Chapter 1

General introduction

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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

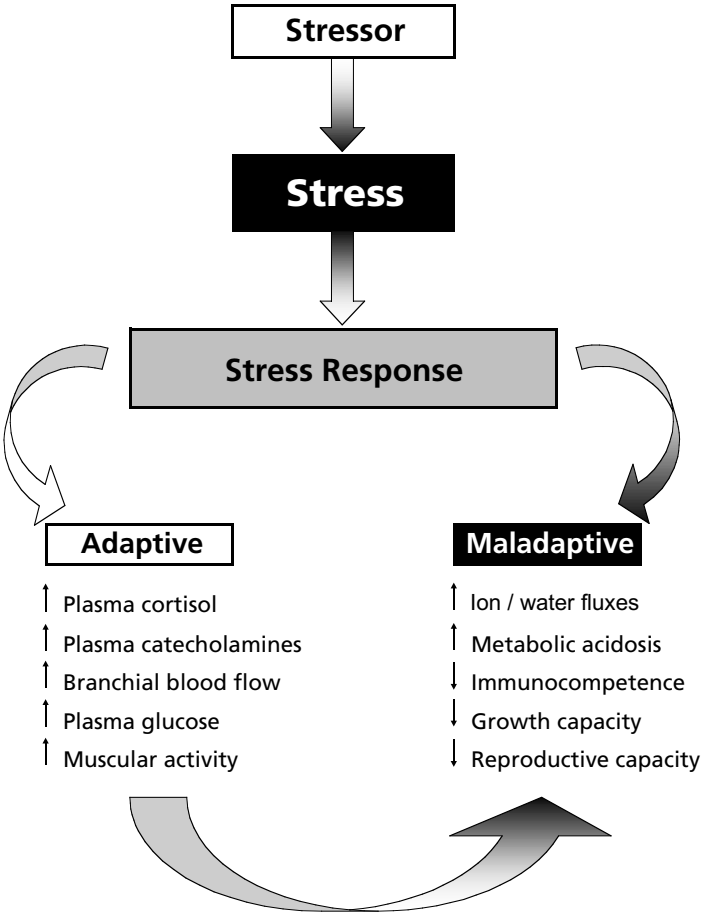

Figure 1. Simplified scheme showing the concept of stress (adapted from Barton & Iwama, 1991).

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, the 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.

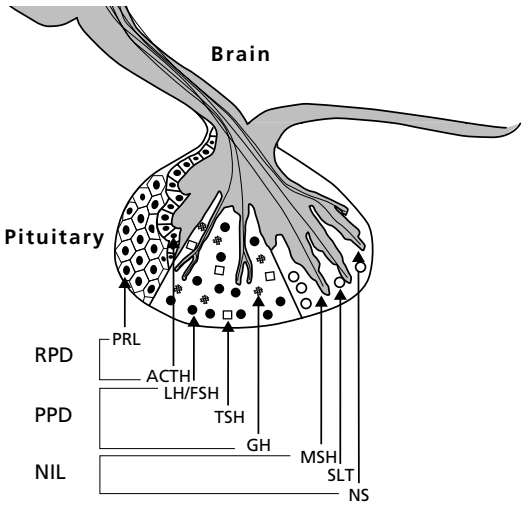

Figure 2. Schematic representation of a fish pituitary. The fish pituitary can be divided into a neuro-intermediate lobe (NIL) and a distal lobe. The NIL contains melanotrophs (MSH), somatolactin cells (SLT) and neuro-secretory nerve endings. The distal lobe or pars distalis can be subdivided into two 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).

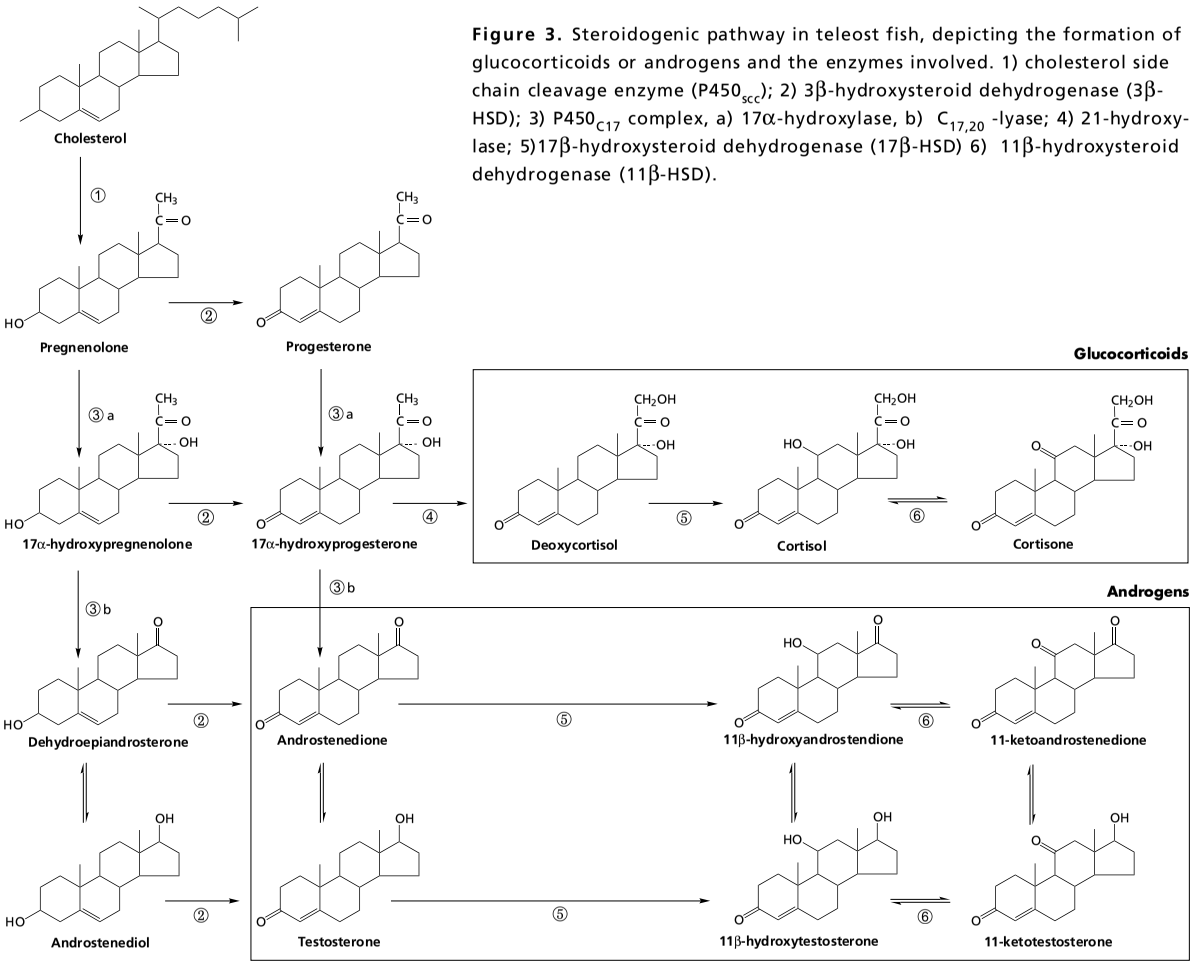

Figure 3. Steroidogenic pathway in teleost fish, depicting the formation of glucocorticoids or androgens and the enzymes involved. 1) cholesterol side chain cleavage enzyme ($P450_{scc}$); 2) 3β-hydroxysteroid dehydrogenase (3β-HSD); 3) $P450_{C17}$ complex, a) 17α-hydroxylase, b) $C_{17,20}$ -lyase; 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-

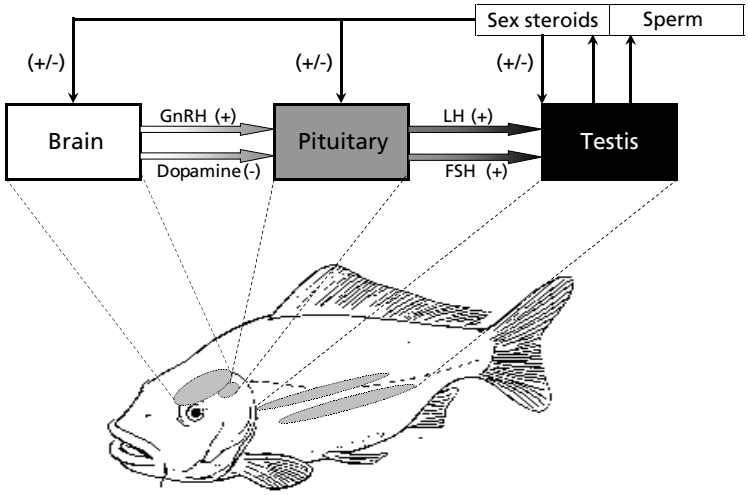

Figure 4. Schematic representation of the Brain-Pituitary-Gonad (BPG) axis. For an explanation, see paragraph 1.4.

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 neuroendocrine 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 hypothalamic 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 neuromodulator has been suggested. The last GnRH class, GnRH3 or the telencephalic form, is found in the forebrain and its function also remains to be clarified (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 conserved 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**	**Ly**s	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

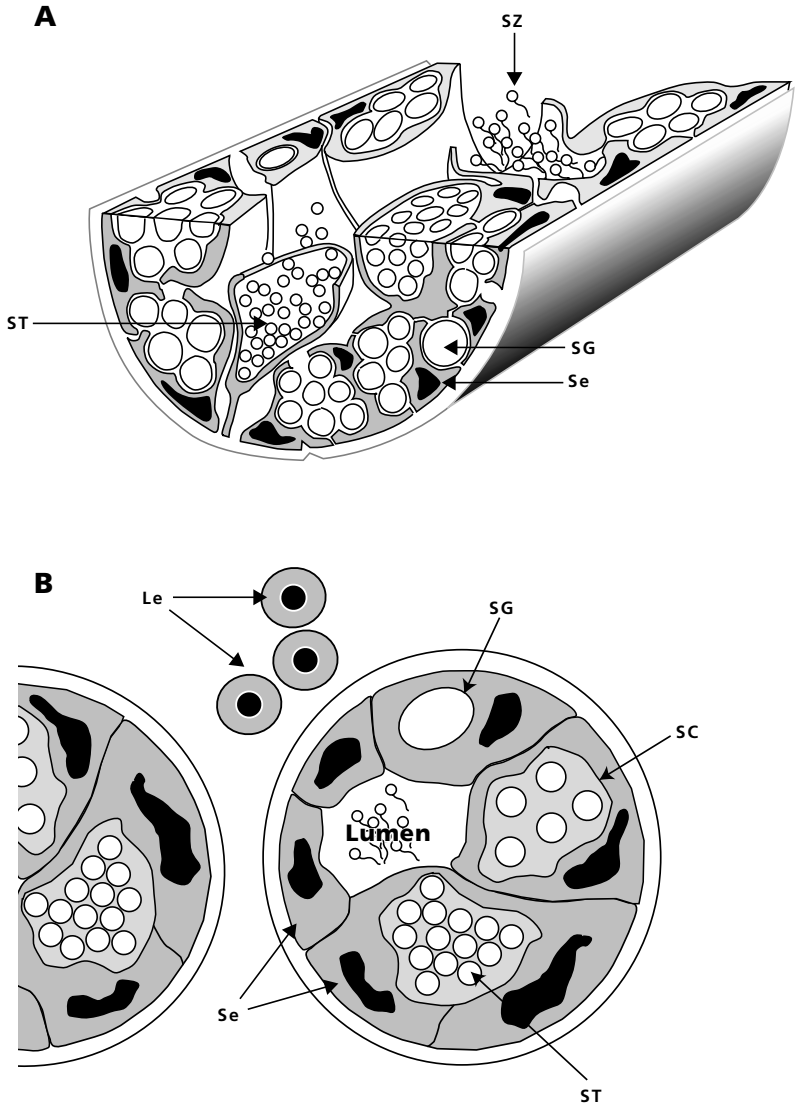

Figure 5. Schematic representation of the anatomical structure of a fish testis. (a) A testicular lobule; (b) a cross section of two lobules. Inside the lobules, Sertoli cells (Se) support the developing germ cells in different stages of development; spermatogonia (SG), spermatocytes (SC), spermatids (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

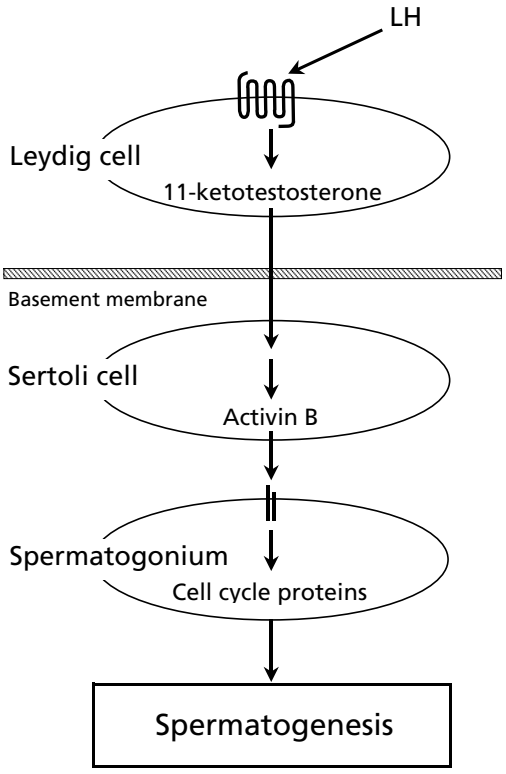

Figure 6. Hormonal regulation of spermatogenesis in fish (adapted from Nagahama, 1994)

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 hypothalamic 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.

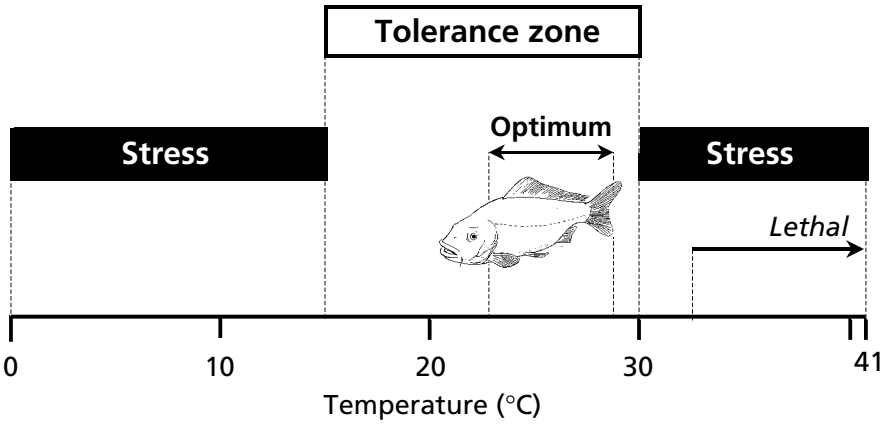

Figure 7. Thermal requirements for the common carp (adapted from Elliot, 1981).

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

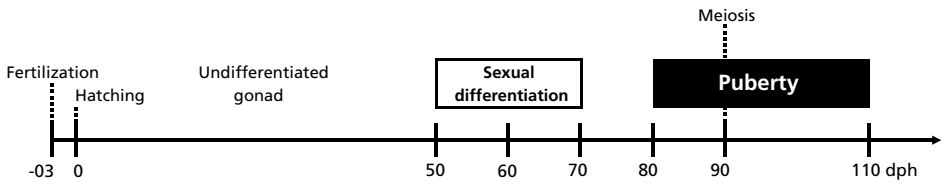

Figure 8. Life span of the common carp strain used in this study (E4xR3R8). The period of sexual differentiation and pubertal development is indicated.

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.