Characterization of the stress response in 17α-hydroxylase deficient common carp

(*Cyprinus carpio L.*)

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Abstract

In this thesis we describe interrenal hyperplasia in a teleost fish, the common carp, *Cyprinus carpio L,* caused by 17α-hydroxylase deficiency. Two homozygous, XX male inbred strains, "E5" and "E7", consistently show 5-10 fold less plasma cortisol in response to net confinement compared to normal male and female carp strains. Both E5 and E7 fish have enlarged head kidneys, which is caused by an increase in size and number of interrenal cells. The experiments and results described in this thesis aim to provide further insight into the genetic background of the 17α-hydroxylase deficiency, and the expression of genes involved in the stress response in P450c17 deficient common carp during and after stress. First, we describe the sequencing of the 17α-hydroxylase/17, 20 lyase gene, *CYP17a1*, in a normal, standard strain ("STD") and one of the 17α-hydroxylase deficient strains (E5) of common carp. Two variants of the *CYP17a1* gene were found. The nucleotide coding region of the CYP17a1.I variant contained 8 exons and 7 introns which is identical to the human *CYP17*. In total 3122 base pairs of the nucleotide sequence were determined. Variant I showed no differences between STD and E5, while variant II is missing exon 7 and part of exon 8 and contains a 3 bp insertion and 8 homozygous SNP's downstream of exon 6. Next, we describe the stress response and gene expression in 17α-hydroxylase deficient E5 and normal STD common carp. The results show a significant increase of corticosterone levels in E5 carp in response to stress. Normal STD carp produce only small amounts of corticosterone. STD carp show a correlation between cortisol production and 11β-HSD2 mRNA expression during the stress response. In contrast, 11β-HSD2 expression is significantly lower in E5 fish and stays at a constant level during confinement. Real-time PCR analysis of StAR and 3β-HSD show a significantly higher expression of these enzymes in E5 during and after net confinement, but P450c21 not, suggesting that transcription of P450c21 is not a limiting step in corticosterone production. Messenger RNA levels in P450c17a1.I are quite variable with a trend towards lower expression levels in E5, suggesting a dysfunction at the transcriptional level. In the last part of this thesis, we investigate the inheritance of interrenal hyperplasia and low cortisol response using backcross and gynogenetic progeny of heterozygote carriers of common carp. Six-month old backcross (BC5 and BC7) and double haploid (DH) progenies were sacrificed after one hour net-confinement, blood sampled, and dissected to determine sex and head kidney-somatic index. Values for cortisol and head kidney index showed a continuous distribution in BC and DH progeny. Values for corticosterone, on the other hand showed a very clear segregation pattern in high and low responders, consistent with a single gene model. There was a significant difference in mean cortisol level between high (H) and low (L) corticosterone responders in BC and DH progeny groups. Surprisingely, H responders were predominantly male, while L responders were female or intersex. These results confirm that 17α-hydroxylase deficiency is inherited as a recessive mutation, and that 17α-deficiency and sex reversal are either caused by the same mutation (pleiotropy) or by closely linked genes.

This thesis is dedicated **:**

To my loving wife **Farahnaz,**

To our dearest daughter **Neshat,**

To our beloved **parents**

And

To my all **Teachers**

Table of contents

Chapter One:

General Introduction

1.1 Introduction

By definition, stress is a condition in which homeostasis, the dynamic equilibrium of an organism, is threatened or disturbed in response to a stressor (Chrousos, Gold, 1992). Through the stress response an animal tries to cope with a stressor by readjusting its biological activities. The stress response begins in the nervous system, (e.g., the Sympathetic-Adrenal Medullary (SAM) system which reacts almost immediately) and in the endocrine system, (e.g., Hypothalamic-Pituitary-Adrenal (HPA) axis, which reacts more slowly). These two systems function to create a precise homeostatic balance. The HPA axis is mainly involved in the long-term stress response. The HPA axis begins in the hypothalamus, which is located at the base of the brain. The hypothalamus produces hormones that either stimulate or inhibit the release of other hormones from the anterior pituitary. When the brain detects a stressor, the pituitary releases adrenocorticotropin hormone (ACTH). ACTH goes on to stimulate a part of the body located just above the kidneys called the adrenal cortex to synthesize and release chemicals known as glucocorticoids, mineralocorticoids and sex steroids. Glucocorticoids are a class of steroid hormones that includes cortisone, cortisol, and corticosterone. Glucocorticoids regulate blood pressure and cardiovascular function, as well as the body's use of proteins, carbohydrates and fats. Glucocorticoid release increases in response to any type of stressor (physical or emotional) and causes the breakdown of stored nutrients into usable forms of energy. Glucocorticoids also inhibit non-essential functions such as growth, reproduction, and inflammation.

A number of disorders of the HPA stress pathway have been described. In Cushing's syndrome, patients produce massive amounts of glucocorticoids (Orth, 1995), while in Addison's disease, an adrenal insufficiency is described (Oelkers, 1996). Congenital adrenal hyperplasia (CAH) is the inherited inability to synthesize cortisol. CAH arises from a dysfunction of one of the enzymes involved in the adrenal steroid synthesis pathway (Deaton et al., 1999). Insufficient cortisol synthesis signals the hypothalamus and pituitary to increase corticotropin releasing hormone (CRH) and adrenocorticotropin releasing hormone (ACTH), respectively (Fig.1). Continued secretion of ACTH causes consecutive stimulation of the adrenal cortex and enlargement of the adrenal glands. All forms of CAH involve excessive or

defective production of sex steroids and can deviate or impair development of primary or secondary sex characteristics in affected infants, children, and adults. Normally adrenal gland secrets only trace amounts of sex hormones, particularly androgens, but in CAH, excessive prenatal production of androgens in affected females results in masculinization of the reproductive tract and ambiguous (male-like) genitalia in the newborn. The clitoris enlarges into a penis-like structure and the labiae of the vulva may fuse to produce a scrotum (Austin, Short, 1982). In human, more than 90% of CAH cases are caused by a 21-hydroxylase deficiency (White, Speiser, 2000). The verilizing form of CAH is seen in approximately 25% of those patients with 21-hydroxylase deficiency.

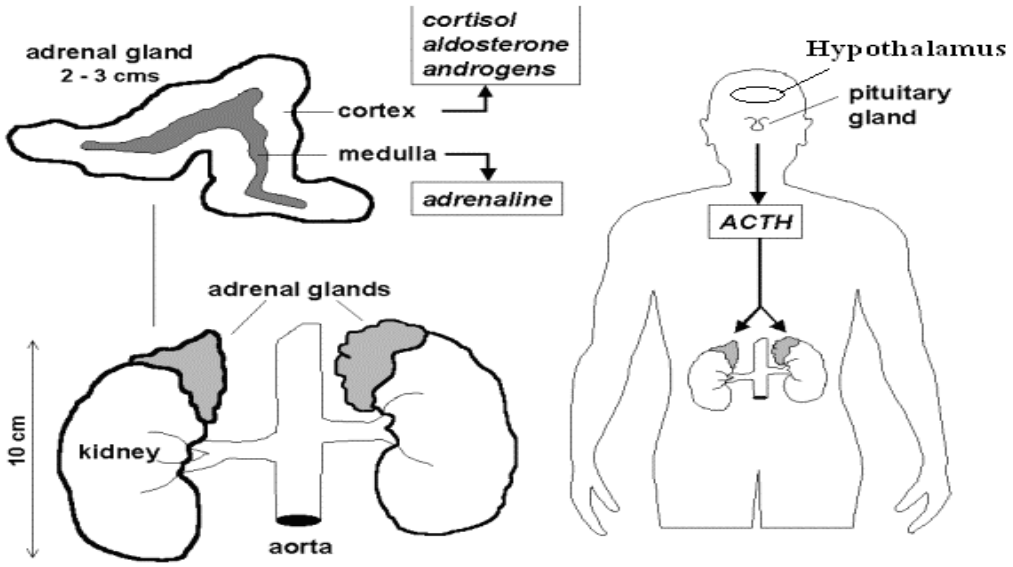

Figure 1.1 Schematic representation of the Hypothalamus-Pituitary-Adrenal (HPA) axis in human (after Conway, 1999).

1.2 Congenital Adrenal Hyperplasia (CAH) in human

1.2.1 Adrenal gland

In mammals generally and human particularly the adrenal glands are a couple of bodies located at the anterior poles of two metanephric kidneys (Fig.1.1). The adrenal consists of two parts, medulla and cortex. The chromaffin cells are located in the medulla and produce

catecholamine (adrenaline). The adrenal cortex is divided into three layers (Fig.1.2): 1) the *zona glomerolusa*, which is the outer zone and produces mineralocorticoids like aldosterone which regulate the body levels of sodium and potassium; 2) The middle-inner part or *zona fasciculata*, which mostly produces glucocorticoids like cortisol and corticosterone. They regulate carbohydrate metabolism. 3) the most inner part or *zona reticularis* that produces androgens, whose actions are similar to that of steroids produced by the male gonads. These steroids are secreted into the blood stream and are necessary for normal health.

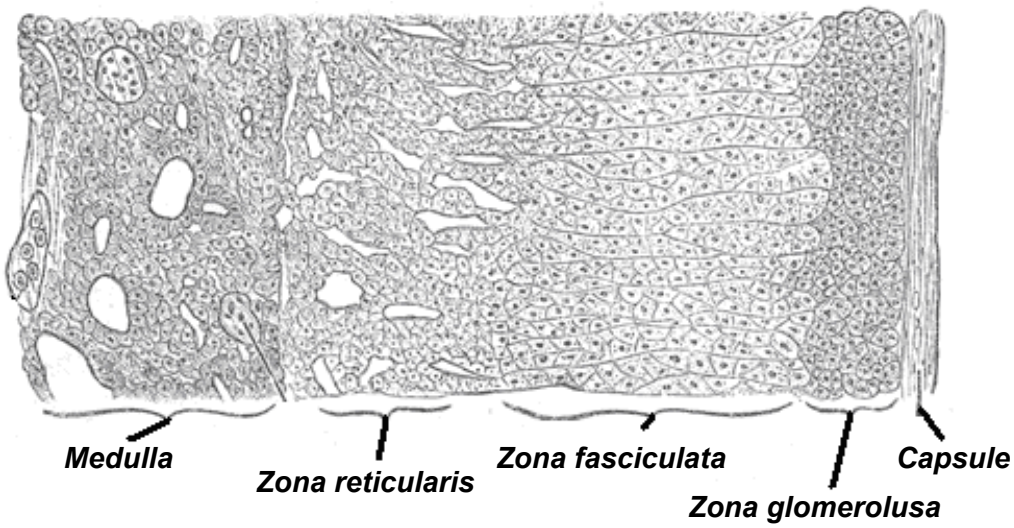

Figure 1.2 Layers of cortex and medulla in adrenal glands (Anatomy of human body, Gray's 1918).

1.2.2 Steroidogenesis in the adrenal gland

Aldosterone, cortisol and androgens are the 3 main steroids involved in congenital adrenal hyperplasia. Availability of cholesterol as the first precursor in the steroidogenic pathway (Fig.1.3) is controlled by steroidogenic acute regulatory protein (StAR). Transport of cholesterol from cellular stores to the inner membrane of mitochondria, where the cholesterol side chain cleavage (P450scc) is located, is the rate limiting step in steroid biosynthesis. The next step in steroidogenesis is the conversion of cholesterol to the C21 steroid pregnenolone. This is catalyzed by the mitochondrial cytochrome P450scc. 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum and mitochondria converts pregnenolone to progesterone. To produce the mineralocorticoid aldosterone, progesterone is

hydroxylated to deoxycorticosterone (DOC) by 21-hydroxylase (P450c21) in the endoplasmic reticulum. Aldosterone is produced by the 11β-hydroxylation of DOC to corticosterone, followed by 18-hydroxylation and 18 oxidation of corticosterone (Hanukoglu, 1992). To produce the glucocorticoid cortisol, P450c17, located in the endoplasmic reticulum of the *zona fasciculata* and *zona reticularis* converts pregnenolone to 17α-hydroxypregnenolone, which is converted into 11 deoxycortisol and finally cortisol by 3β-HSD, P450c21 and P450c11, respectively. In the zona reticularis, androgens are produced by P450c17. P450c17 has a key role in synthesis of sex steroids with dual activities: 17α-hydroxylation and C17, 20 lysis. 17α-hydroxyprogesterone is produced by the hydroxylase activity of P450c17 and androstenedione is produced from 17α-hydroxyprogesterone by the lyase activity of P450c17. Androstenedione is converted in androgens (in males) and estrogens (in females).

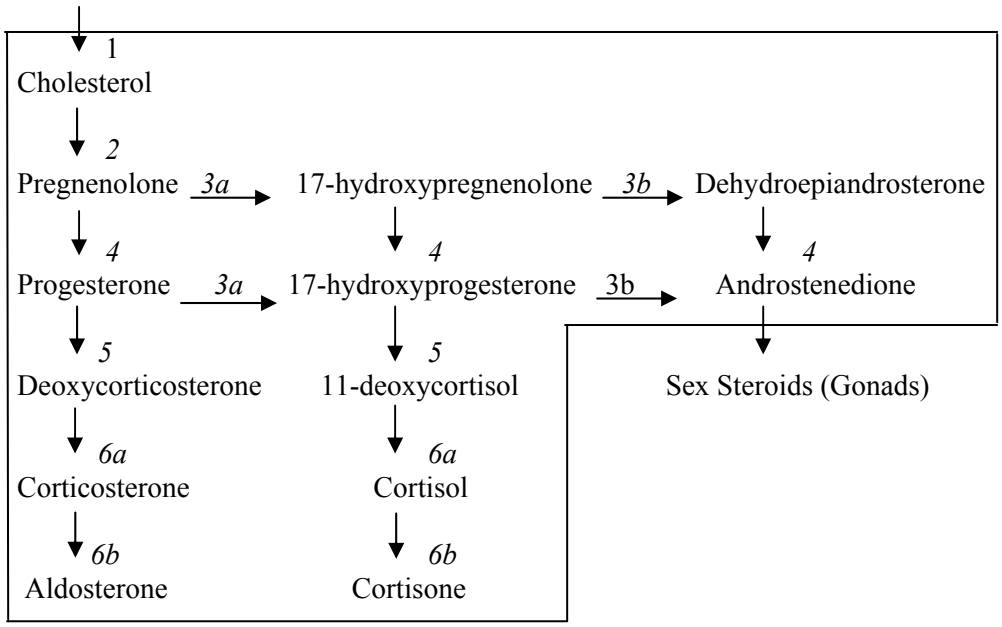

Figure 1.3 Biosynthesis of steroids in Adrenal Cortex. All steroid hormones will be synthesized from cholesterol and the end products can be classified according to their principal effects; mineralocorticoids (aldosterone), glucocorticoids (cortisol in human), sex steroids, androgens and estrogens. Legend: 1) StAR, 2) P450scc, 3a) P450c17 (hydroxylase), 3b) P450c17 (lyase), 4) 3β-HSD, 5) P450c21, 6a) P450c11b1, 6b) 11β-HSD2.

The enzymes required to synthesize the major classes of steroid hormones are delineated in Table 1.1.

Table 1.1 Enzymes that catalyze the initial reactions in the pathways of steroid hormone biosynthesis in human and fish (Hagen et al., 2006; Hanukoglu, 1992; Li et al., 2003a; Lyssimachou, Arukwe, 2007; Zhou et al., 2007b) related to the Fig.1.3 and Fig.1.5.

Common name	Enzyme (Human)	Enzyme (Fish)	Gene
Steroidogenic Acute Regulatory protein	StAR	StAR	*StAR*
Side-chain cleavage enzyme; desmolase	$P450_{SCC}$	$P450_{SCC}$	*CYP11A*
3β-hydroxysteroid dehydrogenase	3β-HSD	3β-HSD	*HSD3B*
17α-hydroxylase/17,20 lyase	P450c17	P450c17a1 P450c17a2	*CYP17-I* *CYP17-II*
21-hydroxylase	P450c21	P450c21	*CYP21*
11β-hydroxylase	P450c11b1	P450c11b1	*CYP11β1*
18β-hydroxylase, 11β-hydroxysteroid dehydrogenase	P450c11b2, 11β-HSD2	P450c11b2, 11β-HSD2	*CYP11β2*

1.2.3 Congenital adrenal hyperplasia (CAH)

Several autosomal recessive mutations have been identified in *CYP21, CYP11B, CYP17, HSD3B, CYP11A* and *StAR* genes. These mutations generally lead to adrenal hyperplasia and partial sex reversal.

CYP21: In 1865, Luigi De Crecchio, an Italian anatomist, first documented CAH in a female patient with enlarged adrenal glands, male appearing genitals but no testes, and an internal female reproductive system. In 1950, researchers discovered that CAH is caused by a disturbance in the normal feedback mechanism which produces cortisol in the adrenal glands. In 1962, the 21-hydroxylase deficiency form of CAH was first discovered. In addition, recessive nature of the genetic trait and identification of hormonal abnormalities were recognized (Bongiovanni, 1969).

More than 90% of the cases of CAH are the result of deficiency in the enzyme steroid 21-hydroxylase (Deaton et al., 1999). The classic form of 21-hydroxylase deficiency has an incidence of 1 in 5000 to 1 in 15000 live births. The structural gene encoding human CYP21 and a pseudogene (CYP21P) are located on chromosome 6p21.3 approximately 30 kb apart. CYP21 and CYP21P each contain 10 exons spaced over 3.1 kb. Their nucleotide sequences are 98% identical in exons and approximately 96% identical in introns (Higashi et al., 1986; White et al., 1986). More than 50 different mutations of CYP21 have been identified. Most mutations appear to be the result of a recombination between CYP21 and the pseudogene CYP21P (White, Speiser, 2000). These mutations alter the structure or production of the enzyme and cause 21-hydroxylase deficiency. Generally, mutations that only partially impair enzyme levels or function will have milder effects than more severe mutations that eliminate or greatly reduce the level of 21-hydroxylase enzyme. The consequence of this multitude of mutations is that there is considerable variability in the clinical presentation of CAH in humans, ranging from severe salt wasting to much more milder syndromes.

In other mammals, reports on CAH are rare. In mice, newborn *aw18* homozygous pups are deficient in 21-hydroxylase activity, and that homozygosity for the *aw18* haplotype directly causes death at the early postnatal stage (Gotoh et al., 1988). A case of congenital adrenal hyperplasia (CAH) produced by 21-hydroxylase deficiency was first observed in dogs in 2002 (Castillo, Ortemberg, 2002).

CYP11B: Congenital adrenal hyperplasia due to 11β-hydroxylase deficiency (11β-OHD) is found in 8 to 9% of the patients with CAH and occurs 1 in 100,000 live births (White, Speiser, 1994). In people affected by this type of CAH, the mutation causes verilization of female fetus because of over secretion of adrenal androgens. The postnatal signs and symptoms of this disorder are similar to 21-OHD; unique to 11β-OHD is that patients frequently suffer from hypertension as a result of accumulation of salt-retaining aldosterone precursors. The first report of congenital adrenal hyperplasia in a domestic cat caused by 11β-OHD was made in 2004 (Knighton, 2004).

CYP17; The first report of deficiency of adrenal 17α-hydroxylase activity in human was published in 1966 (Biglieri et al., 1966). The gene for the cytochrome P450c17 enzyme

resides on chromosome 10q24.3. Homozygous mutations in the gene CYP17 may cause 17α-hydroxylase deficiency (17α-OHD), 17,20 lyase deficiency, or both (Wong et al., 2006). The disorder is inherited in an autosomal recessive manner and accounts for approximately 1% of all CAH cases. Clinical effects of this condition include overproduction of mineralocorticoids and deficiency of prenatal and pubertal sex steroids. In females, P450c17 is necessary for both cortisol and estrogen synthesis. Lack of these hormones results in increases in ACTH and FSH (Follicle Stimulating Hormone) levels. 17α-OHD can cause deviations in secondary sexual characteristics, such as pseudohermafrodism with ambiguous external genitalia in women, and prominent breast development at puberty in man.

CAH due to mutations in HSD3B, StAR and CYP11A are rare in human and animals. The structures of two types of human HSD3B cDNA clones encoding the type I and type II 3β-HSD isoenzymes, respectively, have been recently characterized. The complete nucleotide sequence of the corresponding genes shows that both consist of four exons and three introns included within a DNA fragment of approximately 7.8 kb and assigned to chromosome 1 p13. An elevated ratio of A5- to A4-steroids is considered to be the best biological parameter for the diagnosis of 3β-HSD deficiency. In male newborns, the 3β-HSD defect in the testis leads to pseudohermaphroditism with incomplete masculinization of the external genitalia, while affected females show normal sexual differentiation or mild verilization (Whorwood et al., 1990).

Congenital lipoid adrenal hyperplasia (lipoid CAH) is the most severe form of CAH, eventually destroying all adrenal and gonadal steroidogenesis. Lipoid CAH is caused by mutations in the steroidogenic acute regulatory protein (StAR), which facilitates the entry of cholesterol into mitochondria to initiate steroidogenesis. Patients with lipoid CAH typically suffer from a salt-losing crisis in the first 2 months of life (Chen et al., 2005).

The CYP11A gene, encoding P450scc, is located on chromosome 15q23-24 and contains 9 exons (Katsumata, 2007; Morohashi et al., 1987). Recently, inactivating mutations in the CYP11A gene were described in a 46, XX patient with adrenal deficiency and in 46, XY patients with sex reversal and adrenal insufficiency (Tajima et al., 2001). In rabbits, an inherited feminizing lethal form of CAH caused by P450scc deficiency was first reported

(Fox, Crary, 1978). Further a naturally occurring deletion in the gene encoding P450scc has been reported in rabbits. Rabbits, homozygous for the deletion are affected by congenital lipoid adrenal hyperplasia, while heterozygous animals are phenotypically normal (Yang et al., 1993).

1.3 CAH in Fish

1.3.1 Stress response in fish

In fish culture, many kinds of stressors like netting, handling and transporting are common and unavoidable threats.

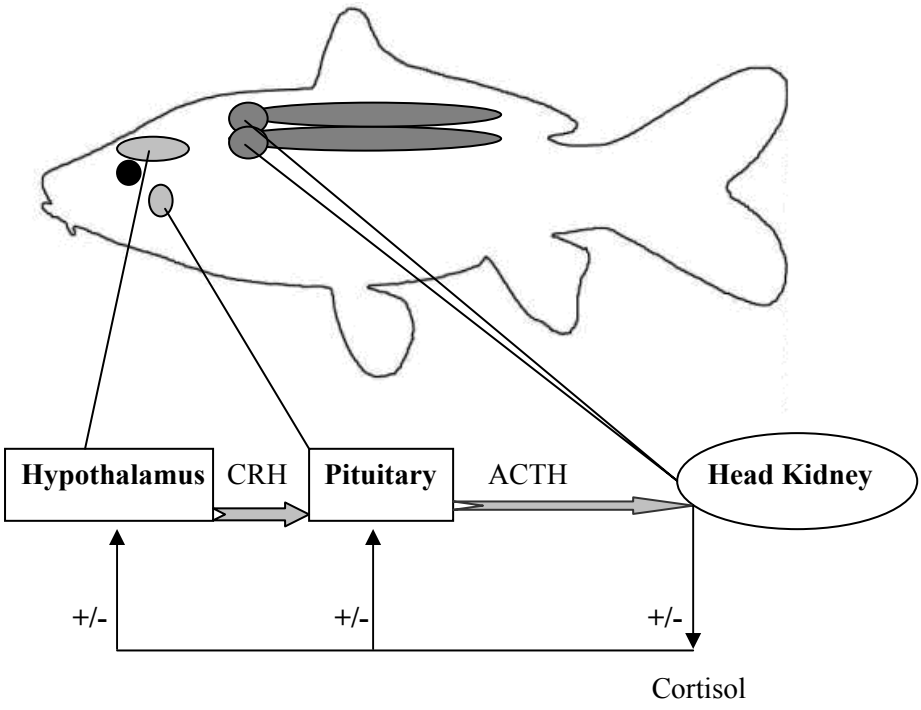

Figure 1.4 Schematic representation of the Hypothalamus-Pituitary-Interrenal (HPI) axis in fish (Consten et al., 2001; Ruane et al., 2001).

The stress response in fish is similar to that of mammals and primary and secondary responses of fish to stress have been described in several species (Iwama et al., 1997; Rotllant

et al., 2003; Wendelaar Bonga, 1997), including carp (Ruane et al., 2001). Once a stressor is recognized, the signal is transferred to hypothalamus by sensory neurons (Fig. 1.4). Then the neuro-endocrine cells of the hypothalamus release corticotropin releasing hormone, CRH, and thyrotropin releasing hormone (TRH). These are major factors that stimulate release of adrenocorticotropic hormone (ACTH) and other peptides from the pituitary (Vale et al., 1981; Van Enckevort et al., 2000). The fish pituitary can be divided into a neuron-intermediate lobe (NIL) and a distal lobe. The distal lobe or pars distalis can be subdivided into two parts; the rostral pars distalis (RPD) and the proximal pars distalis PPD). The corticotrophs are mostly located at the border between the rostral and proximal pars distalis (Cavaco et al., 1998) and control the production of cortisol from interrenal cells located in head kidney through secretion of ACTH (Barton, 2002; Consten et al., 2001).

1.3.2 Head kidney in fish

The adrenal homolog of teleost is not a compact organ as the adrenal glands of most vertebrates, but is composed of chromaffin and interrenal steroidogenic cells surrounding the post cardinal vein and its branches inside the head kidney (Gallo, Civinini, 2003). The right post cardinal vein is generally larger in section than the left one and so the amount of glandular tissue is greater in the right head kidney. The two tissues can be mixed, adjacent, or completely separated. The chromaffin cells secrete catecholamine into the general circulation immediately following any kind of stress, while interrenal cells mainly produce cortisol in response to stress (Wendelaar Bonga, 1997). Interrenal cells can be identified by 3β-HSD staining which is carried out on frozen sections of head kidney tissue.

1.3.3 Steroid biosynthesis

Cortisol is the main product of interrenal secretion in fish. Due to lack of aldosterone in teleosts, cortisol exerts both gluco and mineralocorticoid functions, which are related to energy metabolism balance and control of hydromineral balance, respectively. The basal plasma cortisol level varies from 2 to 42 ng /ml in different species (Gamperl et al., 1994). The plasma cortisol level can increase from 20 to 500 ng/ml under a variety of factors. Cyprinids have a very high cortisol response to stress (Pottinger et al., 2000) although they are more domesticated fish.

As in other animals, steroids in fish are synthesized from a precursor, cholesterol. The first step in this chain reaction is the formation of pregnenolone by side chain cleavage mediated by the enzymatic activity of the P450scc complex located in the inner mitochondrial membrane (Fig.1.5).

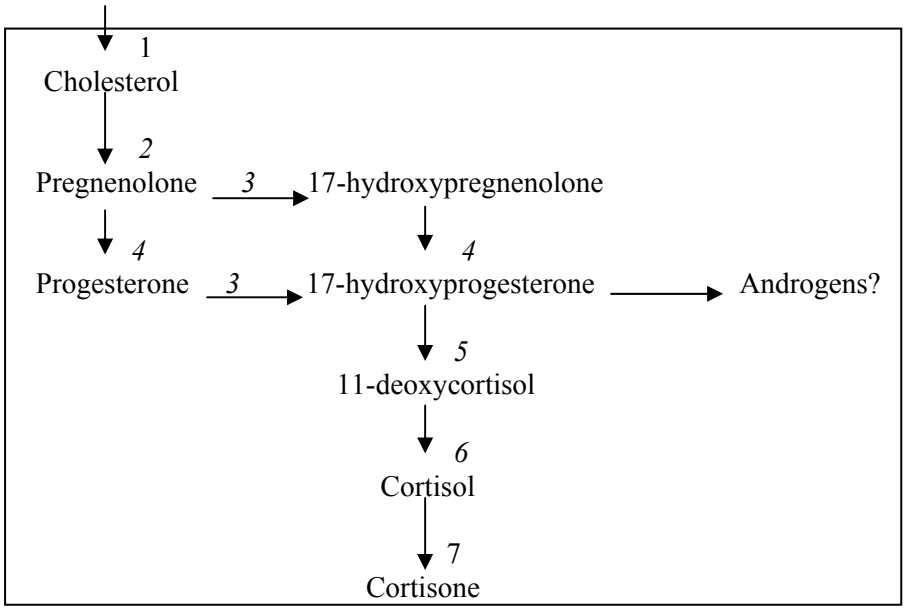

Figure 1.5 Biosynthesis of steroids in Head Kidney of fish. All steroid hormones will be synthesized from cholesterol. The end product is cortisol. Legend: 1) StAR, 2) P450scc, 3) P450c17-II (hydroxylase), 4) 3β-HSD, 5) P450c21, 6) P450c11, 7) 11β-HSD2.

Pregnenolone then undergoes a series of isomerizations and hydroxylations by 17α-hydroxylase (P450c17), 3β-HSD, 21-hydroxylase (P450c21) and 11β-hydroxylase (P450c11) to produce cortisol. Cortisol is inactivated to cortisone by 11β-HSD2, finally. A novel second P450c17 (P450c17-II), lacking the lyase activity has been recently discovered in fish. It is the only P450c17 existing in the head kidney. It has only hydroxylase activity and is responsible for the production of cortisol in fish. However, in gonads, both P450c17-I and II are expressed. Due to the difference in gene structure and enzymatic functions between P450c17-I and -II, androgens are probably not produced in the head kidney (Zhou et al., 2007a; Zhou et al., 2007b) .

1.3.4 CAH in common carp

Isogenic strains of common carp have been produced using andro and gynogenetic manipulations (Bongers et al., 1998; Komen, Thorgaard, 2007; Komen et al., 1988) and plasma cortisol levels in a number of the strains have been determined in response to a standard 20 min net confinement stressor. Most strains typically show a peak response of 200 -300 ng/ml plasma cortisol after around 30 minutes to one hour net confinement. However, two strains E5 and E7, consistently show 5-10 fold less plasma cortisol in response to net confinement compared to other male and female strains (Ruane et al., 2007; Ruane et al., 2005). To determine whether the different cortisol stress response was due to reduced sensitivity of the interrenal cells to ACTH, head kidney tissues isolated from two strains, E5 and a normal all male carp strain "STD", were stimulated with ACTH in an *in vitro* superfusion experiment.

Results showed an increase of cortisol release which was again significantly lower in E5 fish. Morphometric analysis of head kidney tissue demonstrated that head kidneys in E5 fish were enlarged when compared to head kidneys of STD fish. Interrenal cells stained by 3β-HSD showed that the larger head kidney was partially due to the 2-3x increased number of interrenal cells in E5 head kidneys. Steroid analysis in head kidney homogenates indicated that per gram tissue, E5 adrenals synthesize 50% less products during in vitro incubation with precursor's pregnenolone and progesterone. Further analysis on substrate conversions revealed no difference in the ability E5 and STD fish to synthesize 11-deoxycortisol and cortisol from 17α-hydroxy progesterone. However, production of 17α-hydroxyprogesterone from pregnenolone was significantly lower in E5 fish compare to STD fish. Based on these results, Ruane et al., (2005) concluded that the reduced capacity of E5 fish to produce cortisol is caused by a deficiency in 17α-hydroxylase activity. Cortisol normally exerts a negative feedback on ACTH output from the pituitary. A reduced cortisol output leads to increased stimulation of adrenals by ACTH, resulting in increased head kidney size, similar to what is seen in humans (Fig.1.6).

E5 carp show sex reversal from female to male. The cause of this sex reversal is unknown but the working hypothesis is that it is related to interrenal hyperplasia. Both carp strains E5 and

E7 are genetically female. Earlier observations on crossings of E5 carp with normal wild type carp had shown that the sex reversal was inherited as a recessive trait (Komen et al., 1990). Normal, XY male carp are not affected. In humans, masculinization in CAH patients is caused by adrenal androgen production. However, Ruane et al., (2006) concluded that masculinization in E5 carp was not caused by increased androgen production from the head kidney.

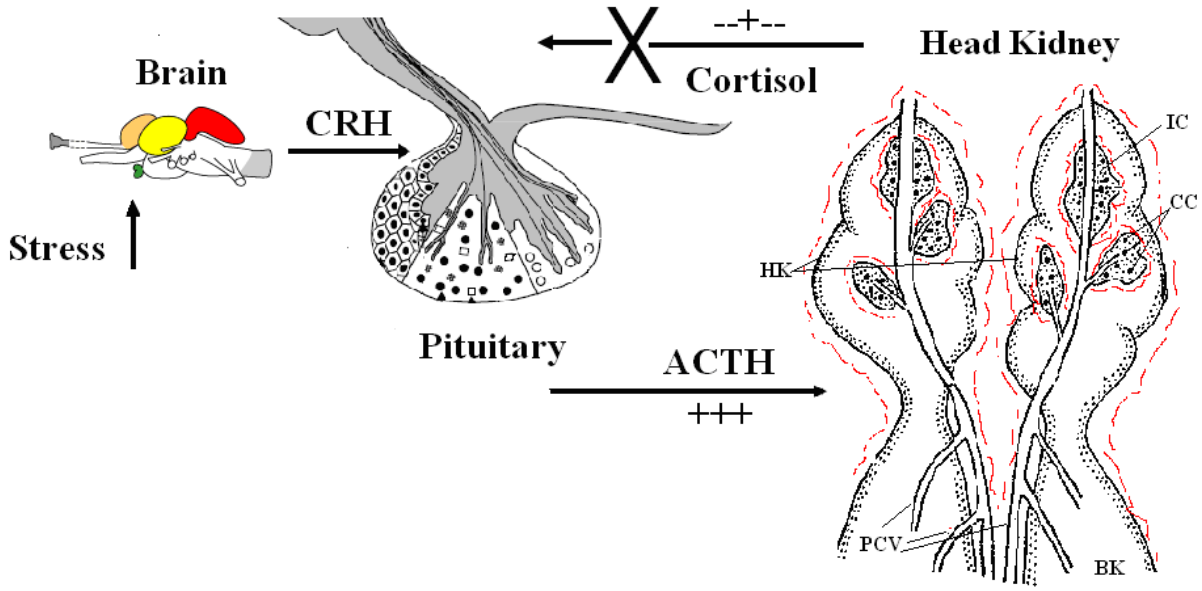

Figure 1.6 The mechanism of CAH in common carp; HK; Head Kidney, BK; Body Kidney, PCV; left and right Post Cardinal Vein, CC; Chromaffin Cells the black and large spots, IC; Interrenal Cells the black and small spots, CRH; Corticosteroid releasing hormone, ACTH; Adrenocorticotropic hormone.

1.4 Aim and outline of the thesis

The general aim of this thesis is to study the expression of genes involved in steroidogenic pathway in head kidney of common carp using real time RT-PCR. The experiments and results described in this thesis provide insights into the physiological responses of P450c17 deficient common carp during and after stress. In the General introduction (**chapter1**) we describe current knowledge on congenital adrenal hyperplasia in humans and common carp. We summarize the concepts stress and stress response, define the steroidogenic pathway and

introduce the steroid producing enzymes, and describe the interrenal tissue and head kidney in human and fish. In **chapter 2**, the molecular characterization of P450C17-I in common carp is studied to identify mutations in the 17α-hydroxylase deficient strain. To do so, we sequence the 17α- hydroxylase/17, 20 lyase gene in both a standard and mutant strain of common carp. In **chapter 3**, we investigate the expression of Steroidogenic acute regulatory protein (StAR), 17α- hydroxylase (P450c17-II), 3β-hydroxysteroid dehydrogenase (3β-HSD), 21-hydroxylase (P450c21) and 11β- hydroxysteroid dehydrogenase (11β-HSD), five genes involved in the cortisol production in head kidneys of normal (i.e. wild type) common carp (*Cyprinus carpio L.*) in response to a 3 h net confinement stress followed by a 22 h recovery course. In this chapter, we will seek the relationship of mRNA expression of the genes involved and plasma level of cortisol in head kidney of wild type common carp to see if net confinement has any influence on transcription of these genes. In **chapter 4,** we analyze the differences in expression of mRNA of the same steroidogenic enzymes in 17α-hydroxylase deficient (E5) common carp during and after stress, to confirm the hypothesis that P450c17 mRNA expression in E5 carps is reduced. We also measure corticosterone as a product of the direct conversion of progesterone by 21 hydroxylase. In **chapter 5,** we study the segregation of interrenal hyperplasia in backcross and gynogenetic progeny of E5 and E7 carp, using corticosterone as a marker. We also investigate the co-segregation of corticosterone production with sex reversal. Finally, General Discussion **(chapter 6)**, gives an overall view of the results presented in chapters 2 to 5, and the implications of the results are discussed.

References

Austin, C.R., Short, R.V., 1982. Reproduction in mammals, Book 2: Embryonic and fetal development. Cambridge University Press, 189 pp.

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integrative and Comparative Biology 42, 517-525.

Biglieri, E.G., Herron, M.A., Brust, N., 1966. 17α-hydroxylation deficiency in man. Journal of Clinical Investigation 45, 1946-1954.

Bongers, A.B.J., Sukkel, M., Gort, G., Komen, J., Richter, C.J.J., 1998. Development and use of genetically uniform strains of common carp in experimental animal research. Laboratory Animals London 32, 349-363.

Bongiovanni, A.M., 1969. Congenital adrenal hyperplasia. New England Journal of Medicine 280, 619-620.

Castillo, V., Ortemberg, L., 2002. Addison disease's in puppy, congenital adrenal hyperplasia (adrenal 21 alpha hydroxylase deficiency) and hypoparathyroidism in dogs. Revista Cientifica de la Facultad de Ciencias Veterinarias de la Universidad del Zulia 12, 175-181.

Cavaco, J.E.B., Vilrokx, C., Trudeau, V.L., Schulz, R.W., Goos, H.J.T., 1998. Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). American Journal of Physiology-Regulatory Integrative and Comparative Physiology 275, R1793-R1802.

Chen, X., Baker, B.Y., Abduljabbar, M.A., Miller, W.L., 2005. A genetic isolate of congenital lipoid adrenal hyperplasia with atypical clinical findings. Journal of Clinical Endocrinology and Metabolism 90, 835-840.

Chrousos, G.P., Gold, P.W., 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. The Journal of the American Medical Association 267, 1244-1252.

Consten, D., Lambert, J.G.D., Goos, H.J.T., 2001. Cortisol affects testicular development in male common carp (*Cyprinus carpio L.*) but not via an effect on LH secretion. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology 129, 671-677.

Deaton, M.A., Glorioso, J.E., McLean, D.B., 1999. Congenital adrenal hyperplasia: not really a zebra American Family Physician 59, 1190–1196.

Fox, R., Crary, D., 1978. Genetics and pathology of hereditary adrenal hyperplasia in the rabbit. Journal of Heredity 69, 251-254.

Gallo, V.P., Civinini, A., 2003. Survey of the Adrenal Homolog in Teleosts. International Review of Cytology 230, 89-187.

Gamperl, A.K., Vijayan, M.M., Boutilier, R.G., 1994. Experimental control of stress hormone levels in fishes: techniques and applications. Reviews in Fish Biology and Fisheries 4, 215-255.

Gotoh, H., Sagai, T., Hata, J., Shiroishi, T., Moriwaki, K., 1988. Steroid 21-hydroxylase deficiency in mice. Endocrinology 123, 1923-1927.

Hagen, I.J., Kusakabe, M., Young, G., 2006. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11β-hydroxylase messenger RNAs in rainbow trout interrenal cells: Relationship with in vitro cortisol production. General and Comparative Endocrinology 145, 254-262.

Hanukoglu, I., 1992. Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. Journal of Steroid Biochemistry and Molecular Biology 43, 779-804.

Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O., Fujii-Kuriyama, Y., 1986. Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene. Proceedings of the National Academy of Sciences USA 83, 2841–2845.

Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., 1997. Fish Stress and Health in Aquaculture. University Press, Cambridge, 278 pp.

Katsumata, N., 2007. Cholesterol side-chain cleavage enzyme (SCC) deficiency. Clinical Pediatric Endocrinology 16, 63-68.

Knighton, E.L., 2004. Congenital adrenal hyperplasia secondary to 11β-hydroxylase deficiency in a domestic cat. Journal of the American Veterinary Medical Association 225, 238-241.

Komen, H., Thorgaard, G.H., 2007. Androgenesis, gynogenesis and the production of clones in fishes: A review. Aquaculture 269, 150-173.

Komen, J., Duynhouwer, J., Richter, C.J.J., Huisman, E.A., 1988. Gynogenesis in common carp (*Cyprinus carpio L.*) I. Effects of genetic manipulation of sexual products and incubation conditions of eggs. Aquaculture 69, 227-239.

Li, Y.Y., Inoue, K., Takei, Y., 2003a. Interrenal steroid 21-hydroxylase in eels: primary structure, progesterone-specific activity and enhanced expression by ACTH. Journal Of Molecular Endocrinology 31, 327-340.

Lyssimachou, A., Arukwe, A., 2007. Alteration of brain and interrenal StAR protein, P450scc, and CYP11β mRNA levels in Atlantic salmon after nominal waterborne exposure to the synthetic pharmaceutical estrogen ethynylestradiol. Journal Of Toxicology And Environmental Health-Part A-Current Issues 70, 606-613.

Morohashi, K., Sogawa, K., Omura, T., Fujii-Kuriyama, Y., 1987. Gene structure of human cytochrome P450scc, cholesterol desmolase. Journal of Biochemistry 101, 879-887.

Oelkers, W., 1996. Adrenal insufficiency. The New England Journal of Medicine 335, 1206-1212.

Orth, D.N., 1995. Cushing's syndrome. The New England Journal of Medicine 332, 791-803.

Pottinger, T.G., Carrick, T.R., Appleby, A., Yeomans, W.E., 2000. High blood cortisol levels and low cortisol receptor affinity: Is the chub (*Leuciscus cephalus*) a cortisol-resistant teleosts. General and Comparative Endocrinology 120, 108-117.

Rotllant, J., Ruane, N.M., Caballero, M.J., Montero, D., Tort, L., 2003. Response to confinement in sea bass (*Dicentrarchus labrax*) is characterised by an increased biosynthetic capacity of interrenal tissue with no effect on ACTH sensitivity.

Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology 136, 613-620.

Ruane, N.M., Huisman, E.A., Komen, J., 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. Journal of Fish Biology 59, 1-12.

Ruane, N.M., Goos, H.J.T., Komen, J., 2007. Stress-induced facilitation of the cortisol response in 17a-hydroxylase deficient XX mas-1/mas-1 carp (*Cyprinus carpio*). General and Comparative Endocrinology 150, 473-479.

Ruane, N.M., Lambert, J.G.D., Goos, H.J.T., Komen, J., 2005. Hypocorticism and interrenal hyperplasia are not directly related to masculinization in XX mas-1/mas-1 carp, (*Cyprinus carpio)*. General and Comparative Endocrinology 143, 66-74.

Tajima, T., Fujieda, K., Kouda, N., Nakae, J., Miller, W.L., 2001. Heterozygous mutation in the cholesterol side chain cleavage enzyme (P450scc) gene in a patient with 46,XY sex reversal and adrenal insufficiency. Journal of Clinical Endocrinology and Metabolism 86, 3820-3825.

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

Van Enckevort, F.H.J., Pepels, P.P.L.M., Leunissen, J.A.M., Martens, G.J.M., Wendelaar Bonga, S.E., Balm, P.H.M., 2000. *Oreochromis mossambicus* (tilapia) corticotropin-releasing hormone: cDNA sequence and bioactivity. Journal of Neuroendocrinology 12, 177-186.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiological Reviews 77, 591-625.

White, P., New, M., Dupont, B., 1986 Structure of human steroid 21-hydroxylase genes. Proceedings of the National Academy of Sciences USA 83, 5111–5115.

White, P.C., Speiser, P.W., 1994. Steroid 11β-hydroxylase deficiency and related disorders. Endocrinology and metabolism clinics of North America 23, 325-339.

White, P.C., Speiser, P.W., 2000. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocrine Reviews 21, 245-291.

Whorwood, C.B., Montalto, J., Sandars, S.R., Connelly, J.F., 1990. Urinary 5-ene-steroid excretion in non-classical congenital adrenal hyperplasia due to 3B-hydroxysteroid dehydrogenase deficiency (NC-3BHSD). Journal of Steroid Biochemistry 35, 735-739.

Wong, S.L., Dhu, S.G., Tsai, C.R., 2006. Seventeen Alpha hydroxylase deficiency. Journal of Formos Medical Association 105, 177-181.

Yang, X., Iwamoto, K., Wang, M., Artwohl, J., Mason, J.I., Pang, S., 1993. Inherited congenital adrenal hyperplasia in the rabbit is caused by a deletion in the gene encoding cytochrome P450 cholesterol side-chain cleavage enzyme. Endocrinology 132, 1977-1982.

Zhou, L.-Y., Wang, D.-S., Shibata, Y., Paul-Prasanth, B., Suzuki, A., Nagahama, Y., 2007a. Characterization, expression and transcriptional regulation of P450c17-I and -II in the medaka (*Oryzias latipes)*. Biochemical and Biophysical Research Communications 362, 619-625.

Zhou, L.-Y., Wang, D.-S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., Nagahama, Y., 2007b. A Novel Type of P450c17 Lacking the Lyase Activity Is Responsible for C21-Steroid Biosynthesis in the Fish Ovary and Head Kidney. Endocrinology 148, 4282-4291.

Chapter Two:

Sequence characterization of *CYP17* gene in common carp

(*Cyprinus carpio L.*)

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Abstract

The *CYP17a1* gene codes for the cytochrome P450c17 enzyme, which is involved in the biosynthesis of cortisol in head kidneys and sex steroids in gonads in vertebrates. Mutations in the *CYP17* gene impair steroid biosynthesis, leading to adrenal hyperplasia. In fish one example of adrenal hyperplasia has been described for carp. The results indicate that interrenal hyperplasia in this mutant strain is caused by a dysfunction of the 17α-hydroxylase activity of the enzyme P450c17 in the interrenal. To identify mutations in this strain, we sequenced the *CYP17a1* gene in a standard and mutant strain of common carp. PCR amplifications were performed with genomic and cDNA of head kidney and gonads as template. Two variants of the *CYP17a1* gene were found in carp. The nucleotide coding region of the *CYP17a1.I* variant contained 8 exons and 7 introns. In total 3122 base pairs of the nucleotide sequence were determined. The intron-exon structure of this variant was identical to the human *CYP17* gene. Variant *CYP17a1.II* is missing exon 7 and exon 8. Variant *I* showed no differences between STD and E5, while variant *II* showed 8 homozygous SNP's (one of them specific for E7 carp) and a 3 bp insertion downstream of exon 6. The gonad cDNA sequence is in accordance with the intron-exon structure of the *CYP17a1.I* variant, indicating that variant *CYP17a1.II* is non-functional. In head kidneys only a cDNA fragment containing exon 3 could be amplified, suggesting that the *CYP17* variant found in gonads is different from the variant in head kidneys. These results are in accordance with the recently published results by Zhou et al. They identified a novel type *CYP17a2*. This gene differs in gene structure from our variant *I* and *II* and the enzyme possesses only the 17α-hydroxylase activity. In situ hybridization strongly suggests that this *CYP17a2* is responsible for the synthesis of cortisol in interrenals.

Keywords: sequencing, interrenal hyperplasia, mutation, 17α-hydroxylase deficiency, *CYP17*, common carp.

2.1 Introduction

CYP17a1 gene encodes cytochrome P450c17, an enzyme that mediates both 17α-hydroxylase and 17, 20-lyase activity in the adrenal cortex and gonads of vertebrates. It catalyzes both 17α-hydroxylation of pregnenolone and progesterone and 17, 20 lysis of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (Lin et al., 1993). These metabolites are important precursors for the production of adrenal cortisol and gonadal androgens or estrogens in vertebrates. Mutations in the *CYP17* gene impair steroid biosynthesis, leading to adrenal hyperplasia. To date, many cases of *CYP17* deficiency in humans have been reported (Costa-Santos et al., 2004). Most mutations are found in the structural gene of *CYP17* and some mutations are located the introns or regulatory elements of the *CYP17* gene (Biglieri et al., 1966). The human *CYP17* gene consists of 8 exons and 7 introns.

One example of interrenal hyperplasia has been described for common carp. Results indicate that interenal hyperplasia in this strain of common carp is caused by a dysfunction of the 17a-hydroxylase activity of the enzyme P450c17 in the interrenal. In order to investigate if mutations in the *CYP17* gene of this mutant carp cause adrenal hyperplasia, we sequenced almost the complete *CYP17a1* gene of a standard and mutant strain of common carp.

Although the cDNA sequence of *CYP17a1* mRNA from gonads has been elucidated for several fish species: rainbow trout (Sakai et al., 1992), fathead minnow (Halm et al., 2003), dogfish (Trant, 1996), Japanese eel (Kazeto et al., 2000), rice field eel (Yu et al., 2003) and zebra fish (Wang, Ge, 2004), the complete genomic sequence has been only determined for fathead minnow and zebra fish and medaka. Primers for sequencing were designed based on the cDNA sequence of mRNA from gonads of medaka (*Oryzias latipes)* and fathead minnow (*Pimephales promelas*) and based on the intron-exon structure of the human genomic *CYP17a1* gene.

2.2 Materials and Methods

2.2.1 Animal production and net confinement

Isogenic standard strains E4Y8, R3R8-yy and mutant strains E5 and E7 (17aOHD) of common carp were obtained from the Wageningen University Fish Culture Facility. Strains E4Y8, R3R8-yy were produced by conventional procedures (Bongers et al., 1998). E5 and E7 clones are reproduced by androgenesis (Komen et al., 1992). Sampling of fish was done at ~200 dph.

2.2.2 Blood and tissue collection

For each strain two fish were sampled and quickly anesthetized in an overdose 2-phenoxyethanol (1:500). Blood was collected from the caudal blood vessels and placed in cooled 1.5 ml plastic tubes containing 3 mg Na2EDTA/3000 IU Aprotinin (Sigma), mixed and centrifuged at 2400g (5min, 4ºC) and stored at -20ºC (Ruane et al., 2001). The head kidney and gonad tissues were collected in RNA-later and snap-frozen in liquid nitrogen and stored at -80ºC.

2.2.3 RNA isolation and cDNA synthesis

Total RNA was extracted from whole head kidneys and gonads of two individual fish according to the manufacturer's instructions (RNeasy Midi kit QIAGEN, 2001). Quantification of the RNA was performed with UV spectroscopy (Nano drop ND-1000) and quality of the RNA was tested on a 1% agarose gel by estimation of the 2:1 ratio between the 28S and 18S rRNA bands. .

The purified RNA was used immediately for RT-PCR or was stored at -80ºC for future use. Total RNA (100 ng/µl) was reversed transcribed with the SuperScript^{TM} III transcriptase kit using random hexamers (First-Strand Synthesis SuperMix Kit, Invitrogen). The cDNA generated by RT was amplified in a PCR reaction containing gene specific forward and reverse primers for *CYP17a1.I.*

2.2.4 Genomic DNA preparation

Genomic DNA was isolated from 25 µl blood with the Puregene Kit (Gentra systems, Minneapolis USA). DNA concentrations were measured as described above and checked on a

0.8% agarose gel for quality. Genomic DNA was diluted to 5 ng/µl with T (5mM) E (0.1mM) buffer.

Table 2.1 Primers used. Med = based on medaka sequence (*Oryzias latipes*, gi 1507677, NCBI), Fm = based on fathead minnow sequence (*Pimephales promelas*, gi 14041613, NCBI), Carp = based on carp sequence (this chapter). The 3rd column shows the sequence of primers used. F = forward primer, R = reverse primer, Ex = Exon, intr = intron.

No.	Primers	Sequence (5'-3')
1	MedCYP17F1	CATGGAGCTCTGTGCATGTTTGG
2	MedCYP17R1	CGTTGGTGACGGCCCGGGTCAGCTC
3	FmCYP17F1-ex1	AGAGCTGGTGGACAACTTGG
4	FmCYP17R1-ex1	GCATGATGGTGGTTGTTCAC
5	FmCYP17R2-ex1	ACCTCTTTCGCATGATGGTG
6	FmCYP17R4-ex4	CCGCTGGACGTTATCACTG
7	FmCYP17F7-ex7	GGAATGGAAGAATCCTGAGC
8	CarpCYP17R2-ex2	CCTTCACCAAACATGCACAG
9	FmCYP17F5-ex5	GCACTTCTGAGGGCCAAAC
10	FmCYP17F4-ex4	CAGTGATAACGTCCAGCGG
11	FmCYP17R6-ex6	ACATGAGGGATGAGGAGTGG
12	CarpCYP17-F1-ex1	GTGAACAACCACCATCATGC
13	CarpCYP17-F4-ex4	GTGTTTCAATCAGAGACAAACTGC
14	FmCYP17-R9-ex8	CGGGCATCTCTAGCGTAAAC
15	FmCYP17-F6-ex6	CCACTCCTCATCCCTCATGT
16	FmCYP17-R7A-ex7	GCTCAGGATTCTTCCATTCC
17	CarpCYP17-F10-ex7	GCAGAAAGGAACCCGAGTC
18	Carp (F) ex 6	AGGTGCAGAGAAAGATTCAG
19	Fm (R) ex 8	GCAGTGTCTGCATTAGTGGGG
20	Carp (R) ex 7	TCGGGTTCCTTTCTGCACAG
21	Carp (F) ex 7	TGTGCAGAAAGGAACCCGAG
22	Carp (F) intr. 5	GTAGATATTCATTGCATTTGAC
23	Carp (F) ex 6	AGCTGGACAATAAGATTGGG
24	CarpE5/E7 spec. (R)	CTGATTTGCTGCATGCTCAC
25	CarpE5/E7 spec. (F)	GTGAGCATGCAGCAAATCAG
26	Carp (F) exon 8	CCCCACTAATGCAGCACTGC

2.2.5 Primer development

Primers were designed by comparing the nucleotide sequences from medaka (*Oryzias latipes*, gi 1507677, NCBI) and fathead minnow (*Pimephales promelas*, gi 14041613, NCBI) cDNA *CYP17a1* sequences. The intron-exon organisation of human genomic *CYP17* (*Homo sapiens*, gi 13904854, NCBI) was used to predict the intron-exon structure in the other fish species. The ORF of *CYP17a1* was amplified from cDNA of the gonads by PCR according to the genomic sequence of carp. All primers are listed in Table 2.1.

2.2.6 Sequence analysis

PCR products were purified either from gel or directly with the Ultra free®-DA vials (Millipore Corporation, Bedford, MA, USA). Sequencing of the purified PCR product was performed with the half big Dye™ (Version 2, Applied Biosystems, Perkin Elmer, Warrington, and GB).

Sequence products were purified with MAHVN45 96 well filter plates (Millipore Corporation, Bedford, MA, USA). The products were analysed on the ABI prism 3730 DNA analyser (Applied Biosystems, Foster city, California, USA). Sequence data were checked for quality with sequence analysis 3.7 and fragments were run through Pregap 4 and aligned with Gap 4. Consensus sequences were blasted with a standard nucleotide blast (BLAST N) on NCBI.

2.3 Results

2.3.1 CYP17a1.I variant

We sequenced two wild type (E4Y8 and R3R8-yy) and two 17α-deficient strains (E5 and E7) of common carp.

Primers used for *CYP17* sequencing based on medaka (*Oryzias latipes*, gi 1507677, NCBI), fathead minnow (*Pimephales promelas*, gi 14041613, NCBI) and carp sequence are listed in

table 2.1. Eight overlapping fragments were sequenced (Fig. 2.1). Exons are indicated in bold. Exons 1-8 have a length of 369, 138, 230, 89, 206, 154, 104 and 279 bp, respectively.

Introns 1-7 have a length of 73, 117, 380, 258, 91, 555 and 79 bp, respectively. The *CYP17a1.I* gene in carp has a total length of 3122 bp including the introns. Except for 19 bp at the 5' end of exon 1 and 130 bp at the 3'end of exon 8 which are not sequenced the total length of the cDNA sequence of CYP17a1.I is 1569 bp. Sequence is submitted to Genbank.

The gonadal cDNA sequence (nucleotide 19-1490) of carp is in accordance with the intron-exon structure of the carp *CYP17* genomic sequence of variant *CYP17a1.I.*

Alignments of carp *CYP17a1.I* genomic sequence (Fig.2.2) with fathead minnow (Genbank Acc.No. AJ277867), zebra fish (Genbank Acc.No. BC117612.1) and channel catfish (Genbank Acc No. AF063837.1) *CYP17a1*'s showed 90, 89 and 77.2 % homology, respectively.

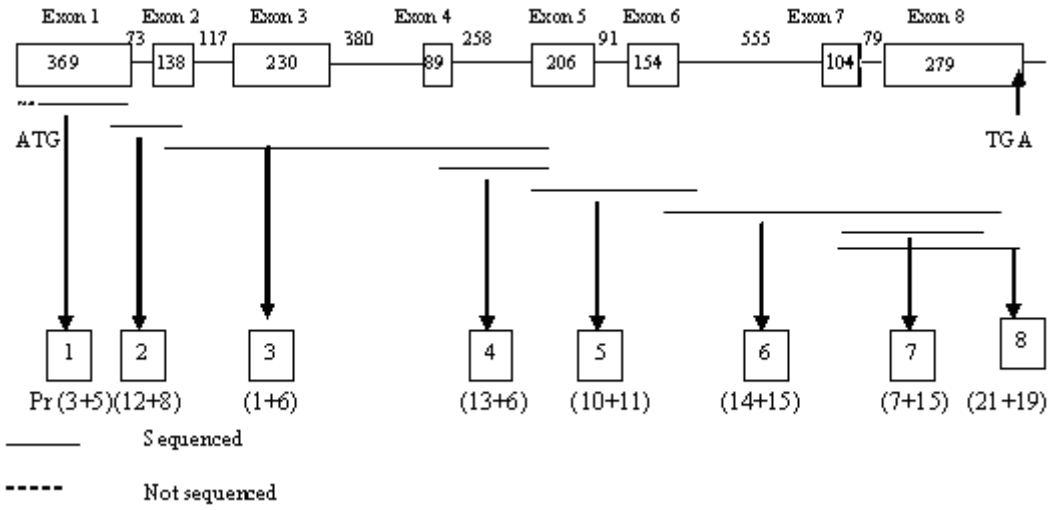

Figure 2.1 Overview of the wild type carp *CYP17a1.I* gene (variant 1). The coding sequence exists of 1569 bp. PCR fragments sequences are indicated in blocks (1 to 8) with primers numbers (Pr) indicated between brackets.

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Carp    AGAGCTGGTGGACAACTTGGTTGAATGTAGCT-GACAATGTCTGACTCACTCATCCTGCC
FHM     AGAGCTGGTGGACAACTTGGATGAATGTATATTGACAATGTCTGAACCACTCATCCTACC
ZF      AGAGCTGGAGGACA-CTCAGTTGAATGTAGCT-GACAATGGCTGAAGCACTCATCCTGCC

Carp    ATGGCTTTTCTGTTCATGCCTGTTCTCTGCAGTAACTCTAGCAGCACTGTATCTCAGAAG
FHM     ATGGCTTCTCTGTTCATGTCTGTTATCTGCAGTAACTCTGGCTGCACTGTATCTCAAAAG
ZF      CTGGCTGCTCTGTTTAAGCCTGTTCTCAGCAGTGACTCTGGCAGCTCTGTATCTCAAACA

Carp    GAAGATGAATGGATTTGTGCCTGGCG---ACAGATCTCCTCCAAGCCTCCCATCACTCCC
FHM     GAAGATGAATGGATTTGTGCCAGGGA---ACAGATCTCCTCCAAGCCTCCCCTCGCTGCC
ZF      GAAGATGAATGGATTTGTGCCAGCAGGAAACAGATCTCCTCCAAGTCTCCCTTCACTGCC

Carp    CATCGGGAGTCTCCTCAGCCTCATTACTGACAGTCCTCCACACATCTACTTTCAGCAGCT
FHM     CATTGGAAGTCTCCTCAGCCTCGTGAGTGACAGTCCACCACACATCTTCTTTCAACAACT
ZF      CATCGGGAGTCTGCTGAGCCTGGTGAGCGACAGCCCTCCGCACATCTTCTTTCAGGACCT

Carp    GCAGAAGAAATACGGAGATCTTTATTCCCTCATGATGGGCTCCCACAAAGTCCTGATTGT
FHM     GCAGGAGAAATACGGAGATATTTATTCCCTCATGATGGGCTCCCACAAAGTCCTTATCGT
ZF      GCAGAAGAAATACGGAGATCTGTATTCCCTCATGATGGGCTCCCACAAACTGCTCATTGT

Carp    GAACAACCACCATCATGCGAAAGAGATCCTGATCAAAAAAGGAAAAATATTTGCAGGCAG
FHM     GAACAACCACCATCATGCGAAAGAGGTTCTGATCAAAAAAGGAAAAATATTTGCAGGGAG
ZF      GAACAACCACCATCATGCGAAGGAGATCCTGATCAAAAAAGGAAAAATATTTGCAGGGAG

Carp    GCCACGAACTGTGTTACAACAGACATGTTAACTCGAGATGGGAAAGATATAGCCTTTGGT
FHM     GCCACGAACTGTGTTACAACAGACTTGTTAACTCGAGATGGGAAAGATATAGCATTTGCT
ZF      GCCACGGACTGTGTTACAACAGACTTGTTAACTCGAGATGGGAAAGATATAGCGTTTGCT

Carp    GACTACAGTTCCACATGGAAGTTCCATCGGAAAATGGTGCATGGA-CTCTGTGCATGTTT
FHM     GACTACAGTTCCACATGGAAGTTCCATCGGAAAATGGTTCATGGAGCTCTTTGTATGTTT
ZF      GACTACAGTTCCACATGGAAGTTTCACCGCAAAATGGTGCATGGAGCTCTTTGCATGTTT

Carp    GGTGAAGGTTCAGTTTCTATTGAGAAGATAATGCAGAGAGGCCAGCTCTATGTGTGACAT
FHM     GGTGAAGGTTCAGTTTCTATTGAGAAGATAATGCAGGGAGGCGAGCTCTATGTGTGAAGT
ZF      GGAGAAGGTTCAGTTTCTATTGAGAAGATAATGCAGGGAGGCGAGCTCTATGTGTGAAGT

Carp    GTTGACTGAATCCCAGAACAGCGCTGTGGATCTGGCACCGGAGCTGACGCGTGCCGTCAC
FHM     GTTGACTGAAACCCAGAACAGCGCTGTGGATCTGGCACCGGAGCTGACCCGTGCCGTCAC
ZF      GCTGACTGAAAGCCAAAACAGTGCTGTGGATCTGGGACCGGAGTTGACACGCGCTGTCAC

Carp    AAACGTAGTGTGTGCCTTGCGTTTCAACTCCTCATACAAGCGGGGAGATGCTGAGTTTGA
FHM     AAACGTGGTGTGTGCTTTGTGTTTCAACTCCTCGTACAAACGTGGAGATGCTGAGTTTGA
ZF      AAATGTGGTGTGTGCCTTGCGTTTTAACTCCTCGTATAAACGTGGAGATGCTGAGTTTGA

Carp    GTCCATGCTCCAGTACAGTCAGGGAATCGTGGATACAGACGCTAAGGACAGCCTGGTGGA
FHM     GTCCATGCTCCAGTACAGCCAGGGTATCGTGGATACAGTTGCTAAAGACAGCTTGGTGGA
ZF      GTCCATGCTCCAGTACAGCCAGGGAATCGTGGATACAGTTGCAAAGGACAGCTTGGTGGA

Carp    TATTTTCCCATGGCTGCAGAGATATTCCCAAACAAAGACCTCAAAATCCTAAGAAAGTGT
FHM     TATTTTCCCATGGCTGCAGAGATATTCCCAAATAAAGAACTCGGAATCTTAAGACAGTGT
ZF      TATTTTCCCATGGCTGCAGAGATTTTCCCAAATAAAGACCTCACAATTCTAAGACAATGC

Carp    GTTTCAATCAGAGACAAACTGCTTCAAAAGAAATACGAGGAACACAAGGTAGGTGGATTA
FHM     GTTTCCATCAGAGACAATTTGCTTCAAAAGAAATATGAAGAACACAAGGTAGGTGGATTA
ZF      ATTTCCATCAGAGATAAACTGCTTCAGAAGAAATATGAGGAACACAAGGTAGGTGACCTA
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Carp    CAGTGATAACGTCCAGCGGGACCTCTTGGATGCACTTCTGAGGGCCAAACGCAGCTCAGA
FHM     CAGTGATAACGTCCAGCGGGACCTCTTGGACGCACTTCTGAGGGCCAAACGCAGTTCAGA
ZF      CAGTGATAACGTGCAGCGGGATCTCCTGGATGCTCTTCTGAGGGCGAAACGCAGCTCAGA

Carp    GAATAACAACACCAGCACTCATGATTAGTTGGTTTAACTAAAGACCACGTGCTCATGACT
FHM     GAATAACAACACCAGCTCTCACGAT--GTTGGTTTAACTGAAGACCACCTGCTCATGACT
ZF      AAACAATAACAGCAGCACTCGTGAT--GTCGGTTTAACTGAAGATCATGTGCTCATGACT

Carp    GTGGGGGACATCTTCGAGGCTGGGGTGGAAACCACTACCACTGTTCTGAAATGGTCAATA
FHM     GTAGGGGACATCTTCGGAGCAGGGGTGGAAACCACGAGCACTGTACTGAAATGGTCAATA
ZF      GTGGGGGACATCTTCGAGGCTGGGGTGGAAACCACTACCACTGTTCTGAAATGGTCCATA

Carp    G-TTACCTTGTACATAACCCACCGGTAGGTGCAGAGAAAGATTCAGCAGAAGCTGGACAA
FHM     GCATACCTTGTCCATTTTCCACAGGTAGGTGCAGAGAAAAATTCAGGAGGAGCTAGACAA
ZF      GCTTACCTCGTCCACAATCCACAGGTAGGTTCAGAGAAAGATTCAAGAGGAGCTCGACAG

Carp    TAAGATTGGGAAAGA-----ATCTTCAA-------------GGAATCTGCCCTATCTAGA
FHM     TAAGATTGGAAAAGACAGACACCCTCAGCTCAGTGATAGAGGGAATTTGCCTTATCTAGA
ZF      TAAGATTGGGAAAGAAAGACACCCTCAGCTCAGCGACAGGGGGAATCTACCTTATCTAGA

Carp    GGCCACTATAAGAGAGGTTCTGAGGATCCGACCCGTCTCACCACTTCTCATTCCTCATGT
FHM     GGCCACTATAAGAGAAGTCCTGAGGATCCGACCCGTCTCCCCACTCCTCATCCCTCATGT
ZF      GGCCACTATTAGAGAAGTGCTGAGGATTCGACCTGTTTCTCCGCTCCTCATCCCTCATGT

Carp    GGCGCTCCAAGACTCCAGCAGTGTGGGAGAATACACTGTGCAGAAAGGAACCCGAGTCAT
FHM     GGCGCTCCAAGACTCCAGCAGTGTGGGAGAATACGCTGTGCAGAAAGGAACCAGAGTCAT
ZF      GGCGCTGCAGGATTCCAGCAGTGTTGGCGAATACACAGTGCAGAAAGGGACGCGGGTCGT

Carp    TATTAACCTGTGGTCTTTACATCATGATGAGAAGGAATGGAAGAATCCTGA-CTGTTTGA
FHM     TATTAATCTGTGGTCTTTACATCATGATGAGAAGGAATGGAAGAATCCTGAGCTATTTGA
ZF      TATTAACCTCTGGTCTTTACATCATGATAAGAAGGAATGGAAGAACCCTGAGCTCTTTGA

Carp    CCCAGGCAGGGCGATTTCTGAACGAGGAGGGTAATGGTTTGTGCTGCCCTTCGGCCAGCT
FHM     CCCAGGCAGGACGATTTCTGAACGACGAGGGTGATGGTTTGTGCTGCCCATCGGCCAGCT
ZF      CCCAGGCAGGACGCTTTCTGAATGAGGAGGGTGATGGTTTGTGCTGCCCGTCGGGCAGTT

Carp    ATCTGCCCTTCGGCGCAGGGGTTCGAGTTTGTCTTGGCGAGGCT-TGGCGAAGATGGAAC
FHM     ACCTGCCATTCGGTGCTGGGGTACGCGTTTGTCTCGGTGAGGCTCTGGCAAAGATGGAAC
ZF      ATCTGCCATTCGGCGCAGGGGTGCGTGTTTGTCTCGGCGAGGCCCTGGCAAAGATGGAGC

Carp    TATTCCTCTTCCTGTCATGGATTCTGCAAAGGTTTACGCTAGAGATGCCCGCTGGCCAGC
FHM     TCTTCCTCTTCCTGTCATGGATTTTGCAAAGGTTTACGCTAGAGATGCCCGCTGGCAAGC
ZF      TCTTCCTCTTCCTGGCGTGGATTTTGCAAAGGTTCACCCTGGAGATGCCCACTGGCCAGC

Carp    CTCTGCCTGACCTCCAGGGCAAGTTTGGTGTGGTTCTTCAACCCAAGAAATTCAAAGTCG
FHM     CTCTGCCCGACCTCCAGGGCAAGTTTGGTGTGGTTCTTCAACCTCAGAAATACAAGGTTA
ZF      CTCTGCCTGACCTCCAGGGCAAGTTTGGCGTGGTTCTTCAACCCAAGAAATTTAAGGTTG

Carp    TTGCTAAACTAAGAGCAGATTGGGAAAAATCCCCACTAATGCAGCACTGC
FHM     TTGCTAAACTGAGAGCAGACTGGGAAAAATCCCCACTAATGCAGCACTGC
ZF      TTGCTAAAGTAAGAGCAGACTGGGAGAAATCCCCACTCATGCAGCACTGC
```

Figure 2.2 Alignment of the coding sequence of carp *CYP17* with fathead (minnow) and zebra fish *CYP17*; Clustal X alignment of *CYP17a1.I* coding sequence for carp, fathead minnow and zebra fish for exons 1-8. The nucleotide differences compare to carp are indicated by the gray boxes.

2.3.2 CYP17a1.II variant

Amplification of exon 6 to 8 (primers 14 and 15) in both wild type and *CYP17* deficient carp lines showed two different PCR products. One product did contain exon 6 to exon 8 as indicated above (*CYP17a1.I*). Sequence analysis of the second PCR product revealed that this product contains exon 6 and a sequence of 796 bp of which two small parts (164 and 165 bp) are homologous to intron 6 of variant 1. In order to verify the sequence of *CYP17a1.II* a specific primer (primer 24) within the *CYP17a1.II* specific region was made in order to amplify fragments with the forward primers of exon 1, 4 and 5. Amplicons (not sequenced) with sizes corresponding to what was calculated were obtained, which indicated that exons 1 to 6 were directly upstream of this fragment. Differences between the two variants were found by blast 2 sequences (NCBI). A schematic view of both variants starting in exon 6 is shown in Fig. 2.3.

Sequence analysis of primers 14 and 15 of *CYP17a1.II* specific fragment revealed pronounced differences between the wild type carp (E4Y8 and R3R8-yy) and the *CYP17* deficient carp (E5 and E7). In total, 7 SNP's and one insertion/deletion of 3 bases was detected between the wild type and mutants E5 and E7 (Table 2.2 and Fig.2.4)). One additional SNP was observed at position 2150 that was homozygote T/T in R3R8-yy, E4Y8 and E5 and G/G in animal E7.

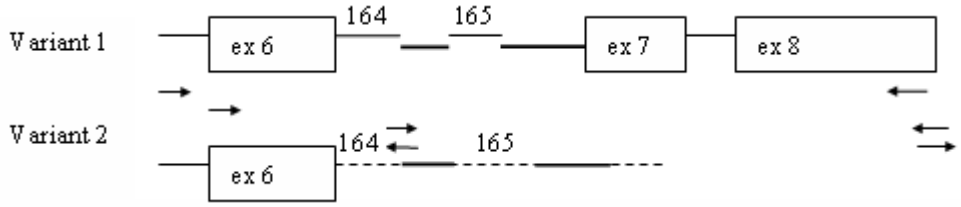

Figure 2.3 Schematic view of exon 6 and intron 6 of both variants of *CYP17* found in E5, E7, E4Y8 and R3R8-yy carp. Exons 7 and 8 were not traced in variant 2, despite using primer (19R), which is located at the 3' end of exon 8. The primer19R was also the reverse primer that amplified (together with 22F or 23F) this aberrant fragment in variant 2. Primers are indicated by arrows. The spaced lines are sequences which were not found in variant 1. These two small parts (164 and 165 bp, respectively) are homologous to intron 6 of variant 1.The fat lines after exon 6 in variant 2 represent original parts of intron 6 (also) indicated and positioned in variant 1. The thin lines represent introns. Primers from left to right: (22F), (23F), (24R), (25F, variant 2 specific), (14R), (19R) / (26F).

Table 2.2 Differences between *CYP17a1.II* sequences (variant 2) of wild type carp and *CYP17* deficient carp.

Nucleotide position.	Fragment	Mutation	E4Y8, R3R8-yy	E5, E7
2150	aberrant sequence	SNP	T/T	G/G*
2278-2280	intron 6 like sequence	Insertion	---	TTA
2348	intron 6 like sequence	SNP	A/A	G/G
2354	intron 6 like sequence	SNP	A/A	C/C
2367	intron 6 like sequence	SNP	C/C	A/A
2396	aberrant sequence	SNP	G/G	T/T
2398	aberrant sequence	SNP	T/T	G/G
2467	aberrant sequence	SNP	C/C	T/T
2584	intron 6 like sequence	SNP	A/A	T/T

*Only for E7. E5 contains a T at position 2150

```
wt      AGGTGCAGAGAAAGATTCAGCAGAAGCTGGACAATAAGATTGGGAAAGAATCTTCAAGGA
E5/E7   AGGTGCAGAGAAAGATTCAGCAGAAGCTGGACAATAAGATTGGGAAAGAATCTTCAAGGA

wt      ATCTGCCCTATCTAGAGGCCACTATAAGAGAGGTTCTGAGGATCCGACCCGTCTCACCAC
E5/E7   ATCTGCCCTATCTAGAGGCCACTATAAGAGAGGTTCTGAGGATCCGACCCGTCTCACCAC

wt      TTCTCATTCCTCATGTGGCGCTCCAAGACTCCAGGTAAGATTTAAAAAACAAACAAACAA
E5/E7   TTCTCATTCCTCATGTGGCGCTCCAAGACTCCAGGTAAGATTTAAAAAACAAACAAACAA

wt      ACAAACAAACAAAAAACT AATATATATTTATTTTATTTAGCAAAGATGCATTAAATTATT
E5/E7   ACAAACAAACAAAAAACG*AATATATATTTATTTTATTTAGCAAAGATGCATTAAATTATT

wt      CAAAAGTGAAAGAAAAGACATAATGGTACAAAAGGACATTAATAACAATAGGAAATGTTT
E5/E7   CAAAAGTGAAAGAAAAGACATAATGGTACAAAAGGACATTAATAACAATAGGAAATGTTT

wt      TGTGAGCATGCAGCAAATCAGCATA---GAATGATTTCTGAAGGATCATGTGACACTGAA
E5/E7   TGTGAGCATGCAGCAAATCAGCATATTAGAATGATTTCTGAAGGATCATGTGACACTGAA

wt      GACTGGAGCAGTGATGCTGAAAATTCAGCTTTACATCAAAGGAATAAATTACATTTTAAA
E5/E7   GACTGGAGCAGTGATGCTGAAAATTCAGCTTTGCATCACAGGAATAAATTAAATTTTAAA

wt      ATATATTCAAATAGAAGTTGGTTTTTTTTTTTAATCAATTGTAGTATTTCATAATATTATT
E5/E7   ATATATTCAAATAGAAGTTGTTGTTTTTTTTTAATCAATTGTAGTATTTCATAATATTATT

wt      GTGTACTGTATTTTGATCAAATAAATGCAGCCGTAATGAGCATAAGAGACTTCTTTCAAA
E5/E7   GTGTACTGTATTTTGATCAAATAAATGCAGCTGTAATGAGCATAAGAGACTTCTTTCAAA

wt      AATGTTAAAATATCTCACCAACCTCAAACTTTTGAATATCATTACATTTTATTTATTCTT
E5/E7   AATGTTAAAATATCTCACCAACCTCAAACTTTTGAATATCATTACATTTTATTTATTCTT
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wt      TAATCTTATAATTATTTGCTATTAATTTAGATCTTTAATGCTCATTGTTTGAAAATTTAA
E5/E7   TAATCTTATAATTATTTGCTATTAATTTTGATCTTTAATGCTCATTGTTTGAAAATTTAA

wt      AAAACACCCAAATTAGGGTTTTAAACTTCCAAATTTTACTCTAAGGGTTGCAGAAAGTTC
E5/E7   AAAACACCCAAATTAGGGTTTTAAACTTCCAAATTTTACTCTAAGGGTTGCAGAAAGTTC

wt      AGTAGTTTACCTAAAATACTGTCCAGTCGTGATGTCTTACCCTAAGAAATTAAAGTCATG
E5/E7   AGTAGTTTACCTAAAATACTGTCCAGTCGTGATGTCTTACCCTAAGAAATTAAAGTCATG

wt      CCAAACTA
E5/E7   CCAAACTA
```

Figure 2.4 Alignment of wild types E4Y8 and R3R8-yy (wt) and E5/E7 carp *CYP17* variant 2 from exon 6 toward. Sequences of both wild types were completely the same. E5 and E7 carp also contained the same *CYP17* sequence. SNP's and the insertion are indicated by the gray boxes which are located at the intron 6 region. Spaced underlining represent fragments of intron 6 of variant II homologous to fragments of intron 6 of variant I. * = specific for E7 only.

2.4 Discussion

We started to determine the genomic and cDNA sequence of *CYP17a1* in standard and mutant strains of common carp in order to determine the mutations that are responsible for the impaired function of 17α-hydroxylase activity in the mutant strain.

We elucidated the genomic structure of the *CYP17a1* gene in common carp. Two variants of the *CYP17a1* gene were identified. The intron-exon structure of variant I was identical to the human *CYP17*. The nucleotide coding region of *CYP17a1* determined from mRNA isolated from gonads was consistent with the 8 exons found in the carp genomic DNA sequence. A blast analysis showed that carp *CYP17a1.I* cDNA sequence was highly homologous with fathead minnow (90%) and zebra fish (89%) *CYP17a1*s. Both species are classified as Cyprinids. The exon-intron structure in the human and bovine *CYP17a1* gene is identical to those described here for carp *CYP17a1.I*, indicating that the gene structure of *CYP17* is highly conserved. Gene conservation over species makes it possible to identify the exon-intron boundaries.

If variant II is a structural gene is still unclear. In rice field eel the *CYP17* gene is alternatively spliced and one *CYP17* transcript was generated by alternative polyadenylation. If these alternative transcripts exits in carp and if they are transcribed from variant II has to

be determined. If these transcripts really exist in carp mutations in variant II may contribute to an impaired steroid biosynthesis.

The nucleotide sequence of gonadal cDNA was in accordance with the genomic sequence of the *CYP17* variant I. However, in head kidneys, only a PCR fragment could be amplified with primers based on the sequence of exon 3 of variant I. These results suggest that the *CYP17* type I expressed in gonads is different from the variant expressed in head kidneys. Recently a novel type of *CYP17*, lacking the lyase activity, has been identified in head kidneys of zebra fish and tilapia (Zhou et al., 2007). Mutations in this type *CYP17a2* could also be responsible for an impaired steroid biosynthesis.

Acknowledgment

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References

Biglieri, E.G., Herron, M.A., Brust, N., 1966. 17-hydroxylation deficiency in man. Journal of Clinical Investigation 45, 1946-1954.

Bongers, A.B.J., Sukkel, M., Gort, G., Komen, J., Richter, C.J.J., 1998. Development and use of genetically uniform strains of common carp in experimental animal research. Laboratory Animals 32, 349-363.

Costa-Santos, M., Kater, C.E., Auchus, R.J., 2004. Two Prevalent *CYP17* Mutations and Genotype-Phenotype Correlations in 24 Brazilian Patients with 17-Hydroxylase Deficiency. Journal of Clinical Endocrinology and Metabolism 89, 49-60.

Halm, S., Kwon, J.Y., Rand-Weaver, M., Sumpter, J.P., Pounds, N., Hutchinson, T.H., Tyler, C.R., 2003. Cloning and gene expression of P450 17α-hydroxylase,17,20-lyase cDNA in the gonads and brain of the fathead minnow (*Pimephales promelas)*. General and Comparative Endocrinology 130, 256-266.

Kazeto, Y., Ijiri, S., Todo, T., Adachi, S., Yamauchi, K., 2000. Molecular cloning and characterization of Japanese eel ovarian P450c17 (*CYP17*) cDNA. General and Comparative Endocrinology 118, 123-133.

Komen, J., de Boer, P., Richter, C.J.J., 1992. Male sex reversal in gynogenetic XX females of common carp (*Cyprinus carpio L.*) by a recessive mutation in a sex-determining gene. Journal of Heredity 83, 431-434.

Lin, D., Black, S.M., Nagahama, Y., Miller, W.L., 1993. Steroid 17a-hydroxylase and 17,20-lyase activities of P450c17: Contributions of serine106 and P450 reductase. Endocrinology 132, 2498-2506.

Ruane, N.M., Huisman, E.A., Komen, J., 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. Journal of Fish Biology 59, 1-12.

Sakai, N., Tanaka, M., Adachi, S., Miller, W.L., Nagahama, Y., 1992. Rainbow trout cytochrome P450c17 (17α-hydroxylase/17,20-lyase) cDNA cloning, enzymatic properties and temporal pattern of ovarian P450c17 mRNA expression during oogenesis. FEBS Letters 301, 60-64.

Trant, J.M., 1996. Functional expression of recombinant spiny dogfish shark (*Squalus acanthias*) cytochrome P450c17 (17α-hydroxylase/C17,20-lyase) in yeast (*Pichia pastoris*). Archives of Biochemistry and Biophysics 326, 8-14.

Wang, Y., Ge, W., 2004. Cloning of zebra fish ovarian P450c17 (*CYP17*, 17a-hydroxylase/17, 20-lyase) and characterization of its expression in gonadal and extra-gonadal tissues. General and Comparative Endocrinology 135, 241-249.

Yu, H., Cheng, H., Guo, Y., Xia, L., Zhou, R., 2003. Alternative splicing and differential expression of P450c17 (*CYP17*) in gonads during sex transformation in the rice field eel. Biochemical and Biophysical Research Communications 307, 165-171.

Zhou, L.Y., Wang, D.S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., Nagahama, Y., 2007. A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology 148, 4282-4291.

Chapter Three:

Quantitative expression analysis of genes involved in the stress response in head kidneys of common carp (*Cyprinus carpio L.*) following net confinement

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Abstract

In this study the expression of five genes involved in the cortisol production in head kidneys of common carp (*Cyprinus carpio L.*) has been investigated in response to net confinement stress. Fish were raised for 6 months and subjected to a net confinement stressor for 3 hours, followed by a recovery period of 22 hours. Blood was collected to determine plasma cortisol and head kidneys were sampled at different time points to isolate total RNA. Cortisol was measured by ELISA and mRNA levels StAR, P450c17a1.I, 3β-HSD, P450c21 and 11β-HSD2 were quantified by real-time RT-PCR. The results showed that the plasma cortisol level was 85-fold higher than in the unstressed control at one hour post-stress and quickly returned to normal after 4 hours recovery. This increase and decrease in cortisol levels was correlated with mRNA levels of 11β-HSD2 during and after confinement, suggesting that 11β-HSD2, which oxidizes cortisol to cortisone, is involved in the regulation of cortisol concentrations. StAR, P450c17a1.I and P450c21 mRNA levels did not change during net confinement stress and recovery. 3β-HSD levels were increased 20 minutes after confinement. These results suggest that cortisol biosynthesis activated by net confinement stress in common carp resulting in high cortisol levels, is at least partly regulated at transcriptional level for 3β-HSD.

Keywords: common carp, stress, cortisol, steroidogenic pathway, net confinement, gene expression

3.1 Introduction

Cortisol is the main steroid hormone produced in the interrenal tissue located in the head kidneys of teleost fish species. It plays an important role in maintaining physiological homeostasis through effects on glycaemia, growth and osmoregulation, especially during the primary stress response (Iwama et al., 1997; Wendelaar Bonga, 1997). In fish, many studies have shown rapid rises in plasma cortisol levels in response to acute stressors (Balm, Pottinger, 1995; Barton, Iwama, 1991; Rotllant et al., 2003; Ruane et al., 2001; Vijayan et al., 1997; Waring et al., 1996). The steroidogenic cytochromes P450 and several hydroxysteroid dehydrogenase are enzyme responsible for the biosynthesis of cortisol (Fig. 3.1). Compared to mammals, the understanding of the regulation of cortisol biosynthesis in fish is limited, because gene or gene products encoding the steroidogenic enzymes have only been isolated from few species.

In mammals, it has been shown that acute changes in cortisol levels are mediated by the mobilization of cholesterol via the steroidogenic acute regulatory protein (StAR). In the inner mitochondrial membrane cholesterol than becomes available for the first step in the steroid biosynthesis, the cholesterol side chain cleavage, resulting in the formation of pregnenolone. StAR transcription levels are the limiting step in cortisol biosynthesis. Gene expression studies in fish suggest that the transcription levels of StAR, P450scc, P450c21 and 11β-hydroxylase (CYP11β) are positively correlated with the synthesis of cortisol in head kidneys. For example, severe acute stress in rainbow trout resulted in elevated plasma cortisol levels and increase in StAR and P450scc transcripts (Geslin, Auperin, 2004; Hagen et al., 2006; Kusakabe et al., 2002; Li et al., 2003b). Infusion of adrenocorticotrophin (ACTH) stimulated cortisol production through enhanced interrenal expression of StAR and P450c21 genes in eel (Li et al., 2003a and 2003b) and *in vitro* ACTH infusion in head kidneys of rainbow trout increased cortisol and CYP11β transcript levels (Hagen et al., 2006). However, acute stress induced by capture in rainbow trout had no effect on StAR, 3β-hydroxysteroid dehydroxylase and 11β-hydroxylase transcript levels (Geslin, Auperin, 2004; Hagen et al., 2006).

All these reports include different species, kind of stressors and number of genes investigated. To further characterize the acute stress response in teleost fish, we present the mRNA levels of five genes involved in cortisol biosynthesis in head kidneys of an isogenic line of common carp after exposure to a net confinement stressor. The mRNA levels of StAR, 17α-hydroxylase (P450c17a1.I), 3β-hydroxysteroid dehydrogenase (3β-HSD), 21 hydroxylase (P450c21), and P450c11b2 (11β-HSD2) are quantified by real-time PCR.

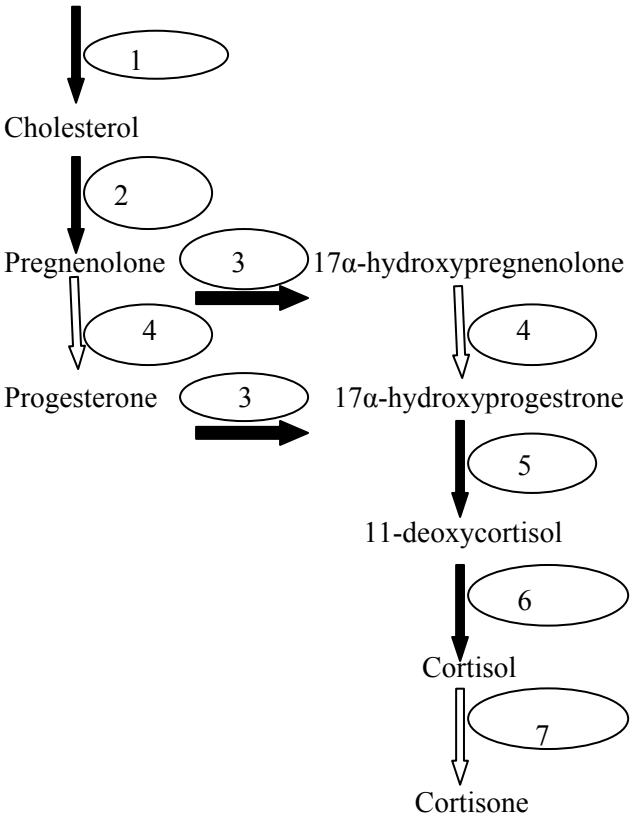


Fig. 3.1 Biosynthesis of cortisol in interrenal tissues of head kidney. Solid black arrows indicate that the enzyme required for that step has been cloned in fish and displays appropriate activity (Kusakabe et al., 2002; Li et al., 2003a; Lyssimachou, Arukwe, 2007; Zhou et al., 2007), Unfilled arrows show steps for which confirmation of enzyme activity is required (Gilmour, 2005). Legends; 1) StAR, 2) P450scc, 3) P450c17, 4) 3β-HSD, 5) P450c21, 6) P450c11b1, 7) 11β-HSD2.

3.2 Materials and Methods

All procedures approved by the Wageningen University committee on ethics in animal experimentation.

3.2.1 Animal production and net confinement procedure

A homozygous inbred male strain was produced by the conventional breeding of an E4E5 (XX) female with a R3R8 (YY) male, that is an androgenetic male strain of Polish/Hungarian (R3R8) origin (Komen et al., 1988). Fish production was done as described previously (Bongers et al., 1998b). Fish were sampled at 195 days post hatching (dph).

The net confinement stressor was performed as described previously (Ruane et al., 2005). Fish were sampled at 0 min (unstressed control), 5 min, 20 min, 1 h and 3 hours confinement, and followed by 1 h, 4 h and 22 hours recovery.

3.2.2 Blood collection and head kidney sampling

At each time point 5 stressed and unstressed fish were sampled and quickly anaesthetized with 3 g Tricaine methyl sulphonate (MS_{222}) and 6 g bicarbonate (NaHCO3) in 10 L water, Crescent Research Chemicals. Blood was collected as previously described by (Ruane et al., 2001). After blood collection fish were killed with 6 g MS_{222} + 12 g NaHCO3 in 10 L water. Total body weight and standard length of fish were measured and the fish were then dissected. Head kidneys (both right and left) were weighed to calculate head kidney somatic index, as follows: HKSI% = Head kidney weight/Body weight X 100 (Ruane et al., 2005). For RNA extraction head kidneys were frozen in liquid nitrogen.

3.2.3 Plasma Analysis

3.2.3.1 Cortisol Measurement

The concentration of cortisol was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY) validated for common carp. Cross reactivity of the antiserum with other steroids in the cortisol production pathway are: cortisone (15.77%), 11-deoxycortisol (15%), corticosterone (4.81%), progesterone (0.06%), and 17α-hydroxyprogesterone (1.36%).

3.2.3.2 Glucose, Lactate and FFA measurements

Plasma glucose was determined by the GOD-Perid method (Boehinger), plasma lactate was measured using a commercial kit (sigma), and plasma total non esterified fatty acids were measured by the ACS-ACOD method (Wako).

3.2.4 RNA Isolation

Total RNA was extracted from whole head kidneys of five individual fish according to the manufacturer's instructions (RNeasy Midi kit QIAGEN, 2001). UV spectroscopy (Nano drop ND-1000) was used to quantify RNA. The concentration was calculated using the Beer-Lambert law. The 2:1 ratio between the 28S and 18S rRNA on a 1% agarose gel was used as an indication that the RNA was intact. The purified RNA was used immediately for RT-PCR or was stored at -80 ºC for future use.

3.2.5 Real-*time PCR*

Total RNA (100 ng/µl) was reversed transcribed with the SuperScript^{TM} III transcriptase kit using random hexamers (First-Strand Synthesis SuperMix Kit, Invitrogen). The cDNA generated by RT was amplified in a PCR reaction containing gene specific forward and reverse primers for StAR, P450c17a1.I , 3β-HSD , P450c21, 11β-HSD2 and 18S rRNA. Primers for 3β-HSD and 11β-HSD were designed based on sequences obtained from Gene bank (accession numbers: *Danio rerio;* NM 212797.1 for 3β-HSD and NM 212720.1 for 11β-HSD type 2). Primers for StAR, P450c17a1.I and P450c21 are designed based on available sequences for carp (H.van Pelt-Heerschap, unpublished results). Primer sequences are listed in Table 3.1.

To detect the expression of StAR, P450c17a1.I, 3β-HSD, P450c21, 11β-HSD2 genes, we used generic double stranded DNA Dye (Syber Green I). The Dye was detected using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). For the PCR reactions we used a total volume of 25 µl reaction mixture containing 2 µl of cDNA template, 12.5 µl ABI Universal PCR SYBER green Universal Master Mix 2x (Applied Biosystems), 1.25 µl (10 µM) of each primer, and 8 µl of milliQ water. The PCR conditions were as follows; denaturation at 95 ºC for 10 min, 40 cycles at 95 ºC; 15 s, 54 ºC (3β-HSD, P450c17a1.I, P450c21 and 11β-HSD2) or 60 ºC (StAR) for 30 s; and 72 ºC 36 s, a 72 ºC for 7 min. 18S rRNA was detected using TaqMan Ribosomal RNA (Applied Biosystems) control Reagents kit according to the manufacturer's instructions. The dilution ranges for the transcripts for quantitative accuracy were examined before running the assay. As controls, the reaction mixture containing no cDNA was used to verify the specificity of the PCR product, whereas RNA was used in the PCR to rule out DNA contamination. Amplification products were verified by melting temperature, agarose gel electrophoresis and direct sequencing (ABI PRISM 3730 DNA Analyzer). Two different PCR assays were conducted in duplicate for each sample and a mean value was used to calculate the transcript levels. The efficiency was determined from the slopes of the curves and the coefficient variation of all slopes was calculated between different runs (Table 3.2). To quantify transcripts of the genes, we monitored 18S rRNA levels as quantitative control and each sample was normalized on the basis of its 18S rRNA content.

Table 3.1 Primers used and the optimal conditions for real-time PCR analysis. The concentration for all primers is 10 µM.

Gene	Annealing ºC	Primer (forward) Sequence (5'-3')	Primer (Reverse) Sequence (5'-3')
StAR	60	ACAGGTGGGTCCATTCTCAG	GTGGAACCCCAATGTCAAAC
P450c17a1.I	54	GAGGCCAGCTCTATGTGTGA	GCATGGACTCAAACTCAGCA
3β-HSD	54	ATTAACGGCGATGAGGACAC	ATCGACAGCCTGGTCCATAG
P450c21	54	GATCCACTGTGGTCATGTGC	CTTGGACCAGGGTTTCTCAA
11β-HSD2	54	GCTGAGCTCTCCCTCATGTC	AGATTCAGGGCAGCTTTTGA
18S rRNA	60	CGTCTGCCCTATCAACTTTCG	TGCCTTCCTTGGATGTGGTAG

Table 3.2 Slopes, correlation coefficients (r^2) and % efficiency for the genes quantified by real-time PCR.

Gene	Slope	r^2	%E	Slope	r^2	%E
StAR	-3.39	0.998	97.4	-3.4	0.99	96.8
P450c17a1.I	-3.61	0.996	89.2	-3.39	0.986	97
3β-HSD	-3.27	0.997	102.3	-3.4	0.989	97
P450c21	-3.60	0.998	89.6	-3.58	0.99	90.3
11β-HSD2	-3.50	0.992	92.9	-3.59	0.985	90

3.2.6 Statistical Analyses

Data were expressed as mean ± SE and were log transformed when necessary. The difference between mRNA levels of the genes studied in each time point over the control was analyzed using a student *t*-test ($P < 0.05$). The differences in plasma cortisol, glucose, lactate, FFA values in response to net confinement were also analyzed using a student t-test ($P < 0.05$).

3.3 Results

3.3.1 The effect of net confinement on cortisol and metabolite profiles

Plasma cortisol, glucose, lactate and free fatty acids (FFA) levels were determined in male common carp subjected to net confinement stress for 5 min, 20 min, 1 h and 3 h net confinement followed by 1 h, 4 h and 22 h recovery. The results are shown in Fig. 3.2 (A-D).

Plasma cortisol levels were significantly ($P < 0.001$) increased after 5 min, 20 min, 1 h and 3 h net confinement 68, 69, 85 and 45 folds, respectively. Cortisol levels quickly returned to the normal levels following release of the fish from the nets in the pre-stress tanks (Fig. 3.2A). Glucose levels were elevated during confinement points of 20 min, 1 h and 3 h. However, the elevation of glucose in the plasma was more gradual and continued to increase for at least another 4 h after the fish were returned to the recovery tanks (Fig. 3.2B). Lactate

levels were significantly increased during 5 and 20 min confinement and then returned to a normal level (Fig. 3.2C). Free fatty acid levels (Fig. 3.2D) were significantly increased after 3 h confinement ($P < 0.01$). Then with a reduction in 1 h recovery again the levels were increased in the rest of the recovery points which were significant in 22 h recovery point ($P < 0.01$).

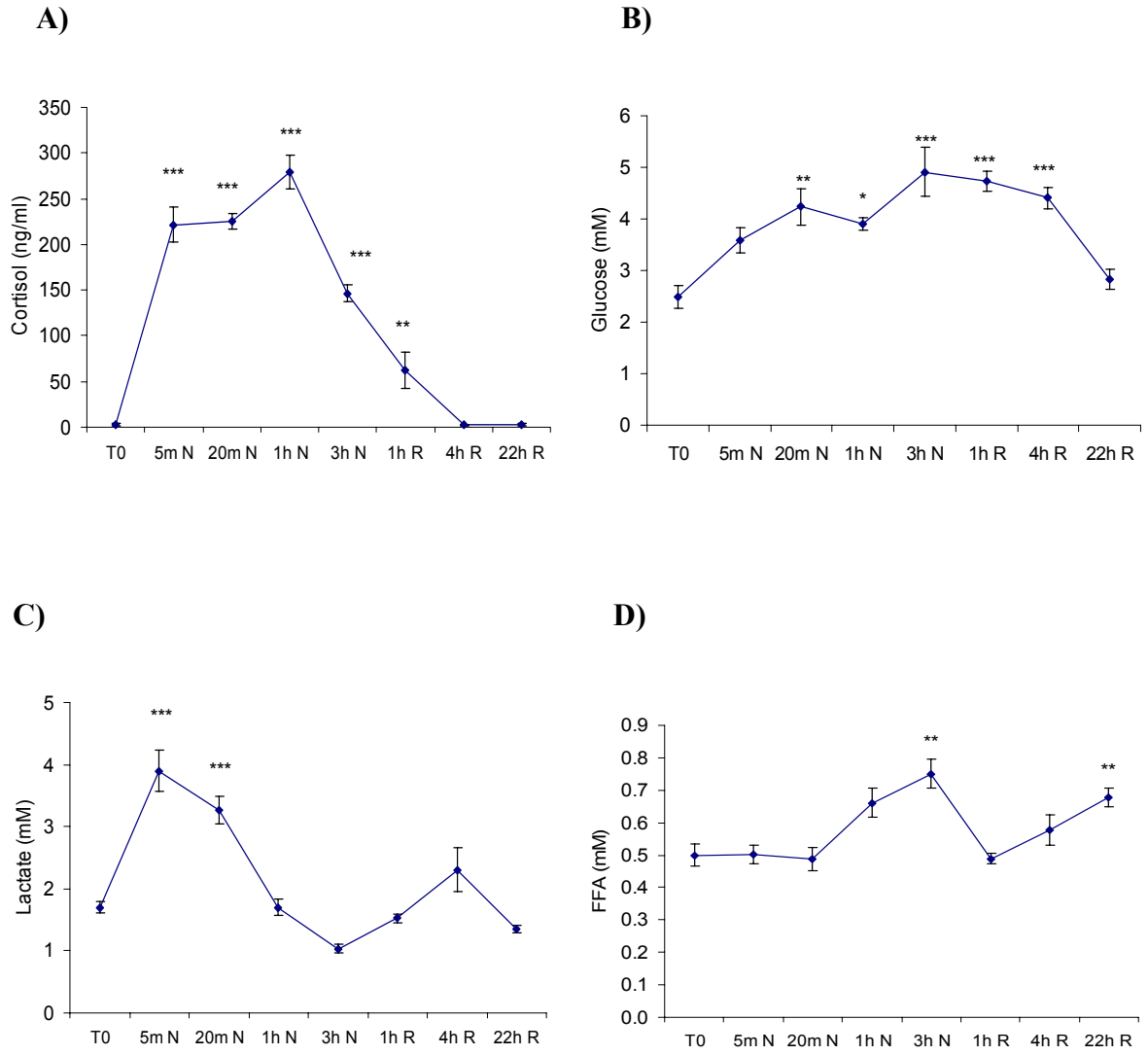


Fig. 3.2 The effect of a 3 h net confinement and subsequent recovery on plasma cortisol (**A**), Glucose (**B**), Lactate (**C**) and FFA (**D**) levels in STD male of common carp. Values represent mean ± SE (n = 5), *, ** and *** indicate statistical significance from the T0-unstressed (Student t-test; $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively).

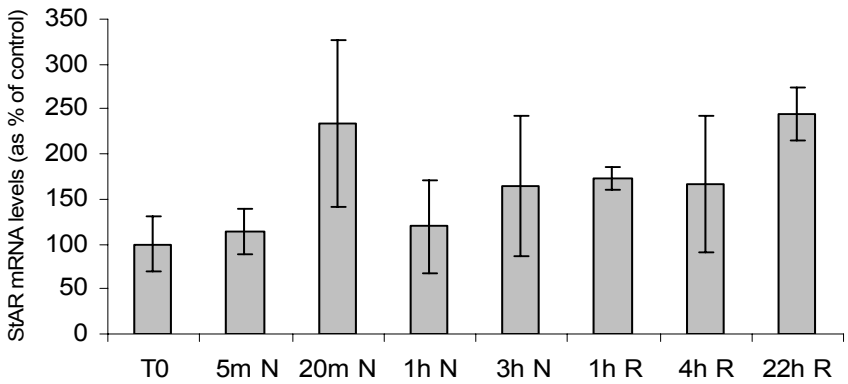
3.3.2 Characterization of StAR, P450c17a1.I , 3β-HSD , P450c21 and 11β-HSD2 transcripts expressed in head kidneys of common carp in response to net confinement stress

StAR, P450c17a1.I, 3β-HSD , P450c21 and 11β-HSD2 mRNA levels were analyzed using real-time PCR in head kidney. The results are shown in Fig. 3.3 (A-E).

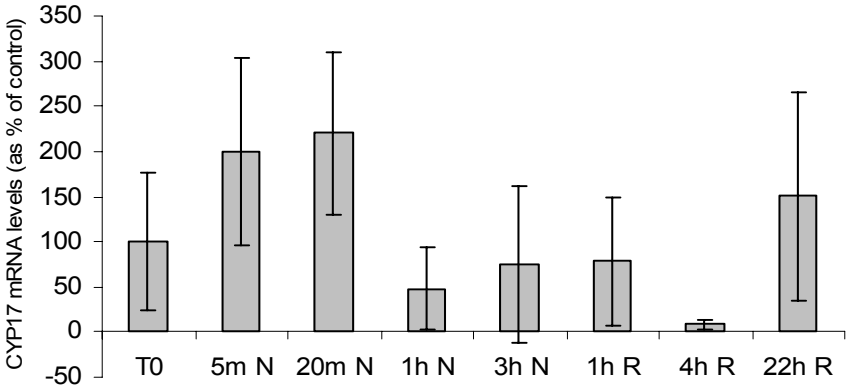
Gene expression of StAR, P450c17a1.I and P450c21 mRNA showed no significant differences compared to unstressed control fish whatever time after net confinement and recovery (Fig. 3.3A, B and D). 3β-HSD revealed a significant difference ($P < 0.028$) between the stressed fish after 20 min of net confinement and the control (Fig.3C). Expression was 2.2 times higher than the control.

Interestingly, 11β-HSD2 gene expression was significantly different between stressed fish after application of 3 h net confinement followed by 22 h recovery compared to the unstressed control. Expression in stressed fish was 2.3 times higher ($P< 0.008$) than in the control after 3 h net confinement. The results show that transcription levels of 3β-HSD and 11β-HSD2 change after exposure to net confinement stress.

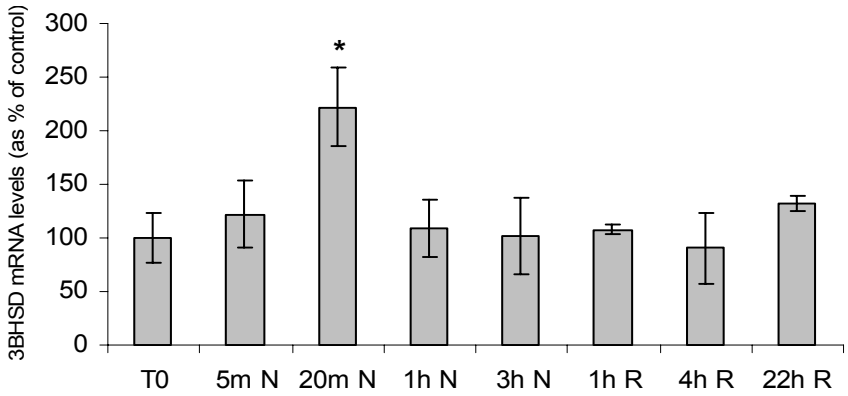
A)



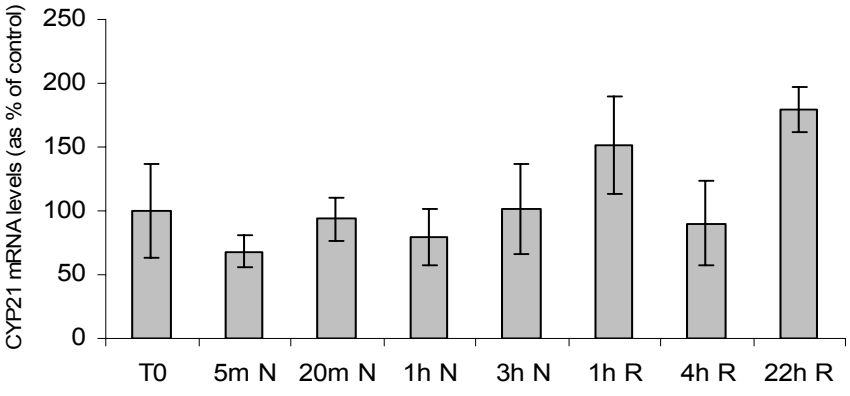
B)


CYP17 mRNA levels (as % of control)
350
300
250
200
150
100
50
0
-50
T0
5m N
20m N
1h N
3h N
1h R
4h R
22h R

C)


3BHSD mRNA levels (as % of control)
300
250
200
150
100
50
0
*
T0
5m N
20m N
1h N
3h N
1h R
4h R
22h R

D)


CYP21 mRNA levels (as % of control)
250
200
150
100
50
0
T0
5m N
20m N
1h N
3h N
1h R
4h R
22h R

E)

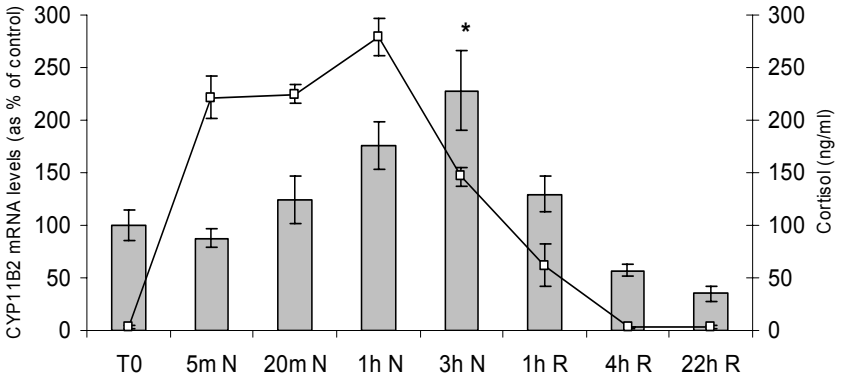


Fig. 3.3 The effect of a 3 h net confinement and subsequent recovery on changes in relative mRNA levels of StAR (**A**), P450c17a1.I (**B**), 3β-HSD (**C**), P450c21 (**D**) and 11β-HSD2 (**E**) in head kidney analyzed by real-time RT-PCR in STD male of common carp. The mRNA levels were normalized against 18S ribosomal RNA. Values represent mean ± SE (n = 5), * indicates statistical significance from the T0-unstressed (Student t-test; $P < 0.05$).

3.3.3 Correlation of changes in plasma cortisol levels with changes in mRNA levels

Changes in plasma cortisol levels (log cortisol) after 3 hours net confinement stress and recovery were positively correlated with 11β-HSD2 mRNA levels ($r = 0.65$, $P < 0.0007$, $n = 23$) (Fig. 3.3E). There was no correlation with the mRNA levels of StAR, P450c17a1.I, 3β-HSD and P450c21.

3.4 Discussion

In this study we describe plasma cortisol, glucose, lactate, free fatty acid and mRNA levels of five enzymes involved in cortisol production in head kidneys of common carp after exposure to net confinement stress. The plasma cortisol level were 85-fold higher than in the unstressed control at one hour post-stress and quickly returned to normal after 4 hours

recovery. The glucose, lactate and plasma free fatty acid levels followed the same pattern as earlier described by Ruane et al. (2001) and are comparable with patterns found in carp (Pottinger, 1998; Ruane et al., 2001), Atlantic salmon (Waring et al., 1996) and other species (Wendelaar Bonga, 1997). StAR, P450c17a1.I and P450c21 mRNA levels did not change during net confinement stress and recovery.

In mammals, it is known that StAR is the rate limiting step in cortisol production (Stocco, 2000). StAR mRNA and protein are rapidly elevated in response to stress. A high degree of conservation in StAR proteins among vertebrates suggests the presence of the same limiting mechanism in all vertebrates (Burns et al., 2000; Kusakabe et al., 2002).

In fish several studies have been undertaken to analyse the relationship between steroidogenic gene expression and cortisol production. Different species, kind of stressors and numbers of genes involved in the cortisol metabolic pathway have been analyzed. In rainbow trout, the data suggest that stressors inducing high levels of cortisol need an activation of StAR and P450scc gene expression and stressors inducing low levels of cortisol do not need activation of gene expression. For example, an acute stress induced by handling and anaesthesia (Geslin, Auperin, 2004) resulted in a 30-fold increase in plasma cortisol levels and an increase in StAR and P450scc enzymes (3β-HSD and CYP11β not analyzed). After 5 minutes chase with a net, the plasma cortisol level was only 3-fold higher than in control rainbow trout and no changes in StAR, P450scc, 3β-HSD and CYP11β mRNA levels were detected. Moreover, hybridization experiments indicated the presence of StAR mRNAs in head kidneys of unstressed rainbow trout, suggesting that pools of StAR mRNA are ready to be translated and transcription is not necessary for production of low concentrations of cortisol.

In contrast, our results with common carp did not show a significant activation of StAR gene expression after induction of high levels of cortisol. Only mRNA levels of 3β-HSD and 11β-HSD2 were elevated 20 minutes and 3 hours post-stress, respectively. These results suggest that a raise in 3β-HSD mRNA levels could contribute to an increase in cortisol production, but it has still to be shown that this enzyme has the appropriate activity in fish in the steroidogenesis pathway.

For 11β-HSD2 mRNA there is a trend in increase during net confinement and decrease after recovery with a significant increase 3 hours after confinement. These changes in 11β-HSD2 transcript levels were correlated with cortisol concentrations. These results suggest that 11β-HSD2, which oxidizes cortisol to cortisone, is involved in the regulation of cortisol concentrations. In mammals, the 11β-HSD2 inactivates cortisol to cortisone, which is crucial for aldosterone action because the glucocorticoid receptor and mineralocorticoid receptor have similar affinities for cortisol. If 11β-HSD2 in head kidneys has the same function in mammals as in fish has to be elucidated (discussion chapter 4). To summarise, our results suggest that cortisol biosynthesis activated by net confinement stress in common carp resulting in high cortisol levels, is at least partly regulated at transcriptional level for 3β-HSD and that 11β-HSD2 is involved in the regulation of cortisol concentrations.

Acknowledgement

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References

Balm, P.H.M., Pottinger, T.G., 1995. Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). General and Comparative Endocrinology98, 279-288.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphsis on the response and effects of corticosteroids. Annual Review of Fish Disease 1, 3-26.

Bongers, A.B.J., Sukkel, M., Gort, G., Komen, J., Richter, C.J.J., 1998. Development and use of genetically uniform strains of common carp in experimental animal research. Laboratory Animals 32, 349-363.

Burns, C.J., Gyles, S.L., Persaud, S.J., Sugden, D., Whitehouse, B.J., Jones, P.M., 2000. Phosphoprotein phosphatases regulate steroidogenesis by influencing StAR gene transcription. Biochemical and Biophysical Research Communication. 273, 35-39.

Geslin, M., Auperin, B., 2004. Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). General and Comparative Endocrinology. 135, 70-80.

Gilmour, K.M., 2005. Mineralocorticoid receptors and hormones: Fishing for answers. Endocrinology 146, 44-46.

Hagen, I.J., Kusakabe, M., Young, G., 2006. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11 beta-hydroxylase messenger RNAs in rainbow trout interrenal cells: Relationship with in vitro cortisol production. General and Comparative Endocrinology. 145, 254-262.

Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., 1997. Fish Stress and Health in Aquaculture. University Press, Cambridge. 278 pp.

Komen, J., Duynhouwer, J., Richter, C.J.J., Huisman, E.A., 1988. Gynogenesis in common carp (*Cyprinus carpio L.*) I. Effects of genetic manipulation of sexual products and incubation conditions of eggs. Aquaculture 69, 227-239.

Kusakabe, M., Nakamura, I., Young, G., 2003. 11B-Hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in rainbow trout: Cloning, sites of expression, and seasonal changes in gonads. Endocrinology 144, 2534-2545.

Kusakabe, M., Todo, T., McQuillan, H.J., Goetz, F.W., Young, G., 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinology 143, 2062-2070.

Li, Y.Y., Inoue, K., Takei, Y., 2003a. Interrenal steroid 21-hydroxylase in eels: primary structure, progesterone-specific activity and enhanced expression by ACTH. Journal of Molecular Endocrinology 31, 327-340.

Li, Y.Y., Inoue, K., Takei, Y., 2003b. Steroidogenic acute regulatory protein in eels: cDNA cloning and effects of ACTH and seawater transfer on its mRNA expression. Zoological Science 20, 211-219.

Lyssimachou, A., Arukwe, A., 2007. Alteration of brain and interrenal StAR protein, P450scc and CYP11β mRNA levels in Atlantic salmon after nominal waterborne exposure to the synthetic pharmaceutical estrogen ethynylestradiol. Journal of Toxicology And Environmental Health-Part A-Current Issues 70, 606-613.

Pottinger, T.G., 1998. Changes in blood cortisol, glucose and lactate in carp retained in anglers' keepnets. Journal of Fish Biology 53, 728-742.

Rotllant, J., Ruane, N.M., Caballero, M.J., Montero, D., Tort, L., 2003. Response to confinement in sea bass (*Dicentrarchus labrax*) is characterised by an increased biosynthetic capacity of interrenal tissue with no effect on ACTH sensitivity. Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology 136, 613-620.

Ruane, N.M., Huisman, E.A., Komen, J., 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. Journal of Fish Biology 59, 1-12.

Ruane, N.M., Lambert, J.G.D., Goos, H.J.T., Komen, J., 2005. Hypocorticism and interrenal hyperplasia are not directly related to masculinization in XX mas-1/mas-1 carp, *Cyprinus carpio*. General and Comparative Endocrinology 143, 66-74.

Stocco, D., 2000. The role of the StAR in steroidogenesis: challenges for the future. Journal of Endocrinology 164, 247-253.

Vijayan, M.M., Pereira, C., Grau, E.G., Iwama, G.K., 1997. Metabolic responses associated with confinement stress in tilapia: The role of cortisol. Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology 116, 89-95.

Waring, C.P., Stagg, R.M., Poxton, M.G., 1996. Physiological responses to handling in the turbot. Journal of Fish Biology 48, 161-173.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiological Reviews 77, 591-625.

Zhou, L.-Y., Wang, D.-S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., Nagahama, Y., 2007b. A Novel Type of P450c17 Lacking the Lyase Activity Is Responsible for C21-Steroid Biosynthesis in the Fish Ovary and Head Kidney. Endocrinology 148, 4282-4291.

Chapter Four:

Stress response in head kidneys of a 17α-hydroxylase deficient common carp (*Cyprinus carpio L.):* cortisol, corticosterone, chloride and gene expression levels

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Abstract

We analyzed the cortisol, corticosterone, chloride, head kidney-somatic index and steroidogenic gene expression levels during net confinement stress and recovery in a 17a-deficient common carp (E5). The results show low cortisol, high corticosterone and enlargement of the head kidney in E5 fish. This is the first report of a significant increase of corticosterone levels in response to stress in low vertebrates due to a dysfunction of 17α-hydroxylase activity. Standard carp shows a correlation between cortisol production and 11β-HSD2 mRNA expression during the stress response. In contrast, 11β-HSD2 expression is significantly lower in E5 fish and stays at a constant level during confinement. Real-time PCR analysis of StAR and 3β-HSD showed significantly higher expression in E5, for StAR at 1 hour post stress and for 3β-HSD 5, 20 minutes, 1 hour post stress and 4 hours recovery. However, no differences in expression levels of P450c21 were found between E5 and standard fish, suggesting that transcription of P450c21 is not a limiting step in corticosterone production. Finally, we analyzed the changes in mRNA levels in P450c17a1.I between E5 and standard fish during confinement. Although the data are quite variable, expression in E5 fish resulted in a trend towards lower accumulation levels in E5, suggesting a dysfunction at transcriptional level. However, we only studied the expression using primers based on exon 3 of variant P450c17a1.I. Results from Zhou et al. (2007) suggest that the P450c17a2 variant, which posses only the hydroxylase activity, is responsible for the synthesis of cortisol in the head kidneys. Sequencing variant P450c17a2 mRNA from carp is in progress.

Keywords: common carp, cortisol, corticosterone, steroidogenic pathway, gene expression, 17α-hydroxylase deficiency, Congenital Adrenal Hyperplasia (CAH)

4.1 Introduction

Corticosteroids play an important role in maintaining physiological homeostasis through effects on glycaemia, growth and osmoregulation, especially during the primary stress response (Iwama et al., 1997; Wendelaar Bonga, 1997). The main corticosteroids in vertebrates are the adrenal glucocorticoids (cortisol and corticosterone), which regulate carbohydrate metabolism and manage stress, and the mineralocorticoids (aldosterone), which regulate salt and water balance and maintain blood pressure by promoting renal sodium chloride and water resorption. Since, no mineralocorticoid receptors and aldosterone had been found in teleost fish, the general view was that cortisol acts in fish as a mineral as well as a glucocorticoid. However, the recent discovery of a mineral corticoid receptor (MR) and two distinct functional glucocorticoid receptors (GR) in rainbow trout suggest that the corticoid signaling in fish is more complex and acts through multiple receptors (Greenwood et al., 2003). Ligand binding studies suggest that deoxycorticosterone could be the physiological ligand of MR in fish.

Congenital adrenal hyperplasia (CAH), a group of disorders in the biosynthesis of cortisol, caused by an enzymatic deficiency in the conversion of cholesterol to cortisol has been described for mammals (Gotoh et al., 1988; Miller, 1991; New, 1992; Pang et al., 1992). Impaired function of steroidogenic enzymes, involved in cortisol production, often results in an increased production of steroid precursors, proximal to the deficient enzymatic step , like overproduction of deoxycorticosterone and corticosterone (Biglieri et al., 1966). The most common form of CAH is 21-hydroxylase deficiency (White, Speiser, 2000). 11β-hydroxylase (White, Speiser, 1994), 17α-hydroxylase/17, 20-lyase (Biglieri et al., 1966) and cholesterol desmolase deficiency (Katsumata, 2007; Morohashi et al., 1987) are rare. Many studies have been devoted to genotyping affected patients, allowing the assignment of specific mutations. Many mutations have been found in the steroidogenic genes resulted in impaired transcriptional activity and/or reduced or destroyed enzymatic activity.

In fish, only one case of interrenal insufficiency has been described (Ruane et al., 2005). An isogenic line of common carp (E5) demonstrates the classic symptoms of adrenal hyperplasia

such as interrenal hyperplasia, and a low production of cortisol in response to ACTH stimulation. Steroid production studies in head kidney homogenates indicate that interrenal hyperplasia in this strain is caused by a dysfunction of the enzyme P450c17.

In this report we further characterized this fish example of interrenal hyperplasia; we have demonstrated that acute stress in this E5 fish results in low production of cortisol and high production of corticosterone. Gene transcription analysis showed differences in the expression levels of the steroidogenic enzymes StAR, P450c17a1.I, 3β-HSD and 11β-HSD type 2 between wild type and E5 fish. A possible role for corticosterone as a ligand for MR and/or GR will be discussed.

4.2 Materials and Methods

All procedures approved by the Wageningen University committee on ethics in animal experimentation.

4.2.1 Animal production and net confinement procedure

Two isogenic strains of common carp were produced for the experiment. Wild type (STD) male was produced by the conventional breeding of an E4E5 (XX) female with a R3R8 (YY) male that is an androgenetic male strain of Polish/Hungarian origin (Komen et al., 1988). E5, a sex reversed XX male strain which is characterized by enlarged head kidney and a low cortisol stress response (Ruane et al., 2005) was produced by androgenesis in the University hatchery (De Haar Vissen, Wageningen University). Fish production was done according to the protocol (Bongers et al., 1998). The net confinement stressor was performed as described previously (Ruane et al., 2005) at time points of T0-unstressed (control), 5 min, 20 min, 1 h and 3 h confinement followed by 1 h, 4 h and 22 hours recovery. Sampling of fish occurred at 195 dph.

4.2.2 Blood collection and head kidney sampling

At each time point the 5 fishes were removed from the tank and quickly anaesthetized with 3 g Tricaine methyl sulphonate (MS_{222}) and 6 g bicarbonate (NaHCO3) in 10 l water, Crescent Research Chemicals. Blood was collected as previously described by (Ruane et al., 2001). After blood collection fish were killed with 6 g MS_{222} + 12 g NaHCO3 in 10 l water. Total body weight and standard length of fish were measured and the fish were then dissected. Head kidneys (both right and left) were weighed to calculate head kidney-somatic index, as follows; HKSI% = Head kidney weight/Body weight X 100

The head kidneys were then collected and immediately frozen in liquid nitrogen.

4.2.3 Plasma Analysis

4.2.3.1 Cortisol Measurement

The concentration of cortisol was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY) validated for common carp. Cross reactivity of the antiserum with other steroids in the cortisol production pathway are: cortisone (15.77%), 11-deoxycortisol (15%), corticosterone (4.81%), progesterone (0.06%) and 17α-hydroxyprogesterone (1.36%).

4.2.3.2 Corticosterone measurement

The concentration of corticosterone was measured using an assay designed correlated–Enzyme Immunoassay corticosterone Kit (Assay designs, Ann Arbor, MI) validated for common carp according to the manufacturer's instructions. All assays were performed in duplicate. Cross reactivity of the antiserum with other steroids in the corticosterone production pathway are: deoxycorticosterone (21.3%), desoxycorticosterone (21.00%), progesterone (0.46%), cortisol (0.046%) and cortisone (0.03%).

4.2.3.3 Chloride measurements

Plasma chloride concentrations were determined using a chloride meter (Jenway).

4.2.4 RNA Isolation

Total RNA was extracted from whole head kidneys of five individual fish according to the manufacturer's instructions (RNeasy Midi kit QIAGEN, 2001). UV spectroscopy (Nano drop ND-1000) was used to quantify RNA. The concentration was calculated using the Beer-Lambert law. The 2:1 ratio between the 28S and 18S rRNA on a 1% agarose gel was used an indication that the RNA was intact. The purified RNA was used immediately for RT-PCR or was stored at -80 ºC for future use.

4.2.5 Real-time PCR

Total RNA (100 ng/µl) was reversed transcribed with the SuperScript^{TM} III transcriptase kit using random hexamers (First-Strand Synthesis SuperMix Kit, Invitrogen). The cDNA generated by RT was amplified in a PCR reaction containing gene specific forward and reverse primers for StAR, P450c17a1.I, 3β-HSD, P450c21, 11β-HSD2 and 18S rRNA. Primers for 3β-HSD and 11β-HSD were designed based on sequences obtained from Gene bank (accession numbers: *Danio rerio;* NM 212797.1 for 3β-HSD and NM 212720.1 for 11β-HSD type 2). Primers for StAR, P450c17a1.I and P450c21 are designed based on available sequences for carp (H.van Pelt-Heerschap, unpublished results). Primer sequences are listed in Table 4.1.

To detect the expression of StAR, P450c17a1.I, 3β-HSD, P450c21 and 11β-HSD genes, we used generic double stranded DNA Dye (Syber Green I). The Dye was detected using the ABI PRISM 7900HT sequence Detection system (Applied Biosystems). For the PCR reactions we used a total volume of 25 µl reaction mixture containing 2 µl of cDNA template, 12.5 µl ABI Universal PCR Syber Green Universal Master Mix 2x (Applied Biosystems), 1.25 µl (10 µM) of each primer, and 8 µl of milliQ water. The PCR conditions were as follows; denaturation at 95 ºC for 10 min, 40 cycles at 95 ºC; 15 s, 54 ºC (P450c17a1.I, 3β-HSD, P450c21 and 11β-HSD2) or 60 ºC (StAR) for 30 s; and 72 ºC 36 s, a 72 ºC for 7 min. 18S rRNA was detected using TaqMan Ribosomal RNA (Applied Biosystems) control Reagents kit according to the manufacturer's instructions. The dilution ranges for the transcripts for quantitative accuracy were examined before running the assay. As controls, the reaction mixture containing no cDNA was used to verify the specificity of the PCR product,

whereas RNA was used in the PCR to rule out DNA contamination. Amplification products were verified by melting temperature, agarose gel electrophoresis and direct sequencing (ABI PRISM 3730 DNA Analyzer). Two different PCR assays were conducted in duplicate for each sample and a mean value was used to calculate the transcript levels. The efficiency was determined from the slopes of the curves and the coefficient variation of all slopes was calculated between different runs (Table 4.2).

To quantify transcripts of the genes, we monitored 18S rRNA levels as quantitative control and each sample was normalized on the basis of its 18S rRNA content.

Table 4.1 Primers used and the optimal conditions for real-time PCR analysis. The concentration for all primers is 10 μM.

Gene	Annealing ºC	Primer (forward) Sequence (5'-3')	Primer (Reverse) Sequence (5'-3')
StAR	60	ACAGGTGGGTCCATTCTCAG	GTGGAACCCCAATGTCAAAC
P450c17a1.I	54	GAGGCCAGCTCTATGTGTGA	GCATGGACTCAAACTCAGCA
3β-HSD	54	ATTAACGGCGATGAGGACAC	ATCGACAGCCTGGTCCATAG
P450c21	54	GATCCACTGTGGTCATGTGC	CTTGGACCAGGGTTTCTCAA
11β-HSD2	54	GCTGAGCTCTCCCTCATGTC	AGATTCAGGGCAGCTTTTGA
18S rRNA	60	CGTCTGCCCTATCAACTTTCG	TGCCTTCCTTGGATGTGGTAG

Table 4.2 Slopes, correlation coefficients (r^2) and % efficiency for the genes quantified by real-time RT-PCR.

Gene	Slope	r^2	%E	Slope	r^2	%E
StAR	-3.39	0.998	97.4	-3.4	0.99	96.8
P450c17a1.I	-3.61	0.996	89.2	-3.39	0.986	97
3β-HSD	-3.27	0.997	102.3	-3.4	0.989	97
P450c21	-3.60	0.998	89.6	-3.58	0.99	90.3
11β-HSD2	-3.50	0.992	92.9	-3.59	0.985	90

4.2.6 Statistical Analyses

The difference in relative transcript values of the genes studied and plasma cortisol, corticosterone, chloride values and HKSI% in response to net confinement followed by recovery time points were analyzed using a student *t*-test followed by Tukey's test ($P<0.05$).

4.3 Results

4.3.1. The effect of net confinement on plasma cortisol, corticosterone, chloride levels and HKSI% changes

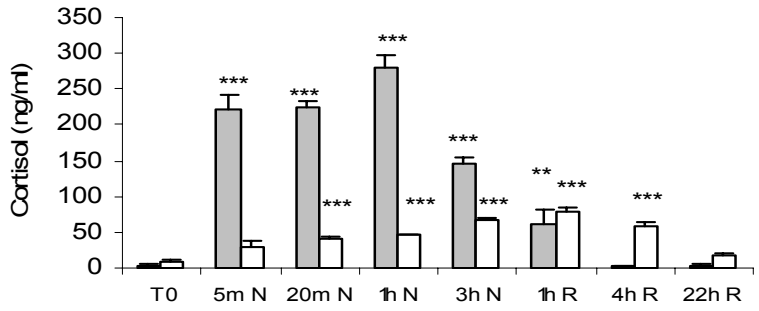
During the net confinement stress, a significantly different cortisol and corticosterone response was found between the STD and E5 fish.

Plasma cortisol levels in STD fish were significantly ($P < 0.001$) increased at time points 5 min, 20 min, 1 h (peak 85-fold) and 3 h after net confinement in the STD line. Cortisol levels quickly returned to the normal levels following release of the remaining fish from the nets to the pre-stress tanks. Plasma cortisol levels in E5 fish were significantly elevated at 20 min after net confinement and reached to a peak at 1 h recovery (~ 8-fold), then returned to the normal level after 22 h recovery (Fig. 4.1A).

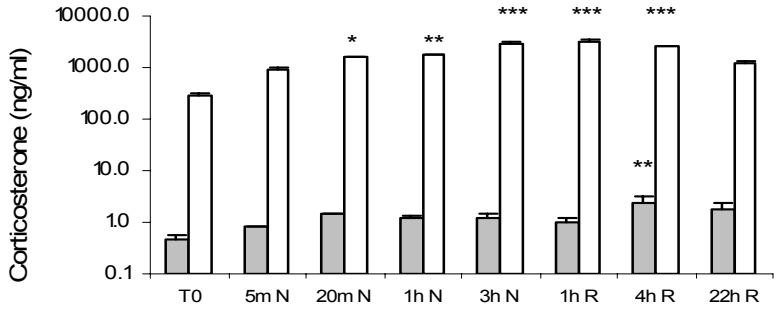
Plasma corticosterone levels (Fig. 4.1B) were significantly ($P<0.0001$) increased in E5 after net confinement with a peak at 1 h recovery (11-fold). Conversely, plasma corticosterone levels in STD did not show great differences at all time points. In E5, changes in cortisol plasma levels (log cortisol) were positively correlated with changes in plasma corticosterone levels (log corticosterone) ($r = 0.88$, $P < 0.0001$, $n = 21$) whereas no correlation was shown in STD fish.

Further, when E5 and STD plasma chloride levels were compared, a significantly ($P < 0.01$) reduced level (0.84-fold) was observed in E5 after 3 h net confinement (Fig. 4.1C).

A)



B)



C)

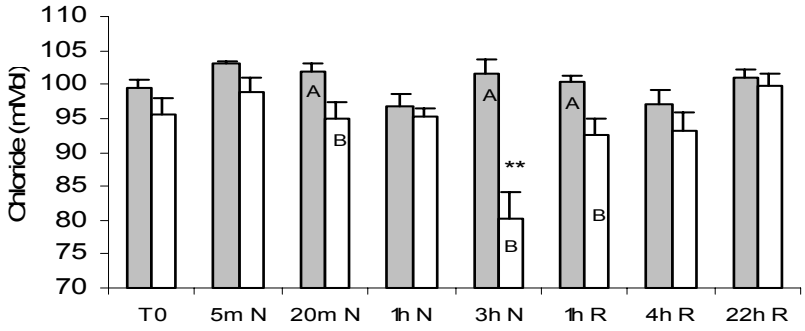


Fig. 4.1 Plasma levels of (A) cortisol, (B) corticosterone and (C) chloride in two isogenic strains of common carp (▒ STD; □ E5), during a 3 h net confinement. Values are means ± SE, $n = 2$ (duplicate tanks, five fish per tank).*P < 0.05, **P < 0.01, ***P<0.001 indicate a significant difference to the T0. Strain differences at a time point are indicated by different letters (A, B, $P < 0.05$). There are significant differences between the strains in all time points for cortisol and corticosterone.

Morphometric analysis of head kidney tissue indicated that the head kidney somatic index was significantly larger in E5 fish than in STD fish (Fig. 4.2).

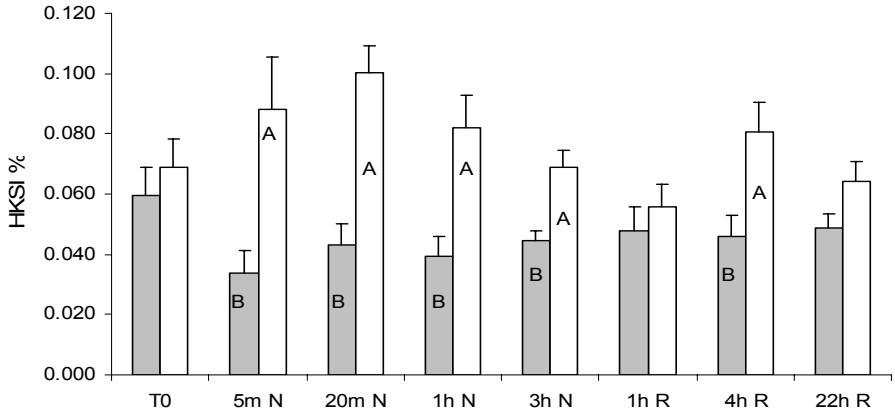


Fig. 4.2 Morphometric analysis of head kidney tissues, HKSI%, from two strains of carp (STD ; E5) subjected to a 3 h net confinement. Differences at a time point are indicated by different letters (A, B, $P < 0.05$). Weight of head kidney (according to Ruane et al, 2005) expressed as a % of the total body weight. Values are means ± SE, $n = 5$

4.3.2. The effect of net confinement on StAR, P450c17a1.I, 3β-HSD, P450c21 and 11β-HSD2 mRNA levels

We investigated the gene expression levels of StAR, P450c17a1.I, 3β-HSD, P450c21 and 11β-HSD2 in head kidneys of STD and E5 fish by real-time PCR. The results are shown in Fig. 4.3A-E, respectively.

Real-time PCR analysis of P450c21 did not reveal any differences between STD and E5 in whatever the time during and after net confinement (Fig. 4.3D) whereas StAR, P450c17a1.I, 3β-HSD and 11β-HSD2 gene expression showed significant differences between STD and E5 fish (Fig. 4.3A, B, C and E).

StAR levels were significantly higher 1 h after confinement in E5 (2.3-fold, $P < 0.038$, Fig. 4.3A).

P450c17a1.I gene expression showed no detectable expression in 25% of the STD and 60% of E5 fish at various time points during and after confinement, resulting in high standard deviations. Despite these variations, P450c17a1.I levels were significantly higher (83-fold) in STD than in E5 fish ($P < 0.04$) 20 minutes after confinement.

3β-HSD levels were significantly higher 20 minutes after confinement in both E5 (3.4-fold, $P < 0.045$) and STD fish (2.2-fold, $P < 0.028$) compared to their control points. The level in E5 was higher than in STD fish in 5, 20 minutes and 1 h after confinement and 4 h recovery.

Real-time PCR analysis of P450c21 did not reveal any differences between STD and E5 in whatever the time during and after net confinement (Fig. 4.3D).

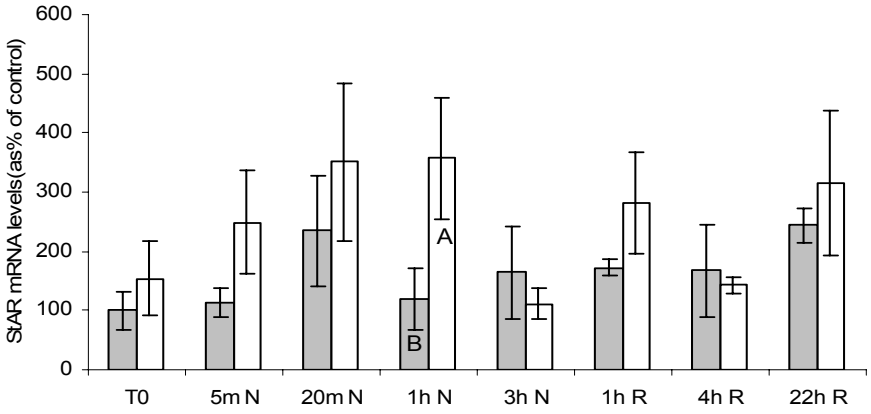
Finally, expression of 11β-HSD2 showed an increase (2.1-fold) compare to the control time point in mRNA levels during net confinement in STD fish with a peak at 3 h netting ($P < 0.008$). The levels at this time point are 5.6 fold higher than in E5 fish ($P < 0.005$). Following release of the fish from the nets 11β-HSD2 transcription levels quickly dropped down in STD fish (Fig.3E).

4.3.3 Relationship of gene expression with cortisol and corticosterone concentrations

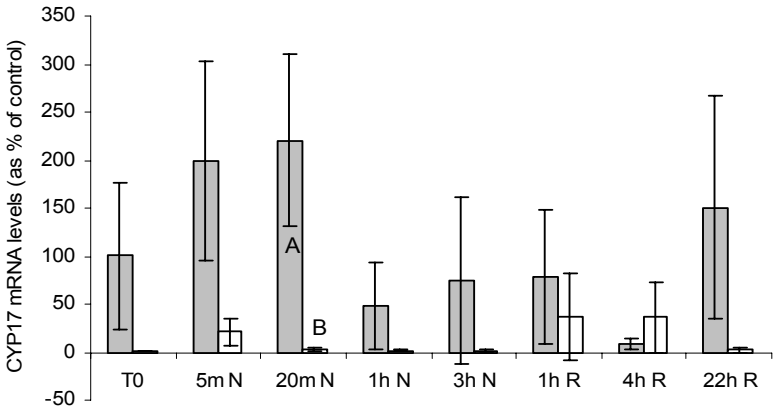
*11β-HS*D2 was strongly correlated with cortisol levels (log cortisol) in STD fish during net confinement and recovery ($r = 0.65$, $P < 0.0007$, $n = 23$).

Levels of StAR and 3β-HSD gene expression during net confinement and recovery were correlated in STD ($r = 0.76$, $P< 0.0001$, $n = 37$) and E5 fish ($r = 0.69$, $P < 0.0003$, $n = 23$).

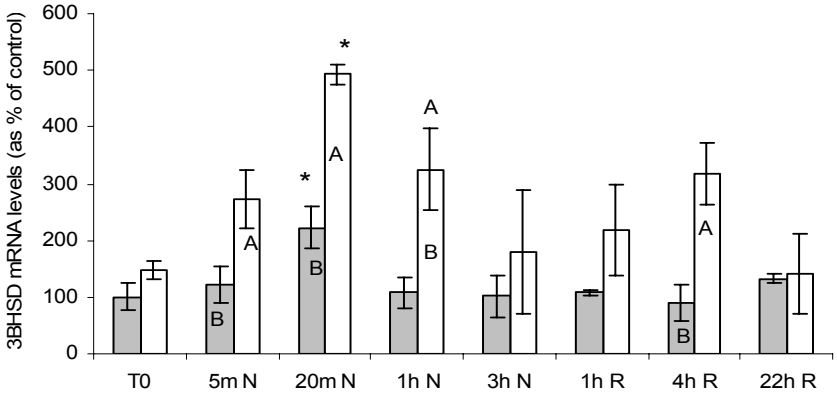
(A)


StAR mRNA levels(as% of control)
600
500
400
300
200
100
0
B
A
T0
5m N
20m N
1h N
3h N
1h R
4h R
22h R

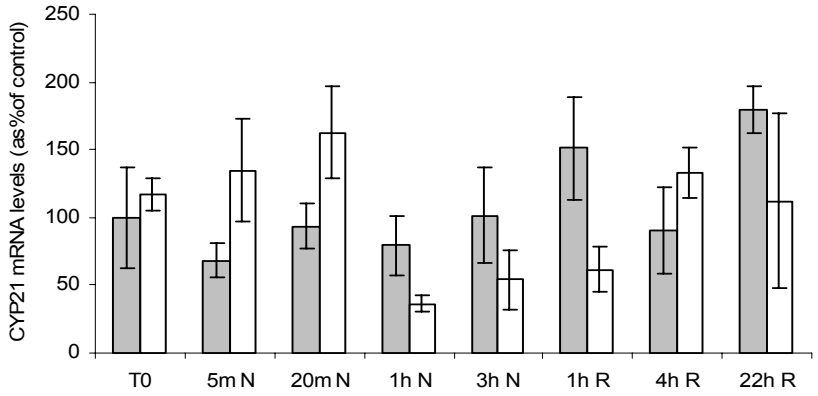
(B)


CYP17 mRNA levels (as % of control)
350
300
250
200
150
100
50
0
-50
A
B
T0
5m N
20m N
1h N
3h N
1h R
4h R
22h R

C)


3BHSD mRNA levels (as % of control)
600
500
400
300
200
100
0
*
*
B
A
B
A
B
A
B
A
T0
5m N
20m N
1h N
3h N
1h R
4h R
22h R

(D)



(E)

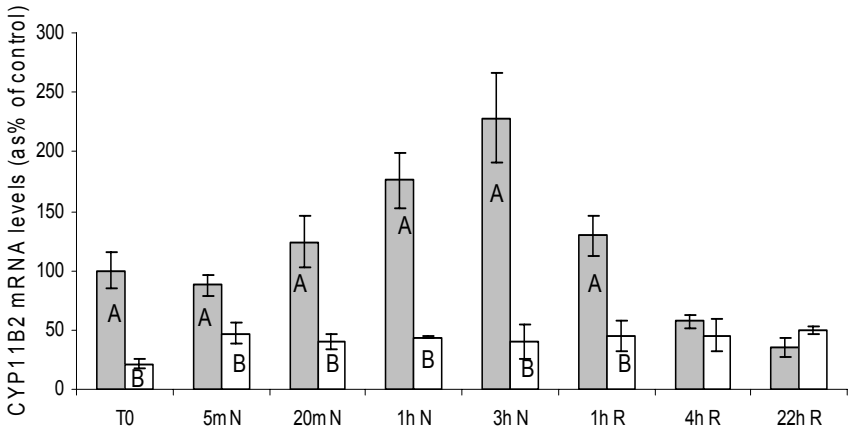


Fig. 4.3 The effect of stressor of 3h net confinement on variation in (A)StAR (B) P450c17a1.I (C) 3β-HSD (D) P450c21 and (E) 11β-HSD2 head kidney mRNA levels analysed by real-time PCR in (STD ▒; E5 □) common carp. The amount of mRNA was corrected by dividing by the amount of 18S RNA. The value at time 0 for STD fish was used arbitrary fixed at 100%. Each point is the means ± SE, n = 5. Significant differences between each time point and T0 are denoted by *P<0.05. Strain differences at a time point are indicated by different letters (A, B, $P < 0.05$).

4.4 Discussion

The aim of this study was to further characterize the stress response in a 17α-hydroxylase deficient common carp (Ruane et al., 2005). Cortisol, corticosterone, chloride, head kidney-somatic index and steroidogenic gene expression levels were analyzed.

The results show low cortisol, high corticosterone and enlargement of head kidney in E5 fish. This is the first report of a significant increase of corticosterone levels in response to stress in low vertebrates due to a dysfunction of 17α-hydroxylase activity. It suggests that reduced cortisol levels caused by P450c17a1.I deficiency leads to increased stimulation of the adrenals by ACTH, resulting in an increased production of corticosterone by increased numbers and sizes of the steroidogenic cells.

The question is if corticosterone exerts any biological activity in fish or is the result of build up of intermediates of steroid synthesis in E5 fish, due to dysfunction of P450c17a1.I.

In tetrapod vertebrates metabolic functions are regulated by glucocorticoid hormones cortisol and /or corticosterone. Aldosterone is the main mineralocorticoid, playing a role in sodium transport. Most fish appear to lack aldosterone (Bern, Madsen, 1992). Despite the absence of this mineralocorticoid, mineral receptors (MR) have been cloned in fish and their activation by various steroids studied (Greenwood et al., 2003; Sturm et al., 2005). It appeared that cortisol, 11β-deoxycorticosterone and aldosterone have affinities for the fish MR (Bury, Sturm, 2007).

If corticosterone can activate the fish MR or GR is not yet investigated. At least, our results suggest that corticosterone does not activate MR or GR, since the behavior during stress response of E5 fish is different from standard fish, indicating that corticosterone cannot compensate for the lack of cortisol. Moreover, high affinity of corticosterone for MR would lead to continuous activation of the MR's, resulting in a sodium in-balance. We only found a small difference (0.84-fold) in plasma chloride levels between E5 and STD after 3 h confinement, indicating that there are no large differences in plasma chloride levels.

On the other hand, if selective mineralocorticoid activation of the fish MR is possible, the same mechanism described below for mammals for activation of the MR and GR could be present in fish.

In mammals, cortisol has a high affinity for both the MR and GR receptor. The free levels of cortisol are 100-fold higher in blood than those of aldosterone (Baker, 2003; Funder, 2004). Despite these high concentrations of glucocorticoids, aldosterone can bind to the MR receptor, due to co-expression of the MR's with 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which inactivates cortisol, but not aldosterone. In this regard, our results in standard carp show a correlation between cortisol production and 11β-HSD2 mRNA expression during the stress response, suggesting a similar mechanism of controlling the access of glucocorticoids to fish MR and GR. In contrast, 11βHSD2 expression stays at a constant level during confinement in the E5 carp mutant, showing low cortisol production.

Additionally, differences in gene expression of enzymes involved in the conversion of cholesterol to corticosterone/cortisol between E5 and standard fish were analyzed. We expected a higher expression of enzymes involved in the steroidogenic pathway like StAR, 3β-HSD and P450c21 in E5 compared to standard fish, due to an enlarged head kidney in E5 fish, containing relative more steroidogenic cells that produce corticosterone. Indeed, for StAR we found a significantly higher expression in E5 fish 1 h after confinement and for 3β-HSD 5, 20 minutes and 1 h after stress and 4 h after recovery. However, no differences in expression level of P450c21 were found between E5 and standard fish, suggesting that transcription of P450c21 is not a limiting step in corticosterone production. To further accurately determine the changes in the individual head kidney cells in situ hybridization studies are necessary.

Finally, we analyzed the changes in mRNA levels in P450c17a1.I between E5 and standard fish during confinement. Previous results indicated that the interrenal hyperplasia in E5 fish is caused by a dysfunction of the 17a-hydroxylase activity of the enzyme P450c17a1.I. Although the data are quite variable, expression in E5 fish resulted in a trend towards lower accumulation levels in E5, suggesting a dysfunction on transcriptional level. However, we only studied the expression using primers based on exon 3 of variant P450c17a1.I (Chapter

2). Results from Zhou et al., (2007) suggest that the P450c17a2 variant, which posses only the hydroxylase activity, is responsible for the synthesis of cortisol in the head kidneys. Sequencing variant P450c17a2 mRNA from carp is underway to further analyze the transcription of this enzyme in E5 fish.

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References

Baker, M.E., 2003. Evolution of Glucocorticoid and Mineralocorticoid Responses: Go Fish. Endocrinology 144, 4223-4225.

Bern, H.A., Madsen, S.S., 1992. A selective survey of the endocrine system of the rainbow trout (*Oncorhynchus mykiss*) with emphasis on the hormonal regulation of ion balance. Aquaculture 100, 237-262.

Biglieri, E.G., Herron, M.A., Brust, N., 1966. 17-hydroxylation deficiency in man. Journal of Clinical Investigation 45, 1946-1954.

Bongers, A.B.J., Sukkel, M., Gort, G., Komen, J., Richter, C.J.J., 1998. Development and use of genetically uniform strains of common carp in experimental animal research. Laboratory Animals 32, 349-363.

Bury, N.R., Sturm, A., 2007. Evolution of the corticosteroid receptor signaling pathway in fish. General and Comparative Endocrinology 153, 47-56.

Funder, J.W., 2004. Is aldosterone bad for the heart? Trends in Endocrinology and Metabolism 15, 139-142.

Gotoh, H., Sagai, T., Hata, J., Shiroishi, T., Moriwaki, K., 1988. Steroid 21-hydroxylase deficiency in mice. Endocrinology 123, 1923-1927.

Greenwood, A.K., Butler, P.C., White, R.B., Demarco, U., Pearce, D., Fernald, R.D., 2003. Multiple Corticosteroid Receptors in a Teleost Fish: Distinct Sequences, Expression Patterns, and Transcriptional Activities. Endocrinology 144, 4226-4236.

Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., 1997. Fish Stress and Health in Aquaculture. University Press, Cambridge, 278 pp.

Katsumata, N., 2007. Cholesterol side-chain cleavage enzyme (SCC) deficiency. Clinical Pediatric Endocrinology 16, 63-68.

Komen, J., Duynhouwer, J., Richter, C.J.J., Huisman, E.A., 1988. Gynogenesis in common carp (*Cyprinus carpio L.*) I. Effects of genetic manipulation of sexual products and incubation conditions of eggs. Aquaculture 69, 227-239.

Miller, W., 1991. Congenital adrenal hyperplasia. Endocrinol Metab Clin North Am 20, 721-749.

Morohashi, K., Sogawa, K., Omura, T., Fujii-Kuriyama, Y., 1987. Gene structure of human cytochrome P450scc, cholesterol desmolase. Journal of Biochemistry 101, 879-887.

New, M.I., 1992. Genetic disorders of adrenal hormone synthesis. Hormon Research. 37, 22-33.

Pang, S., Yang, X., Wang, M., Tissot, R., Nino, M., Manaligod, J., Bullock, L., Mason, J., 1992. Inherited congenital adrenal hyperplasia in the rabbit, absence cholesterol side-chain cleavage cytochrome P450 gene expression. Endocrinology 131, 181-186.

Ruane, N.M., Huisman, E.A., Komen, J., 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. Journal of Fish Biology 59, 1-12.

Ruane, N.M., Lambert, J.G.D., Goos, H.J.T., Komen, J., 2005. Hypocorticism and interrenal hyperplasia are not directly related to masculinization in XX mas-1/mas-1 carp (*Cyprinus carpio)*. General and Comparative Endocrinology. 143, 66-74.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E., Prunet, P., 2005. 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146, 47-55.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiological Reviews 77, 591-625.

White, P.C., Speiser, P.W., 1994. Steroid 11 beta hydroxylase deficiency and related disorders. Endocrinology and metabolism clinics of North America 23, 325-339.

White, P.C., Speiser, P.W., 2000. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocrine Reviews 21, 245-291.

Zhou, L.Y., Wang, D.S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., Nagahama, Y., 2007. A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology148,4282-4291

Chapter Five:

Co-Segregation of interrenal hyperplasia and sex in backcross nd doubled haploid populations of common carp (*Cyprinus carpio L.*)

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Abstract

The aim of this study was to investigate the inheritance of interrenal hyperplasia and low cortisol response using backcross and gynogenetic progeny of heterozygote carriers of common carp. The inbred strains used in this experiment were E4 (XX, normal), E5 (XX, interrenal hyperplasia) and E7 (XX, interrenal hyperplasia). Heterozygote carriers were produced by crossing E4 with E5 (E4E5: XX). E4E5 females were crossed with E5 and E7 animals (BC5 and BC7), and or reproduced by gynogenesis to produce doubled haploids (DH). Progeny were raised and at 6 months subjected to a net confinement stressor. Fish were sacrificed after one hour net-confinement and blood was collected to determine cortisol and corticosterone values. Fish were dissected to determine sex, and head kidney was weighed to head kidney-somatic index. Values for cortisol and head kidney index showed a continuous distribution in BC and DH progeny. Values for corticosterone, on the other hand showed a very clear segregation pattern, consistent with a single gene model. There was a highly significant difference in mean cortisol level between high (H) and low (L) corticosterone responders in BC and DH progeny groups. Mean values for H responders in the 3 groups BC5, BC7 and DH were respectively: corticosterone 1706, 1760 and 1366 ng/ml, and for cortisol: 21.3, 24 and 17.3 ng/ml. For L responders values were: corticosterone 1.5, 1.1 and 0.6 ng/ml, and for cortisol: 66.7, 105.1 and 65.2 ng/ml respectively. ead kidney index was not significantly different between H and L groups, but sex was. H responders were predominantly male, while L responders were female or intersex.

Keywords: sex determination, interrenal hyperplasia, stress, cortisol, corticosterone, common carp.

5.1 Introduction

Congenital Adrenal Hyperplasia (CAH) represents a group of inherited disorders that are all characterized by impaired production of cortisol by the adrenal gland. CAH occurs because of a deficiency in any one of the enzymes involved in the conversion of cholesterol to cortisol in human (Miller, 1991; New, 1992), mammal (Deaton et al., 1999; Gotoh et al., 1988; Pang et al., 1992) and recently in fish (Ruane et al., 2005).

In common carp, Cyprinus carpio, L., interrenal insufficiency is observed in two homozygous XX male strains, E5 and E7 (Ruane et al., 2007; Ruane et al., 2005). These homozygous strains are produced by androgenesis (Komen, Thorgaard, 2007) and produce 3-5x less cortisol during a net confinement stress test than normal carp. The reduced production of cortisol is not caused by a reduced sensitivity of interrenal cells to ACTH, but by a reduced conversion of progesterone into 17α-hydroxyprogesterone, the precursor for cortisol. Head kidneys of E5 fish are enlarged, and have significantly more and larger steroid producing cells than kidneys of normal carp (Ruane et al., 2005). More recently, it was shown that E5 carp also produce copious amounts of corticosterone, presumably as a consequence of progesterone accumulation and subsequent conversion by 21-hydroxylase (Nematollahi et al., 2006).

In 1992, the discovery of female to male sex reversal in the E5 strain led to the postulation of a recessive mutation in a putative autosomal sex determining gene, termed mas^{-1}, that induced masculinization in XX, E5 females (Komen et al., 1992a). In this model, homozygous XX (mas^{-1}/ mas^{-1}) animals develop as males (e.g. E5, E7) while heterozygous XX, (mas^{+}/mas^{-1}) animals develop as normal females. Normal XY males are not affected. A cross between heterozygous XX, (mas^{+}/mas^{-1}) females and homozygous XX, (mas^{-1}/mas^{-1}) males again produces 50% males and 50% females in the offspring (Komen et al., 1992a).

The fact that E5 carp are sex reversed XX males, and suffer from interrenal hyperplasia, suggests that these two phenomena are related, and that these carp show all the symptoms of congenital adrenal hyperplasia, as seen in humans. However, a recent study by Ruane et al.

(2005) showed that sex reversal was not caused by androgen production from the head kidney, as is normally the case in humans.

The first objective of the present study was to investigate the inheritance pattern of CAH in common carp using backcross and gynogenetic doubled haploid progenies of presumed heterozygous animals.

More specifically we analyzed the segregation of interrenal hyperplasia in crosses of E5 and E7, two XX sex reversed clones of common carp, with presumed heterozygote carriers, using plasma cortisol and plasma corticosterone during net confinement as marker traits. The aim was to see if interrenal hyperplasia is a true genetic disorder, or whether it is caused by the doubled haploid constitution of E5 and E7 fish. The second objective of this study was to (re-) examine the co segregation of interrenal hyperplasia and sex reversal.

5.2 Materials and Methods

5.2.1 Animals

The homozygote inbred strains used in this experiment were E4 (XX, female, normal plasma cortisol response after stress), E5 and E7 (XX, male, low plasma cortisol response after stress). Both E5 and E7 clones are reproduced by androgenesis (Komen et al., 1992a). Heterozygote E4E5 animals had been produced earlier from a cross between females of a homozygous XX clonal strain (E4) and E5 males. E4 females show a normal cortisol response during stress. Mature heterozygote E4E5 animals were backcrossed to E5 and E7 animals (BC5 and BC7) or reproduced by homozygous gynogenesis to produce doubled haploids (DH).

All crosses E4xE5, BC5 and BC7 were produced by conventional fertilization techniques by mixing freshly ovulated eggs with undiluted milt before adding tap-water for activation and fertilization.

Doubled haploid (DH) offspring were produced by fertilizing eggs with irradiated sperm and giving eggs a heat shock (at 40 ^{o}C for 2 min, 30 min after fertilization) at a prometaphase of the first mitosis, to induce endomitosis (EM gynogenesis: (Komen et al., 1991).

Fish larvae from BC5, BC7 and DH groups were reared in 25-L tanks and fed with freshly hatched Artemia nauplii 4-5 times a day for the first 3-4 weeks. After this period fish were distributed in groups of 100 fish over 6 experimental rectangular tanks (2 tanks / genetic group) with a water volume of 120 l, and a water flow of 5 l min^{-1}. Fish were fed with pelleted food (Provimi) at a feeding level of 20 g/kg metabolic fish weigh per day ($g/kg^{0.8}.d^{-1}$) until 120 dph (days post hatching) and then 10 $g/kg^{0.8}.d^{-1}$ until 200 dph. All tanks were connected to the same recirculation system equipped with a biofiltration unit and received a constant flow of O_2 saturated water at 25ºC under a 12-h light/dark photoperiod. NH_4^+ and NO_2 were monitored daily and never exceeded 1 ppm; pH was maintained at 6.5. Stocking density was decreased to 70 fish per tank on 100 dph and to 50 fish on 150 dph.

5.2.2 Experimental procedure

The net confinement stressor was performed according to the protocol developed by Ruane et al. (2001). Groups of 24 fish were caught in one sweep and distributed over five 5-L nets (4-5 fish per net) and confined for 1 hour. In total a number of 96 BC5 fish, 48 BC7 fish and 96 DH fish were sampled. In addition, 10 E4E5 females were netted and sampled after one hour for cortisol and corticosterone. To avoid sampling effect of one group of fish on cortisol response of remaining fish in the same tank, repeated sampling from the same tank was done on different days. Confinement tanks received a flow of water at 5 l min-1 and were well aerated to avoid oxygen shortage in the nets.

5.2.3 Blood Sampling and Steroid measurements

After 1 hour net confinement fish were removed from the tank and quickly anaesthetized with a mixture of Tricaine methane sulphonate and Sodium bicarbonate (3 gr MS_{222} + 6 gr

$NaHCO_3$10 l^{-1} water, Crescent Research Chemicals). Blood was collected by saturated hypodermic syringe from the caudal blood vessel within 3 min per sample group. The collected blood was placed into cooled 1.5 ml plastic tubes containing 3 mg Na_2EDTA, mixed and centrifuged (2400 g; 4 ºC) for 5 min. The collected plasma was stored at -20 ºC for further analyses.

Plasma cortisol was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY) validated for common carp plasma. Ten µl of plasma was diluted with 990 µl of EIA Buffer which was provided to dilute enzyme conjugate and cortisol standards. The reaction was measured with a microplate reader (Lab systems Multiskan MS, Diagnostic Merlin system BV) with a dual wave length at 450 nm and 690 nm. Cross reactivity of the antiserum to other steroids in the cortisol production pathway are: cortisone (15.77 %), 11-deoxycortisol (15.00 %), corticosterone (4.81 %), progesterone (0.06 %), 17-α hydroxyprogestrone (1.36 %).

Plasma corticosterone was measured using an ELISA kit (Assay Designs, Ann Arbor, MI) validated for common carp plasma. Plasma was diluted 1:100 with a mixture of assay buffer and Steroid Displacement Reagent. The reaction was measured with a micro plate reader as before, using a dual wave length at 420 nm and 570 nm. Cross reactivity of the antiserum to other steroids in the corticosterone production pathway are: deoxycorticosterone (21.00 %), Progesterone (0.46 %), cortisol (0.046 %), cortisone (0.03 %). All assays were performed in duplicate.

5.2.4 Head kidney index

After blood collection, fish were killed with a mixture of Tricaine methane sulphonate and Sodium bicarbonate (6 gr MS_{222} + 12 gr $NaHCO_3$10 l^{-1} water, Crescent Research Chemicals), standard length and weight of fish were measured and then the fish were dissected. Head kidneys (both right and left) were weighted to calculate head kidney-somatic index (HKSI %) as follows:

HKSI % = Head Kidney Weight/Body Weight X 100

5.2.5 Sex ratios

At the age of 200 dph common carp males contain a well developed white testis with mature spermatogonia; females have pale reddish, transparent ovaries with numerous previtellogenic oocytes. Gonads of the fish were scored by visual inspection in 5 different classes: 1) Testis, 2) IS-95; intersex gonads with more than 95% testis tissue 3) IS-50 gonads with equal amount of ovarian and testicular tissue, 4) IS-10 ovaries with less than 10% testis tissue, and 5) Ovaries. Filiform gonads were scored as sterile.

5.2.6 Data Analysis

Due to a very clear segregation of corticosterone values, fish were grouped in high (H) and low (L) corticosterone responders. A chi-square test for goodness of fit was used to compare observed and expected frequencies of H and L numbers in each genetic group.
Data were subjected to analysis of variance (ANOVA) using general linear model procedure (Institute, 1989) to determine differences between H and L fish in BC5, BC7 and DH groups. The effects of corticosterone (L and H) nested within strain (1, 2, and 3), sample day (1, 2 and 3), tank number (1-6), body weight, and HKSI% on cortisol values were tested for significance. Analysis of variance was performed using proc GLM (Institute, 1989) and the least significant factors were removed from the model in a stepwise manner until all remaining effects were significant ($P < 0.05$). The full model used in for the analysis was:

$$Yijk = \mu + St.j\ (CS)\ k + \beta 1\ *HKSI\ ijk + \beta 2*LogCS\ ijk + \varepsilon\ ijk \quad (\text{Model 1})$$

Where, Yijk is an observation of Cortisol, μ is the overall mean, St.*j(CS)k is* effect of the k^{th} corticosterone responder group (1, 2) nested within the j^{th} strain (1, 2, 3), *β1* is the regression coefficient of HKSI %, *HKSI.ijk* is observation of HKSI, *β2* is the regression coefficient of Log corticosterone, *LogCS ijk* is observation of corticosterone , *ε ijk* is the random residual term.

The effects of corticosterone (L and H) nested within strain (1, 2, 3), sample day (1, 2 and 3), tank number (1-6), cortisol value, and body weight, on HKSI% values were tested for

significance. Analysis of variance was performed using proc GLM (Institute, 1989) and the least significant factors were removed from the model in a stepwise manner until all remaining effects were significant (P<0.05). The full model used in for the analysis was:

$Yijk = \mu + S.d\ i + St.j\ (CS)\ k + \beta 1\ *C\ ijk + \beta 2*W\ ijk + \varepsilon\ ijk$ (Model 2)

Where, Yijk is an observation of HKSI, μ is the overall mean, S.d.i is Sample day (i = 1, 2, 3), *St j (CS) k is* effect of the k^{th} Corticosterone responder group (1, 2) nested within the j^{th} Strain (1, 2, 3), β1 is the regression coefficient of cortisol, C.*ijk* is observation on cortisol, *β2* is the regression coefficient of weight, *W ijk* is observation of weight, *ε ijk* is the random residual term. Least square means (LSM) were calculated for the fixed effect classes using the LSMEANS option of proc GLM.

5.3 Results

5.3.1 Steroid measurements

Plasma cortisol and corticosterone levels of E4E5 fish after 1 hour confinement were 177.6 ± 56.6 ng/ml and 2.6 ± 1.2 ng/ml, respectively. Results of plasma corticosterone levels in BC5, DH and BC7 fish after 1 hour confinement are shown in Table 5.1 and Fig. 5.1.

Corticosterone values could be classified into two groups: High (H) with a range of 832-3003 ng/ml and low (L) with a range of 0.141-16.7 ng/ml. The two distributions are not overlapping. Observed frequencies of H and L fish were not significant different from an expected 1:1 ratio in each of the three groups, using a χ^2-test for goodness of fit ($P < 0.05$, $df = 1$). Mean (± sd) values (ng/ml) for high corticosterone responders were 1706 ± 393 in BC5, 1366 ± 274.8 in DH and 1760 ± 359.8 in BC7. Plasma values for low corticosterone responders were 1.5 ± 2.5 in BC5, 0.6 ± 0.6 in DH, and 1.1 ± 0.8 in BC7 groups. There were no significant differences between strains for either high or low responding fish.

Results of plasma cortisol levels in BC and DH groups are shown in Table 5.1 and Fig. 5.2.

Plasma cortisol values showed a more or less continuous distribution in each group (not shown), but mean plasma cortisol values of high (H) corticosterone responders were significantly lower than plasma cortisol values in low (L) corticosterone responders (figure 2). There was a significant effect of strain on plasma cortisol (table 2). For H fish, mean values differed from 17.3 ± 7.1 in DH to 24 ± 13.7 in BC7 groups; for L fish, mean values ranged from 65.2 ± 40.1 in DH to 105.1 ± 43.9 in BC7 groups. Type III mean squares of the effects in model 1 are summarized in Table 5.2.

All effects were highly significant ($P < 0.05$). Corticosterone, HKSI and strain together explain 50% of the variation in cortisol values.

Table 5.1 Mean (± SD) of Corticosterone (ng/ml), Cortisol (ng/ml) and head kidney somatic index (HKSI, in %) in backcross (BC5, BC7) and doubled haploid (DH) progeny groups of common carp (*Cyprinus carpio L.)*. H = High Corticosterone Responders, L = Low Corticosterone Responders.

.

Progeny group			*Corticosterone*		*Cortisol*		*HKSI*	
		N	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
BC5	L	54	1.5	2.5	66.7	46.9	0.136	0.032
	H	42	1706	393	21.3	10.2	0.143	0.039
DH	L	51	0.6	0.6	65.2	40.1	0.128	0.037
	H	45	1366	274.8	17.3	7.1	0.157	0.055
BC7	L	26	1.1	0.8	105.1	43.9	0.121	0.039
	H	22	1760	359.8	24.0	13.7	0.151	0.033

Table 5.2a Type III mean squares (MS), degree of freedom (DF) and significance of effects for plasma cortisol levels (Model 1).

Source	DF	MS
HKSI	1	4692.4*
Log Corticosterone	1	5264.9*
Strain (CS)	5	12055.4***
Error	227	1022
R^2	0.50	

$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

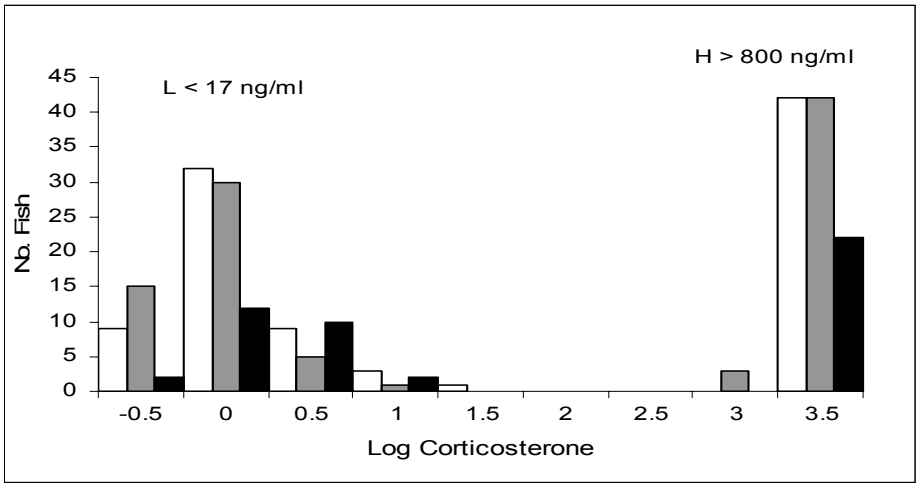

Figure 5.1 Frequency distribution of plasma corticosterone (Log ng/ml) levels in BC5 (□), DH (▒) and BC7 (■) progeny groups.

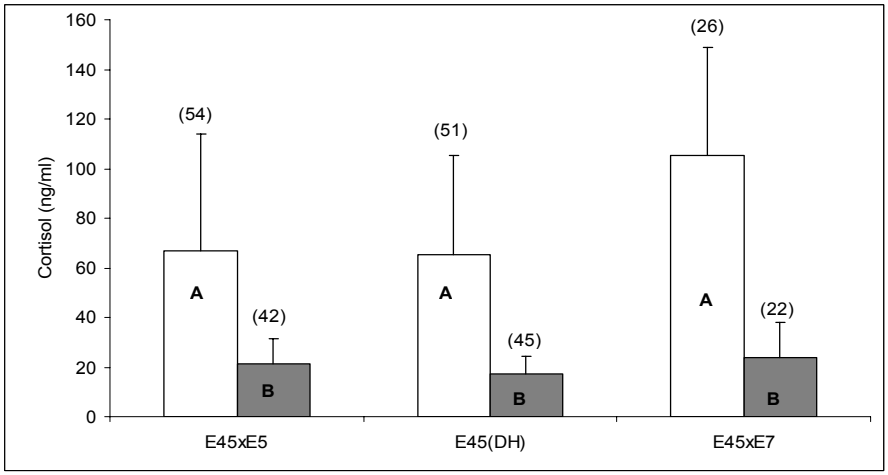

Figure 5.2 Plasma cortisol (ng/ml) of low (□) and high (▒) corticosterone responders in BC5, BC7 and DH progeny groups of common carp. Values are mean ± standard deviation (N in parenthesis); different letters indicate a significant difference ($P < 0.001$) between L and H responders from the same progeny group.

5.3.2 HKSI

Mean (± sd) HKSI values (in %) in H and L corticosterone responders in BC and DH groups are shown in Table 5.1 and Fig. 5.3. Mean values ranged from 0.121 ± 0.04 to 0.157 ± 0.06 in

DH and BC groups. Mean HKSI values in general were higher for H than for L responder fish, but not significantly, except for DH fish (Fig.5.3). Type III mean squares (MS) and the significance of effects for HKSI according to the Model 2 are given in Table 5.3.

Effects of cortisol, body weight, sample date and strain on HKSI were all found to be highly significant. Model 2 explained 16% of the variation in HKSI%.

Table 5.2b Type III mean squares (MS), degree of freedom (DF) and significance of effects for HKSI (Model 2).

Source	DF	MS
Cortisol	1	0.007*
Weight	1	0.006*
Strain (CS)	5	0.008***
Sampl.Date	2	0.008**
Error	230	0.001
R^2	0.16	

$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

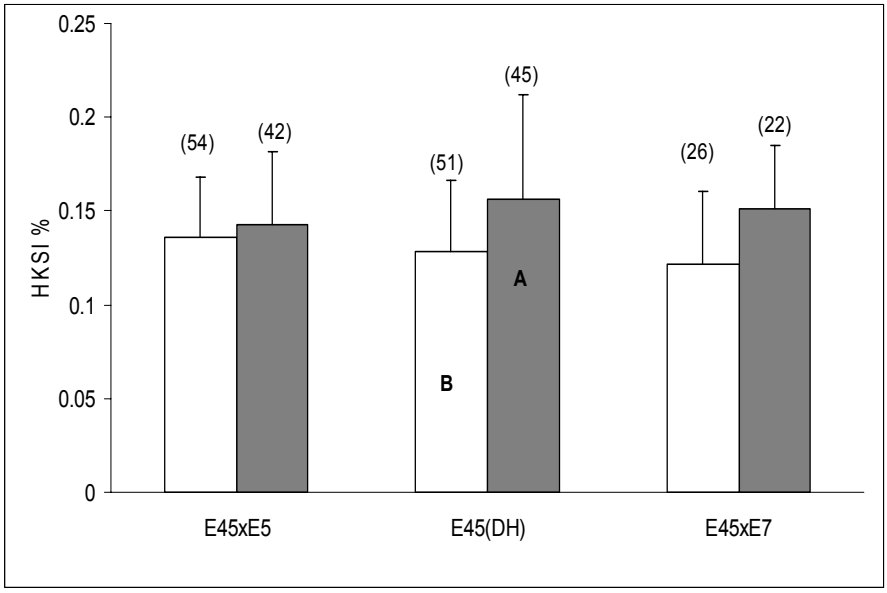

Figure 5.3 Head kidney somatic index (%) in low (□) and high (■) corticosterone responders in BC5, BC7 and DH progeny groups of common carp. Values are mean ± standard deviation (N in parenthesis); different letters indicate a significant difference ($P < 0.05$) between L and H responders from the same progeny group.

5.3.3 Sex ratios

Females in general had clearly distinguishable pale reddish, transparent ovaries, with numerous small previtellogenic oocytes. Males had well developed white testis. Intersex fish had gonads composed of white opaque areas (testicular tissue) and pale transparent areas (ovarian tissue). Sex ratios were grouped according to high or low corticosterone responder (Table 5.3), and tested for significance using a χ^2-test for goodness of fit. Both BC groups contained large numbers of intersex fish with advanced testis development. Only few females were observed in each group. The effect of corticosterone on maleness was significant ($P < 0.05$): H fish in BC5 and BC7 groups were almost exclusively male. The highest numbers of females and lowest number of males were observed in the L fish of the DH group: 45.1 % and 2 % respectively. In this group the effect of corticosterone on maleness was highly significant ($P < 0.01$): in the H responding fish only 4.4 % females and 51 % males were observed.

Table 5.3 Numbers (and %) of males, intersex and females in H and L corticosterone responders in BC and DH progeny groups of common carp, *Cyprinus carpio L.*

Progeny group		N	Male	IS-95	IS-50	IS-10	Female	ST
BC5	L	54	4 (7.4)	32 (59.3)	6 (11.1)	8 (14.8)	4 (7.4)	0
	H	42	41 (97.6)	1 (2.4)	0	0	0	0
DH	L	51	1 (2)	6 (11.8)	9 (17.6)	5 (9.8)	23 (45.1)	7 (13.7)
	H	45	23 (51.1)	12 (26.7)	5 (11.1)	1 (2.2)	2 (4.4)	2 (4.4)
BC7	L	26	2 (7.7)	11 (42.3)	1 (3.8)	3 (11.5)	8 (30.8)	1 (3.8)
	H	22	19 (86.4)	3 (13.6)	0	0	0	0

H = High Corticosterone Responder, L = Low Corticosterone responder; IS-95: intersex gonad with predominantly testicular tissue and some oocytes; IS-50: intersex gonad with more or less equal amounts of testicular and ovarian tissue; IS-10: intersex gonad with predominantly ovarian tissue and small areas of testicular tissue; ST: Filiform gonad, presumably sterile.

5.4 Discussion

The main finding of this study was that approximately half of the backcross and double haploid progenies produced large amounts of corticosterone. In an earlier study (chapter 4, this thesis) we reported that E5 fish produced high plasma corticosterone after confinement stress. It is assumed that corticosterone is produced as a result of impairment of 17α-hydroxylase activity in the head kidney. This enzyme is responsible for the conversion of progesterone into 17α-hydroxyprogesterone, which is subsequently converted into cortisol. When 17α-hydroxylase is impaired, the excess progesterone is converted by 21-hydroxylase into deoxycorticosterone and corticosterone. Here we provide evidence that the over-production of corticosterone is inherited as a monogenic recessive trait. First, both E4 and E4E5 fish are normal, i.e. they produce normal levels of cortisol after stress and very little amounts of corticosterone. Second, both backcrosses of E4E5 fish with either E5 or E7 (high corticosterone producers) contained nearly 50 % fish with normal stress responses, and 50 % fish with abnormal high levels of corticosterone. Furthermore, high and low corticosterone producers were also found in the DH group, again in equal numbers. This indicates that E4E5 fish are heterozygous for the recessive trait. Heterozygote parents produce DH progeny which are homozygous for either the wild type allele or the mutant allele. Together, these observations confirm that high plasma corticosterone after stress is a heritable, recessive trait, and is not caused by the doubled haploid constitution of E5 or E7 fish.

The levels of cortisol observed in this study were highly variable and lower than with those found in other studies on carp (Ruane et al., 2001; Tanck et al., 2000). However, grouping of fish in H and L responders showed that the two groups indeed have different mean cortisol responses with overlapping distributions. This is in contrast with earlier studies, using clonal strains (Ruane et al., 2001; Ruane et al., 2005) where all fish of the same clone showed a fairly homogenous response to confinement stress. In this study, backcrosses and DH progenies were tested, i.e. groups of fish which were not genetically uniform, but segregating for heterozygous parental alleles. The increased variation in cortisol responses might reflect the increased genetic variation in these backcross and DH progenies, as observed earlier by

(Tanck et al., 2001; Tanck et al., 2002) and point to other as yet unknown factors influencing the amplitude of the cortisol response in carp.

It is not clear why the average cortisol response of L corticosterone responders, i.e. "normal" fish, was lower than previously observed. One difference with earlier studies was the age at which fish were tested (> 200 dph). It is known that in fish, the stress response tends to go down with age (Wendelaar Bonga, 1997). It is also possible that the ELISA used in this experiment produces lower readings for cortisol than the previously used RIA for cortisol. Cross reactivity of the antibody used was 15.8 % with cortisone, which is higher than the RIA used previously by Ruane et al. (2001). We were not able to compare the ELISA with the RIA, using the same carp plasma.

There was little difference in head kidney index between the different groups. However, H fish consistently had higher mean index than L fish, although not significantly so. Head kidney was also very variable in size, both in H and L fish. This shows that, as with cortisol, head kidney index is not a reliable indicator to identify 17α-hydroxylase deficient carp.

The most important finding in this study was the observation that H corticosterone responders were predominantly male, while L responders were mainly intersex and female. Sex determination in common carp is primarily controlled by a, as yet unidentified, male dominant gene, termed "Y", as in mammals (Bongers et al., 1999; Komen et al., 1991). Males are heterozygous XY while females are homozygous recessive "XX". Sex determination is labile however, and sex reversal can be induced by endocrine factors (Gimeno et al., 1996) and other, as yet unknown, environmental factors (Devlin, Nagahama, 2002). In 1992, a new case of sex reversal was described in which a putative recessive mutation mas^{-1} caused female to male sex reversal in homozygous condition (Komen et al., 1992a). The inheritance of this mutation was described in the same clones and backcrosses as used in the present study. Crossing a mas^{+}/mas^{-1} (E4E5) female with a homozygous mas^{-1}/mas^{-1} (E5) male resulted in approximately 50% male mas^{-1}/mas^{-1} and 50% (pooled) intersex fish and females (mas^{+}/ mas^{-1}). In this study we confirm that corticosterone has a significant effect on sex determination, suggesting that the two traits (i.e. "high corticosterone" and "male sex reversal") are either linked or caused by pleiotropic effects of the mutation responsible for

17α-hydroxylase deficiency. In an earlier study, Ruane et al. (2005) concluded that masculinization was not caused by hypocorticism and interrenal hyperplasia, but this conclusion was based on the fact that these authors could not convincingly demonstrate the production of androgens by the interrenal.

It is unlikely that corticosterone is directly responsible for sex reversal. Corticosteroids do not play a role during sex reversal (Devlin and Nagahama, 2002) and most teleosts only produce minute if any, amounts of corticosterone (Sturm et al., 2005). The mechanism by which 17α-hydroxylase deficiency causes sex reversal therefore remains elusive. In a recent paper, Zhou et al., (2007) identified a second CYP17-II (P450c17a2) which is expressed in the Nile tilapia gonad during the onset of meiosis. The expression pattern is sex specific as the onset of male and female meiosis differs by 50 days ph. P450c17a1 on the other hand is already expressed in males and females from hatch. If 17α-hydroxylase deficiency is caused by a defect in P450c17a2 (chapter 4, this thesis), then it is likely that the onset in meiosis in common carp gonads is also affected. Earlier we have shown that meiosis in XX, *mas-1/mas-1* animals follows a male specific pattern (Komen et al., 1992b).

In conclusion, the observed masculinization (Komen et al., 1992a), low amount of plasma cortisol after stress, enlarged head kidney (Ruane et al., 2005) and high amount of plasma corticosterone after stress (Nematollahi et al., 2006) in E5 and E7 fish together strongly suggest that common carp suffering from 17α-hydroxylase deficiency show all the classical symptoms of congenital adrenal hyperplasia as seen in humans (Miller, 1991).

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References

Bongers, A.B.J., Zandieh Doulabi, B., Richter, C.J.J., Komen, J., 1999. Viable androgenetic YY genotypes of common carp (*Cyprinus carpio L.*). Journal of Heredity 90, 195-198.

Deaton, M.A., Glorioso, J.E., McLean, D.B., 1999. Congenital adrenal hyperplasia: not really a zebra American Family Physician 59, 1190-1196.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208 191-364.

Gimeno, S., Gerritsen, A., Bowmer, T., Komen, J., 1996. Feminization of male carp. Nature 384, 221-222.

Gotoh, H., Sagai, T., Hata, J., Shiroishi, T., Moriwaki, K., 1988. Steroid 21-hydroxylase deficiency in mice. Endocrinology 123, 1923-1927.

Institute, S., 1989. User's Guide; Statistics, Version 6.12 Edition. SAS institute, Cary, NC.

Komen, H., Thorgaard, G.H., 2007. Androgenesis, gynogenesis and the production of clones in fishes: A review. Aquaculture 269, 150-173.

Komen, J., de Boer, P., Richter, C.J.J., 1992a. Male sex reversal in gynogenetic XX females of common carp (*Cyprinus carpio L.*) by a recessive mutation in a sex-determining gene. Journal of Heredity. 83, 431-434.

Komen, J., Yamashita, M., Nagahama, Y., 1992b. Testicular development induced by a recessive mutation during gonadal differentiation of female common carp. Development, Growth & Differentiation 34, 535-544.

Komen, J., Bongers, G., Richter, C.J.J., Huisman, E.A., 1991. Gynogenesis in common carp (*Cyprinus carpio L.*): II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92, 127-142.

Miller, W., 1991. Congenital adrenal hyperplasia. Endocrinology and metabolism clinics of North America 20, 721-749.

Nematollahi, M.A., van Pelt, H., Komen, J., 2006. Stress response during and after confinement in 17-a hydroxylase deficient common carp (*Cyprinus carpio L*), VII th International Congress on the Biology of Fish, Newfoundland, Canada, P170.

New, M.I., 1992. Genetic disorders of adrenal hormone synthesis. Hormone Research. 37, 22-33.

Pang, S., Yang, X., Wang, M., Tissot, R., Nino, M., Manaligod, J., Bullock, L., Mason, J., 1992. Inherited congenital adrenal hyperplasia in the rabbit, absence cholestrol side-chain cleavage cytochrome P450 gene expression. Endocrinology 131, 181-186.

Ruane, N.M., Huisman, E.A., Komen, J., 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. Journal of Fish Biology 59, 1-12.

Ruane, N.M., Goos, H.J.T., Komen, J., 2007. Stress-induced facilitation of the cortisol response in 17?-hydroxylase deficient XX mas-1/mas-1 carp (*Cyprinus carpio*). General and Comparative Endocrinology 150, 473.

Ruane, N.M., Lambert, J.G.D., Goos, H.J.T., Komen, J., 2005. Hypocorticism and interrenal hyperplasia are not directly related to masculinization in XX mas-1/mas-1 carp (*Cyprinus carpio*). General and Comparative Endocrinology 143, 66-74.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E., Prunet, P., 2005. 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146, 47-55.

Tanck, M.W.T., Vermeulen Kees, J., Bovenhuis, H., Komen, J., 2001. Heredity of stress-related cortisol response in androgenetic common carp (*Cyprinus carpio L.*). Aquaculture 199, 283-294.

Tanck, M.W.T., Claes, T., Bovenhuis, H., Komen, J., 2002. Exploring the genetic background of the stress response using isogenic progenies of common carp selected for high or low stress related cortisol. Journal of Aquaculture 204, 419-434.

Tanck, M.W.T., Booms, G.H.R., Eding, E.H., Wendelaar Bonga, S.E., Komen, J., 2000. Cold shocks: A stressor for common carp. Journal of Fish Biology 57, 881-894.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiological Reviews 77, 591-625.

Zhou, L.Y., Wang, D.S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., Nagahama, Y., 2007. A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology 148, 4282-4291.

Chapter Six:

General Discussion

The overall objective of this thesis was to generate a better understanding of the causes and consequences of cortisol insufficiency in a lower vertebrate, the common carp (*Cyprinus carpio* L.). Cortisol insufficiency is the main characteristic of the congenital adrenal hyperplasia (CAH) syndrome in humans. CAH is defined as the inability of the adrenal gland to synthesize cortisol in response to stress. Adrenal hyperplasia results from continued ACTH stimulation of the adrenal gland, caused by the absence of the negative feedback exerted by cortisol. CAH happens when one of the enzymes involved in the steroidogenic pathway to produce cortisol doesn't work properly. In humans, adrenal hyperplasia is often accompanied with an increase in the production of sex steroids, mainly androgens, which causes masculinization of external genitalia in (baby) girls.

In lower vertebrates, adrenal (interrenal) hyperplasia was until recently unknown. Interrenal hyperplasia was first described in a female to male sex reversed XX common carp strain, "E5" (Ruane et al 2005; 2007). In this isogenic strain, 17α-hydroxylase was found to be dysfunctional, inhibiting the production of cortisol, and causing interrenal hyperplasia. This thesis describes a series of experiments investigating the cause (s) and effects of 17α-hydroxylase insufficiency in this strain. The aims were:

- To sequence the P450c17 gene in head kidney of common carp and search for functional mutations.

-To further characterize the stress response at the gene regulation level in the head kidney of a 17α-hydroxylase deficient common carp.

-To study the inheritance of 17α-hydroxylase insufficiency in backcross and gynogenetic progenies of common carp and look for a causative relation with sex reversal.

Here, we will discuss the current views concerning the structure, regulation and function of P450c17 in teleost head kidneys and gonads (1) and explore ways in which P450c17 could cause sex reversal in common carp and teleost fish in general (2, 3).

6.1 One or more *CYP17* genes

P450c17 is a microsomal enzyme, catalyzing at least two distinct activities, 17α-hydroxylase and 17, 20 lyase, essential for the biosynthesis of glucocorticoids in the adrenals and sex steroid precursors in the gonads. This dual activity of P450c17 has been subject of many studies. In mammals, this tissue and developmental specific action of P450c17 has been attributed to specific posttranslational modifications of the enzyme. Alternatively, the existence of P450c17 isoenzymes has been proposed to explain this dual function. However, in mammals, there appeared to be only one P450c17a1, encoded by a single gene *CYP17-I*, expressed both in adrenals and gonads.

In teleost fish species additional types of P450c17 have been identified. Recently, two *CYP17* genes were found by an *in silico* analysis from the genomes of six species (Zhou et al., 2007). *CYP17-I* is expressed in the gonads and possesses both the 17α-hydroxylase and 17, 20 lyase activities. *CYP17-II* is expressed in head kidney and gonads and is only involved in the hydroxylation reaction. The spatial and temporal expression patterns suggest that in teleosts, P450c17a2 is responsible for the synthesis of C21 steroids, such as cortisol in the head kidney.

In rice field eel, 4 different isoforms of *CYP17a1* gene transcripts have been identified in the gonads (Yu et al., 2003). Rice field eel has the characteristic of natural sex reversal from female via intersex into male during its life. Northern blot and in situ hybridization studies indicate that the expression *CYP17a1* is differentially regulated during gonad development, suggesting that these 4 transcripts play an important role in gonad differentiation during sex reversal. Three transcripts were generated by alternative splicing and one by alternative polyadenylation. Alternative spicing occurred only in the 3' region of the gene. These 4 isoforms may encode different length of proteins, missing the C-terminal part of the complete P450c17 enzyme. In particular, the C-terminal region of P450c17 is involved in the heme-binding and absolutely required for hydroxylase and lyase activity, suggesting that proteins translated from the 4 transcripts in eel have different functions. For example in mice, P450c17 possesses in addition to the 17a-hydroxylase/17, 20 lyase activity, squalene monooxygenase activity, involved in cholesterol synthesis.

Interestingly, in common carp we identified an additional variant (*CYP17a1.II*) of the *CYP17a1* gene. This *CYP17a1.I* gene consists of 8 exons and 7 introns. The *CYP17a1.II* variant is missing exon 7 and 8.

Due to essential functions the *CYP17* genes are evolutionary conserved among vertebrates. Two whole genome duplications in the evolutionary history of vertebrates and the additional duplication in the common carp genome may explain the existence of three *CYP17* gene variants in common carp. Analysis of all known *CYP17* sequences revealed at least that the *CYP17a2* gene exists only in teleost fish (Zhou et al., 2007), and is the result of neo-functionalization of an ancestral duplicated gene. The two forms of CYP17a1 might be the result of a more recent allotetraploidization event that gave rise to the common carp genome. Whether the two forms a1.I and a1.II are both functional variants of *CYP17*, or whether the truncated form is an inactive pseudogene, remains to be investigated. Variant *CYP17a1.I* of carp is expressed in the head kidney and in gonads. Whether variant *CYP17a1.II*, missing exon 7 and 8, is a structural gene has to be investigated. Like in rice field eel, different isoforms of *CYP17* transcripts could be generated from this variant and play a role in cortisol synthesis and sex steroid synthesis.

6.2 Sex determination and sex reversal in common carp

It is thought that sex determination in fish is polygenic, with major and minor genes located on the sex and autosomal chromosomes. As in mammals, sex determination in common carp seems to be primarily controlled by a male dominant gene, termed “Y” (Bongers et al., 1999; Komen et al., 1991). Males are heterozygous XY while females are homozygous recessive “XX”. Homozygous YY males can be produced by androgenesis and are fertile. They produce all male progeny when crossed with normal females. However, sex differentiation is labile, particularly in females, and sex reversal can be induced by endocrine manipulation (Gimeno et al., 1996) and by environmental factors like temperature (Devlin, Nagahama, 2002). In 1992, a second, minor, gene was identified that causes male sex reversal in female carps. A genetic study indicated that this sex reversal was inherited as a recessive mutation,

which was termed *mas-1* (Komen et al., 1992a*)*. The *mas-1* gene does not affect male sex differentiation but homozygous females develop as males or intersexes. Heterozygous XX, *mas-1/+* animals are female or sometimes intersex, depending on rearing conditions (crowding, temperature).

In chapter 5, we described the results of backcrosses of E5 fish on heterozygous E4E5 animals. In terms of *mas*, this is a cross of *mas-1/mas-1* males with *mas-1/+* females, and should produce 50 % *mas-1*/*mas-1* males and 50 % *mas-1/+* females. However, progeny was mostly intersex or male. This means that either the *mas-1/+* genotype is also sensitive to sex reversal, or sex reversal is more common in highly inbred carp. Our results are different from the original results described in (Komen et al., 1992a), in which E5 males were crossed with either *mas-1/+* or *+/+* outbred females. In these crosses, *mas-1/+* offspring were predominantly female. This suggests that part of the observed sex reversal is caused by the highly inbred condition of the progeny. Doubled haploid progeny of *mas-1/+* females are also homozygous and expected to consist of 50 % *mas-1/mas-1* males and 50 % *+/+* females. Although there were significantly more females in this group than in the backcross groups, the sex ratio was again skewed towards males and intersexes. However, the linkage between high and low corticosterone and sex in this group was highly significant (chapter 5). This strongly suggests a role of 17α-hydroxylase deficiency in sex reversal in *mas-1/mas-1* carp.

6.3 P450c17 and sex reversal in fish

Steroid production during sexual differentiation in carp is characterized by the production of estrogens in ovaries and the production of 11 hydroxylated androgens in testes, from day 60 onwards (at 25 ºC). Androstenedione is the precursor of these sex steroids and is produced by the 17α-hydroxylase and 17, 20 lyase activities of P450c17.

Most of our knowledge on the action of P450c17 however comes from studies on the steroidogenic shift in teleost ovarian follicles prior to ovulation: in follicles surrounding vitellogenic oocytes, granulosa cells produce estrogens, but for a brief period just before ovulation they produce 17, 20 dihydroxy-4-pregnen-3-one, which is the maturational steroid

in teleost fish. This shift is caused by a reduction in 17, 20 lyase activity and a concomitant increase in 20β-hydroxylase activity (Fig. 6.1).

Recently, Zhou et al. (2007) showed that P450c17a2 lacks 17, 20 lyase activity and is expressed in the interrenal. In tilapia gonads, expression of P450c17a1 is confined to granulosa cells surrounding growing oocytes. Granulosa cells from mature follicles produce mainly P450c17a2. This confirms the idea that the steroidogenic shift is orchestrated by up- and down regulation of two different P450c17 genes.

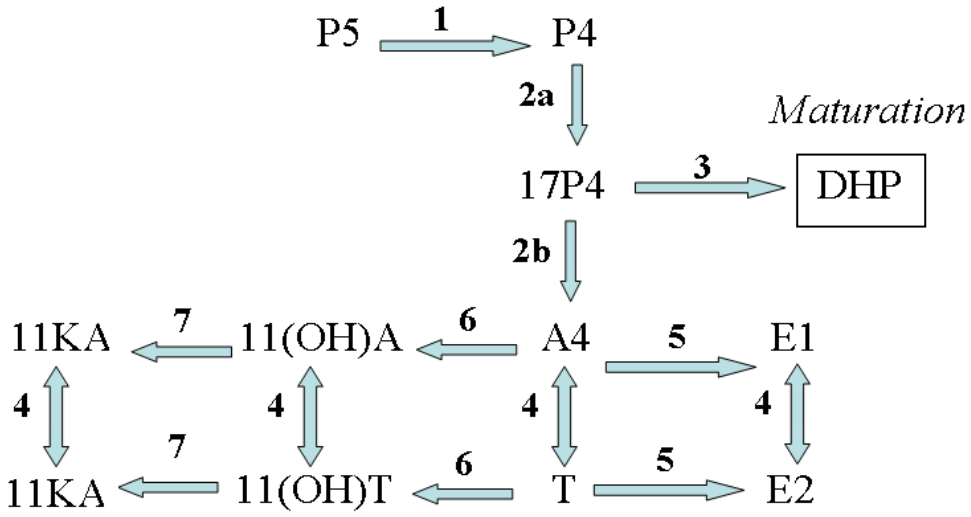

Fig. 6.1 The major steroid biosynthetic pathways in the gonads. The substrate abbreviations; P5: Pregnenolone, P4: Progesterone, 17P4: 17α-hydroxyprogesterone, DHP: 17α, 20β-dihydroxy-4-pregnen-3-one, A4: Andrestenedione, E1: Estrone, E2: Estradiol, T: Testosterone, 11 (OH) A: 11-hydroxyandrostenedione, 11 (OH)T : 11β-hydroxytestosterone, 11KA:11-keto Androstenedione, 11KT: 11-ketoTestosterone. The enzyme numbers; 1: 3β-HSD, 2a: 17a-hydroxylase, 2b: C17, 20 lyase, 3: 20β-HSD, 4: 17β-HSD, 5: Aromatase, 6: 11β-hydroxylase, 7: 11β-HSD.

During sexual differentiation, P450c17a1 was expressed in gonads of XX and XY fish from 5 dph and continued to be expressed strongly in gonads both in adult male and female. The expression of P450c17a2 however was sexual dimorphic, with early expression in females and later expression in males, matching the pattern of sex specific meiosis (Zhou et al., 2007). Interestingly, 17α, 20β dihydroxy-4-pregnen-3-one has been found to be an essential factor in the initiation of meiosis in Japanese eel (Miura et al., 2006).

If we generalize the findings to carp oogenesis and spermatogenesis, according to (Komen et al., 1992b) onset of meiosis in carp female and male should coincide with a peak in the expression of P450c17a2. When we assume that P450c17a2 exhibits only 17α-hydroxylase activity in common carp, and that in E5 carp, expression of P450c17a2 is defective, then meiosis in common carp gonads should also be affected. However, this seems to be not the case.

Sex differentiation in carp can be described based on the morphology of the ovarian lumen and timing of meiotic entry and sexual phenotype of germ cells. In female gonads, sexual differentiation is first seen by the formation of an oviduct: the club-shaped gonad forms a proximal ridge which fuses with the coelomic wall to form an enclosed tube-like cavity. At 25 ºC, meiosis (oogenesis) commences between 60-70 dph and is first observed in the anterior part of the gonad. Normal male gonads do not form an oviduct, and meiosis (spermatogenesis) starts about one month later. By day 100, most germ cells have entered spermatogenesis, and between 110 and 120 dph the gonad has developed into a typical testis, made up of numerous cysts containing spermatocytes or spermatids and fused cysts filled with mature spermatozoa.

Gonads of *mas-1/mas-1* animals initially differentiate as a normal female gonad with the development of a proximal extension of stromal cells indicating the formation of an ovarian cavity. However, the ovarian lumen is often only partly closed. Meiosis starts approximately at the same time as in normal females but, next to cysts with prophase oocytes, cysts containing spermatogonia type B also appear, and by 90 dph (at 25 ºC), the intersex development of this type of gonad is evident. Due to active spermatogenesis these gonads rapidly increased in size, becoming twice the size of a normal gonad of that age. Mature spermatozoa are observed at 100 dph while in the same area many pre-vitellogenic oocytes can be found (Komen et al., 1992b). The main difference between *mas-1* testis development and normal testis development is the onset of meiosis, which is earlier, compared to normal males. These observations do not point towards a defect in the production of a meiosis inducing substance like e.g. 17, 20 dihydroxy-4-pregnen-3-one. It is therefore unlikely that 17α-hydroxylase deficiency, if caused by a defect in the regulation of P450c17a2 expression,

is also responsible for the observed sex reversal through a sex specific failure to induce meiosis.

In the rice field eel, P450c17 alternative splicing (see earlier) in gonads is observed to accompany sex reversal from female to male in this species. In this paper (Yu et al., 2003), the authors speculate that sex determination and differentiation cascade is regulated by alternative splicing at the level of transcription. The expression patterns of the P450c17 (dominantly expressed in testis, less in ovary and least in ovotestis) are consistent with the sex reversal process of the rice field eel. Moreover, the expression of the gene is restricted to a key region of sex differentiation, the germinal lamella (the gonadal epithelium), from where different germ cells will differentiate (Yu et al., 2003). It is not known whether rice field eel also possess P450c17a2 or whether the authors only observed P450c17a1.

Further studies are needed to clarify if alternative splicing in P450c17a1.I in gonads of common carp and if these patterns are different between normal and *mas-1* carp.

The most interesting finding of this thesis was that 17α-hydroxylase deficient carp produce huge amounts of plasma corticosterone. Although some studies introduced corticosterone and possibly deoxycorticosterone (DOC) as the ancestral ligand for mineralocorticoids (Baker, 2003) and DOC as the most potent activator of mineralocorticoid receptors in trout (Sturm et al., 2005), there is still a question if corticosterone exerts any endocrine or physiological function in common carp.

It is tempting to speculate that the accumulation of progestins like progesterone (Ruane et al., 2005) and corticosterone cause sex reversal either directly or via local conversion in the gonad. Female gonads produce relatively little estrogen, while *mas-1* gonads are capable of producing both 11 hydroxy androgens and estrogens. Sex reversal in mas-1 gonads might be caused by dosage dependent conversion of progesterone in androstenedione but it is not clear why this should be converted into androgens and not estrogens. Alternatively corticosterone might be directly responsible for sex reversal. However, there are no studies in fish that have tested this hypothesis.

6.4 Conclusions

Our isogenic strains of common carp E5 and E7, which show hypocorticism and interrenal hyperplasia, are a useful model for studying the Congenital Adrenal Hyperplasia syndrome in fish. Our results confirm the finding of (Ruane et al., 2005) that hypocorticism in E5 carp is caused by 17α-hydroxylase dysfunction. Expression patterns of several genes involved in the steroidogenic partway for cortisol were all up-regulated in E5. Expression of P450c17a1 however was ambiguous. This suggests that the 17α-hydroxylase dysfunction might be caused by a failure to express sufficient amount of the enzyme, rather than by a defect in the enzyme itself.

We furthermore showed that E5 carp produce large amounts of corticosterone during confinement stress. Thus, corticosterone is a reliable marker to distinguish CAH animals from normal carp. Using corticosterone as a marker, we were able to show that high corticosterone is inherited as a recessive trait, and that it co-segregates with the hypothetical *mas-1* factor, which causes female to male sex reversal. However, at present we do not have a clear hypothesis linking 17α hydroxylase deficiency to sex reversal in *mas-1/mas-1* animals.

Due to the complexity of the P450c17 gene and the finding of a new variant of P450c17a1.II in carp, it was not possible to relate the 17a-hydroxylase deficiency to a specific mutation in the gene. Sequencing of the recently discovered P450c17a2 in common carp is in progress. Knowing the structure and following the expression of this P450c17a2 gene in head kidneys during stress and in gonads during sex differentiation in normal and E5 carp will eventually determine the cause of hypocorticism and its relation with sex reversal in carp.

References

Baker, M.E., 2003. Evolution of Glucocorticoid and Mineralocorticoid Responses: Go Fish. Endocrinology 144, 4223-4225.

Bongers, A.B.J., Zandieh Doulabi, B., Richter, C.J.J., Komen, J., 1999. Viable androgenetic YY genotypes of common carp (*Cyprinus carpio L.*). Journal of Heredity 90, 195-198.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208 191-364.

Gimeno, S., Gerritsen, A., Bowmer, T., Komen, J., 1996. Feminization of male carp. Nature 384, 221-222.

Komen, J., de Boer, P., Richter, C.J.J., 1992a. Male sex reversal in gynogenetic XX females of common carp (*Cyprinus carpio L.*) by a recessive mutation in a sex-determining gene. Journal of Heredity. 83, 431-434.

Komen, J., Yamashita, M., Nagahama, Y., 1992b. Testicular development induced by a recessive mutation during gonadal differentiation of female common carp. Development, Growth & Differentiation 34, 535-544.

Komen, J., Bongers, G., Richter, C.J.J., Huisman, E.A., 1991. Gynogenesis in common carp (*Cyprinus carpio L.*): II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92, 127-142.

Miura, T., Higuchi, M., Ozaki, Y., Oath, T., Miura, C., 2006. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. Proceedings of the National Academy of Sciences of the United States of America 103, 7333-7338.

Ruane, N.M., Lambert, J.G.D., Goos, H.J.T., Komen, J., 2005. Hypocorticism and interrenal hyperplasia are not directly related to masculinization in XX mas-1/mas-1 carp (*Cyprinus carpio)*. General and Comparative Endocrinology 143, 66-74.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E., Prunet, P., 2005. 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146, 47-55.

Yu, H., Cheng, H., Guo, Y., Xia, L., Zhou, R., 2003. Alternative splicing and differential expression of P450c17 (CYP17) in gonads during sex transformation in the rice field eel. Biochemical and Biophysical Research Communications 307, 165-171.

Zhou, L.Y., Wang, D.S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A., Sakai, F., Nagahama, Y., 2007. A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. Endocrinology 148, 4282-4291.

Appendices

Summary

In humans, Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive inherited defect in the production of cortisol. The low level of cortisol in the blood stimulates the pituitary gland to secrete the hormone ACTH, Adrenocorticotropic Hormone, in an attempt to restore the level of cortisol to normal. Because of the block in production of cortisol this does not occur but the stimulation causes enlargement of the adrenal glands and an excessive production of androgen

In this thesis we describe adrenal hyperplasia in a teleost fish, the common carp, *Cyprinus carpio, L,* caused by 17α hydroxylase deficiency. Two homozygous, XX male strains, "E5" and "E7", consistently show 5-10 fold less plasma cortisol in response to net confinement compared to other male and female strains. They have enlarged head kidneys, which is caused by a 2-3 fold increase in number of interrenal cells. The experiments and results described in this thesis aim to provide further insight into the genetic background of the 17α-hydroxylase deficiency, and the expression of genes involved in the stress response in P450c17 deficient common carp during and after stress.

The *CYP17a1* gene codes for the cytochrome P450c17 enzyme. In **Chapter 2,** we describe the sequencing of the 17α-hydroxylase/17, 20 lyase gene, CYP17a1, in a standard (STD) and the mutant strain (E5) of common carp. PCR amplifications were performed with genomic and cDNA as template. Two variants of the CYP17a1 gene were found. The nucleotide coding region of the *CYP17*a1.I variant contained 8 exons and 7 introns. In total 3122 base pairs of the nucleotide sequence were determined. The intron-exon structure of this variant was identical to the human CYP17. Variant CYP17a1.II is missing exon 7 and part of exon 8 and contains an insertion and SNP's in intron 6. Variant I showed no differences between STD and E5, while variant II showed 8 homozygous SNP's (one of them was specific for E7 carp) and a 3 bp insertion downstream of exon 6. In **Chapter 3,** we investigated the expression of five genes involved in the cortisol production in head kidneys of normal ("standard ") common carp in response to a 3 hr net confinement stress. mRNA levels of StAR, P450c17a1.I, 3β-HSD, P450c21 and 11β-HSD2 were quantified by real time RT-PCR. Results show that the plasma cortisol level was 85-fold higher than in the unstressed control

at one hour post-stress and quickly returned to normal after 4 hours recovery. This increase and decrease in cortisol levels was correlated with mRNA levels of 11β-HSD2 during and after confinement, suggesting that 11β-HSD2, which oxidizes cortisol to cortisone, is involved in the regulation of cortisol concentrations. StAR, P450c17a1.I and P450c21 mRNA levels did not change during net confinement stress and subsequent recovery. 3β-HSD levels increased 20 minutes after confinement. These results suggest that cortisol biosynthesis at the transcriptional level is partly regulated by 3β-HSD. In **Chapter 4**, we describe the stress response and gene expression in 17α-hydroxylase deficient common carp. The results show a significant increase of corticosterone levels in response to stress due to dysfunction of 17α-hydroxylase activity. Standard carp shows a correlation between cortisol production and 11β-HSD2 mRNA expression during the stress response. In contrast, 11β-HSD2 expression is significantly lower in E5 fish and stays at a constant level during confinement. Real-time PCR analysis of StAR and 3β-HSD show significantly higher expression in E5, for StAR at 1 hour and for 3β-HSD at 5 and 20 minutes, and 1 hour net confinement, and at 4 hours recovery. No differences in expression levels of P450c21 were found between E5 and standard fish, suggesting that transcription of P450c21 is not a limiting step in corticosterone production. Finally, we analyzed the changes in mRNA levels in P450c17a1.I between E5 and standard fish during confinement. Although the data are quite variable, there was a trend towards lower accumulation levels in E5, suggesting a dysfunction at the transcriptional level. In **Chapter 5,** we investigate the inheritance of interrenal hyperplasia and low cortisol response using backcross and gynogenetic progeny of heterozygote carriers of common carp. The inbred strains used in this experiment were E4 (XX, normal), E5 (XX, interrenal hyperplasia) and E7 (XX, interrenal hyperplasia). Heterozygote carriers were produced by crossing E4 with E5 (E4E5: XX). E4E5 females were crossed with E5 and E7 animals (BC5 and BC7), and or reproduced by gynogenesis to produce doubled haploids (DH). Progeny were raised and at 6 months subjected to a net confinement stressor. Fish were sacrificed after one hour net-confinement, blood sampled, and fish dissected to determine sex, and head kidney-somatic index. Values for cortisol and head kidney index showed a continuous distribution in BC and DH progeny. Values for corticosterone, on the other hand showed a very clear segregation pattern, consistent with a single gene model. There was a significant difference in mean cortisol level between high (H) and low (L) corticosterone responders in

BC and DH progeny groups. Mean values for H responders in the 3 groups BC5, BC7 and DH were respectively: corticosterone 1706, 1760 and 1366 ng/ml, and for cortisol: 21.4, 24 and 17.3 ng/ml. For L responders values were: corticosterone 1.5, 1.1 and 0.6 ng/ml, and for cortisol: 66.7, 105.1 and 65.2 ng/ml respectively. Head kidney index was not significantly different between H and L groups, but sex was. H responders were predominantly male, while L responders were female or intersex. These results confirm that 17α-hydroxylase deficiency is inherited as a recessive mutation, and that 17α deficiency and sex reversal are either caused by the same mutation (pleiotropy) or by closely linked genes.

In conclusion, 17α-hydroxylase deficient common carp show all the classic symptoms of congenital adrenal hyperplasia (CAH): masculinization, low amounts of plasma cortisol and high amounts of plasma corticosterone after stress and enlarged head kidney. We also confirmed that 17α hydroxylase deficiency is inherited as a recessive mutation. However, we did not find the mutation responsible for 17α-hydroxylase deficiency. Recently (2007), a novel type CYP17a2 has been identified in several teleost fish (medaka, zebra fish, fugu, stickleback and tilapia). This gene differs in gene structure from our variant CYP17a1.I and CYP17a1.II and possesses only the 17α-hydroxylase activity. In situ hybridizations show that this CYP17a2 is responsible for the synthesis of cortisol in the interrenal of medaka and Nile tilapia. CYP17a2 also appears to be involved in the onset of meiosis in tilapia. These observations suggest that 17α-hydroxylase deficiency in common carp is caused by a mutation in CYP17a2, and that this mutation also causes sex reversal by interfering with the onset of meiosis in female gonads. Cloning attempts to identify CYP17a2 in common carp are currently in progress.

Samenvatting

Adrenogenitaal syndroom (AGS) is een autosomaal recessief verervend defect in de productie van cortisol. Het lage niveau van cortisol in het bloed stimuleert de hypofyse tot de secretie van ACTH, adrenocorticotroop hormoon, in een poging het cortisol niveau te herstellen tot een normale waarde. Door het defect in de productie van cortisol gebeurt dit niet, maar de stimulatie veroorzaakt wel een vergroting van de bijnieren en een overmatige productie van androgeen.

In dit proefschrift beschrijven wij een door 17α hydroxylase deficiëntie veroorzaakt adrenogenitaal syndroom in een beenvis, de karper, *Cyprinus carpio L*. Twee homozygoot mannelijke XX lijnen, "E5" en "E7", vertonen in vergelijking met andere mannelijke en vrouwelijke lijnen consistent een 5-10 maal lager plasma cortisol niveau in reactie op stress. Deze twee lijnen hebben vergrote kopnieren, veroorzaakt door een 2-3 maal sterke toename van hormoon producerende bijnier cellen. De experimenten en resultaten, zoals beschreven in dit proefschrift, hebben als doel een verder inzicht te verschaffen in enerzijds de genetische achtergrond van de 17α hydroxylase deficiëntie en anderzijds de expressie van genen betrokken bij de stress respons van 17α hydroxylase deficiënte karper, zowel tijdens als na stress.

Het CYP17a1 gen codeert voor het cytochroom P450c17 enzym, ook wel bekend als 17α hydroxylase / C17-20 lyase. In **Hoofdstuk 2** beschrijven wij het bepalen van de base volgorde van CYP17a1, in een standaard (STD) en gemuteerde (E5) lijn van de karper. PCR amplificatie werden gedaan met genomisch en cDNA als template. Twee varianten van het CYP17a1 gen werden gevonden. De coderende regio van de CYP17a1.I variant bevatte 8 exonen en 7 intronen. In totaal werden 3122 base paren van de nucleotide volgorde bepaald. De intron-exon structuur van deze variant was identiek aan de humane CYP17. Variant CYP17a1.II mist exon 7 en een deel van exon 8 en bevatte een insertie en SNP's in intron 6. Variant 1 toonde geen verschillen tussen STD en E5, terwijl variant II 8 homozygote SNP's (waarvan 1 specifiek voor E7 karper) en een 3 bp insertie na exon 6 toonde. In **Hoofdstuk 3** hebben we de expressie onderzocht van 5 genen betrokken in de cortisol productie in kopnieren van normale ("standaard") karper in reactie op een 3-uur durende opsluiting in een net. Messenger RNA niveaus van StAR, P450c17a1.I, 3β-HSD, P450c21 en 11β-HSD2

werden gekwantificeerd door middel van RT-PCR. De resultaten toonden dat het niveau van plasma cortisol een uur na de stressor 85 maal hoger was dan in niet gestreste controle dieren en dat dit plasma cortisol na een 4-uur durend herstel weer op een normaal niveau was. De toe- en afname in cortisol niveau was gecorreleerd aan het RNA niveau van 11β-HSD2, zowel tijdens als na opsluiting. Dit suggereert dat 11β-HSD, wat cortisol oxideert tot cortison, betrokken is bij de regulatie van cortisol concentraties. StAR, P450c17a1.I en P450c21 mRNA niveaus veranderden niet tijdens de stress door opsluiting in een net en het daaropvolgende herstel. Het 3β-HSD niveau steeg 20 minuten na opsluiting. Deze resultaten suggereren dat cortisol biosynthese op transcriptie niveau deels gereguleerd wordt door 3β-HSD. In **Hoofdstuk 4** beschrijven wij de stress respons en gen expressie in 17α hydroxylase deficiënte karper. De resultaten tonen een significante toename in corticosteron niveau in bloed plasma in reactie op stress, veroorzaakt door een niet functionerende 17α-hydroxylase activiteit. Standaard karper vertoont een significante correlatie tussen cortisol productie en 11β-HSD2 mRNA expressie tijdens de stress respons. Echter, de 11β-HSD2 expressie is significant lager in E5 dieren en blijft op een constant niveau tijdens opsluiting. RT-PCR analyse van StAR en 3β-HSD toonde een significant hogere expressie in E5; voor StAR na 1 uur en voor 3β-HSD na 5, 20 en 60 minuten opsluiting en na 4 uur herstel. Tijdens opsluiting werden er geen verschillen in expressie van P450c21 gevonden tussen E5 en standaard dieren, wat er op kan duiden dat de transcriptie van P450c21 geen limiterende stap is in corticosteron productie. Tot slot hebben we de veranderingen geanalyseerd in mRNA niveaus van P450c17a1.I tussen E5 en standaard dieren tijdens opsluiting. Ondanks dat er vrij veel variatie in de data aanwezig was, was er een trend naar lagere accumulatie niveaus in E5; dit suggereert dat er een probleem op transcriptie niveau is. In **Hoofdstuk 5** onderzoeken we de overerving van bijnier hyperplasie en lage cortisol respons door gebruik te maken van terugkruisingen en gynogenetische nakomelingen van heterozygote dragers. De inteelt lijnen, gebruikt in dit experiment, zijn E4 (XX, normaal), E5 (XX, interrenale hyperplasie) en E7 (XX, interrenale hyperplasie). Heterozygote dragers werden geproduceerd door E4 met E5 te kruisen (E4E5: XX). Vrouwelijke E4E5 werden gekruist met E5 en E7 dieren (BC5 en BC7) en/of verkregen door gynogenese om dubbele haploiden te produceren (DH). Nakomelingen werden op een leeftijd van 6 maanden blootgesteld aan stress door opsluiting in een net. De vissen werden gedood na een uur opsluiting. Een bloedmonster werd genomen waarna de

dieren werden ontleed om het geslacht en de kopnier-somatische index te kunnen bepalen. De waarden van cortisol en kopnier-somatische index vertoonden een continue verdeling in BC en DH nakomelingen. Echter, waarden voor corticosteron vertoonden een duidelijk segregatie patroon, consistent met een enkel gen model. Er was een significant verschil tussen gemiddelde cortisol niveaus van hoog (H) en laag (L) corticosteron producerende dieren in BC en DH nakomelingen. Gemiddelde waarden van H dieren in de drie groepen, BC5, BC7 en DH, waren achtereenvolgens: corticosteron 1706, 1760, en 1366 ng/ml en voor cortisol: 21.4, 24 en 17.3 ng/ml. De volgende waarden werden gevonden voor L dieren: corticosteron 1.5, 1.1, en 0.6 ng/ml en voor cortisol: 66.7, 105.1 en 65.2 ng/ml. De kopnier-somatische index was niet significant verschillend tussen H en L groepen, maar het geslacht was dat wel. H dieren waren voornamelijk mannelijk, terwijl L dieren vooral vrouwelijk of hermafrodiet waren. Deze resultaten bevestigen dat 17α hydroxylase deficiëntie overerft als een recessieve mutatie en dat 17α hydroxylase deficiëntie en geslachtsomkeer veroorzaakt worden door dezelfde mutatie (pleiotropie) of door sterk gekoppelde genen.

Concluderend, 17α hydroxylase deficiënte karpers vertonen alle klassieke symptomen van het adrenogenitaal syndroom (AGS): geslachtsomkeer, lage niveaus van plasma cortisol en hoge niveaus van plasma corticosteron na stress, en vergrote kopnieren. We hebben ook bevestigd dat 17α hydroxylase deficiëntie overerft als recessieve mutatie. Echter, we hebben niet de mutatie gevonden die verantwoordelijk is voor 17α hydroxylase deficiëntie. Onlangs (2007) is een nieuw type CYP17a2 geïdentificeerd in een aantal beenvissen (medaka, zebravis, fugu en tilapia). Dit gen verschilt in genstructuur van onze varianten CYP17a1.I en CYP17a1.II en bezit alleen 17α hydroxylase activiteit. In situ hybridisatie laat zien dat dit CYP17a2 verantwoordelijk is voor de synthese van cortisol in de interrenale cellen van medaka en Nijl tilapia. Daarnaast lijkt CYP17a2 ook betrokken bij de meiose in tilapia. Deze bevindingen suggereren dat 17α hydroxylase deficiëntie in karper veroorzaakt wordt door een mutatie in CYP17a2 en dat deze mutatie ook geslachtsomkeer veroorzaakt door middel van verstoring van de meiose in vrouwelijke gonaden. Pogingen om CYP17a2 in karper te kloneren worden momenteel ondernomen.

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Mohammad Ali Nematollahi

Wageningen, The Netherlands

March 2008, Esfand 1386

List of Publications

Peer-Reviewed Papers

Nematollahi, MA., Crooijmans, R.P.M.A., Bergsma, J., van Pelt-Heerschap, H., Komen, H., Sequence characterization of CYP17 gene in common carp (*Cyprinus carpio L.), to be submitted.*

Nematollahi, MA., van Pelt-Heerschap, H., Komen, H., Quantitative expression analysis of genes involved in the stress response in head kidneys of common carp (*Cyprinus carpio L.*) following net confinement, *to be submitted.*

Nematollahi, MA., van Pelt-Heerschap, H., Komen, H., Stress response in head kidneys of a 17α-hydroxylase deficient common carp (*Cyprinus carpio L.*): cortisol, corticosterone, chloride and gene expression levels, *to be submitted.*

Nematollahi, MA., van Pelt-Heerschap, H., Komen, H., Co-Segregation of interrenal hyperplasia and sex in backcross and doubled-haploid populations of common carp (*Cyprinus carpio L.*), *to be submitted.*

Proceeding and Abstracts

Nematollahi, MA., van Pelt-Heerschap, H., Komen, H., 2006, Co-segregation of sex reversal and adrenal hyperplasia in backcross and gynogenetic populations of common carp (*Cyprinus carpio L.*). IX International Symposium for Genetics in Aquaculture (ISGA), Montpellier, France, pp 76.

Nematollahi, MA., van Pelt-Heerschap, H., Komen, H., 2006. Stress response during and after confinement in 17 Alpha hydroxylase deficient common carp (*Cyprinus carpio L*).VII th International Congress on the Biology of Fish, St John's, Newfoundland, Canada, pp170.

Nematollahi, MA., van Pelt-Heerschap, H., Komen, H**,** Quantitative analysis of genes involved in stress response in head kidney of CYP17 deficient common carp (*Cyprinus carpio L.*). Conference Genomics for Animal Health**.** Utrecht. The Netherlands, pp -.

Awards

Best Student Oral Presentation; Nematollahi, MA., van Pelt-Heerschap, H., Komen, H., 2006. Stress response during and after confinement in 17α-hydroxylase deficient common carp (*Cyprinus carpio L*).VII th International Congress on the Biology of Fish, St John's, Newfoundland, Canada, pp170**.**

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The Basic Package		year	credits*
WIAS Introduction Course (mandatory)		2006	
Course on philosophy of science and/or ethics (mandatory)		2006	
SUBTOTAL			**3**
Scientific Exposure		year	
International conferences			
International Symposium Genetics in Aquacullture IX. France		2006	
VII International Congress on the Biology of Fish. Canada (The best Oral Presentation Award)		2006	
Conference "Genomics for Animal Health".The Netherlands		2007	
Seminars and workshops			
WIAS Science Day 2004, 2005, 2006, 2007		2004-7	
RNAi Technical Seminar.The Netherlands		2007	
Presentations			
International Symposium Genetics in Aquacullture IX. France (Poster)		2006	
VII International Congress on the Biology of Fish. Canada (Oral)		2006	
WIAS Science Day (Poster)		2007	
Conference "Genomics for Animal Health".The Netherlands (Poster)		2007	
SUBTOTAL			**10**
In-Depth Studies		year	
Disciplinary and interdisciplinary courses			
International Course on Molecular Biology and Ecology In Aquaculture. Finland		2005	
Undergraduate courses			
Genomics		2004	
Advanced Statistics Course		2004	
Modern Statistics in life Sciences		2006	
SUBTOTAL			**22**
Statutory Courses		year	
Use of Laboratory Animals (mandatory when working with animals)		2005	
SUBTOTAL			**5**
Professional Skills Support Courses		year	
Course Techniques for Scientific Writing (advised)		2004	
Information Literacy Course		2005	
Upper-Intermediate English		2004	
Fluency		2003	
SUBTOTAL			**5**
Research Skills Training (optional)		year	
Preparing own PhD research proposal		2004	
SUBTOTAL			**5**
Education and Training Total			**50**
* one ECTS credit equals a study load of approximately 28 hours			

About the Author

Mohammad Ali Nematollahi was born on Jul. 23, 1966 in Toyserkan, Hamedan, Iran. He accomplished his high school at biology science in 1984. He received his B.Sc. degree in the field of Zoology in 1989 at Ferdusi University of Mashhad (Iran). In 1993, he accomplished his M.Sc. in the field of Fishery in The University of Tehran (Iran). The topic of his M.Sc. thesis was "Comparative study of quality and quantity of seminal fluid in cultured Salmonids of Iran" which was accepted as a distinguished thesis. Meantime, he was employed in the Aquaculture Dept. of Iran Fishery Organization as an expert of Aquaculture development in the regional projects during 1990-1994. He was appointed as a lecturer at the Fisheries and Environmental Sciences group, Faculty of Natural Resources, University of Tehran (Karaj/Iran) in 1995. Then, he was granted a scholarship from the Ministry of Science, Research and Technology (MSRT) of Iran, allowing him to do a PhD in abroad. In August 2003, he started his PhD project in Aquaculture and Fisheries Group accompanied with Animal Breeding and Genomic Center of Wageningen University, The Netherlands. The title of his PhD thesis was "Characterization of stress response in 17α-hydroxylase deficient common carp (Cyprinus carpio L.)". He will be back to his job as an academic staff of The University of Tehran.

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