1995) and accelerated the development of the hypothalamic GnRH system (Dubois *et al.*, 1998).

The adaptation to severe and chronic stress has been shown to interfere with processes such as growth, immune response or reproduction. 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). Elevated cortisol levels resulted in an impairment of spermatogenesis, as well as a reduction of the synthesis of the 11-oxygenated androgens. We showed that these effects were not mediated by an effect of cortisol on LH secretion (Consten *et al.*, 2001b). However, cortisol does affect the LH secretion at the level of the pituitary as we observed a smaller LH releasable pool and a reduced salmon GnRHa-stimulated LH secretion *in vitro*. Testosterone has been shown to induce development of pituitary gonadotrophs, leading to an increase in LH content and GnRH-inducible LH release maturation (Cavaco *et al.*, 1995). We hypothesized that cortisol inhibits the testicular testosterone secretion and thereby prevents LH storage, which leads to a reduced GnRH-inducible LH release *in vitro*. Furthermore, we suggested that the reduced steroid hormone secretion not only had its effects on the maturation of pituitary gonadotrophs, but also on the testicular development (Consten *et al.*, 2001a, 2001b).

The aim of the present study was to investigate whether cortisol has a direct effect on the development of pituitary and testis or an indirect effect via the reduced androgen secretion. Therefore we combined cortisol treatment with a replacement of the testicular steroid hormones, testosterone and 11-oxygenated androgens.

Material and Methods

Animals

Isogenic male common carp, 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 61 dph when the experiment started.

Steroid treatment

Three hundred and twenty animals were equally divided over six groups. At 61 dph, two days before the onset of cortisol treatment, three control groups (designated C (control), CK (control + OA) and CT (control + T)) and three

future cortisol groups (designated F (cortisol), FK (cortisol + OA), FT (cortisol + T)) were implanted with cocoa butter containing either no steroid, 11-ketoandrostenedione (OA) or testosterone (T) at a dose of 5 mg/kg bodyweight (dose determined by pilot studies). 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 solidified within the body-cavity of the fish, even if the fish are kept at 25°C (van Malssen et $al.$ 1996). The steroids, T and OA (5 mg/kg fish) were suspended in molten cocoa butter and fish were implanted with the cocoa butter by injecting 100 µl per 20 g body weight with a 1 ml syringe (needle: 21Gx1$^1/_2$"). OA has been shown to be rapidly converted into 11-ketotestosterone (11KT). Pilot studies revealed that steroid levels peaked one day post-injection and were back to control levels ten days post-injection. Therefore all groups were re-implanted every ten days and also one day before the onset of sampling at 89 dph, in order to measure the effectiveness of the implantation. At several time intervals, covering the pubertal development of the common carp, 15 fish per group were sampled.

Cortisol (Steraloids Inc. Wilton, USA) containing food (100 mg/kg food) was prepared as described by Pickering et $al.$ (1987b). Starting at 63 dph, all control groups received control food (C), the other groups the cortisol-containing food (F). 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 all groups (n=15) were sampled at several time-intervals during the pubertal development at 89, 95, 100 and 120 dph. 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 / (bodyweight-testis weight)) and fixed for histological determination of the testicular development.

Testicular histology

For determination of the spermatogenetic 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 germ cells including spermatozoa. The number of animals

per group with the same stage of testicular development are counted and expressed as a percentage of the total group.

Pituitary incubations

Ten pituitaries per group were collected individually and pre-incubated for 18 h in L-15 medium (15mM HEPES buffered, pH 7.4, 26mM sodium bicarbonate, 100,000 U/l penicillin/streptomycin) containing 5% horse serum. The pituitaries were rinsed once and 0.5 ml fresh L-15 medium 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 containing 10 nM salmon GnRHa 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.

Incubation medium and pituitary LH determination

LH was quantified in the incubation medium and the pituitaries using a homologous radioimmunoassay (RIA) (slightly modified from Goos *et al.*, 1986). Ten pituitaries per treatment group were individually homogenized and assayed. 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 the steroids 11-ketotestosterone (11KT), 11-ketoandrostenedione (OA) and testosterone (T) were measured in a RIA as described by Schulz (1985).

Cloning of a carp glucocorticoid receptor partial cDNA

Total RNA was isolated from common carp brains by the method of Chirgwin *et al.* (1979). Oligo dT-primed cDNA was synthesized using Superscript II RNaseH⁻ reverse transcriptase (Life Technologies, Breda, The Netherlands), according to the manufacturer's instructions. A partial 372 bp glucocorticoid receptor (GR) cDNA of the carp was PCR amplified using degenerate primers based on the rainbow trout (Ducouret *et al.*, 1995), tilapia (Tagawa *et al.*, 1997) and Japanese flounder (Tokuda, unpublished results, acc.no. AB013444) GR sequences. The following oligodeoxynucleotide primers were used (Life Technologies, Breda, The Netherlands): carp GR-Fw, 5'-CTGCAGT-GCTCCTGGCTITTYCTIATG-3' and carp GR-Rv, 5'-GTIAGCTGATA-GAAICKCTGCCARTTYTG-3'. The amplified fragment was subcloned into

pGEM-T vector (Promega) and transformed into *Escherichia coli* competent cells. The sequence of the clone was checked by nucleotide sequence analysis.

In situ hybridization

In order to investigate the presence of the GR mRNA in the testis, a non-radioactive *in situ* hybridization, as described by Braat *et al.* (1999), was performed on sections of testis taken from 95 dph old carp, fixed in 4% paraformaldehyde, 5% acetic acid in phosphate buffered saline (PBS). Digoxygenin–rUTP-labeled anti-sense and sense probes were synthesized after linearization of the GR cDNA fragment by performing PCR on the pGEM-T vector with vector-based primers PBS-A and PBS-E, followed by *in vitro* transcription with T7– and SP6 RNA polymerase, respectively.

Statistics

All results are expressed as mean $\pm$ 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

Steroid treatment

Implantation of cocoa butter containing OA leads to a significant elevation of the plasma levels for 11KT at 89 dph, one day post-implantation (Fig. 1A). A slight, not significant increase in plasma OA (data not shown) was observed indicating that the conversion of OA to 11KT is nearly 100%. Implantation of T also leads to elevated plasma T levels at 89 dph (Fig. 1B).
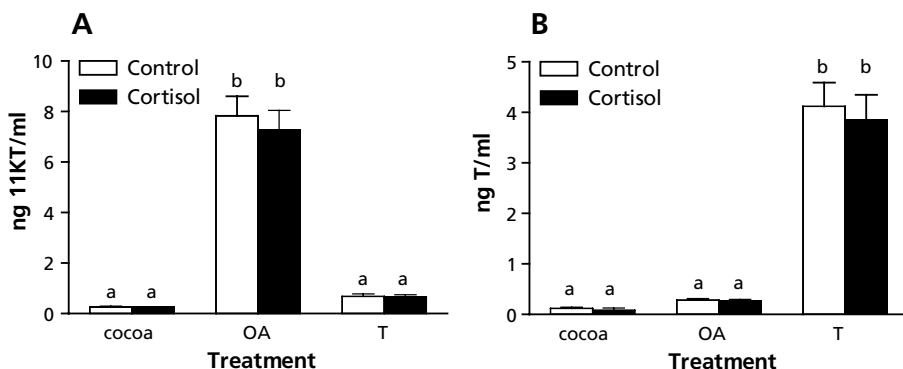
Figure 1. Plasma levels for (A) 11KT and (B) T one day after implantation (89 dph) with cocoabutter alone, OA or T, respectively (n=10). Data sharing the same letter are not significantly different (p<0.05)..

Gonadosomatic index (GSI)

The increase in GSI, as indicated by the increase in the values on the Y-axis, reflects normal testicular development of the control fish (C) during puberty (Fig. 2). In contrast, the cortisol treated fish (F) show an impaired testicular development as follows from the significantly lower GSI at 95, 100 and 120 dph and the histological analysis of the testis at 95 dph (Fig. 2E). Implantation of OA had no significant effect on the gonadal development in the control fed animals (CK) at all days. Cortisol treated animals, implanted with OA (FK), also showed an impaired spermatogenesis, similar to the cortisol treated fish (F). However, only at 120 dph, concomitant cortisol and OA treatment leads to an increase in GSI compared to cortisol treatment alone. Due to the somewhat higher varia-tion in this group, the difference, however, is not statistically significant
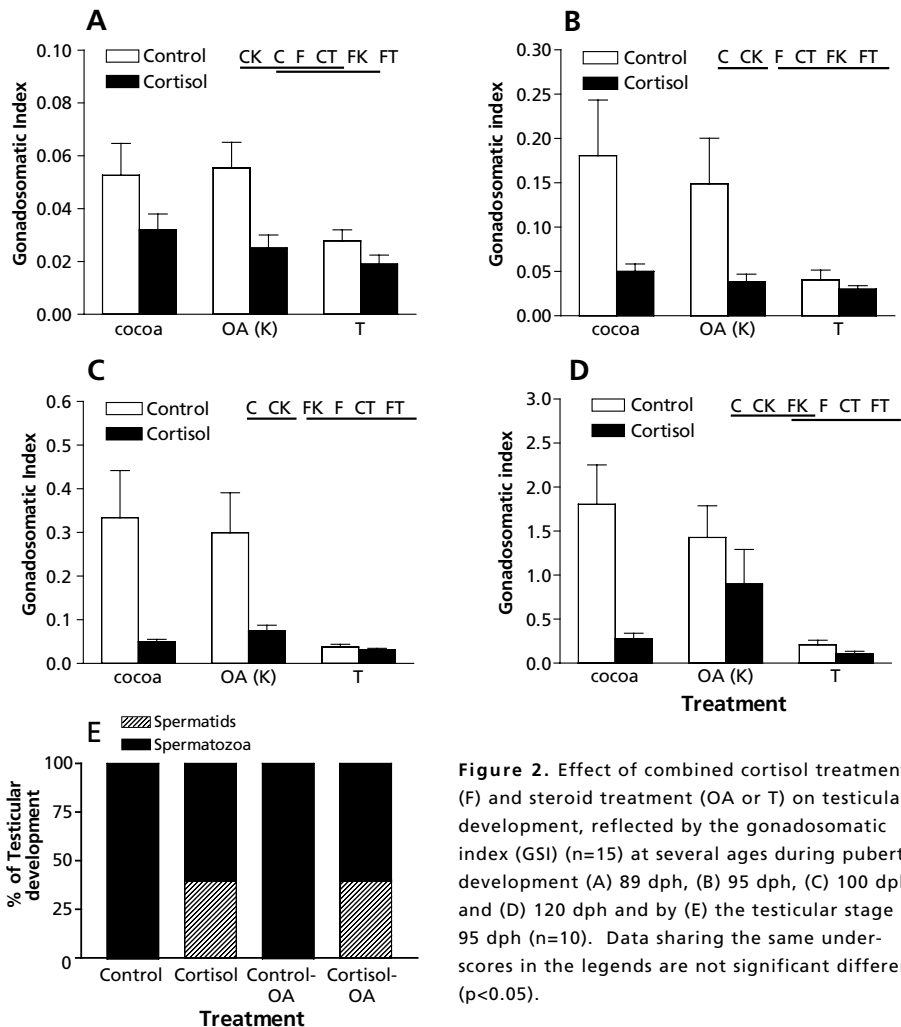
Figure 2. Effect of combined cortisol treatment (F) and steroid treatment (OA or T) on testicular development, reflected by the gonadosomatic index (GSI) (n=15) at several ages during pubertal development (A) 89 dph, (B) 95 dph, (C) 100 dph and (D) 120 dph and by (E) the testicular stage at 95 dph (n=10). Data sharing the same under-scores in the legends are not significant different (p<0.05).

(Fig. 2D). Treatment with T had a similar effect on testicular development as cortisol treatment. At all days sampled both the control (CT) and cortisol (FT) treated group, implantation with T caused a significant inhibition of the testicular development (Fig. 2).

Pituitary content

Pituitary LH content increased significantly during pubertal development (Fig. 3). At 89 and 95 dph no significant difference was observed between control and cortisol treated fish (Fig. 3A, B). However, at 100 dph there is a slight, not significant, difference (Fig. 3C), whereas at 120 dph the LH content of the control fish is significantly higher than in the cortisol treated fish (Fig. 3D). Concomitant treatment with OA resulted in somewhat lower pituitary LH levels at 95 and 100 dph (Fig. 3B, C). However, at 120 dph the inhibitory effect of OA becomes apparent, both OA-treated groups (CK and FK) are significant-
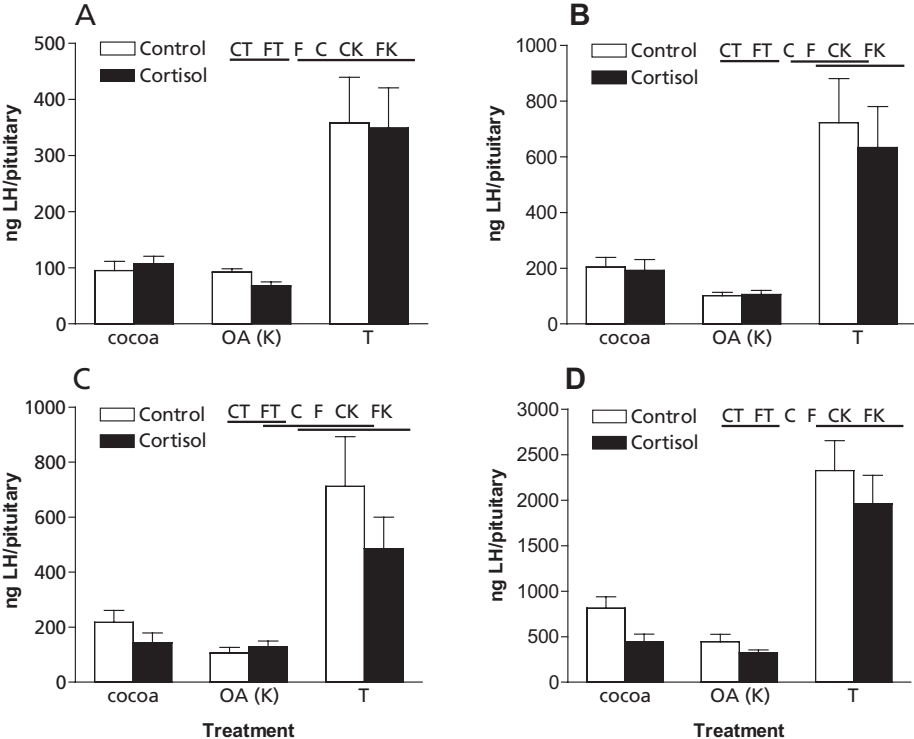
Figure 3. Effect of combined cortisol treatment (F) and steroid treatment (OA or T) on pituitary LH content (n=10) at several ages during pubertal development (A) 89 dph, (B) 95 dph, (C) 100 dph and (D) 120 dph. Data sharing the same underscores in the legends are not significant different (pK0.05).

ly different from the control group (C) (Fig. 3D). Treatment with T strongly elevated the pituitary LH content in both control and cortisol treated groups (CT and FT) at all sampled days. At 100 dph, concomitant treatment with cortisol caused to some extent an inhibition of the T-induced increase in the pituitary LH content (Fig. 3C).

Salmon GnRHa-stimulated LH secretion in vitro

In all groups the *in vitro* LH release was significantly stimulated by 10 nM sGnRHa (Fig. 4). Cortisol treatment alone had no effect on both basal and sGnRHa-stimulated LH release at 94dph (Fig. 4A). At 100 dph basal secretion was unaffected by the cortisol treatment, but the sGnRHa-stimulated release was significantly decreased (Fig. 4B), whereas at 120 dph both basal and sGnRHa-stimulated release were significantly depressed (Fig. 4C). Similar to the pituitary LH content, treatment with OA resulted in a slight reduction of the basal and sGnRHa-stimulated LH release at 95 dph (Fig. 4A). This reduction is significant at100 and 120 dph; both OA-treated groups (CK and FK) are significantly different from the control group (C) (Fig. 4B, C).
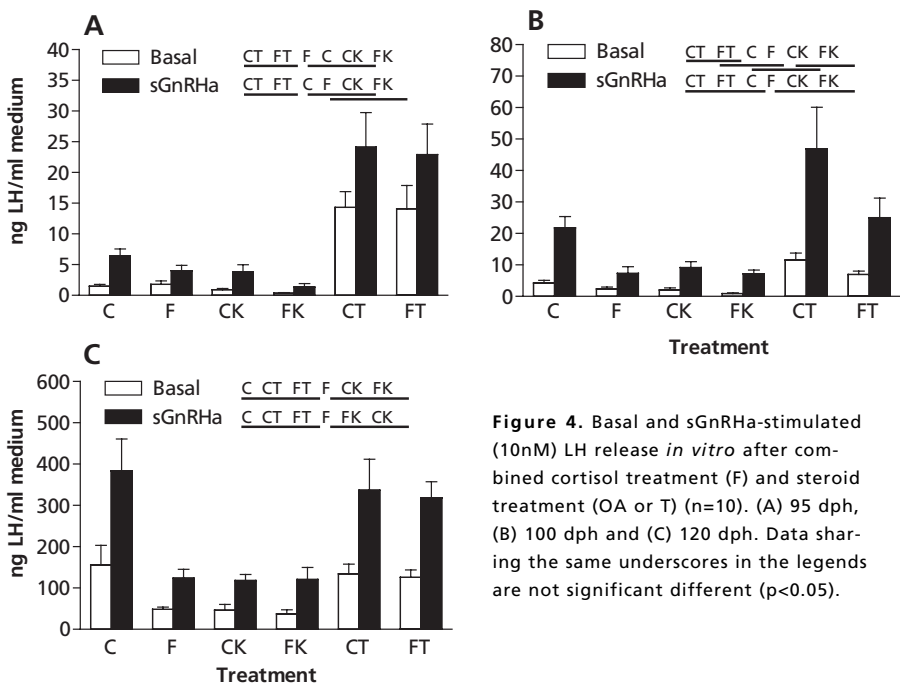
Figure 4. Basal and sGnRHa-stimulated (10nM) LH release *in vitro* after combined cortisol treatment (F) and steroid treatment (OA or T) (n=10). (A) 95 dph, (B) 100 dph and (C) 120 dph. Data sharing the same underscores in the legends are not significant different (p<0.05).

Treatment with T caused a significant increase in the basal as well as the sGnRHa stimulated LH release *in vitro* at 95 and 100 dph (Fig. 4A, B). At 120 dph both T treated groups (CT and FT) are, however, no longer significantly different from the control group (C) (Fig. 4C). Concomitant treatment with cor-

tisol has no significant effect on the basal and sGnRHa-stimulated LH release *in vitro*, although at 100 dph slightly lower levels are found in the combined cortisol and T treated group (FT) compared to the T treated control group (CT) (Fig. 4B)

Carp glucocorticoid receptor

Part of the glucocorticoid receptor was amplified from common carp brain cDNA, using the carp GR-Fw and carp GR-Rv primers. This yielded a PCR product of approximately 370 bp. The amplified fragment was subcloned and identified by DNA-sequence analysis. The sequence showed highest homology with the rainbow trout GR. The nucleotide sequence and the deduced amino acid sequence are shown in figure 5.

```
            S   F   G   L   G   W   R   S   Y   Q   Q   C   N   G
Carp GR-Fw- TCC'TTC'GGC'TTG'GGC'TGG'AGA'TCC'TAC'CAG'CAG'TGT'AAC'GGA'

 G   M   L   C   F   A   P   D   L   V   I   N   E   E   R   M   K
GGC'ATG'CTG'TGT'TTC'GCT'CCA'GAC'CTG'GTC'ATC'AAT'GAG'GAG'AGG'ATG'AAA'

 L   P   Y   M   N   D   Q   C   S   Q   M   L   K   I   T   S   E
CTA'CCC'TAC'ATG'AAT'GAC'CAG'TGT'AGC'CAG'ATG'CTG'AAG'ATC'ACC'AGT'GAC'

 L   V   R   L   Q   V   S   Y   D   E   Y   L   C   M   K   V   L
CTG'GTC'AGG'CTG'CAG'GTG'TCC'TAC'GAC'GAG'TAT'CTC'TGC'ATG'AAA'GTC'CTC'

 P   L   L   S   T   V   P   K   D   G   L   K   S   Q   A   V   F
CCG'CTC'CTC'AGC'ACA'GTA'CCA'AAG'GAC'GGC'CTT'AAA'AGC'CAA'GCT'GTG'TTT'

 D   E   I   R   M   S   Y   I   K   E   L   G   K   A   V   V   K
GAT'GAA'ATC'CGC'ATG'TCG'TAC'ATC'AAG'GAG'CTG'GGC'AAA'GCC'GTT'GTT'AAA'

 R   E   E   N   S   S
CGA'GAG'GAG'AAC'TCC'AGC'-carp GR-Rv
```

Figure 5. Nucleotide sequence of the partial GR cDNA (372 bp) and the deduced amino acid sequence. The depicted sequence is the sequence between the degenerate primers carp Gr-Fw and carp GR-Rv.

In situ hybridization

The *in situ* hybridization on testicular tissue of common carp revealed that the glucocorticoid receptor mRNA is present in several types of germ cells. Spermatogonial stem cells and early spermatogonia showed no staining, whereas late spermatogonia show specific staining for the GR. The most intense staining is found in spermatocytes. In spermatids and spermatozoa the messenger for the GR was not detected. Sections incubated with the cRNA sense probe yielded no signal (Fig. 6).
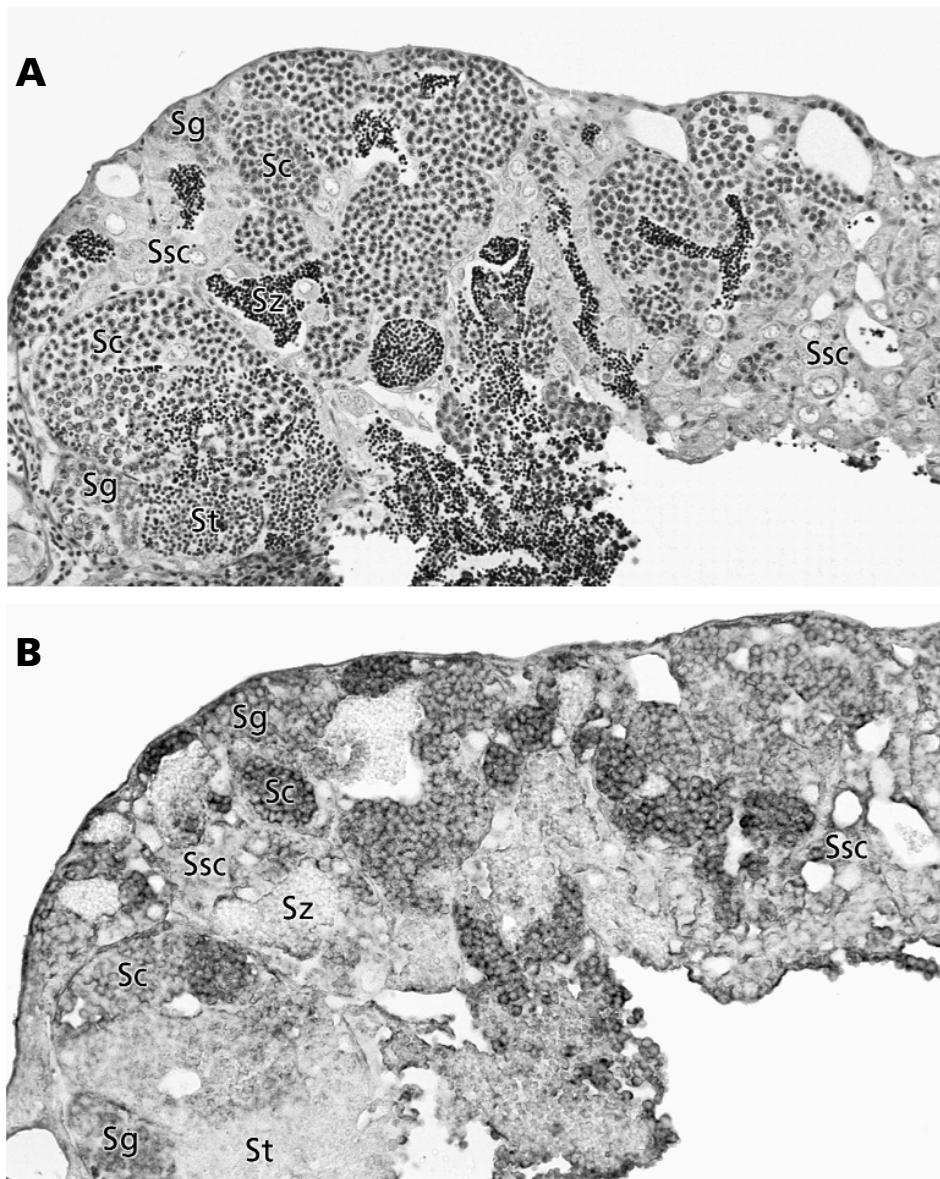
Figure 6. *In situ* hybridization of common carp testis with the GR cRNA probe.(A) Haemaluin eosin staining; (B) *in situ* hybridization. Staining is found in late spermatogonia (Sg) and spermatocytes (Sc), whereas spermatogonial stem cells (Ssc), spermatids (St) and spermatozoa (Sz) show no signal (magn: 240x).

Discussion

In an earlier study (Consten *et al*., 2001a) we observed that the inhibitory action of cortisol on pubertal development of the testis in common carp was accompanied by a suppressed testicular androgen secretion. 11-Oxygenated androgens have been shown to stimulate spermatogenesis in the Japanese eel, *Anguilla japonica* (Miura *et al*., 1991), in the African catfish (Cavaco *et al*., 1998b) and in the common carp (Komen, personal communication). Also in the goldfish, *Carassius auratus*, a close relative of the common carp, 11KT has been shown to induce spermatogenesis (Kobayashi *et al*., 1991). The inhibitory effect of cortisol on spermatogenesis could be a direct one, or its effect may be via the reduced androgen production.

The aim of the present study was to elucidate the role of the androgens, 11KT and T in the cortisol–induced suppression of testicular development in the male common carp. Therefore, in this study we combined cortisol treatment with the replacement of 11KT, by implanting the fish with OA, which is readily converted to 11KT.

In this study, cortisol treatment caused again a retardation of pubertal development, reflected by significantly lower GSIs from 95 dph onwards and the less advanced spermatogenetic stages at 95 dph. However, restoration of the 11KT levels in cortisol treated animals did not result in a testicular development similar to the control animals. Testosterone treatment caused a clear suppression of the testicular growth.

These results indicate that, even when the 11KT levels are restored in the cortisol treated fish, the inhibitory effect of cortisol on testicular development cannot be prevented. Thus, cortisol interferes with spermatogenesis at a lower level of the stimulatory cascade than 11KT. In the endocrine regulation of spermatogenesis, 11KT acts on the Sertoli cells, in which it triggers the production of activin B. Activin B then acts on the spermatogonia to induce mitosis, leading to the formation of spermatocytes (Nagahama, 1994). Possibly, cortisol acts on the Sertoli cell, interfering with the production and secretion of activin B. In mammalian testes, the presence of glucocorticoid receptors (GRs) in Sertoli cells has been demonstrated (Levy *et al*., 1989, Weber *et al*., 2000) and these cells respond to glucocorticoids (Jenkins *et al*., 1986; Lim *et al*., 1996). Therefore, an effect of cortisol on spermatogenesis via Sertoli cells can not be excluded. However, in rat testes, the GRs have also been shown to be localized on spermatogenetic elements (Schultz *et al*., 1993; Weber *et al*., 2000). The labeling of germ cells indicates that a more direct inhibitory effect of glucocorticoids on spermatogenesis may occur. This is supported by the observation that in several tissues glucocorticoids have been shown to interfere with cell cycle proteins, thereby inhibiting the cell cycle progression (Rogatsky *et al*., 1997; Samuelsson

et al., 1999; Smith *et al.*, 2000). We partially cloned the GR of the common carp to investigate the localization of the GR mRNA in the testis by means of *in situ* hybridization. GR mRNA appeared to be present in late spermatogonia and spermatocytes. This indicates that also in fish, cortisol may act directly on the germ cells, interfering with the cell cycle proteins and thereby hampering spermatogenesis.

Similar to the testicular development, also the pituitary gonadotrophs were affected by the cortisol treatment. Cortisol treated fish had lower pituitary LH contents, which was first observed at 100 dph and became pronounced at 120 dph. Furthermore, the *in vitro* studies showed that from 100 dph on the sGnRHa-stimulated LH secretory capacity of the pituitaries of cortisol treated fish was lower compared to controls. From 120 dph on, both basal and sGnRHa-stimulated LH secretion are significantly depressed in cortisol treated fish. These results are comparable to our earlier study (Consten *et al.*, 2001b), in which we suggested that cortisol inhibits the testicular testosterone secretion and thereby prevents LH storage, leading to a reduced GnRH-inducible LH release *in vitro*. Also in other species testosterone has been shown to induce development of pituitary gonadotrophs, leading to an increase in LH content and GnRH-inducible LH release (Cavaco *et al.*, 1995, 1998d). In the present study, we combined cortisol treatment with the replacement of T. T replacement was able to overcome the cortisol-induced inhibition in gonadotroph maturation. In contrast, 11KT treatment had an inhibitory effect on the pituitary development, which is significant on 120 dph. Studies in the African catfish, also indicate an inhibitory effect of 11KT on the pituitary LH levels (Cavaco *et al.*, 1995; Rebers *et al.*, 2000).

In conclusion, we show that cortisol has a direct inhibitory effect spermatogenesis, which is independent of 11KT. The effect of cortisol on the hypophysial LH secretion is, however, is caused by an indirect effect, involving the testicular secretion of testosterone.

Summarizing discussion
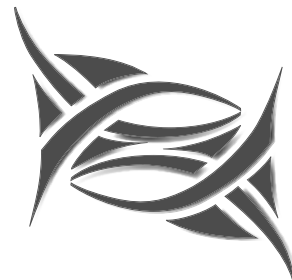
Summarizing Discussion

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 corti-sone. This means that cortisol may inhibit the conversion of 11β-hydroxyan-drostenedione (OHA) into OA and, in this way, may contribute to the inhibi-tion of the testicular androgen secretion. Chapter 6 deals with this question. Our *in vitro* results demonstrated that, indeed, cortisol can compete dose depen-dently 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 admi-nistration, 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 cor-tisol treated fish have the same potency to convert tritiated steroids per weight unit as untreated control fish. Furthermore, by enzymecytochemistry and subse-quent 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 steroido-genic 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 cor-tisol 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 impor-tance 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 cor-tisol 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.

References

Almeida SA, Petenusci SO, Anselmo-Franci JA, Rosa-e-Silva AAM, Lamano-Carvalho TL. 1998 Decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty. Braz. J. Med. Biol. Res. 31(11): 1443-1448.

Amano M, Okuzawa K, Yanagisawa T, Hasegawa Y, Aida K. 1992 Immunoreactive gonadotropin-releasing hormone (ir-GnRH) in the carp brain. Nippon Suisan Gakkaishi 58: 593.

Amoss M, Burgus R, Blackwell R, Vale W, Fellows R, Guillemin R. 1971 Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. Biochem. Biophys. Res. Comm. 44: 205-210.

Attardi B, Tsujii T, Friedman R, Zeng ZW, Roberts JL, Dellovade T, Pfaff DW, Chandran UR, Sullivan MW, DeFranco DB. 1997 Glucocorticoid repression of gonadotropin-releasing hormone gene expression and secretion in morphologically distinct subpopulations of GT1-7 cells. Mol. Cell. Endocrinol. 131: 241-255.

Balm PHM, Hovens MLM, Wendelaar Bonga SE. 1995 Endorphin and MSH in concert form the corticotropic principle released by tilapia (*Oreochromis mossambicus*, Teleostei) melanotropes. Peptides 16: 463-469.

Bambino TH and Hsueh AJW. 1981 Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis *in vivo* and *in vitro*. Endocrinology 108: 2142-2148.

Barry TP, Aida K, Okumura T, Hanyu I. 1990 The shift from C-19 to C-21 steroid synthesis in spawning male common carp, *Cyprinus carpio*, is regulated by the inhibition of androgen production by progestogens produced by spermatozoa. Biol. Reprod. 43: 105-112.

Barton BA and Iwama GK. 1991 Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Diseases, 3-26.

Billard R, Fostier A, Weil C, Breton B. 1982 Endocrine control of spermatogenesis in teleost fish. Can. J. Fisheries Aquat. Sci. 39: 65-79.

Bogerd J, Zandbergen MA, Andersson E, Goos HJTh. 1994 Isolation, characterization and expression of cDNAs encoding the catfish-type and chicken-II-type gonadotropin-releasing-hormone precursors in the African catfish. Eur. J. Biochem. 222: 541-549.

Bongers ABJ, Ben-Ayed MZ, Zandieh-Doulabi B, Komen J, Richter CJJ. 1997 Origin of variation in isogenic, gynogenetic and androgenetic strains of common carp, *Cyprinus carpio*. J. Exp. Zool. 277: 72-79.

Bongers ABJ, Zandieh-Doulabi B, Richter CJJ, Komen J. 1999 Viable androgenetic YY genotypes of common carp, *Cyprinus carpio*, L. J. Heredity 90: 195-198.

Borg B. 1994 Mini review: androgens in teleost fishes. Comp. Biochem. Physiol. 109C(3): 219-245.

Borski RJ. 2000 Nongenomic membrane actions of glucocorticoids in vertebrates. TEM 11(10): 427-436.

Boussès P and Chapuis JL. 1998 Deferred seasonal increase in testes weight under poor nutritional conditions in a sub-Antarctic population of rabbits (*Oryctolagus cuniculus*). J. Zool., Lond. 245(Part 3): 285-291.

Braat AK, Zandbergen MA, de Vries E, van der Burg B, Bogerd J, Goos HJTh. 1999 Cloning and expression of the zebrafish germ cell nuclear factor. Mol. Reprod. Dev. 53: 369-375.

Briski KP and Sylvester PW. 1994 Antagonism of type II, but not type I glucocorticoid receptors in elevated basal luteinizing hormone release in male rats. Neuroendocrinology 60: 601-608.

Briski KP, Vogel KL, McIntyre AR. 1995 The antiglucocorticoid, RU486, attenuates stress-induced decrease in plasma-luteinizing hormone concentrations in male rats. Neuroendocrinology 61: 638-645.

Burzawa-Gérard E and Fontaine YA. 1972 The gonadotropins of lower vertebrates. Gen. Comp. Endocrinol. 3: 715-728.

Cadepond F, Ulmann A, Baulieu E-E. 1997 RU486 (mifepristone): mechanisms of action and clinical uses. Ann. Rev. Medicine 48: 129-156.

Campbell PM, Pottinger TG, Sumpter JP. 1992 Stress reduces the quality of gametes produced by rainbow trout. Biol. Reprod. 47: 1140-1150.

Carolsfeld J, Powell JFF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE, Sherwood NM. 2000 Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology 141(2): 505-512.

Carragher J, Sumpter JP, Pottinger TG, Pickering AD. 1989 The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. Gen. Comp. Endocrinol. 76: 310-321.

Carragher J and Sumpter JP. 1990 The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. Gen. Comp. Endocrinol. 77: 403-407.

Castro WLR and Matt KS. 1997 Neuroendocrine correlates of separation stress in the Siberian dwarf hamster (*Phodopus sungorus*). Physiol. Behav. 61(4): 477-484.

Cavaco JEB, Schulz RW, Trudeau VL, Lambert JGD Goos HJTh. 1995 Sexual steroids and regulation of puberty in male African catfish (*Clarias gariepinus*). In: Goetz FW and Thomas P (Eds.) Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish; 1995; Austin, Texas. p360.

Cavaco JEB, Lambert JGD, Schulz RW, Goos HJTh. 1997 Pubertal development of male African catfish, *Clarias gariepinus*. *in vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol. Biochem. 16(2): 129-138.

Cavaco JEB. 1998a Sexual steroid hormones participate in the control of puberty in male African catfish, *Clarias gariepinus*. Thesis, University of Utrecht, Utrecht, The Netherlands.

Cavaco JEB, Vilrokx C, Trudeau VL, Schulz RW, Goos HJTh. 1998b Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). Am. J. Physiol.; Regulatory Integrative Comp. Physiol. 44(6): R1793–R1802.

Cavaco JEB, van Bemmel A, Zandbergen MA, Schulz RW, Goos HJTh. 1998c Castration of immature African catfish (*Clarias gariepinus*) impairs the meiosis–related activation of pituitary gonadotrophs. In: Cavaco JEB. Sexual steroid hormones participate in the control of puberty in male African catfish, *Clarias gariepinus*. Utrecht, The Netherlands: University of Utrecht, Thesis.

Cavaco JEB, Huck JHJ, Peute J, Schulz RW, Goos HJTh. 1998d Steroid hormones and gonadotroph activation in juvenile male African catfish (*Clarias gariepinus*). In: Cavaco JEB. Sexual steroid hormones participate in the control of puberty in male African catfish, *Clarias gariepinus*. Utrecht, The Netherlands: University of Utrecht, Thesis.

Chandran UR, Attardi B, Friedman R, Dong KW, Roberts JL, DeFranco DB. 1994 Glucocorticoid receptor–mediated repression of gonadotropin–releasing hormone promoter activity in GT1 hypothalamic cell lines. Endocrinology 134: 1467–1474.

Chandran UR, Attardi B, Friedman R, Zheng Z, Roberts JL, DeFranco DB. 1996 Glucocorticoid repression of the mouse gonadotropin–releasing hormone gene is mediated by promoter elements that are recognized by heteromeric complexes containing glucocorticoid receptor. J. Biol. Chem. 271: 20412–20420.

Chang Y, Huang F, Lo T. 1992 Isolation and sequence analysis of carp gonadotropin β–subunit gene. Mol. Mar. Biol. Biotech. 1: 97–105.

Charpenet G, Tache Y, Forest MG, Haour F, Saez JM, Bernier M, Ducharme JR, Collu R. 1981 Effects of chronic intermittent immobilization stress on rat testicular androgenic function. Endocrinology 109(4): 1254–1258.

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294–5300.

Chow MM, Kight KE, Gothilf Y, Alok D, Stubblefield J, Zohar Y. 1998 Multiple GnRHs present in a teleost species are encoded by separate genes: analysis of the sbGnRH and cGnRH-II genes from the striped bass, *Morones saxatilis*. J. Mol. Endocrinol. 21: 277–289.

Chrousos GP and Gold PW. 1992 The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. J. Am. Med. Assoc. 267: 1244–1252.

Coddington EJ and Cree A. 1995 Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female whistling frogs, *Litoria ewingi*. Gen. Comp. Endocrinol. 100: 33–38.

Consten D, Lambert JGD, Goos HJTh. 2000 Inhibitory effects of cortisol on *in vivo* and *in vitro* androgen secretion in male common carp, *Cyprinus carpio*. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (Eds.) Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish; 2000; Bergen, Norway. p192

Consten D, Bogerd J, Komen J, Lambert JGD, Goos HJTh. 2001a Long–term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L. Biol. Reprod. 64(4) in press.

Consten D, Lambert JGD, Goos HJTh. 2001b Cortisol affects testicular development in male common carp, *Cyprinus carpio* L., but not via an effect on LH secretion. Comp. Biochem. Physiol. in press.

Coward K, Bromage NR, Little DC. 1998 Inhibition of spawning and associated suppression of sex steroid levels during confinement in the substrate-spawning *Tilapia zillii*. J. Fish Biol. 52(1): 152-165.

Crim LW, Evans DM. 1979 Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 37: 192-196.

de Man AJM, Hofman JA, Hendriks Th, Rosmalen FMA, Ross HA, Benraad ThJ. 1980 A direct radio-immunoassay for plasma aldosterone: significance of endogenous cortisol. Neth. J. Medicine 23: 79-83.

Donaldson EM. 1981 The pituitary-interrenal axis as an indicator of stress in fish. In: Pickering AD (Eds.) Stress and fish. Academic Press, London, UK, pp11-47.

Ducouret B, Tujague M, Ashraf J, Mouchel N, Servel N, Valotaire Y, Thompson EB. 1995 Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. Endocrinology 136(9): 3774-3783.

Dubois EA, Florijn MA, Zandbergen MA, Peute J, Goos HJTh. 1998 Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish (*Clarias gariepinus*). Gen. Comp. Endocrinol. 112: 383-393.

Dubois EA, Zandbergen MA, Peute J, Hassing GAM, van Dijk W, Schulz RW, Goos HJTh. 2000 Gonadotropin-releasing hormone fibers innervate the pituitary of the male African catfish (*Clarias gariepinus*) during puberty. Neuroendocrinology 72: 252-262.

Elliot JM. 1981 Some aspects of thermal stress on freshwater teleosts. In: Pickering AD (Eds.) Stress and fish. Academic Press, London, UK, pp209-246.

Engelsma MY, Stet RJM, Schipper H, Verburg-van Kemenade BML. 2001 Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. Dev. Comp. Immunol. 25: 195-203.

Fenske M. 1997 Role of cortisol in the ACTH-induced suppression of testicular steroidogenesis in guinea pigs. J. Endocrinol. 154: 407-414.

Fernald RD and White RB. 1999 Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Frontiers in Neuroendocrinology 20(3): 224-240.

Foo JTW and Lam TJ. 1993a Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. Aquaculture 115: 145-158.

Foo JTW and Lam TJ. 1993b Retardation of ovarian growth and depression of serum steroid levels in the tilapia, *Oreochromis mossambicus*, by cortisol implantation. Aquaculture 115: 133-143.

Gielen JT and Goos HJTh. 1983 The brain-pituitary-gonadal axis in the rainbow trout, *Salmo gairdneri*. II. Direct effect of gonadal steroids on the gonadotropic cells. Cell Tissue Res. 233: 377-388.

Goos HJTh, de Leeuw R, Burzawa-Gérard E, Terlou M, Richter CJJ. 1986 Purification of gonadotropic hormone from the pituitary of the African catfish, *Clarias gariepinus* (Burchell), and the development of a homologous radioimmunoassay. Gen. Comp. Endocrinol. 63: 162-170.

Goos HJTh, Facchinetti F, Henderson IW, Pierantoni R, Polzonetti-Magni AM. 1993 Pubertal development: big questions, small answers. Cell. Comm. Reprod. 11-20.

Goos HJTh, Bosma PT, Bogerd J, Tensen CP, Li KW, Zandbergen MA, Schulz RW. 1997 Gonadotropin-releasing hormones in the African catfish: molecular forms, localization, potency and receptors. Fish Physiol. Biochem. 17: 45-51.

Grier HJ. 1981 Cellular organization of the testis and spermatogenesis in fishes. Amer. Zool. 21: 345-357.

Griswold MD. 1998 The central role of Sertoli cells in spermatogenesis. Semin. Cell Dev. Biol. 9(4): 411-416.

Higa M, Kitahashi T, Sasaki Y, Okada H, Ando H. 1997 Distinct promoter sequences of two precursor genes for salmon gonadotropin-releasing hormone in masu salmon. J. Mol. Endocrinol. 19: 149-161.

Huang C, Huang F, Wang Y, Chang Y, Lo T. 1992 Organization and nucleotide sequence of carp gonadotropin β subunit genes. Biochim. Biophys. Acta 1129: 239-242.

Itoh H, Suzuki K, Kawauchi H. 1988 The complete amino acid sequences of β-subunits of two distinct Chum salmon GTHs. Gen. Comp. Endocrinol. 71: 438-451.

Jenkins N and Ellison JD. 1986 Corticosteroids suppress plasminogen activation in the bovine Sertoli cell. J. Endocrinol. 108: R1-R3.

Jimenez-Liñan M, Rubin BS, King JC. 1997 Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. Endocrinology 138(10): 4123-4130.

Joshi SN. 1982 Effect of hydrocortisone acetate on the maturity of male *Labeo gonius* (Ham.). Endokrinologie 80(3): 299-303.

King JA and Millar RP. 1982 Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization. J. Biol. Chem. 257: 10729-10732.

Klungland H, Lorens JB, Andersen O, Kisen GO, Alestrom P. 1992 The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. Mol. Cell. Endocrinol. 84: 167-174.

Kobayashi M, Aida K, Stacey NE. 1991 Induction of testis development by implantation of 11-ketotestosterone in female goldfish. Zool. Sci. 8: 389-393.

Koldras M, Bieniarz K, Kime DE. 1990 Sperm production and steroidogenesis in testes of the common carp, *Cyprinus carpio* L., at different stages of maturation. J. Fish Biol. 37: 635-645.

Komen J, Bongers ABJ, Richter CJJ, van Muiswinkel WB, Huisman EA. 1991 Gynogenesis in common carp (*Cyprinus carpio* L.) II: The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92: 127-142.

Lamers AE, Flik G, Atsma W, Wendelaar Bonga SE. 1992 A role for di-acetyl α-melanocyte-stimulating hormone in the control of cortisol release in the teleost *Oreochromis mossambicus*. J. Endocrinol. 135: 285-292.

Levy FL, Ree AH, Eikvar L, Govindan MV, Jahnsen T, Hansson V. 1989 Glucocorticoid receptors and glucocorticoid effects in rat Sertoli cells. Endocrinology 124: 430-436.

Lim K, Yoon SJ, Lee MS, Byun SH, Kweon GR, Kwak ST, Hwang BD. 1996 Glucocorticoid regulation of androgen binding protein expression in primary Sertoli cell cultures from rats. Biochem. Biophys. Res. Commun. 218: 490-494.

Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM. 1992 Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. Proc. Natl. Acad. Sci. USA 89: 6373-6377.

López-Calderón A, Gonzaléz-Quijano MI, Tresquerres JAF, Ariznavarreta C. 1990 Role of LHRH in the gonadotrophin response to restraint stress in intact male rats. J. Endocrinol. 124: 241-246.

Magri MH, Solari A, Billard R, Reinaud P. 1985 Influence of testosterone on precocious sexual development in immature rainbow trout. Gen. Comp. Endocrinol. 57: 411-421.

Mahmoud IY and Licht P. 1997 Seasonal changes in gonadal activity and the effects of stress on reproductive hormones in the common snapping turtle, *Chelydra serpentina*. Gen. Comp. Endocrinol. 107(3): 359-372.

Matsuo H, Baba Y, Nair NMG, Arimura A, Schally AV. 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Comm. 43: 1334-1339.

McCormick SD, Shrimpton JM, Carey JB, Odea MF, Sloan KE, Moriyama S, Björnsson BT. 1998 Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. Aquaculture 168: 221-235.

McLachlan RI, Wreford NG, O'Donnell L, Kretser DM, Robertson DM. 1996 The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. J. Endocrinol. 148: 1-9.

Metz JR, Van den Burg EH, Wendelaar Bonga SE, Flik G. 2001 Branchial Na^+/K^+-ATPase in *Cyprinus carpio* adapted to three temperatures. In preparation

Michael AE and Cooke BA. 1994 A working hypothesis for the regulation of steroidogenesis and germ cell development in the gonads by glucocorticoids and 11β-hydroxysteroid dehydrogenase (11β-HSD). Mol. Cell. Endocrinol. 100 (1-2): 55-63.

Miura T, Yamauchi K, Takahashi H, Nagahama Y. 1991 Hormonal induction of all stages of spermatogenesis *in vitro* in the Japanese eel (*Anguilla japonica*). Proc. Natl. Acad. Sci. USA 88: 5774-5778.

Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H. 1984 Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc. Natl. Acad. Sci. USA 88: 5774-5778.

Moberg GP. 1985 Biological response to stress: Key to assessment of well-being? In Moberg GP (Eds.) Animal stress. American Physiological Society, Bethesda, MD, USA, pp27-49.

Monder C. 1991 Corticosteroids, receptors, and the organ-specific functions of 11 beta-hydroxysteroid dehydrogenase. FASEB J. 5(15): 3047-3054.

Moore MC, Thomson CW, Marler CA. 1991 Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 81: 217-226.

Mugnier C, Fostier A, Guezou S, Gaignon JL, Quemener L. 1998 Effect of some repetitive factors on turbot stress response. Aquaculture International 6(1): 33-45.

Nagahama Y. 1994 Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Norman RL and Smith CJ. 1992 Restraint inhibits luteinizing hormone and testosterone secretion in intact male rhesus macaques: effects of concurrent naloxone administration. Neuroendocrinology 55: 405–415.

Okubo K, Amano M, Yoshiura Y, Suetake H, Aida K. 2000 A novel form of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. Biochem. Biophys. Res. Comm. 276: 298–303.

Orr TE and Mann DR. 1992 Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rats. Hormones and Behavior 26: 350–363.

Padmanabhan V, Keech C, Convey EM. 1983 Cortisol inhibits and adrenocorticotropin has no effect on luteinizing hormone-releasing hormone-induced release of luteinizing hormone from bovine pituitary cells *in vitro*. Endocrinology 112: 1782–1787.

Pankhurst NW, Van Der Kraak G, Peter RE. 1995a A reassessment of the inhibitory effects of cortisol on ovarian steroidogenesis. In: Goetz FW and Thomas P (Eds.), Reproductive Physiology of Fish, Austin, USA, p195.

Pankhurst NW, Van Der Kraak G, Peter RE. 1995b Evidence that the inhibitory effect of stress on reproduction in teleost fish is not mediated by the action of cortisol on ovarian steroidogenesis. Gen. Comp. Endocrinol. 99: 249–257.

Pankhurst NW and Van Der Kraak G. 2000 Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout *in vivo*, through the action of cortisol. Gen. Comp. Endocrinol. 117: 225–237.

Peter RE and Yu KL. 1997 Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Rev. Fish Biol. Fisheries 7: 173–197.

Pickering AD. 1981 Stress and fish. Academic Press, London, UK.

Pickering AD, Pottinger TG, Carragher J, Sumpter JP. 1987a The effect of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. 68: 249–259.

Pickering AD, Pottinger TG, Sumpter JP. 1987b On the use of dexamethasone to block the pituitary-interrenal axis in the brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. 65: 346–353.

Pickering AD, Pottinger TG, Sumpter JP, Carragher JF, Le Bail P-Y. 1991 Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 83: 86–93.

Pierce JG and Parsons TF. 1981 Glycoprotein hormones: structure and function. Ann. Rev. Biochem. 50: 465–495.

Powell JFF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM. 1996 Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc. Natl. Acad. Sci. USA 93: 10461–10464.

Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA, Sherwood NM. 1994 Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proc. Natl. Acad. Sci. USA 91: 12081–12085.

Rebers FEM, Tensen CP, Schulz RW, Goos HJTh, Bogerd J. 1997 Modulation of glycoprotein hormone α and gonadotropin IIβ subunit mRNA levels in the pituitary gland of mature male African catfish, *Clarias gariepinus*. Fish Physiol. Biochem. 17: 99–108.

Rebers FEM, Hassing GAM, Zandbergen MA, Goos HJTh, Schulz RW. 2000 Regulation of steady-state luteinizing hormone messenger ribonucleic acid levels, *de novo* synthesis and release by sex steroids in primary pituitary cell cultures of male African catfish, *Clarias gariepinus*. Biol. Reprod. 62: 864–872.

Rivier C and Rivest S. 1991 Effects of stress on the activity of the hypothalamic–pituitary–gonadal axis: peripheral and central mechanisms. Biol. Reprod. 45: 523–532.

Rogatsky I, Trowbridge JM, Garabedian MJ. 1997 Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms. Mol. Cell Biol. 17(6): 3181–3193.

Rosen H, Jameel ML, Barkan AL. 1988 Dexamethasone suppresses gonadotropin-releasing hormone (GnRH) secretion and has direct pituitary effects in male rats: differential regulation of GnRH receptor and gonadotropin response to GnRH. Endocrinology 122: 2873–2880.

Samuelsson MK, Pazirandeh A, Davani B, Okret S. 1999 p57Kip2, a glucocorticoid-induced inhibitor of cell cycle progression in HeLa cells. Mol. Endocrinol. 13(11): 1811–1822.

Sankar BR, Maran RRM, Sivakumar R, Govindarajulu P, Balasubramanian K. 2000 Chronic administration of corticosterone impairs LH signal transduction and steroidogenesis in rat Leydig cells. J. Steroid Biochem. Mol. Biol. 72: 155-162.

Schultz R, Isola J, Parvinen M, Honkaniemi J, Wikström A, Gustafsson J.-Å, Pelto-Huikko M. 1993 Localization of the glucocorticoid receptor in testis and accessory sexual organs of male rat. Mol. Cell. Endocrinol. 95: 115-120.

Schulz RW. 1985 Measurement of five androgens in the blood of immature and mature male rainbow trout, *Salmo gairdneri* (Richardson). Steroids 46(2,3): 717-726.

Schulz RW, Bosma PT, Zandbergen MA, van der Sanden MCA, van Dijk W, Peute J, Bogerd J, Goos HJTh. 1993 Two gonadotropin-releasing hormones in the African catfish, *Clarias gariepinus*: localization, pituitary receptor binding, and gonadotropin release activity. Endocrinology 133: 1569-1577.

Schulz RW, van der Corput L, Janssen-Dommerholt J, Goos HJTh. 1994 Sexual steroids during puberty in male African catfish (*Clarias gariepinus*): serum levels and gonadotropin-stimulated testicular secretion in vitro. J. Comp. Neurology 164: 195-205.

Schulz RW, Lubberink K, Zandbergen MA, Janssen-Dommerholt C, Peute J, Goos HJTh. 1996 Testicular responsiveness to gonadotropic hormone *in vitro* and Leydig and Sertoli cell ultrastructure during pubertal development in African catfish (*Clarias gariepinus*). Fish Physiol. Biochem. 15(3): 243-254.

Schulz RW, Zandbergen MA, Peute J, Bogerd J, van Dijk W, Goos HJTh. 1997 Pituitary gonadotrophs are strongly activated at the beginning of spermatogenesis in African catfish, *Clarias gariepinus*. Biol. Reprod. 57: 139-147.

Schulz RW and Goos HJTh. 1999 Puberty in male fish: concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). Aquaculture 177(1-4): 5-12.

Selye H. 1936 A syndrome produced by diverse nocuous agents. Nature 138: 32.

Sharpe CS, Thompson DA, Blankenship HL, Schreck CB. 1998 Effects of routine handling and tagging procedures on physiological stress responses in juvenile Chinook salmon. Prog. Fish Cult. 60(2): 81-87.

Sherwood NM, Eiden L, Rivier JE, Spiess J, Vale W. 1983 Characterization of a teleost gonadotropin-releasing hormone. Proc. Natl. Acad. Sci. USA 80: 2794-2798.

Sherwood NM, Sower SA, Marschak DR, Fraser BA, Brownstein MJ. 1986 Primary structure of gonadotropin-releasing hormone from lamprey brain. J. Biol. Chem. 261: 4812-4819.

Smith E, Redman RA, Logg CR, Coetzee GA, Kasahara N, Frenkel B. 2000 Glucocorticoids inhibit developmental stage-specific osteoblast cell cycle. Dissociation of cyclin A-cyclin-dependent kinase 2 from E2F4-p130 complexes. J. Biol. Chem. 275(26): 19992-20001.

Sower SA, Chiang YC, Lovas S, Conlon JM. 1993 Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. Endocrinology 132: 1125-1131.

Stouthart AJHX, Lucassen ECHET, van Strien FJC, Balm PHM, Lock RAC, Wendelaar Bonga SE. 1998 Stress responsiveness of the pituitary-interrenal axis during early life stages of common carp (*Cyprinus carpio*). J. Endocrinol. 157, 127-137.

Sumpter JP, Pickering AD, Pottinger TG. 1985 Stress-induced elevation of plasma α-MSH and endorphin in brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. 92: 257-265.

Sumpter JP, Dye HM, Benfey T J. 1986 The effect of stress on plasma ACTH, α-MSH, and cortisol levels in salmonid fishes. Gen. Comp. Endocrinol. 62: 377-385.

Suter DE, Schwartz NB, Ringstrom SJ. 1988 Dual role of glucocorticoids in regulation of pituitary content and secretion of gonadotropins. Am. J. Physiol. 254: E595-E600.

Tagawa M, Hagiwara H, Takemura A, Hirose S, Hirano T. 1997 Partial cloning of the hormone-binding domain of the cortisol receptor in tilapia, *Oreochromis mossambicus*, and changes in the mRNA level during embryonic development. Gen. Comp. Endocrinol. 108(1): 132-140.

Tahirovic HF. 1998 Menarchal age and the stress of war: an example from Bosnia. Eur. J. Pediatr. 157: 978-980.

Takeo J, Hata J, Segawa C, Toyohara H, Yamashita S. 1996 Fish glucocorticoid receptor with splicing variants in the DNA binding domain. FEBS Lett. 389: 244-248.

Tanck MWT, Booms GHR, Eding EH, Wendelaar Bonga SE, Komen J. 2000 Cold shock: a stressor for common carp, *Cyprinus carpio* L. J. Fish Biol. 57: 881-894.

Teitsma CA, Anglade I, Lethimonier C, Le Dréan G, Saligaut D, Ducouret B, Kah O. 1999 Glucocorticoid receptor immunoreactivity in neurons and pituitary cells implicated in reproductive functions in rainbow trout: a double immunohistochemical study. Biol. Reprod. 60: 642-650

Tilbrook AJ, Canny BJ, Serapiglia MD, Ambrose TJ, Clarke IJ. 1999 Suppression of the secretion of luteinizing hormone due to isolation/restraint stress in gonadectomised rams and ewes is influenced by sex steroids. J. Endocrinol. 160: 469-481.

Trudeau VL, Peter RE, Sloley BD. 1991a Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44: 951-960.

Trudeau VL, Lin HR, Peter RE. 1991b Testosterone potentiates the serum gonadotropin response to gonadotropin-releasing hormone in the common carp (*Cyprinus carpio*) and Chinese loach (*Paramisgurnus dabryanus*). Can. J. Zool. 69: 2480-2484.

Turnbull AV and Rivier C. 1997 Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. Proc. Soc. Exp. Biol. Med. 215(1): 1-10.

Vale W, Spiess J, Rivier C, Rivier J. 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213(4514): 1394-1397.

Valli G, Sudha S, Sankar BR, Govindarajulu P, Srinivasan N. 2000 Altered corticosterone status impairs steroidogenesis in the granulosa and thecal cells of Wistar rats. J. Steroid Biochem. Molec. Biol. 73: 153-158.

Van Der Kraak G, Suzuki K, Peter RE, Itoh H, Kawauchi H. 1992 Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85: 217-229.

van Dijk PLM, van den Thillart GEEJM, Balm PHM, Wendelaar Bonga SE. 1993 The influence of gradual water acidification on the acid/base status and plasma hormone levels in carp. J. Fish Biol. 42, 661-671.

van Malssen K, Peschar R, Brito C, Schenk H. 1996 Real-time X-ray powder diffraction investigations on cocoa butter. III. Direct β-crystallization of cocoa butter: occurrence of a memory effect. JAOCS 73(10): 1225-1230.

van Oordt PGWJ and Peute J. 1983 The cellular origin of pituitary gonadotropins in teleosts. In: Hoar WS, Randall DJ, Donaldson EM (Eds.) Fish Physiology. Academic Press, New York, USA, pp137-186.

Van Weerd JH, Komen J. 1998 The effects of chronic stress on growth in fish: a critical appraisal. Comp. Biochem. Physiol. [A] 120: 107-112.

Vijayan MM and Leatherland JF. 1992 In vivo effects of the steroid analogue RU486 on some aspects of intermediary and thyroid metabolism of brook charr, *Salvelinus fontinalis*. J. Exp. Zool. 263: 265-271.

Vijayan MM, Reddy PK, Leatherland JF, Moon TW. 1994 The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. Gen. Comp. Endocrinol. 96: 75-84.

Weber MA, Groos S, Höpfl U, Spielmann M, Aumüller G, Konrad L. 2000 Glucocorticoid receptor distribution in rat testis during postnatal development and effects of dexamethasone on immature peritubular cells in vitro. Andrologia 32(1): 23-30.

Weyts FAA, Verburg-van Kemenade BML, Flik G, Lambert JGD, Wendelaar Bonga SE. 1997 Conservation of apoptosis as an immune regulatory mechanism: Effects of cortisol and cortisone on carp lymphocytes. Brain Beh. Imm. 11(2): 95-105.

Weyts FAA, Verburg-van Kemenade BML, Flik G. 1998 Characterisation of glucocorticoid receptors in peripheral blood leukocytes of carp, *Cyprinus carpio* L.. Gen. Comp. Endocrinol. 111(1): 1-8.

Weyts FAA, Cohen N, Flik G, Verburg-van Kemenade BML. 1999 Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. Fish Shellfish Immunol. 9: 1-20.

Wendelaar Bonga SE. 1997 The stress response in fish. Physiol. Rev. 77(3): 591-625.

Wolfenson D, Roth Z, Meidan R, 2000 Impaired reproduction in heat-stressed cattle: basic and applied aspects. Animal Reproduction Science 60-61: 535-547.

Yaron Z. 1995 Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129: 49-73.

Yoo MS, Kang HM, Choi HS, Kim JW, Troskie BE, Millar RP, Kwon HB. 2000 Molecular cloning, distribution and pharmacological characterization of a novel gonadotropin-releasing hormone ([Trp8] GnRH) in frog brain. Mol. Cell. Endocrinol. 164: 197-204.

Zohar Y. 1980 Dorsal aorta catheterization in rainbow trout (*Salmo gairdneri*) I. Its validity in the study of blood gonadotropin patterns. Reprod. Nutr. Dev. 20: 1811-1823.

NWO-ALW Programme:

Physiological strategies during acclimation to temperature-shock in fish
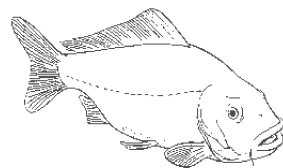
Selective breeding for stress response in common carp (*Cyprinus carpio* L.) using androgenesis.

M.W.T. Tanck and J. Komen

Fish Culture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

The aim of our research was to explore the genetic background of the stress response in common carp (*Cyprinus carpio* L.) and produce isogenic strains with divergent stress responses. As stressor a rapid temperature decrease (= cold shock) was used. As a preparatory step, a number of experiments were carried out to investigate the validity of the cold shock as a stressor and define a selection criterion for the selection experiment. The stress response of common carp was studied by evaluating plasma cortisol, glucose and lactate after a rapid temperature drop (ΔT: 7, 9 or 11°C). All three amplitudes used induced a significant rise in plasma cortisol levels. Peaks occurred within 20 min after onset of the cold shock. However, no stress-related related secondary metabolic changes were observed in any of the experiments described: plasma glucose levels remained unaffected and plasma lactate levels dropped. Based on these results, the plasma cortisol concentration at 20 min after onset of a 9°C cold shock was set as selection criterion in our selection experiment.

The first step in the actual selection experiment was the formation of the base population. This base population was an F_1 cross between six sires from a wild strain originating from the Anna Paulowna (AP) polder and a highly domesticated homozygous E4 dam already present in our laboratory. Thirty-three randomly picked sires from these six E4×AP full-sib families (F_1) were androgenetically reproduced to create the F_2 generation, which thus consisted of 33 doubled haploids (DH) progeny groups. These 33 DH progeny groups (566 individuals) were subjected to the 9°C cold shock, enabling us to estimate a heritability (h^2) for the height of the cortisol stress response. A high h^2 estimate of 0.60 was found, which clearly shows that the stress response due to a cold shock is hereditary in the carp population used.

Because the model used to estimate the h^2 assumed a complete homozygous state of the DH individuals and to ensure that only homozygous individuals would be used for subsequent reproduction, all individuals within the 33 DH androgenetic progeny groups were analysed using 11 microsatellite markers. In total, 92% of the androgenetic DH individuals proved to be homozygous at all 11 loci. Forty-three out of the 47 heterozygous individuals were heterozygous at a single locus only. This heterozygosity was probably due to DNA fragments caused by UV-irradiation of the eggs, although the maternal origin of the fragments could not be proved beyond doubt. Screening with 11 microsatellites also revealed two linkage groups, a segregation distortion at two microsatellite loci and possible association of some microsatellites with weight, length, stress-related plasma cortisol levels and basal plasma glucose levels.

Selection of individual fish from the 33 DH progeny groups based on the response at 4 months was not possible. Therefore, three DH progeny groups with a high (H1-3) and three with a low (L1-3) mean plasma cortisol concentration were selected. The 154 DH fish in these six groups were individually tagged, mixed and subjected to a second cold shock at an age of 15 months. For each individual fish, a breeding value was estimated (EBV) for stress-related cortisol. Two homozygous sires (two high and two low) and dams (high and low) were selected based on their EBV and used to produce four homozygous (HomIso) and eight heterozygous isogenic (HetIso) strains. These were used in two separate experiments to examine the genetic background of the stress-related cortisol response. In both experiments, the strains were subjected to the 9°C cold shock at an age of 5 months. The ranking in plasma cortisol levels of the HomIso strains was identical to the ranking in EBV of the sires and the maximal difference of 350 nmol/l was similar to the expected difference based on these EBV's. Differences between the HetIso strains were smaller than expected, and influence of non-additive genetic effects could not be detected.

Apart from the isogenic strain used in the first experiments, no complete profiles of the cortisol, glucose and lactate dynamics had been examined in other isogenic strains. Therefore, an additional experiment, parallel to the selection experiment, was carried out to investigate the "complete" cortisol, glucose and lactate dynamics during the cold shock in four, readily available, isogenic. The experiments showed that stress-related cortisol response patterns can differ consistently between genotypes of common carp. The observed differences in plasma glucose and lactate dynamics between control and shocked fish were most likely temperature related.

Based on the results of the experiments performed, it can be argued that the best method to change the stress response of common carp would be through selective breeding (exploiting additive genetic effects) rather than through cross-breeding (exploiting non-additive genetic effects). The selection and the "parallel" experiments resulted in several isogenic strains of common carp with at least

two types of cortisol stress responses. Type I showed a relative short cortisol response with either a high or low peak at 20 min after onset of the shock. Type II showed a similar cortisol level at 20 min but no significant decrease in this level during the cold shock. These different isogenic strains will be valuable tools in future research into the stress response itself and its effects on other traits like growth, reproduction and health. This way, some of the problems related to the use of stress response as selection criterion in commercial breeding programmes in fish could be solved in the near future.

Residual heterozygosity was demonstrated to occur in androgenetic progenies, most likely due to maternal DNA fragments induced by the UV irradiation of the eggs. Improved control measures were implemented in the androgenesis procedure, but androgenetic progenies destined for further reproduction purposes should be screened for residual heterozygosity. Androgenetic reproduction proved to be a useful tool for dissection of phenotypic variance and heritability estimations for traits, especially in combination with selection experiments aimed at development of isogenic strains for this trait. Androgenesis might result in reduced fertility in female progeny, but the advantages are such that inclusion of androgenetic reproduction within larger commercial breeding programmes for faster dissemination of genetic progress and product protection should be considered as a promising option.

The role of the HPI-axis of the common carp in response to rapid changes in temperature

E.H. van den Burg, S.E. Wendelaar Bonga, and G. Flik
Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

When fish face stressful conditions, the hypothalamus–pituitary–interrenal (HPI) axis is activated to enable the individual to cope with the stressor and to realise homeostasis. A key function in the functioning of the HPI axis is attributed to proopiomelanocortin (POMC)-derived hormones that are produced by the corticotrope cells in the pituitary pars distalis and the melanotrope cells in the pituitary pars intermedia. These hormones include adrenocorticotropic hormone (ACTH), α–melanocyte-stimulating hormone (α–MSH), and β–endorphin. ACTH is a potent stimulator of cortisol release by the interrenal cells in the head kidney, but in the Mozambique tilapia, also α–MSH has corticotropic activity (Lamers *et al.*, 1992), which can be potentiated by β–endorphin (Balm *et al.*, 1995). Cortisol is the end product of the HPI-axis and it reallocates energy away from investment activities, such as reproduction, growth and immune functioning, to adaptation to stress, e.g. by restoring ionic balance.

To investigate the role of α-MSH and ß-endorphin in the stress response, we set up a series of experiments in which common carp (*Cyprinus carpio*) were subjected to a 9°C cold shock. As temperature influences virtually all physiological processes, it is expected that a sudden drop in ambient water temperature from 25°C to 16°C induces a stress response. At different time points after the onset of the temperature shock, blood and pituitary glands were taken and analysed for cortisol, α-MSH and β-endorphin contents.

Indeed, a 9°C cold shock induces a stress response, as evidenced by rapid elevating plasma cortisol levels from 14 ± 13 to 247 ± 45 ng/ml (mean±sd, n=10) after 20 minutes. Three hours after the start of the shock, the plasma cortisol concentration had declined to 63 ± 27 ng/ml. At this point, the shock was stopped and the water temperature was elevated to 25°C. Plasma cortisol levels subsequently returned to basal levels. There was no effect of the temperature shock on pituitary content and plasma concentrations of α-MSH and β-endorphin, indicating that there is no specific role for these peptides in the response of this fish to a temperature shock.

In subsequent experiments, we determined the effects of the temperature shocks on brain activity. We applied functional Magnetic Resonance Imaging (fMRI) to study how a cold shock influences cerebral blood flow. Using this *in vivo* approach, we demonstrated that the blood flow decreased in the brain, but that the opposite was true in the hypothalamic/pituitary region. Whether this observation is a stress- or temperature-induced phenomenon is unclear at present.

The rise in plasma cortisol levels and the changes in blood flow in the brain appear both to be very sudden effects rather then a gradual response in parallel to the decline in ambient water temperature. This may indicate that a temperature change itself is the stressor rather than the magnitude of the temperature drop. As ambient temperature influences virtually every process in poikilotherms, fish have to readjust their physiology. For instance, we recently demonstrated that carp adapted to 15°C have double the amount of Na^+/K^+-ATPase copies compared to 29°C-adapted fish (Metz *et al.*, 2001) to compensate for the lower activity of the enzyme at low temperatures.

In conclusion, a rapid drop in ambient water temperature induces a stress response in the common carp to counteract temperature-induced effects on its physiology. The rise in plasma cortisol levels is likely an ACTH-mediated event, as both α-MSH and β-endorphin are not clearly involved in this stress response.

The influence of temperature-induced stress on the development and function of the immune system of the common carp *Cyprinus carpio* L.

M.Y. Engelsma, Van Muiswinkel, W.B. and Verburg-van Kemenade, B.M.L.
Cell Biology & Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, The Netherlands.

Stress induced immuno-suppression in fish is mostly attributed to actions of steriod hormones released upon activation of the hypothalamus-pituitary-interrenal (HPI)-axis. As in mammals the neuro-endocrine and immune system in fish co-operate in a bi-directional way, sharing regulatory molecules and receptors. This project focuses on possible neuro-endocrine modulation of immune functioning through HPI-axis hormones during acute stress. Moreover, the interesting hypothesis is investigated that hormone secretion is regulated by interleukins from immune-cell origin.

Like mammals fishes possess a complex and well developed immune system. Roughly the immune system can be divided in two types of responses: an innate or a-specific response and an acquired or specific response. In the innate immune response, phagocytic cells (macrophages and neutrophilic granulocytes) play a key role, while in the specific response T- and B-lymphocytes are the important mediators.

So far we studied the effects of acute temperature stress and the effects of cortisol, a major product of the HPI-axis, on the immune system. Previous work with cells cultured *in vitro* showed that especially activated B-lymphocytes were sensitive to cortisol, leading to programmed cell death, apoptosis. *In vivo*, after repeated temperature shocks the relative number of circulating B-lymphocytes (precursors of antibody producing cells) was significantly decreased. The decrease was even more pronounced after challenging the immune system (Engelsma *et al.*, in preparation). This drop in relative number can either be caused by the redistribution of cells to other body compartments or by apoptosis. In line with this, antibody titers of TNP-LPS immunized carp were lower in the stressed group compared to the control. Together these results suggest impairment of the acquired immune system after acute mild stress.

Cells of the innate immune system turned out to be less sensitive to cortisol. Of the leukocyte cell types neutrophilic granulocytes were least affected by application of temperature stress. This is in agreement with previous *in vitro* experiments where neutrophilic granulocytes were even rescued from apoptosis by cortisol.

Cytokine molecules, like interleukin-1 beta (IL-1β), play a pivotal role in

the regulation of different processes within the immune system. Cells of the immune system release IL–1β as a result of infection or tissue damage. Moreover, as deduced from mammalian studies, they are important candidates able to affect the HPI-axis by altering the release of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH).

In fish, most interleukin molecules await identification but the IL–1β sequences of several teleost fishes were recently elucidated. In the tetraploid carp we identified two IL–1β genes (Engelsma *et al.*, in preparation). The two carp mRNA sequences share about 74% amino acid identity. Interestingly, the IL–1β2 sequence has an extensive polymorphism not found in the IL–1β1 sequence. In contrast to some other fish species, in carp a constitutive expression of IL–1β RNA was seen in predominantly the immune organs head kidney and spleen.

In vitro, in head kidney phagocytes, the IL–1β RNA expression could be upregulated by stimuli such as for example lipopolysaccharide (LPS), a major constituent of the cell wall of gram-negative bacteria. In contrast, cortisol could inhibit the basal expression of IL–1β RNA. However, when cells were pre-stimulated with cortisol or when cortisol was added simultaneously with LPS, cortisol could not inhibit LPS induced expression. Probably LPS can overrule the glucocorticoid receptor mediated inhibition via the nuclear factor-kB pathway (Engelsma *et al.*, 2001). This might imply that cortisol cannot suppress IL–1β activation during infection.

Currently we are investigating the effect of recombinant IL–β on immune functions, under stress and non-stress conditions. Together with our partners at the Department of Animal Physiology in Nijmegen we study the effects of IL–β on release of pro-opiomelanocortin (POMC)-derived peptides and cortisol. To evaluate genetic differences in stress-related immune modulation we will measure leukocyte activities and interleukin release in the two carp lines for high and low cortisol response.

Forkortet udgave for udenforstående

"Stress, pubertet........ og det med fisk??" Det er en opmærkning jeg har hørt flere gange. Og prøv at forklare det. I det følgende afsnit håber jeg at gøre mig forståelig i hvad jeg de sidste 4 år har været optaget af og hvorfor.

Lige fra begyndelsen af sin oprindelse har fisk været brugt som et vigtigt ernæringsprodukt. Først i mindre mængder, nok til daglig forbrug, men med den større stigning i handel er fiskefangsten blevet en vigtig industri. Nu for tiden kan vi købe fisk overalt og i store mængder, og som følge heraf er visse fiskesorter truet. Derfor investere vi stadig mere og mere i at dyrke disse fiskesorter. Ved dyrkning af fisk må man først vide hvordan man holder dem i live, og her spiller stress en vigtig rolle, herefter skal man også vide hvordan man kan lade fisk formere sig.

Stress er ikke andet end en forandring i dens leveforhold, feks. legemlig anstrengelse, men også en pludselig forandring af temperatur. Stress kan ikke udelukkes ved dyrkelse af fisk, og på længere tid er der stor risiko for at fisk ikke udvikler sig, samt sygdomme, så det er ikke kun fiskenes sundhed der er på retur, men også avlernes indtægt. Det samme gælder for forplantningen, p.g.a. den skadelige virkning af stress. Evnen til at forplante sig er en proces der bliver udviklet i perioden som vi kalder puberteten, i denne periode sker der ved mennesker en forandring fysisk og psykisk, kønsorganerne udvikler sig, og vi indstiller os på vores nye opgave, forplantning. For fisk er det præcis det samme, for mænd (menneske eller fisk) sker der i puberteten en udvikling af kønsceller i testiklerne, som senere bliver til sædceller. Denne process bliver kaldt spermatogenese.

I min undersøgelse har jeg studeret indflydelsen af stress i pubertetsudviklingen af den mandlige karpe. Her har jeg brugt karper før de kom i puberteten, 3 gange om ugen blev de skiftevis flyttet til vandtemperatur fra (25°C til 14°C). Det viste sig at udviklingen af kønsceller blev forsinket i de stressede karper, og som følge heraf kom de senere i puberteten. Ved stress bliver der forskellige hormoner produceret i kroppen, et af disse hormoner kaldes

cortisol. Min undersøgelse viste sig at cortisol var ansvarlig for en senere pubertetsudvikling, derfor begyndte vi at undersøge hvordan kan cortisol gøre det.

Ved regulering af forplantning er en antal hormonprodukserende organer vigtige. Hjernen produkser hormonet "gonadotropin-releasing hormone" (GnRH). Dette hormon stimulerer cellerne i hypofysen (en lille kirtel under hjernen) som afgiver gonadotropines (luteïniserend hormon, LH og follikel stimulerende hormon, FSH). I testiklerne sørger disse to hormoner for produktionen af kønsceller og steroidhormon. Steroidhormoner sørger for ved produktionen af kønsceller også for kommunikationen tilbage til hjernen og hypofysen, så systemet kan kontrolere sig selv. Ved at behandle unge karper med cortisol har vi fundet ud af at herved blev puberteten også forsinket, ligesom ved stress. Derfor var udstedelse af alle hormoner i hjerne-hypofyse-gonade systemet (HHG-systemet) nedskåret. Ud af min undersøgelse, som beskrevet i dette dokterdisputats, viser at cortisol har en indflydelse på testiklerne, og som følge heraf bliver kønscelle-udviklingen direkte hæmmet. Herved bliver kønsorganerne mindre udviklet, samt udstedelsen af steroide i blodet er mindre. Fra en tidligere undersøgelse, viste det sig at fisk i puberteten og som stammer fra testikler med sterioden, spillede en vigtig rolle ved udviklingen af HHG-systemet. Så p.g.a. stress bliver udstedelse af steroiden mindre, som igen har indflydelse på udviklingen af denne as. Vores resultater viser at den hæmmet hormon produktion i hypofysen er en følge af en mindre udstedelse af testosteron i testiklerne.

Populært udtrykt sidder stress ikke mellem ørerne, men imellem benene.

Samenvatting voor de leek

"Stress, puberteit……. en dat bij vissen ??" Dat is een opmerking die ik veel te horen heb gekregen. Probeer het maar eens uit te leggen. In het komende stukje hoop ik dan ook in begrijpelijke taal duidelijk te maken, wat ik in de afgelopen 4 jaar gedaan heb en waarom.

Al vanaf het begin van zijn ontstaan heeft de mens vis gebruikt als een belangrijke voedselbron. Eerst kleinschalig, voldoende om te voorzien in de eigen behoefte. Maar met de opkomst van de handel ontwikkelde de visvangst zich tot een belangrijke industrie. Tegenwoordig is vis overal en altijd te koop. Dit heeft echter tot gevolg dat een aantal commerciëel belangrijke vissoorten overbevist dreigen te raken. Vandaar dat men steeds meer investeert in het kweken van deze soorten. Om vissen te kunnen kweken moet je weten hoe je ze in goede conditie kan houden. Het is daarbij van groot belang stress zoveel mogelijk te voorkomen. Daarnaast zal je moeten weten hoe je de vissen kunt laten voortplanten.

Ter verduidelijking, stress is in wezen niets anders dan elke verstoring van de rust-situatie waarin het dier zich bevindt, veroorzaakt door een stressor. Dat kan bijvoorbeeld een zware lichamelijke inspanning zijn, maar ook een abrupte verandering in de omgevingstemperatuur. Bij het kweken van vissen kan stress niet altijd voorkomen worden. Op de lange termijn kan dit leiden tot een verminderde groei en en verhoogde gevoeligheid voor ziekten, waardoor het welzijn van de vis terugloopt maar ook de opbrengst voor de kweker. Dat laatste geldt ook voor de nadelige gevolgen van stress op de voorplanting.

In mijn studie ging het om het laatste: wat is het nadelige gevolg van stress op voortplanting, maar vooral hoe komt het tot stand.

Vissen, net als mensen en alle andere dieren, zijn in hun jeugd nog niet geslachtsrijp en nog niet in staat zich voort te planten. Het vermogen tot voortplanting ontwikkelt zich tijdens de periode die puberteit genoemd wordt. In deze periode ontwikkelen de geslachtorganen zich en het voortplantingsgedrag. In mannelijke individuen wordt de puberteit gekenmerkt door de ontwikkeling

van de geslachtscellen in de testes (de zaadballen), met als eindresultaat de beweeglijke zaadcellen. Dit proces wordt spermatogenese genoemd.

In mijn onderzoek heb ik gekeken naar de effecten die stress heeft op de puberteitsontwikkeling van mannelijke karpers. Hiervoor gebruikte ik karpers die op speciale wijze gekweekt werden door de afdeling Visteelt en Visserij van de Universiteit Wageningen. Het speciale was dat alle dieren genetisch gelijk waren (allemaal tweeling-broertjes), waardoor hun puberteitsontwikkeling zeer synchroon verliep. Ik heb de jonge karpers, nog voor de puberteit begon, 3 keer per week op een onverwacht moment blootgesteld aan een snelle verlaging van de watertemperatuur (van 25°C naar 14°C). Het blijkt dat in gestresste karpers de ontwikkeling van de geslachtscellen is vertraagd, waardoor ze dus later dan normaal in de puberteit komen.

Zoals al eerder vermeld hebben we stress gedefiniëerd als een verstoring van de rusttoestand. Gelukkig heeft het lichaam de mogelijkheid om deze verstoring te herstellen. Het kan zich aanpassen aan de stressor: stressadaptatie. Bij deze stressadaptatie komen een aantal hormonen vrij in het lichaam. Eén van deze hormonen is cortisol. Mijn onderzoek laat op de eerste plaats zien dat cortisol niet alleen gebruikt wordt voor het herstel van het verstoorde evenwicht, maar ook verantwoordelijk is voor de vertraagde puberteitsontwikkeling. Daarom ben ik vervolgens gaan onderzoeken hoe cortisol dit kan doen.

Bij de regulatie van de voortplanting zijn een aantal hormoon-produc- erende organen belangrijk. De hersenen produceren het hormoon "gonadotropin-releasing hormone" (GnRH). Dit hormoon stimuleert bepaalde cellen in de hypofyse (een klein kliertje onderaan de hersenen), die daarop de gonadotropines (luteïnizerend hormoon, LH en follikel stimulerend hormoon, FSH) afgeven. In de testes zorgen deze twee hormonen voor de aanmaak van geslachtscellen en steroidhormonen. De steroidhormonen dragen bij aan de aan- maak van de geslachtscellen en verzorgen ook de communicatie terug naar de hersenen en de hypofyse zodat het systeem zichzelf kan controleren. Dit hor- monale sytseem noemen we de hersenen-hypofyse-gonade as (HHG-as).

De puberteitsontwikkeling van jonge karpers werd, net als bij temperatu- urstress, vertraagd door de vissen te behandelen met cortisol. Daarnaast was de afgifte van alle hormonen in de HHG-as verminderd en daarmee zou de ver- traging in de puberteitsontwikkeling verklaard kunnen worden. Uit mijn onder- zoek, beschreven in dit proefschrift, is echter gebleken dat cortisol met name inwerkt op de testes, waarbij het mogelijk zorgt voor een directe remming van de geslachtscel-ontwikkeling. Hierdoor blijft de groei van de geslachtsorganen achter en ook de afgifte van steroiden aan het bloed is daardoor verminderd. Uit eerder onderzoek was al gebleken dat in vissen met name de steroiden afkom- stig van de testes een belangrijke rol spelen bij de onwikkeling van de HHG-as tijdens de puberteit. Doordat stress (cortisol) de afgifte van deze steroiden ver-

mindert, heeft dit ook gevolgen voor de ontwikkeling van de HHG-as. Onze resultaten laten bijvoorbeeld zien dat de geremde hormoonproductie in de hypofyse een gevolg is van de verminderde afgifte van testosteron uit de testes.

Om het nu populair samen te vatten zit de stress wat dit betreft dus niet tussen de oren!

Dankwoord

Na 4 jaar, een heleboel vissen, een slordige 50.000 kilometers werkverkeer en een ontelbaar aantal buisjes, eppjes en puntjes is het dan eindelijk zover. De klus is (bijna) geklaard, het proefschrift is een feit. Hoewel het voorblad anders doet vermoeden, is dit proefschrift geen eenmans-actie geweest. Middels dit stukje wil ik een ieder bedanken die bijgedragen heeft aan dit proefschrift, op wat voor manier dan ook.

Allereerst wil ik Henk Goos noemen, mijn promoter en directe begeleider. Ik weet nog goed dat in de zomer van 1996 het verlossende telefoontje kwam. Ik zou voor mijn scriptie 5 maanden naar Portugal gaan. Dit werden uiteindelijk maar 3 maanden omdat jullie met mij "in zee wilden gaan". Ik heb in al die tijd nooit spijt gehad van deze beslissing. Henk, ik wil je bedanken voor het vertrouwen. Je hebt altijd met een wakend oog toezicht gehouden op dit project. In het begin heb je de jonge hond in mij aardig weten te temmen. Je hebt me geleerd de rode draad te vinden in het onderzoek en je daar dan ook echt aan te houden. Jouw goede zorgen, zowel voor mij alsook voor de groep, zijn erg bepalend geweest voor het plezier dat ik in mijn werk gehad heb. Je hebt daarom, denk ik, de bijnaam "Mother Goos(e)" terecht verdiend. Als ik terug kijk op deze 4 jaar, dan heb ik erg veel van je geleerd, zowel op wetenschappelijk vlak alsook op het organisatorische vlak. Verder ben ik erg blij geweest met je taalkundige correcties in al mijn manuscripten. Dacht ik dat ik het aardig verwoord had, dan wist jij het toch nog anders te brengen waardoor het net wat eleganter overkwam. Aan het eind van je loopbaan als actieve professor heb je niet veel vrije tijd gehad. Drie promoties in zes weken tijd, brengen ontzettend veel werk met zich mee. Daarnaast had je ook nog een hoop andere beslommeringen. Ondanks dat heb je ons alledrie naar de finish weten te helpen. Henk, nogmaals bedankt voor al je goede zorgen en ik hoop dat je nu wat meer tijd over hebt voor je zelf.

Jan Lambert, hoewel ik aan het einde van mijn 4 jaar pas echt gebruik kon maken van jouw specialisme, was jij toch aangewezen als mijn co-promoter.

Jouw algemene kennis van de biologie en jouw gave om "advocaat van de duivel" te spelen zijn erg nuttig geweest bij de vele werkbesprekingen die we gehad hebben. Samen met Henk vormde je, wat mij betreft, een ideaal team. Ook de vele gezamenlijke tochtjes naar Wageningen, soms afgesloten met een hapje of een drankje bij jou thuis, heb ik erg gewaardeerd. Jan, bedankt.

Uiteraard moet ik mijn onzichtbare werkgever bedanken, de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) en ik wil daarbij met name mevrouw den Rooden noemen. Bedankt voor alle vriendelijke hulp bij de verschillende administratieve verrichtingen (reisdeclaraties, ouderschapsverlof, etc.).

Zonder de inzet van extra mankracht (sorry Astrid, maar het is echt "man"kracht), begin je als oio niet veel. In mijn geval heb ik erg veel aan mijn studenten gehad. Tom Groeneveld, Edward Kuijer en Eelco Keuning, zonder jullie hulp zou dit allemaal echt nooit gelukt zijn. In de wandelgangen werden jullie gekscherend de "stress-boys" genoemd, maar ik hoop dat ik jullie niet al te veel gestressd heb. Ik heb het zelf niet zo ervaren, alhoewel er soms wel erg drukke dagen bij zaten. Bedankt voor alle gezelligheid en vooral voor jullie inzet. Met plezier heb ik voor jullie begeleiding gezorgd en ik wens jullie succes met jullie carrières.

Coby Janssen-Dommerholt en Wytske van Dijk, mijn paranimfen. Tijdens de vier jaar tijd heb ik op de werkvloer het meeste met jullie te maken gehad, zeker gezien de hoeveelheid assays die ik gedraaid heb. Alhoewel er in mijn project geen analytische ondersteuning ingepland was, waren jullie toch altijd bereid om mij te helpen. Ik wil jullie bedanken voor alle gezelligheid op het lab en ook voor jullie interesse in mijn project, maar ook in ondergetekende persoonlijk. Ook onze samenwerking tijdens de jaarlijkse Endo-cursus was een succes, als we het tenminste niet over de resultaten van de studenten hebben. Ik vind het geweldig dat jullie allebei zo enthousiast reageerden toen ik vroeg of jullie mijn paranimfen wilden zijn. Eigenlijk was dit ook wel nodig, want dit project was met name een mannenproject (zowel wat betreft proefdier, als ook wat betreft direct betrokken personen) en op dit punt vind ik vrouwelijke inbreng en ondersteuning toch prettiger.

Ook de andere analisten en vaste medewekers, ontzettend bedankt; Thijs Zandbergen (voor de in situ, je hulp bij het hypofyses verwijderen en uiteraard, de thee), Ine Hassing (kamergenoot en altijd bereid tot een praatje over van alles en nog wat), Joke Granneman (koud hè, Antarctica), Rüdiger Schulz (jouw enorme kennis heeft me regelmatig goed aan het denken gezet), Jan Bogerd (bedankt voor de lessen in moleculaire biologie, die komen nu goed van pas), Jan Peute (is sporten nou echt nog wel gezond?), Astrid Freriksen (moeder overste?).

Verder wil ik mijn collega-aio's in Utrecht noemen, die net als ik in het zelfde schuitje zitten of zaten. Jullie weten hoe het is als er iets fout gaat, of toch niet helemaal gaat zoals verwacht. Frank Rebers (je bent toch wel een beetje mijn "grote" voorbeeld geweest), Eduardo Cavaco (your work was inspiring, muito obrigado), Koen Braat en Maarten Blom (allebei mede-aio-vaders, geweldig toch!), Eline Dubois, Henry Vischer, Marion Blomenröhr en Cristina Teves. Allemaal bedankt voor de gezelligheid.

Naast collega-aio's in Utrecht waren er ook nog de illustere "partners in stress" in Wageningen en Nijmegen. Ook jullie zitten of zaten geregeld tot over je oren in de stress. Mijn motto was altijd dat je de stress het beste bij de vissen kon laten en zolang ik vissen had viel de persoonlijke stress inderdaad mee, maar dit had eigenlijk een andere reden. Voor mijn geheim, zie het laatste stukje van dit dankwoord. Michael Tanck, Erwin van den Burg, Marc Engelsma, Neil Ruane, Jurriaan Metz en Jeroen Saeij, bedankt voor jullie steun en interesse in de gezamenlijke projecten. Ook bedankt voor jullie lessen over de andere aspecten van de stressadaptatie.

Uiteraard werden ook deze mensen gecoacht. De 3-maandelijkse werkbesprekingen, hebben veel betekend voor mijn onderzoek. Sjoerd Wendelaar Bonga en Gert Flik, bedankt voor jullie kritische opmerkingen en interesse. Hans Komen, bedankt voor alles, ennuh..... als jij in de stress zit, kom je maar langs in Noorwegen. Wim van Muiswinkel en Lidy Verburg, van jullie heb ik veel geleerd over het immuunsyteem van de karper.

No fish, no glory. Oftewel, wat had ik moeten beginnen zonder vissen. Daarom mijn speciale dank voor alle dierverzorgers. De karpers werden in Wageningen gekweekt. Sietse Leenstra, Aart Hutten en Menno ter Veld (hoeveel vissen heb je nodig?). Jullie hebben er altijd voor gezorgd dat ik keurig op tijd mijn vissen kreeg en altijd in perfecte conditie (Michael en Hans, jullie ben ik niet vergeten, ook hiervoor bedankt). De vissen werden vervolgens vervoerd naar Utrecht en ook daar moest goed voor ze gezorgd worden. Meneer van Oostrum, Henk Schriek, Job Jansen (goed, m'neer!) en Ko van Rootselaar. Bedankt voor jullie hulp bij het voeren, met name in het weekend.

Gerard Stooker en Ed van der Veen van het atelier, door jullie kwam er tenslotte schot in de zaak.

Angela de Lange, je staat er ook in, hoor. Angela, gewoon bedankt voor alles. Je nuchtere kijk op de dingen des levens en je luisterend oor heb ik erg kunnen waarderen, en uiteraard mag ik je dropjes niet vergeten te vermelden.

Cor Nijhof, bedankt voor de gezelligheid, ennuh.... ik drink nog steeds geen koffie.

Verder wil ik de mensen van Zuid 3, de afdelingen Beeldverwerking & Vormgeving en de fotografie bedanken, en dan met name Maarten Terlou en

Pieter van Dorp van Vliet. Maarten, bedankt voor je hulp met de IBAS. Pieter, ontzettend bedankt voor de uiteindelijke verwerking van dit proefschrif. Het eindresultaat is werkelijk schitterend geworden.

Als laatste wil ik een aantal mensen uit de persoonlijke hoek bedanken.

Mormor Jensen, tusind tak for alt. Som du kan se har jeg fået hjælp af Jette med at oversætte til dansk. Du har fortjent dette, fordi du altid er der, hvis vi har brug for hjælp, samt din dør står altid åben. Jeg håber at vi længe endnu må have denne fornøjelse. Tak for din gæstfrihed, og din interesse i mit arbejde, samt i mig personligt. Som Michelle altid siger, I love you too!

Jette, ontzettend bedankt voor je hulp met het Deens.

Ten slotte wil ik mijn ouders bedanken. Wie had gedacht dat ik het nog eens tot dr. Consten zou schoppen.

Als allerlaatste wil ik uiteraard mijn thuisfront bedanken.

Michelle, je hebt me altijd in alles gesteund. Veel belangrijker nog, je hebt me laten zien wat werkelijk het belangrijkste in het leven is, namelijk je gezin. Er staat ons voor de toekomst een spannende tijd te wachten en ik ben erg blij dat jij die met mij wilt delen. Ik kan je alleen niet beloven dat ik in de week-enden niet naar die guppies van me moet. Jeg elsker dig.

Jeremy, zonder jou zou ik het een stuk moeilijker gehad hebben de laatste tijd. Jij bent mijn geheim tegen stress en ik ben blij dat ik de mogelijkheid gehad heb om veel tijd met jou door te brengen. Er is niets ontspannender dan na een drukke dag thuis te komen en dat ondeugende koppie van jou te zien. Werkelijk alle stress valt dan van je af en er is nog maar één ding dat dan telt. Jij !!

Dimitri

Curriculum vitae

Dimitri Consten was born on February 22th, 1973 in Utrecht. After finishing his secondary education in 1991 at College Blaucapel in Utrecht, he started reading Biology at the University of Utrecht in 1992. He passed his freshman year with honors and continued his education in a physiological/molecular direction. As an undergraduate student, he participated in two research projects. His major research project he performed at the Research group for Comparative Endocrinology under supervision of dr. F.E.M. Rebers, studying the regulation of gonadotropin subunit mRNAs in the African catfish. As a second research project, he worked at the Hubrecht Laboratory under supervision of dr. J. Charité and dr. J. Deschamps, during which he investigated a regulatory element in one of the mouse *Hox* genes. He obtained a grant from the Erasmus programme (the present Socrates programme) of the EC to do a three month research project at the University of Algarve, Portugal, where he participated in a study of the discards of commercial fisheries from the South coast of Portugal. After graduating in November 1996, he worked as a PhD student at the Research Group for Comparative Endocrinology at the University of Utrecht. During this period he performed the research described in this thesis and obtained his licenses for the use of laboratory animals (art. 9) and radioactive materials (Radiation Hygiene, level 4B). In addition, he contributed to the education of undergraduate students in Biology. The author was a member of the Research School for Developmental Biology.

Publications

Charité J, de Graaf W, Consten D, Reijnen MJ, Korving J, Deschamps J. 1998 Transducing positional information to the *Hox* genes: critical interaction of *cdx* gene products with position-sensitive regulatory elements. Development 125: 4349-4358

Consten D, Lambert JGD, Goos HJTh. 2000 Inhibitory effects of cortisol on *in vivo* and *in vitro* androgen secretion in male common carp, *Cyprinus carpio*. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (Eds.) Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish; 2000; Bergen, Norway. p192

Consten D, Bogerd J, Komen J, Lambert JGD, Goos HJTh. 2001 Long-term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L. Biol. Reprod. 64(4): 1063-1071

Consten D, Lambert JGD, Goos HJTh. 2001 Cortisol affects testicular development in male common carp, *Cyprinus carpio* L., but not via an effect on LH secretion. Comp. Biochem. Physiol. *In press*.

Consten D, Kuijer EE, Groeneveld TWL, Engelsma MY, Tanck MWT, ter Veld M, Komen J, Lambert JGD, Goos HJTh. Cortisol mediates the inhibitory effect of cold shock stress on pubertal development in male common carp, *Cyprinus carpio* L. *Submitted*.

Consten D, Lambert JGD, Goos HJTh. Corticosteroids affect the testicular androgen production in male common carp, *Cyprinus carpio* L. *Submitted*.

Consten D, Keuning ED, Terlou M, Lambert JGD, Goos HJTh. Cortisol effects on 11β-hydroxysteroid dehydrogenase and the testicular androgen synthesizing capacity in common carp, *Cyprinus carpio* L. *In preparation*

Consten D, Keuning ED, Bogerd J, Zandbergen MA, Lambert JGD, Goos HJTh. Sex steroids and their involvement in the cortisol-induced inhibition of pubertal development in male common carp, *Cyprinus carpio* L. *In preparation*.