Experimental hyperthyroidism and central mediators of stress axis and thyroid axis activity in common carp (*Cyprinus carpio* L.)

Edwin J W Geven, Folkert Verkaar, Gert Flik and Peter H M Klaren

Department of Animal Physiology, Faculty of Science, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

(Requests for offprints should be addressed to P H M Klaren; Email: p.klaren@science.ru.nl)

(E J W Geven and F Verkaar contributed equally to this work)

Abstract

The effect of experimental hyperthyroidism, realized by T_4 injection, on central mediators of the hypothalamo–pituitary–interrenal axis (HPI-axis) in common carp (*Cyprinus carpio* L.) was studied. Our results show that hyperthyroidism evokes a marked 3·2-fold reduction in basal plasma cortisol levels. Corticotropin-releasing hormone-binding protein (CRH-BP) mRNA levels in the hypothalamus, measured by real-time quantitative PCR, were significantly elevated by 40%, but CRH, urotensin-I, prepro-TRH, prohormone convertase-1 (PC1), and POMC mRNA levels were unchanged. In the pituitary pars distalis, PC1, CRH receptor-1, and POMC mRNA levels were unaffected, as was ACTH content. Plasma α -MSH concentrations were significantly elevated by 30% in hyperthyroid fish, and this was reflected in PC1 and POMC mRNA levels in pituitary pars intermedia that were increased 1·5- and 2·4-fold respectively. The α -MSH content of the pars intermedia was unchanged. Hyperthyroidism has profound effects on the basal levels of a central mediator, i.e., CRH-BP, of HPI-axis function in unstressed carp *in vivo*, and we conclude that HPI- and hypothalamo–pituitary–thyroid-axis functions are strongly interrelated. We suggest that the changes in plasma cortisol, thyroid hormone, and α -MSH levels reflect their concerted actions on energy metabolism.

Journal of Molecular Endocrinology (2006) **37**, 443–452

Introduction

The synthesis and the secretion of thyroxine (T_4) by the thyroid gland are stimulated by thyroid-stimulating hormone (TSH) from the pituitary gland. In mammals, TSH secretion is under positive hypothalamic control by thyrotropin-releasing hormone (TRH), and thyroid hormones exert a classical negative feedback on the expression of TSH (Shupnik 2000) and TRH (Koller *et al.* 1987, Kakucska *et al.* 1992, Guissouma *et al.* 1998). In teleosts also, inhibitory regulation by thyroid hormones of pituitary TSH expression has been confirmed (Larsen *et al.* 1997, Pradet-Balade *et al.* 1997, Yoshiura *et al.* 1999, Chatterjee *et al.* 2001), but, to the best of our knowledge, the only non-mammalian study showing an inhibitory effect of T_3 and T_4 on the expression of TRH was conducted on chicken (Lezoualc'h *et al.* 1992).

Studies on bighead carp (*Aristichthys nobilis*), arctic charr (*Salvelinus alpinus*), and Japanese eel (*Anguilla japonica*) indicate a role for TRH in fishes similar to that in mammals (Eales & Himick 1988, Chatterjee *et al.* 2001, Han *et al.* 2004). However, in the lungfish (*Protopterus ethiopicus*), Nile tilapia (*Oreochromis niloticus*), and common carp (*Cyprinus carpio*) TRH does not stimulate

TSH secretion (Gorbman & Hyder 1973, Melamed *et al.* 1995, Kagabu *et al.* 1998). In several non-mammalian vertebrates, including teleosts, factors other than TRH were found to be thyrotropin-releasing factors. Indeed, in coho salmon (*Oncorhynchus kisutch*), corticotropin-releasing hormone (CRH) exerts thyrotropic effects on cultured-pituitary cells (Larsen *et al.* 1998). A direct stimulatory action of CRH was also demonstrated for amphibian, reptilian, and avian pituitary thyrotropes (reviewed by De Groef *et al.* (2006)).

The principal action of hypothalamic CRH in teleosts is the regulation of the stress response (Huising *et al.* 2004, Flik *et al.* 2006). In teleostean fishes, that lack a median eminence, neurons from the nucleus preopticus (NPO) send axons directly to the pituitary gland where they release CRH upon registration of a stressor. CRH stimulates the release of adrenocorticotrophic hormone (ACTH) from corticotrope cells in the pituitary pars distalis (Metz *et al.* 2004) by the activation of the CRH receptor-1 (CRH-R1; Huising *et al.* 2004). ACTH is derived from pro-opiomelanocortin (POMC) through the action of prohormone convertase-1 (PC1; Benjannet *et al.* 1991, Castro & Morrison 1997), and stimulates the secretion of cortisol from the interrenal cells in the head kidney (Flik *et al.* 2006).

The effect of CRH on pituitary corticotropes is modulated by CRH-binding protein (CRH-BP), a hypothalamic factor that binds CRH with a higher affinity than does CRH-R1 (Potter *et al.* 1991, Cortright *et al.* 1995). The primary action of CRH-BP is generally thought to be inhibitory on the CRH-induced ACTH release from pituitary corticotropes (Potter *et al.* 1991, Cortright *et al.* 1995, Westphal & Seasholtz 2006). In common carp, CRH-BP immunoreactivity is localized in perikarya in the recessus opticus, a hypothalamic region distinct from the NPO, and colocalizes in CRH-immunopositive nerve fibers projecting from the NPO to pituitary corticotropes (Huising *et al.* 2004). Beside CRH, urotensin-I (UI), and CRH-R2, two other members of the teleostean CRH system (Flik *et al.* 2006), have also been implicated in the stress response of teleosts (Fryer *et al.* 1983, Bernier & Craig 2005). UI has a potent thyrotropic effect on coho salmon pituitary cells, similar to CRH (Larsen *et al.* 1998). However, the exact role of UI in the stress response and thyroid metabolism of teleosts has scarcely been addressed. Other members of the teleostean CRH-family are urocortin-2 and -3 orthologs (Hsu & Hsueh 2001, Lewis *et al.* 2001, Boorse *et al.* 2005), but, as yet, no data are available on their function in fish.

The dual function of CRH as a corticotropin- and a thyrotropin-releasing hormone suggests a functional relationship between the hypothalamo-pituitary-interrenal axis (HPI-axis) and the hypothalamo-pituitary-thyroid axis (HPT-axis). This is further corroborated by the finding, in fishes, that experimentally elevated plasma cortisol or thyroid hormone levels correlate negatively with plasma T_4 and cortisol respectively (Redding *et al.* 1984, Mustafa & MacKinnon 1999). These findings, and in particular the absence of a thyrotropic effect of TRH in carp and the thyrotropic effect of CRH and UI in coho salmon, led us to postulate that thyroid hormones may have important regulatory effects on mediators of stress axis activity in the central nervous system of common carp.

Materials and methods

Animals and animal procedures

Common carp (*Cyprinus carpio* L.), hereafter called carp, of the all-male E4×R3R8 isogenic strain (Bongers *et al.* 1997) and with a body weight of 41 ± 12 g (mean $\pm$ s.d.) were obtained from the Department of Fish Culture and Fisheries of Wageningen University (The Netherlands). Fish were kept in stock in 150 l tanks, with circulating filtered Nijmegen city tap water at 22 °C, at a photoperiod ratio of 16 h light:8 h darkness. Fish were fed commercial fish food (Trouwit; Trouw,

Putten, The Netherlands) at a ration of 1.5% of the estimated body weight per day.

Fish were injected intraperitoneally (i.p.) on day 1 of the treatment regimen with 10 µg T_4 (from Sigma Chemical Co.) per gram body weight in saline (0.9% NaCl), followed by i.p. injections of 1 µg T_4 per gram body weight every third day for a period of 2 weeks. Control fish were injected with vehicle only. Fish were sampled on the first day after administration of the final injection. To this end, fish were deeply anesthetized in 0.1% 2-phenoxyethanol; mixed arterial and venous blood was collected by puncture from the caudal vessels using heparinized 23 gauge needles. Animals were then killed by spinal transection, and pituitary gland, hypothalamus and preoptic region (hereafter referred to as hypothalamus), and head kidneys were dissected. Plasma was obtained from whole blood by centrifugation (15 min, 15 000 g, 4 °C), and stored at -20 °C until further analysis. Pituitary glands used for pituitary hormone content measurements, and those used for measurements of tissue POMC, PC1, CRH-R1, and CRH-R2 mRNA levels were bisected into pars distalis and pars intermedia. Pituitary glands used for measurements of TSH β -subunit mRNA were left intact. Tissues were immediately frozen in dry ice and stored at -80 °C until further processing.

In vitro superfusion and static incubation of carp head kidney

Head kidneys from two untreated carp were removed directly after anesthesia and placed individually in a superfusion chamber. Tissues were superfused with a Hepes-Tris-buffered medium (pH 7.4) saturated with carbogen (95% O_2 -5% CO_2), at a rate of 30 µl/min as described in detail by Metz *et al.* (2005). When a stable baseline of cortisol secretion was obtained, T_4 (at 1 µM), 3,5,3'-triiodothyronine (T_3 ; 1 µM), or human ACTH₍₁₋₃₉₎ (hACTH; 0.1 µM), were administered for a period of 30 min. Ten- or twenty-minute fractions were collected, stored on ice, and analyzed for cortisol content as described later. Thyroid hormones and hACTH were obtained from Sigma Chemical Co.

Head kidneys from five untreated carp were obtained. The head kidneys obtained from one animal were pooled, diced into approximately 1 mm³ sized fragments, and suspended in the same buffer that was used for superfusion experiments. The suspension was distributed in a 12-well plate in 2 ml volumes. Ten nanomoles of T_4 were added; control incubations received the saline vehicle only. After 16 h, the incubation medium was sampled for cortisol, after which 30 nM hACTH were added and left to incubate for 1 h, subsequently, the medium was sampled again.

RNA extraction and cDNA synthesis

To extract total RNA, selected tissues were removed from seven animals from the control and T₄-treated group respectively, and homogenized in 500 µl TRIzol reagent (Invitrogen) by ultrasonification. Following a DNase treatment (Invitrogen), 1 µg RNA was reverse transcribed to cDNA in a 20 µl reaction mixture containing 300 ng random primers, 0.5 mM dNTPs, 10 mM dithiothreitol, 10 U RNase inhibitor, and 200 U Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 37 °C and stored at –20 °C.

Cloning and sequencing of carp PC1 cDNA

Primer sequences used are shown in Table 1; oligonucleotides were purchased from Biolegio (Malden, The Netherlands). PC1 sequences were retrieved from the puffer fish (*Takifugu rubripes*) and zebrafish (*Danio rerio*) genomes via a BLAST search at the Ensembl site (<http://www.ensembl.org>) using mouse PC1 cDNA as a query sequence. Mouse PC1 cDNA was aligned with the retrieved puffer fish and zebrafish sequences, and degenerate primers PC1-fw and PC1-rv were designed

based on conserved sequences. To obtain a carp PC1 nucleotide sequence, a PCR was carried out on carp pituitary pars distalis cDNA using primers PC1-fw and PC1-rv. PCR products were separated by electrophoresis on a 1% agarose–ethidium bromide gel, ligated into a pCR4-TOPO vector, and introduced into competent TOP10 *Escherichia coli* cells (TOPO TA Cloning Kit; Invitrogen). Successfully transfected cells were selected on LB–kanamycine agar and checked for appropriately sized inserts using primers T3 and T7. Successfully transfected plasmids were extracted (Miniprep; Bio-Rad), sequenced using primers T3 and T7, and analyzed on an ABI Prism 310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). The 5'- and 3'-ends were obtained by rapid amplification of cDNA ends (RACE)-PCR using the GeneRacer kit (Invitrogen). One microgram of RNA from carp pituitary pars distalis was processed according to the GeneRacer protocol (Invitrogen) to obtain RACE-ready cDNA. PCR was performed using gene-specific primers (PC1-3'RACE and PC1-5'RACE) and the GeneRacer ligated primers. Template DNA was mixed with 600 nM GeneRacer primer and 200 nM gene-specific primers in

Table 1 Primer sequences with corresponding GenBank accession numbers

	Accession number	Primer	Sequence 5' → 3'
Gene product			
PC1	AM236095	PC1-fw PC1-rv PC1-3'RACE PC1-5'RACE PC1-3'RACEn PC1-5'RACEn qPC1-fw qPC1-rv	AGGCTCCATYTGKTGGG CCASCCGGGGTTATTGGC GCACTGGCTTTGGAGCAGAATCCT GGAGATGGTGTACGGACTGTCTGT CCTGGCGAGACCTTCAGCACTTAG CAGTTGTCGCCCTGACGACCACCG GCTGGTTTGATGGTCAACAG GACTTTGGGATCAGCCAGAT ATTAACCCCTCACTAAAGGGA TAATACGACTCACTATAGGG AGTACCGAACGGCCATCTTG TGCCACCGGATAGGTGAAGT TTGGCTCTGGCTGTTCTGTGT TCATCTGTCAGATCAGACCTGCATA CCCTGCTGATCGCCTTCAT GCAGGATAAATGCTGTAATCAGTT GTTGGAGCTCTTGTTATTGCCTTTA GAAAGTCGTAATTAAGTCCAGTGGAT CATCCGGCTCGGTAACAGAA CCAACAGACGCTGCGTTAACT GCACCTGTGTCCAGCATGAA GGTGCTCAGCGGGATGTG TTACCGGTCGCTTTCACGTT AGCTGTGTGCCAAACCAAAC ACAATGATCTCAAGCGGTCCAT CCACCCAGAAGCTCGACAAA CAACAGGGGAAAAGATGACACAGATC GGGACAGCACAGCCTGGAT CCGTGGGTGACATCGTTACA TCAGGACATTGAACCTCACTGTCT
Vector		T3 T7	
TSH-β	AB003585	qTSHβ-fw qTSHβ-rv	
POMC	Y14618	qPOMC-fw qPOMC-rv	
CRH-R1	AJ576244	qCRH-R1-fw qCRH-R1-rv	
CRH-R2	AJ781795	qCRH-R2-fw qCRH-R2-rv	
CRH	AJ317955	qCRH-fw qCRH-rv	
UI	M11671	qUI-fw qUI-rv	
pp-TRH	AB179818	qTRH-fw qTRH-rv	
CRH-BP	AJ490881	qCRH-BP-fw qCRH-BP-rv	
β-Actin	M24113	qACT-fw qACT-rv	
40S	AB012087	q40S-fw q40S-rv	

Y: C or T/U; S: C or G; K: G or T/U.

a volume of 50 µl containing 1.75 mM MgCl₂, 350 µM dNTPs, and 3.75 U *Taq* polymerase-*Tgo* polymerase mix (Roche Applied Science). The 5'- and 3'-nested PCRs (primers: PC1-3'RACEn and PC1-5'RACEn) were then performed on the PCR products to further amplify the PC1 cDNA fragments. Nested PCR products were analyzed on agarose gel, cloned into a TOPO vector, and sequenced as described previously. The authenticity of the consensus sequence from four to six clones was confirmed from alignments with PC1 cDNAs from angler fish (Roth *et al.* 1993) and mouse, and the putative PC1 cDNAs from the puffer fish and zebrafish genomes. The carp PC1 nucleotide sequence thus obtained has been submitted to the DDBJ-EMBL-GenBank databases under accession number AM236095.

Hormone measurements

Pituitary pars distalis and pars intermedia tissues, obtained from five to seven animals, were homogenized on ice in 200 µl 10 mM HCl in a Potter-Elvehjem device. Homogenates were diluted in demineralized water 200-fold before analysis. Blood plasma, obtained from 19 control animals and 17–21 T₄-treated animals, was diluted fivefold in RIA buffer prior to cortisol measurements, plasma analyzed for α-melanocyte-stimulating hormone (MSH) was not diluted. ACTH was measured using RIA as described by Metz *et al.* (2004). A commercially available polyclonal rabbit antiserum against human ACTH_{1–24} (Biogenesis, Poole, UK) was used. The α-MSH RIA was performed as described by van den Burg *et al.* (2003). The antiserum had full cross-reactivity with des-, mono-, and di-acetylated α-MSH (Vaudry *et al.* 1978). Radio-labeled ACTH and α-MSH were prepared by ¹²⁵I-addition using the Iodogen method (ICN, Costa Mesa, CA USA), and purified through solid-phase extraction (octadecyl Bakerbond column, J.T. Baker, Phillipsburg, NJ, USA). Cortisol was measured by RIA, as described by Metz *et al.* (2005), using a commercially available antiserum (Campro Scientific, Veenendaal, The Netherlands) and ¹²⁵I-cortisol (Amersham). Plasma-free T₄ (fT₄) was measured using a commercially available enzyme-linked immunoassay (Research Diagnostics, Inc., Flanders, NJ, USA) according to manufacturer's instructions.

Real-time quantitative PCR

The relative tissue expression of POMC, PC1, CRH-R1, CRH-R2, and TSH β-subunit mRNA in pituitary pars distalis, pars intermedia, and intact pituitary gland, obtained from seven control and seven T₄-treated animals, was measured by use of real-time quantitative

PCR (RQ-PCR). In hypothalamus, the relative expression of POMC, CRH, UI, prepro-TRH, PC1, and CRH-BP was measured. In general, cDNA was diluted 50-fold, but cDNA from pituitary pars distalis and pars intermedia was diluted 25 000-fold to measure *POMC* gene expression. Five microliters of cDNA were used in a 25 µl reaction mixture consisting of 12.5 µl SYBR Green Master Mix (PE Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3 µl of each primer (600 nM final concentration) were added. The primer sets used for PCR are shown in Table 1. RQ-PCR was performed on a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The reaction mixture was incubated for 10 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 1 min annealing and extension at 60 °C. Melting curves confirmed the identity and specificity of the PCR products. Dissociation plots were analyzed and cycle threshold (C_T) values were determined. The expression of genes of interest was calculated relative to β-actin and 40S ribosomal protein S11 expression. Both control genes yielded similar results and therefore all results in this study are expressed relative to β-actin mRNA expression.

Statistical analysis

All data are presented as mean values ± S.E.M. The number of different preparations (*n*) is given in parentheses. Differences between groups were assessed with Student's unpaired *t*-test. Statistical significance was accepted at *P* < 0.05 (two-tailed), probabilities are indicated by asterisks (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Results

Animal model validation

Plasma-free T₄ concentrations were elevated 5.3-fold (*P* < 0.001) in T₄-treated fish (Fig. 1A) and pituitary TSH β-subunit mRNA levels were 3.9-fold (*P* < 0.001) lower than in control animals (Fig. 1B), which confirmed a successful induction of hyperthyroidism. The validation of our experimental hyperthyroid animal model is further corroborated by the hyperactive behavior of the T₄-injected group, in particular during feeding, compared with the control group (personal observations).

Plasma hormone levels

In control animals, basal plasma cortisol levels were 54 ± 6 ng/ml (equivalent to 149 ± 18 nM; *n* = 19), which is similar to the values measured in unstressed carp reported elsewhere (van den Burg *et al.* 2003,

Figure 1 (A) Plasma-free T₄ concentrations (pM) in saline-injected (closed bar, $n=19$) and T₄-injected (open bar, $n=21$) animals. (B) Expression levels of TSH β -subunit mRNA relative to β -actin in the pituitary glands of saline-injected (closed bar, $n=21$) and T₄-injected (open bar, $n=7$) animals. Student's t -test was used for statistical evaluation.

Metz *et al.* 2004). In hyperthyroid carp, basal plasma cortisol levels were reduced 3.2-fold ($P<0.001$; Fig. 2A). Plasma α -MSH levels in control carp were 0.30 ± 0.11 nM ($n=19$), which values are, again, within the range measured in unstressed carp (van den Burg *et al.* 2003, 2005a, Metz *et al.* 2005). In hyperthyroid carp, basal α -MSH levels had increased by 30% ($P<0.05$; Fig. 2B).

Pituitary pars distalis and hypothalamus

In the pituitary pars distalis, no significant changes in POMC, PC1, and CRH-R1 mRNA expression levels relative to β -actin were detected between saline-injected and T₄-injected animals, whereas CRH-R2 mRNA was not detectable ($n=7$, results not shown). Concomitantly, the ACTH content of the pituitary pars distalis was similar in saline-injected and T₄-injected carp (189 ± 16 and 176 ± 23 pg/gland respectively; $n=7$; $P=0.65$). In the hypothalamus, mRNA levels of POMC, CRH, UI, prepro-TRH, and PC1 were not affected by the T₄ treatment, but CRH-BP mRNA levels

Figure 2 (A) Plasma cortisol and (B) α -MSH concentrations in saline-injected (closed bars, $n=19$) and T₄-injected (open bars, $n=21$ and 17 respectively) animals. Student's t -test was used for statistical evaluation.

were elevated significantly by 40% in hyperthyroid animals ($P<0.05$; Fig. 3).

Pituitary pars intermedia

In the pituitary pars intermedia of hyperthyroid carp POMC and PC1 mRNA expression levels were elevated 1.5-fold ($P<0.05$) and 2.4-fold ($P<0.01$) respectively; whereas the expression of CRH-R1 mRNA was not affected by T₄ treatment. CRH-R2 mRNA was not detectable (Fig. 4). The α -MSH content of the pars intermedia of hyperthyroid animals was 30 ± 5 pmol/gland ($n=5$), and had not changed significantly compared with controls, whose value was 32 ± 4 pmol/gland ($n=7$; $P=0.77$).

In vitro head kidney incubations

In two pilot experiments, carp head kidneys superfused *in vitro* displayed a basal cortisol secretion rate of 58 ± 9 pg/min per head kidney, which is similar to values reported earlier (Metz *et al.* 2005). Following a 20-min exposure to 1 μ M T₄ or T₃, the basal cortisol release was virtually unchanged; the viability of the head kidney

Figure 3 Expression levels of POMC, CRH, urotensin-I (UI), preproTRH (ppTRH), PC1 and CRH-BP mRNA relative to β -actin in hypothalamic tissue of saline-injected (closed bars, $n=7$) and T_4 -injected (open bars, $n=7$) animals. Student's *t*-test was used for statistical evaluation.

tissue was confirmed by stimulation with $0.1 \mu\text{M}$ hACTH, to which the tissue responded with a pronounced rise in the cortisol secretion rate (results not shown). Based on these observations, we decided to perform long-term incubations of head kidneys with thyroid hormones. After a 16-h static incubation, the cortisol concentration in the control incubation medium was $0.26 \pm 0.03 \text{ nM}$ ($n=5$). When head kidney tissue was incubated in the presence of 10 nM T_4 for the same period of time, the cortisol concentration in the incubate was similar: $0.27 \pm 0.07 \text{ nM}$ ($n=5$; $P=0.86$). Upon subsequent incubation with $0.1 \mu\text{M}$ hACTH for 1 h, the cortisol concentration in the control medium had increased to $4.4 \pm 1.3 \text{ nM}$ ($n=5$), and that in the medium containing 10 nM T_4 to $3.1 \pm 1.8 \text{ nM}$ ($n=5$; $P=0.59$), confirming the viability of the head kidney tissue after a prolonged static incubation.

Figure 4 Expression levels of POMC, prohormone convertase-1 (PC1), CRH-R1, and CRH-R2 mRNA relative to β -actin in the pituitary pars intermedia in saline-injected (closed bars, $n=7$) and T_4 -injected (open bars, $n=7$) animals. N.D., not detectable. Student's *t*-test was used for statistical evaluation.

Discussion

Basal plasma cortisol levels in the control group were similar to the values measured in unstressed carp in other studies (van den Burg *et al.* 2003, Metz *et al.* 2004). Moreover, the basal cortisol levels we measured, one day after the final i.p. injection was administered, are considerably lower than the concentrations that were measured in carp subjected to an acute or chronic stressor, which displayed plasma cortisol levels that were increased 5- to 26-fold (Weyts *et al.* 1997, van den Burg *et al.* 2003, Huising *et al.* 2004, Metz *et al.* 2004) and 5- to 10-fold (Huising *et al.* 2004, Metz *et al.* 2005) respectively, compared with control levels. It was also observed that after the removal of an acute stressor, the elevated plasma cortisol levels in carp decrease rapidly, i.e., within 90–120 min, to basal control levels (Weyts *et al.* 1997, Huising *et al.* 2004), and that no changes occur in the hypothalamic expression of CRH and CRH-BP (Huising *et al.* 2004). We judge that our injection protocol *per se* did not constitute a chronic stressor, and did not result in acclimation of the experimental animals. The cortisol concentrations that were measured in the control group reflect basal, unstressed levels.

We here demonstrate in a validated experimental animal model that experimentally induced hyperthyroidism results in a marked hypocortisolemia. The decrease in basal plasma cortisol correlates with an increase in hypothalamic CRH-BP mRNA expression. Since the release of cortisol from viable carp head kidney tissue does not respond to short- and long-term exposure to T_4 *in vitro*, we exclude the interrenal cells as a target for direct thyroid hormone action. Taken together, these results support a role for thyroid hormone as a regulator of the teleostean stress axis, by the modulation of CRH-BP expression and, hence, the bioavailability of ligands from the CRH family. In carp, CRH-BP is expressed in a cell population that is clearly distinct from the CRH-immunoreactive nucleus preopticus (Huising *et al.* 2004), and these neurons could well be the target for thyroid hormone action.

Our experimental design only allowed the determination of end-point measurements. Earlier changes, if present, in the CRH and thyroid systems of our animals would therefore not be detected. Indeed, van den Burg *et al.* (2005b) exposed carp to an acute change in ambient temperature and demonstrated, using functional magnetic resonance imaging, that there is a fast response (i.e., already evident after 30 s) and a clear chronology in the activation and deactivation of brain areas that are involved in the stress response. On a time scale of hours to days, chronically stressed carp differentially express members of the CRH system, and have blunted, but still elevated plasma cortisol levels (Huising *et al.* 2004, Metz *et al.* 2005)

which reflect acclimation of the fish to the stressor. Our results can then be interpreted as the acclimation of the CRH and thyroid systems to a chronic hyperthyroidism. We are presently designing time-course studies to investigate the dynamics of the interactions between the CRH and the thyroid systems.

The activation of the hypothalamo–pituitary–interrenal (HPI) axis in teleosts results in the release of cortisol, the prime stress hormone. The main function of cortisol in the stress response of teleostean fish is the redistribution of energy away from, e.g., growth and reproduction, and toward physiological functions required for coping with the stressor and to restore homeostasis (Wendelaar Bonga 1997, Schreck 2000). Cortisol stimulates gluconeogenesis and lipolysis (Sheridan 1988, van der Boon *et al.* 1991), which result in increased plasma glucose and free fatty acid levels. These catabolic actions of cortisol reflect its glucocorticoid potency that is commensurate to the central role of this hormone in the stress response and the regulation of energy metabolism.

As is cortisol, thyroid hormone is fundamentally implicated in the regulation of energy metabolism in vertebrates. Thyroid hormone stimulates basal metabolic rate and oxygen consumption in several teleostean tissues (Peter & Oommen 1989, Lynshieng & Gupta 2000), and has profound effects on lipid, carbohydrate, and protein metabolism in teleosts (Plisetzkaya *et al.* 1983, Sheridan 1986, Ballantyne *et al.* 1992, Scott-Thomas *et al.* 1992, Shameena *et al.* 2000). In addition, during prolonged fasting, plasma T_3 levels decrease in Nile tilapia (*Oreochromis niloticus*; Van der Geyten *et al.* 1998), which could well represent a mechanism for the adaptation, by downregulating energy expenditure, to caloric and nutritional deprivation.

Since both the HPI- and HPT-axis are fundamentally involved in the regulation of energy expenditure, it can be anticipated that, of necessity, mutual interactions between these two exist. Indeed, European eel (*Anguilla anguilla*) and coho salmon (*Oncorhynchus kisutch*) treated with cortisol showed suppressed plasma T_3 levels (Redding *et al.* 1984, 1986). It is interesting to note that Larsen *et al.* (1998) demonstrated thyrotropic effects of CRH-family peptides in cultured pituitary cells from the latter species. Two other salmonid species, Atlantic salmon (*Salmo salar*) and arctic charr (*Salvelinus alpinus*), treated with cortisol or given iodide supplementation, showed predictably increased plasma cortisol and thyroid hormone levels respectively, and that correlated negatively with plasma thyroid hormone and cortisol levels respectively (Mustafa & MacKinnon 1999). We here suggest that these negative correlations between plasma cortisol and thyroid hormone are caused by the interaction of the HPI- and HPT-axis in the brain of the fish. This would allow the integration of afferent signals

and the coordinated regulation of, ultimately, the interrenal cells and the thyroid gland.

To our knowledge, this is the first study to simultaneously investigate the *in vivo* responsiveness of both the CRH system and TRH for treatment with thyroid hormone in a teleost. The upregulation of hypothalamic *CRH-BP* gene expression in hyperthyroid carp indicates that a member of the CRH family is the principal TSH-releasing factor in this species. This is further corroborated by the non-responsiveness of prepro-TRH expression and PC1 that is involved in the processing of pro-TRH to mature TRH in the hypothalamus (Schaner *et al.* 1997). Indeed, in carp (Kagabu *et al.* 1998) and a number of other teleosts (Gorbman & Hyder 1973, Melamed *et al.* 1995), TRH does not stimulate TSH secretion. Instead, CRH is the thyrotropic factor (Larsen *et al.* 1998). CRH has a widespread distribution in the teleostean brain; hypophysiotropic CRH-positive neurons have been identified in the NPO, and the nucleus lateralis tuberis (NLT) and nucleus recessi lateralis (NRL) in the hypothalamus (Matz & Hofeldt 1999, Pepels *et al.* 2002, Huising *et al.* 2004). Matz & Hofeldt (1999) showed that, in Chinook salmon (*Oncorhynchus tshawytscha*), CRH-immunoreactive fibers, originating from the preoptic region and NLT, terminate with bouton-like swellings in the proximity of TSH-ir cells in the pituitary pars distalis. These anatomical data, together with our results, provide evidence for the existence of a CRH–TSH– T_4 axis in teleosts. However, since UI is very potent in stimulating the secretion of TSH from cultured teleostean pituitary cells (Larsen *et al.* 1998), we cannot exclude UI as a thyrotropic principle. Yulis *et al.* (1986) showed extensive UI-ir staining in hypothalamic perikarya as well as in fibers that innervate the pituitary proximal pars distalis of the white sucker *Catostomus commersoni*. Indeed, in carp (our unpublished results) and other teleosts (Ueda *et al.* 1983), the proximal pars distalis harbors TSH-ir cells. Since CRH and UI have a high affinity for CRH-BP (Baigent & Lowry 2000, Valverde *et al.* 2001), an increased expression of CRH-BP is likely to affect the bioavailability of UI as well as that of CRH. Experimental data clearly point to UI as a thyrotropic factor; and, until matters are conclusively resolved, the teleostean HPT-axis should be represented as a CRH/UI–TSH– T_4 axis.

TRH has also a wide distribution in the teleostean brain, and hypophysiotropic neurons expressing TRH were found in distinct parvocellular and magnocellular regions in the NPO of salmonids (Ando *et al.* 1998, Díaz *et al.* 2001). In carp brain, TRH-ir neurons are located in the NRL, and TRH-ir fibers are present in the NLT and NPO (Hamano *et al.* 1990), which nuclei, in the same animal species, also express CRH (Huising *et al.* 2004). By their anatomical locations, it thus appears

that hypothalamic CRH-ir and TRH-ir neurons can communicate bidirectionally.

We found that experimental hyperthyroidism resulted in an increase in plasma α -MSH, which was corroborated by increased pituitary pars intermedia POMC and PC1 mRNA expression. Although α -MSH is considered to be corticotropic in Mozambique tilapia (*Oreochromis mossambicus*; Lamers *et al.* 1992, Balm *et al.* 1995), no stimulatory effect of α -MSH was observed on carp interrenal cells *in vitro* (Metz *et al.* 2005). It was suggested that elevated plasma α -MSH levels sustain HPI-axis activity through a short-loop positive feedback on CRH neurons in the nucleus preopticus (Metz *et al.* 2005). The opposite changes in plasma cortisol and α -MSH levels in hyperthyroid carp reported here are compatible with a role of α -MSH in the fine-tuning of HPI-axis activity, but our results do not provide conclusive evidence.

We here postulate that the effects observed in this study are probably related via the physiological actions of cortisol and thyroid hormone in the regulation of energy metabolism. α -MSH can fulfill a similar role, although evidence is scarce. In goldfish (*Carassius auratus*), POMC mRNA expression was observed in brain regions known to control food intake, and intracerebroventricular injection of the α -MSH agonist [Nle⁴, D-Phe⁷]- α -MSH (NDP- α -MSH) inhibited food intake (Cerdá-Reverter *et al.* 2003). We interpret our experimental results to reflect the concerted actions of cortisol, thyroid hormone, and α -MSH on energy metabolism in carp.

Acknowledgements

We are grateful to Mr Tom Spanings for excellent animal care and fish husbandry, and to Mr Marcel van den Boogaart and Dr Juriaan Metz for technical assistance. We also express our thanks to Dr Mark Huising (Salk Institute, La Jolla) for providing the CRH, UI, CRH-BP, CRH-R1, and CRH-R2 sense and antisense primers. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Ando H, Ando J & Urano A 1998 Localization of mRNA encoding thyrotropin-releasing hormone precursor in the brain of sockeye salmon. *Zoological Science* **15** 945–953.
- Baigent SM & Lowry PJ 2000 Urocortin is the principal ligand for the corticotrophin-releasing factor binding protein in the ovine brain with no evidence for a sauvagine-like peptide. *Journal of Molecular Endocrinology* **24** 53–63.
- Ballantyne JS, John TM, Singer TD & Oommen OV 1992 Short-term effects of triiodothyronine on the bowfin, *Amia calva* (Holostei), and the lake char, *Salvelinus namaycush* (Teleostei). *Journal of Experimental Zoology* **261** 105–109.
- Balm PHM, Hovens ML & Wendelaar Bonga SE 1995 Endorphin and MSH in concert form the corticotrophic principle released by tilapia (*Oreochromis mossambicus*; Teleostei) melanotropes. *Peptides* **16** 463–469.
- Benjannet S, Rondeau N, Day R, Chretien M & Seidah NG 1991 PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *PNAS* **88** 3564–3568.
- Bernier NJ & Craig PM 2005 CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **289** R982–R990.
- Bongers ABJ, Ben-Ayed MZ, Zandieh Doulabi B, Komen J & Richter CJ 1997 Origin of variation in isogenic, gynogenetic, and androgenetic strains of common carp, *Cyprinus carpio*. *Journal of Experimental Zoology* **277** 72–79.
- Boorse GC, Crespi EJ, Dautzenberg FM & Denver RJ 2005 Urocortins of the South African clawed frog, *Xenopus laevis*: conservation of structure and function in tetrapod evolution. *Endocrinology* **146** 4851–4860.
- Castro MG & Morrison E 1997 Post-translational processing of proopiomelanocortin in the pituitary and in the brain. *Critical Reviews in Neurobiology* **11** 35–57.
- Cerdá-Reverter JM, Schioth HB & Peter RE 2003 The central melanocortin system regulates food intake in goldfish. *Regulatory Peptides* **115** 101–113.
- Chatterjee A, Hsieh Y-L & Yu JYL 2001 Molecular cloning of cDNA encoding thyroid stimulating hormone β subunit of bighead carp *Aristichthys nobilis* and regulation of its gene expression. *Molecular and Cellular Endocrinology* **174** 1–9.
- Cortright DN, Nicoletti A & Seasholtz AF 1995 Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein. *Molecular and Cellular Endocrinology* **111** 147–157.
- De Groef B, Van der Geyten S, Darras VM & Kühn ER 2006 Role of corticotropin-releasing hormone as a thyrotropin-releasing factor in non-mammalian vertebrates. *General and Comparative Endocrinology* **146** 62–68.
- Díaz ML, Becerra M, Manso MJ & Anadón R 2001 Development of thyrotropin-releasing hormone immunoreactivity in the brain of the brown trout *Salmo trutta fario*. *Journal of Comparative Neurology* **429** 299–320.
- Eales JG & Himick BA 1988 The effects of TRH on plasma thyroid hormone levels of rainbow trout (*Salmo gairdneri*) and arctic charr (*Salvelinus alpinus*). *General and Comparative Endocrinology* **72** 333–339.
- Flik G, Klaren PHM, van den Burg EH, Metz JR & Huising MO 2006 CRF and stress in fish. *General and Comparative Endocrinology* **146** 36–44.
- Fryer J, Lederis K & Rivier J 1983 Urotensin I, a CRF-like neuropeptide, stimulates ACTH release from the teleost pituitary. *Endocrinology* **113** 2308–2310.
- Gorbman A & Hyder M 1973 Failure of mammalian TRH to stimulate thyroid function in the lungfish. *General and Comparative Endocrinology* **20** 588–589.
- Guissouma H, Ghorbel MT, Seugnet I, Ouatas T & Demeneix BA 1998 Physiological regulation of hypothalamic TRH transcription *in vivo* is T₃ receptor isoform specific. *FASEB Journal* **12** 1755–1764.
- Hamano K, Inoue K & Yanagisawa T 1990 Immunohistochemical localization of thyrotropin-releasing hormone in the brain of carp, *Cyprinus carpio*. *General and Comparative Endocrinology* **80** 85–92.
- Han Y-S, Liao I-C, Tzeng W-N & Yu JYL 2004 Cloning of the cDNA for thyroid stimulating hormone β subunit and changes in activity of pituitary – thyroid axis during silencing of the Japanese eel, *Anguilla japonica*. *Journal of Molecular Endocrinology* **32** 179–194.

- Hsu SY & Hsueh AJW 2001 Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nature Medicine* **7** 605–611.
- Huising MO, Metz JR, van Schooten C, Taverne-Thiele AJ, Hermesen T, Verbarg-van Kemenade BML & Flik G 2004 Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. *Journal of Molecular Endocrinology* **32** 627–648.
- Kagabu Y, Mishiba T, Okino T & Yanagisawa T 1998 Effects of thyrotropin-releasing hormone and its metabolites, cyclo(His-Pro) and TRH-OH, on growth hormone and prolactin synthesis in primary cultured pituitary cells of the common carp, *Cyprinus carpio*. *General and Comparative Endocrinology* **111** 395–403.
- Kakucska I, Rand W & Lechan RM 1992 Thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is dependent upon feedback regulation by both triiodothyronine and thyroxine. *Endocrinology* **130** 2845–2850.
- Koller KJ, Wolff RS, Warden MK & Zoeller RT 1987 Thyroid hormones regulate levels of thyrotropin-releasing-hormone mRNA in the paraventricular nucleus. *PNAS* **84** 7329–7333.
- Lamers AE, Flik G, Atsma W & Wendelaar Bonga SE 1992 A role for di-acetyl α -melanocyte-stimulating hormone in the control of cortisol release in the teleost *Oreochromis mossambicus*. *Journal of Endocrinology* **135** 285–292.
- Larsen DA, Dickey JT & Dickhoff WW 1997 Quantification of salmon α - and thyrotropin (TSH) β -subunit messenger RNA by an RNase protection assay: regulation by thyroid hormones. *General and Comparative Endocrinology* **107** 98–108.
- Larsen DA, Swanson P, Dickey JT, Rivier J & Dickhoff WW 1998 *In vitro* thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology* **109** 276–285.
- Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W *et al.* 2001 Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *PNAS* **98** 7570–7575.
- Lezoualc'h F, Hassan AH, Giraud P, Loeffler JP, Lee SL & Demeneix BA 1992 Assignment of the β -thyroid hormone receptor to 3,5,3'-triiodothyronine-dependent inhibition of transcription from the thyrotropin-releasing hormone promoter in chick hypothalamic neurons. *Molecular Endocrinology* **6** 1797–1804.
- Lynshiang DS & Gupta BB 2000 Role of thyroidal and testicular hormones in regulation of tissue respiration in male air-breathing fish, *Clarias batrachus* (Linn.). *Indian Journal of Experimental Biology* **38** 705–712.
- Matz SP & Hofeldt GT 1999 Immunohistochemical localization of corticotropin-releasing factor in the brain and corticotropin-releasing factor and thyrotropin-stimulating hormone in the pituitary of Chinook salmon (*Oncorhynchus tshawytscha*). *General and Comparative Endocrinology* **114** 151–160.
- Melamed P, Eliahu N, Levavi-Sivan B, Ofir M, Farchi-Pisanty O, Rentier-Delrue F, Smal J, Yaron Z & Naor Z 1995 Hypothalamic and thyroidal regulation of growth hormone in tilapia. *General and Comparative Endocrinology* **97** 13–30.
- Metz JR, Huising MO, Meek J, Taverne-Thiele AJ, Wendelaar Bonga SE & Flik G 2004 Localisation, expression and control of adrenocorticotrophic hormone in the nucleus preopticus and pituitary gland of common carp (*Cyprinus carpio* L.). *Journal of Endocrinology* **182** 23–31.
- Metz JR, Geven EJW, van den Burg EH & Flik G 2005 ACTH, α -MSH and control of cortisol release: cloning, sequencing and functional expression of the melanocortin-2 and melanocortin-5 receptor in *Cyprinus carpio*. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **289** R814–R826.
- Mustafa A & MacKinnon BM 1999 Atlantic salmon, *Salmo salar* L., and Arctic char, *Salvelinus alpinus* (L.): comparative correlation between iodine-iodide supplementation, thyroid hormone levels, plasma cortisol levels, and infection intensity with the sea louse *Caligus elongatus*. *Canadian Journal of Zoology* **77** 1092–1101.
- Pepels PPLM, Meek J, Wendelaar Bonga SE & Balm PHM 2002 Distribution and quantification of corticotropin-releasing hormone (CRH) in the brain of the teleost fish *Oreochromis mossambicus* (tilapia). *Journal of Comparative Neurology* **453** 247–268.
- Peter MC & Oommen OV 1989 Oxidative metabolism in a teleost, *Anabas testudineus* Bloch: effect of thyroid hormones on hepatic enzyme activities. *General and Comparative Endocrinology* **73** 96–107.
- Plisetskaya E, Woo NY & Murat JC 1983 Thyroid hormones in cyclostomes and fish and their role in regulation of intermediary metabolism. *Comparative Biochemistry and Physiology. A, Comparative Physiology* **74** 179–187.
- Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ & Vale WW 1991 Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. *Nature* **349** 423–426.
- Pradet-Balade B, Schmitz M, Salmon C, Dufour S & Querat B 1997 Down-regulation of TSH subunit mRNA levels by thyroid hormones in the European eel. *General and Comparative Endocrinology* **108** 191–198.
- Redding JM, Schreck CB, Birks EK & Ewing RD 1984 Cortisol and its effects on plasma thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology* **56** 146–155.
- Redding JM, deLuze A, Leloup-Hatey J & Leloup J 1986 Suppression of plasma thyroid hormone concentrations by cortisol in the European eel *Anguilla anguilla*. *Comparative Biochemistry and Physiology. A, Comparative Physiology* **83** 409–413.
- Roth WW, Mackin RB & Noe BD 1993 Characterization and regulation of expression of the anglerfish prohormone processing endopeptidase PC1. *Endocrine Journal* **1** 131–140.
- Schaner P, Todd RB, Seidah NG & Nillni EA 1997 Processing of prothyrotropin-releasing hormone by the family of prohormone convertases. *Journal of Biological Chemistry* **272** 19958–19968.
- Schreck CB 2000 Accumulation and long-term effects of stress in fish. In *The Biology of Animal Stress*, pp 147–158. Eds GP Moberg & JA Mench. New York: CABI Publishing.
- Scott-Thomas DA, Ballantyne JS & Leatherland JF 1992 Interactive effects of high stocking density and triiodothyronine-administration on aspects of the *in vivo* intermediary metabolism and *in vitro* hepatic response to catecholamine and pancreatic hormone stimulation in brook charr, *Salvelinus fontinalis*. *Journal of Experimental Zoology* **263** 68–82.
- Shameena B, Varghese S, Leena S & Oommen OV 2000 3,5,3'-triiodothyronine (T_3) and 3',5'-diiodothyronine (T_2) have short-term effects on lipid metabolism in a teleost *Anabas testudineus* (Bloch): evidence from enzyme activities. *Endocrine Research* **26** 431–444.
- Sheridan MA 1986 Effects of thyroxine, cortisol, growth hormone, and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. *General and Comparative Endocrinology* **64** 220–238.
- Sheridan MA 1988 Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry* **90** 679–690.
- Shupnik MA 2000 Thyroid hormone suppression of pituitary hormone gene expression. *Reviews in Endocrine & Metabolic Disorders* **1** 35–42.
- Ueda H, Young G & Nagahama Y 1983 Immunocytochemical identification of thyrotropin (TSH)-producing cells in pituitary glands of several species of teleosts with anti-serum to human TSH β -subunit. *Cell and Tissue Research* **231** 199–204.
- Valverde RA, Seasholtz AF, Cortright DN & Denver RJ 2001 Biochemical characterization and expression analysis of the *Xenopus laevis* corticotropin-releasing hormone binding protein. *Molecular and Cellular Endocrinology* **173** 29–40.
- van den Burg EH, Metz JR, Ross HA, Darras VM, Wendelaar Bonga SE & Flik G 2003 Temperature-induced changes in thyrotropin-releasing hormone sensitivity in carp melanotropes. *Neuroendocrinology* **77** 15–23.

- van den Burg EH, Metz JR, Spanings FAT, Wendelaar Bonga SE & Flik G 2005a Plasma α -MSH and acetylated β -endorphin levels following stress vary according to CRH sensitivity of the pituitary melanotropes in common carp, *Cyprinus carpio*. *General and Comparative Endocrinology* **140** 210–221.
- van den Burg EH, Peeters RR, Verhoye M, Meek J, Flik G & Van der Linden A 2005b Brain responses to ambient temperature fluctuations in fish: reduction of blood volume and initiation of a whole-body stress response. *Journal of Neurophysiology* **93** 2849–2855.
- van der Boon J, van den Thillart GEEJM & Addink ADF 1991 The effects of cortisol administration on intermediary metabolism in teleost fish. *Comparative Biochemistry and Physiology. A, Comparative Physiology* **100** 47–53.
- Van der Geyten S, Mol KA, Pluymers W, Kühn ER & Darras VM 1998 Changes in plasma T_3 during fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in hepatic type II iodothyronine deiodinase. *Fish Physiology and Biochemistry* **19** 135–143.
- Vaudry H, Tonon MC, Delarue C, Vaillant R & Kraicer J 1978 Biological and radioimmunological evidence for melanocyte stimulating hormones (MSH) of extrapituitary origin in the rat brain. *Neuroendocrinology* **27** 9–24.
- Wendelaar Bonga SE 1997 The stress response in fish. *Physiological Reviews* **77** 591–625.
- Westphal NJ & Seasholtz AF 2006 CRH-BP: the regulation and function of a phylogenetically conserved binding protein. *Frontiers in Bioscience* **11** 1878–1891.
- Weyts FAA, Verburg-van Kemenade BML, Flik G, Lambert JGD & Wendelaar Bonga SE 1997 Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. *Brain Behavior and Immunity* **11** 95–105.
- Yoshiura Y, Sohn YC, Munakata A, Kobayashi M & Aida K 1999 Molecular cloning of the cDNA encoding the β subunit of thyrotropin and regulation of its gene expression by thyroid hormones in the goldfish, *Carassius auratus*. *Fish Physiology and Biochemistry* **21** 201–210.
- Yulis CR, Lederis K, Wong K-L & Fisher AWF 1986 Localization of urotensin I- and corticotropin-releasing factor-like immunoreactivity in the central nervous system of *Catostomus commersoni*. *Peptides* **7** 79–86.

Received in final form 20 July 2006

Accepted 4 August 2006

Made available online as an Accepted Preprint

12 September 2006