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Response to Stress in 17α-hydroxylase Deficient Common Carp (*Cyprinus carpio* L.)

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ABSTRACT

The aim of this study was to analyze the stress response during 3 hours net confinement stress and recovery period of 22 hours in normal (STD) and in 17α-hydroxylase deficient common carp (E5). Fish were raised for 6 months and sampled at T_0 (control, unstressed), 5 minutes, 20 minutes, 1 hour and 3 hours of exposure to net confinement, and after 1 hour, 4 hours, and 22 hours of recovery. At every sampling time, blood was collected to determine cortisol, corticosterone, glucose, lactate and free fatty acids (FFA) levels (5 fish per strain). Fish and head kidney were weighed before and after dissection, respectively, to determine head kidney somatic Index (HKSI). Morphometric analysis of head kidney tissues indicated that the head kidney somatic index was significantly higher in E5 fish (0.076±0.021) compared with STD fish (0.045±0.015). Also, significant differences in cortisol and corticosterone as well as in glucose, lactate and FFA values were observed between the two strains of E5 and STD. Moreover, the pattern of changes of glucose and FFA during stress and afterward indicated a significant difference compared to the T_0. Results support the conclusion that the reduced capacity of ill fish to produce cortisol is caused by a deficiency in 17α-hydroxylase activity. A reduced cortisol output leads to increased stimulation of adrenals by adrenocorticotropic Hormone (ACTH), resulting in increased outputs of corticosterone.

Keywords: Congenital interrenal hyperplasia, Corticosterone, Cortisol.

INTRODUCTION

In fish culture, many kinds of stressors like netting, handling, and transporting are common and unavoidable threats. Once a stressor is recognized, the signal is transferred to hypothalamus by sensory neurons. 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 (Iwama *et al*., 1997; Rotllant *et al*., 2003; Vale *et al*., 1981; Van Enckevort *et al*., 2000; Wendelaar Bonga, 1997).

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 (Wendelaar Bonga, 1997). The basal plasma cortisol level varies from 2 to 42 ng ml^{-1} in different species (Gamperl *et al*., 1994). It can increase from 20 to 500 ng ml^{-1} under a variety of factors. Cyprinids have a very high cortisol response to stress (Pottinger *et al*., 2000; Nematollahi 2010) although they are the most domesticated fish.

Isogenic strains of common carp have been produced using andro and gynogenetic

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manipulations (Bongers *et al*., 1998; Komen *et al*., 1988) and plasma cortisol levels in a number of strains have been determined in response to a standard 20 minutes net confinement stressor (Ruane *et al*., 2005). Most strains typically show a peak response of 200-300 ng ml^{-1} plasma cortisol after around 30 minutes to one hour net confinement (Ruane *et al*., 2005). However, 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; Nematollahi *et al*., 2009). 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 significantly lower in E5 fish. Interrenal cells stained by 3*β*-HSD showed that the larger head kidney was partially due to the 2-3x increased number of interrenal cells in E5 head kidneys (Ruane *et al*., 2005). 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. Based on these results, Ruane *et al*. (2005) concluded that the reduced capacity of E5 fish to produce cortisol was 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 observed in humans. Moreover, a novel type of P450c17 (P450c17a2) has been identified recently in teleosts, lacking the lyase activity. It has been shown that P450c17a2 is involved in cortisol production in head kidneys

by converting pregnenolone to 17α-hydroxypregnenolone, which is a precursor in the cortisol biosynthesis pathway (Zhou *et al*., 2007a, b).

In this study we further characterize this fish example of interrenal hyperplasia; we will demonstrate whether or not acute stress in 17α-hydroxylase (P450c17a2) deficient common carp results in low production of cortisol and high production of corticosterone?

MATERIALS AND METHODS

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 Wageningen University. Fish production was done following the protocol described in Bongers *et al*.(1998). The net confinement stressor was performed as described previously (Ruane *et al*., 2005) and fish were sampled at time points of T_0-unstressed (control), 5 minutes, 20 minutes, 1 hour and 3 hours confinement followed by 1 hour, 4 hours, and 22 hours recovery. At the date of sampling the fish used had 195 days after hatching.

Blood Collection and Head Kidney Sampling

At each time point, the 5 fish were removed from the tank and quickly anaesthetized with 0.3

g L^{-1} tricaine methyl sulphonate (MS_{222}, Crescent Research Chemicals, Phoenix, AZ, USA) and 0.6 g/L NaHCO3. Blood was collected as previously described by Ruane *et al*. (2001). After blood collection, the fish were killed with 0.6 g L^{-1} MS_{222}+1.2 g L^{-1} NaHCO3. Total body weight and standard

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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×100*

Plasma Analysis

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 (8.1%), corticosterone (4.5%), progesterone (0.1%) and 17α-hydroxyprogesterone (1%).

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 (19%), progesterone (0.4%), cortisol (4.5%) and cortisone (0%).

Plasma glucose was determined by the GOD-Perid method (Boehringer); plasma lactate was measured using a commercial lactate assay kit. (Sigma Diagnostics, protocol 735), and plasma total non esterified fatty acids were measured by the ACS-ACOD (acyl-coenzyme A synthetase Acyl-CoA oxidase) method (Wako Chemicals, Richmond, Virginia, USA).

Statistical Analyses

A two way ANOVA was used to compare STD and E5 fish followed by Dunnet's post hoc test to determine significant differences compared to T_0 levels of E5 and STD, respectively ($P< 0.05$).

RESULTS

During the net confinement stress, plasma cortisol and corticosterone levels were significantly changed in both STD and E5 fish.

Plasma cortisol levels in STD fish were significantly ($P< 0.001$) increased at time points 5 minutes, 20 minutes, 1 hour, and 3 hours after net confinement. 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 minutes after net confinement and reached a peak at 1 hour recovery, then, returned to the normal level after 22 hours recovery (Table 1).

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

Further, morphometric analysis of head kidney tissue indicated that the head kidney somatic index was significantly higher in E5 fish than in STD fish (Table 1).

Glucose levels in STD males were elevated during confinement periods of 20 minutes, 1 hour, and 3 hours, however the elevation of glucose in the plasma was more gradual and continued to increase for at least another 4 hours after the fish were returned to the recovery tanks. Plasma glucose levels in E5 fish were significantly elevated at 5 minutes after net confinement and reached a peak at 1 hour recovery; it remained at high levels even after 22 hours recovery. During the net confinement stress, a significantly different glucose response was found between the STD and E5 fish in 1 hour net and 22 hours recovery (Figure 1-A).

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Table 1. Plasma level of cortisol and corticosterone and morphometric analysis of head kidney tissues (HKSI %) from two strains of carp (STD and E5) during a 3 h net confinement.

	Fish	T0 control	5 m retting	20 m retting	1 hour retting	3 hours retting	1 hour recovery	4 hour recovery	22 hour recovery
Cortisol (ng/ml)	STD	3.3±2.9	225.4±49.8***a	218.1± 22.1***a	281.6±49.4***a	143.2±16.1***a	42.6±8.2**a	2.3±1.5 a	2.9±3.5 a
	E5	9.6±1.7	22.0±5.3 b	41.9±6.5***b	43.7±9.1***b	64.2±9.5***b	68.9±10.7***b	55.8±10.2***b	14.3±7.6 b
Corticosterone (ng/ml)	STD	0.48±0.13 b	0.82±0.03 b	1.45±0.20 b	1.16±0.52 b	1.22±0.43 b	1.02±0.31 b	2.38±1.45**b	1.83±0.98 b
	E5	285±37.1 a	893.7±302.1 a	1546±289.3*a	1720±173.7**a	2854±591***a	3217±640***a	2536±256***a	1168±351 a
HKSI (%)	STD	0.06±0.018	0.034±0.017 b	0.043±0.015 b	0.039±0.015 b	0.044±0.008 b	0.048±0.018	0.046±0.016 b	0.049±0.011
	E5	0.069±0.021	0.088±0.039 a	0.100±0.008 a	0.082±0.024 a	0.069±0.013 a	0.056±0.016	0.080±0.023 a	0.064±0.015

Values are means ± SE, n = 2 Duplicate tanks, five fish per tank).), *, ** and *** indicate statistically significant differences from the T0-unstressed ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively). Strain differences at a time point are indicated by different letters (a, b, $P < 0.05$).

Lactate levels in STD fish were significantly increased during 5 and 20 min confinement and then returned to a normal level. Plasma lactate levels in E5 fish were significantly elevated at 5 min after net confinement and returned to a normal level. During the net confinement stress, a significantly different lactate response was found between the STD and E5 fish in almost all time points, except 5 min netting (Figure 1-B).

Free fatty acid levels in STD fish were significantly increased after 3 hours confinement ($P < 0.01$). Then, with a reduction in 1 hour recovery again the levels were increased in the rest of the recovery periods, which were significant at the 22 hours recovery point ($P < 0.01$). Plasma FFA levels in E5 fish were significantly elevated at 4 and 22 hours recovery. A significantly different FFA response was found between the STD and E5 fish in 4 h recovery (Figure 1-C).

DISCUSSION

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 and Speiser, 2000). 11β-hydroxylase (White and Speiser, 1994), 17α-hydroxylase/17, 20-lyase (Biglieri *et al*., 1966) and cholesterol desmolase deficiency (Katsumata, 2007; Morohashi *et al*., 1987) are rare.

In fish, only one case of interrenal insufficiency has been described (Ruane *et al*., 2005). An isogenic line of common carp

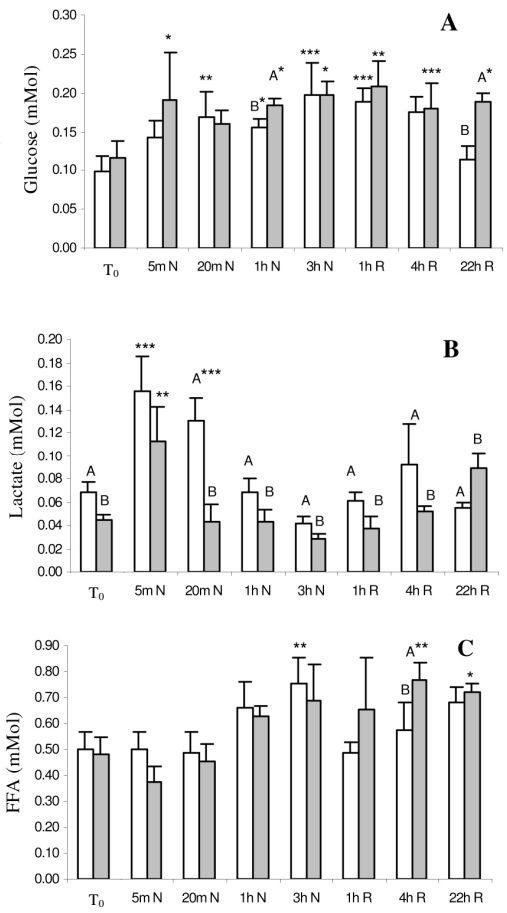

Figure 1. The effect of a 3 h net confinement and subsequent recovery on plasma glucose (A) lactate (B) and FFA (C) levels in STD (□) and E5 (■) males of common carp. Values represent mean±SE (n=5), *, ** and *** indicate statistically significant differences from the T_0-unstressed ($P< 0.05$, $P< 0.01$ and $P< 0.001$, respectively). Strain differences at a time point are indicated by different letters (A, B; $P< 0.05$).

(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 (Ruane *et al*., 2005; Nematollahi *et al*., 2012).

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 (Nematollahi *et al*., 2012). It suggests that reduced cortisol levels caused by P450c17a2 deficiency leads to increased stimulation of the adrenals by ACTH, resulting in an increased production of corticosterone by increase in number and size of the steroidogenic cells (Figure 2).

In this study, we further characterized this fish example of adrenal hyperplasia and demonstrated that acute stress in this E5 fish resulted in low production of cortisol and high production of corticosterone. The results of further characterization of the stress response in 17α-hydroxylase deficient common carp show low cortisol, high corticosterone levels and enlargement of head kidney in E5 fish.

The question is whether 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 P450c17a2.

In Fish, 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).

In tetrapod vertebrates metabolic functions

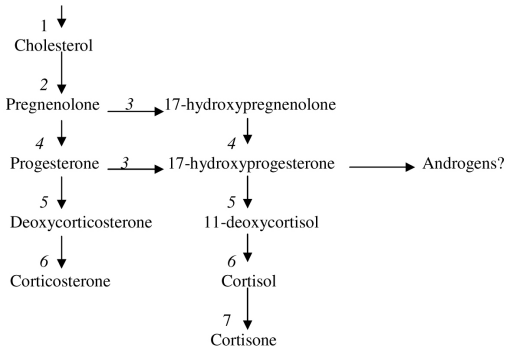

Figure 2. Biosynthesis of steroids in Head Kidney of fish. All steroid hormones will be synthesized from cholesterol. The end product is cortisol. Legend: (1) StAR; (2) P450scc; (3) P450c17a2 (hydroxylase); (4) 3β-HSD; (5) P450c21; (6) 11β1-HSD, (7) 11β2-HSD.

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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 and Madsen, 1992). Since no mineralocorticoid receptors and aldosterone has been found in teleost fish, the general view is that cortisol acts in fish as a mineral as well as a glucocorticoid. 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). Seemingly, cortisol, 11β-deoxycorticosterone and aldosterone have affinities for the fish MR (Bury and Sturm, 2007). It is not yet known whether corticosterone can activate the fish MR or GR.

Although the glucose and lactate levels followed relatively the same pattern as earlier described by Ruane *et al*. (2001), there were significant differences in both metabolites between the two strains in our results. The results are comparable with patterns found in carp (Pottinger, 1998), Atlantic salmon (Waring *et al*., 1996) and other species (Wendelaar Bonga, 1997). It is known that cortisol increase gluconeogenesis in liver as a result of the activation of key gluconeogenic enzymes such as glucose 6-phosphate. Increasing of plasma lactates is primarily due to anaerobic glycolysis in the white muscle and is related to stressors involved in some form of oxygen shortage resulting in muscle anaerobiosis (Dabrowska *et al.,* 1991). Plasma FFA levels showed a difference between both strains during confinement. As it was described, catecholamines in carp may control levels of FFAs in plasma (van den Thillart *et al*., 2001).

Results support the conclusion that the reduced capacity of ill fish to produce cortisol is caused by a deficiency in 17α-hydroxylase activity. A reduced cortisol output leads to increased stimulation of adrenals by Adrenocorticotropic Hormone (ACTH), resulting in increased output of corticosterone.

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پاسخ به تنش در ماهی کپور معمولی فاقد آنزیم ۱۷ الفا هیدروکسیلاز

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چکیده

هدف از این مطالعه آنالیز پاسخ به استرس" اسارت در تور" بمدت ۳ ساعت در ماهی کپور معمولی استاندارد و یک نژاد از کپور معمولی با نقص در آنزیم ۱۷ الفا هیدروکسیلاز بود. ماهیان ۶ ماهه در معرض استرس اسارت در تور در مدت صفر(بدون استرس)، ۵ دقیقه، ۲۰ دقیقه، یکساعت، و ۳ ساعت و سپس یکساعت، ۴ ساعت و ۲۲ ساعت ریکاوری قرار گرفتند. در هر نقطه زمانی، خونگیری از ۵ ماهی برای تعیین سطوح کورتیزول، کورتیکواسترون، گلوکوز، لاکتات، اسیدهای چرب آزاد انجام شد. شاخص سوماتیک غده فوق کلیه با توزین ماهیان قبل از تشریح و غده فوق کلیه پس از تشریح تعیین شد. آنالیز بافت شناسی غده فوق کلیه نشان داد که شاخص سوماتیک غده فوق کلیه بطور معنی داری در ماهی بیمار (۰/۰۲۱ ± ۰/۰۷۶) بزرگتر از ماهی استاندارد (۰/۰۱۵ ± ۰/۰۴۵) بود. اختلافات معنی داری نیز در میزان سطوح کورتیزول، کورتیکواسترون و لاکتات بین دو نژاد نیز وجود داشت. در مورد گلوکوز و اسیدهای چرب آزاد اختلافات معنی دار نبود، اگرچه الگوی تغییرات گلوکوز و اسیدهای چرب آزاد در طی استرس و پس از آن نسبت به حالت بدون استرس اختلاف داشت. نتایج این آزمایش نتیجه گیری قبل را که ظرفیت کاهش یافته ماهیان بیمار برای تولید کورتیزول در اثر نقص در فعالیت آنزیم ۱۷ الفا هیدروکسیلاز است را تایید کرد. این کاهش کورتیزول موجب افزایش تحریک آدرنال بوسیله آدرنوکورتیکوتروپیک هورمون شده که نتیجه آن افزایش تولید کورتیکواسترون است.

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