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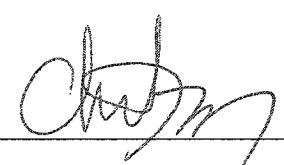
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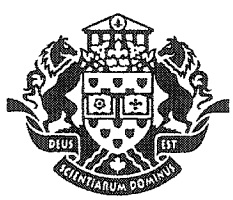

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CORTICOTROPIN-RELEASING FACTOR (CRF) AND NEUROPEPTIDE Y (NPY) mRNA LEVELS ARE MODIFIED BY STRESS AND GLUCOCORTICOIDS IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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ABSTRACT

The studies described in this thesis were designed to address the hypothesis that stress and glucocorticoids modify the levels of corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) mRNA in the rainbow trout (*Oncorhynchus mykiss*) brain. Standard cloning techniques provided the full-length cDNA sequences coding for trout NPY and two CRF paralogues. Using a ribonuclease protection assay (RPA), a tissue distribution demonstrated that both CRF paralogues are primarily abundant in the preoptic area of the trout brain, whereas NPY mRNA is more abundant in the telencephalon.

The effects of social stress on CRF1 and NPY mRNA levels were examined with pairs of sized-matched juvenile female trout. After 72 h of interaction, CRF1 and NPY mRNA levels were respectively 51% and 32% higher in the preoptic area of subordinate trout. These results suggest that CRF and NPY may be involved in the control of the stress axis in trout. However, my experiments could not distinguish between the effects of stress and food deprivation.

The levels of plasma cortisol in isolated trout indicated that confinement in small boxes was more stressful than isolation in a large tank. The elevation in CRF1 mRNA levels was greater and more persistent in confined fish. CRF1 mRNA levels were also elevated in trout physically disturbed to exhaustion but only after repeated chasing events. Together, these results suggest that the duration and intensity of stress are important factors determining the magnitude and persistence of the elevation in CRF mRNA levels.

The effects of glucocorticoids on CRF and NPY mRNA levels were examined by exposing trout to cortisol, a glucocorticoid receptor antagonist (RU-486) or a cytochrome P450 inhibitor that blocks cortisol synthesis (metyrapone). My results suggest that glucocorticoids

modify CRF1 and NPY mRNA levels but only part of the results support the presence of a glucocorticoid-induced negative feedback loop. Further studies are required to clarify the role of glucocorticoids in the regulation of the stress axis activity in fish.

RÉSUMÉ

Les études décrites dans cette thèse ont été conçues afin de tester l'hypothèse que le stress et les glucocorticoïdes modifient les niveaux d'ARNm de la corticolibérine (CRF) et de neuropeptide Y (NPY) dans le cerveau de la truite arc-en-ciel (*Oncorhynchus mykiss*). Des méthodes de clonage standards ont permi d'obtenir les séquences complètes d'ADNc codant pour NPY et deux paralogues de CRF de la truite. En utilisant un essai de protection contre les ribonucléases (RPA), une analyse de distribution dans les tissus a démontré que les deux paralogues de CRF sont principalement abondants dans l'aire préoptique du cerveau, tandis que l'ARNm de NPY est plus abondant dans le télencéphale.

Les effets du stress social ont été examinés à l'aide de truites femelles immatures appariées en fonction de leur taille. Après 72 h d'interaction, les niveaux d'ARNm codant pour CRF1 et NPY étaient respectivement 51% et 32% plus élevés dans l'aire préoptique des truites subordonnées. Ces résultats suggèrent que CRF et NPY pourrait être impliqué dans le contrôle de l'axe du stress chez la truite. Par contre, mes expériences n'ont pas permi de séparer les effets attibuables au stress et au jeûne.

Les niveaux plasmatiques de cortisol chez les truites isolées indiquent que le confinement dans de petites boîtes était plus stressant que l'isolement dans de grands bassins. L'élévation des niveaux d'ARNm de CRF1 était plus importante et plus persistante chez les poissons confinés. Les niveaux d'ARNm de CRF1 étaient également élevés chez des truites perturbées physiquement jusqu'à épuisement mais seulement après plusieurs périodes de pourchasse. Ces résultats suggèrent que la durée et l'intensité du stress sont des facteurs importants déterminant l'ampleur et la persistance de l'augmentation des niveaux d'ARNm de CRF.

Les effets des glucocorticoïdes ont été examinés en exposant des truites au cortisol, à un antagoniste du récepteur des glucocorticoïdes (RU-486) ou à un inhibiteur des cytochromes P450 qui bloque la synthèse du cortisol (metyrapone). Mes résultats suggèrent que les glucocorticoïdes affectent les niveaux d'ARNm de CRF1 et NPY mais seulement une partie des résultats supportent la présence d'un rétrocontrôle négatif par les glucocorticoïdes. D'autres études sont nécessaires pour clarifier le rôle des glucocorticoïdes dans la régulation de l'activité de l'axe de stress chez les poissons.

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LIST OF ABBREVIATIONS

3β-HSD	3β-Hydroxysteroid dehydrogenase Δ^5-Δ^4 isomerase
5-HT	Serotonin or 5-hydroxytryptamine
ACH	Acetylcholine
ACTH	Adrenocorticotropic hormone
ARNm	ARN messager
AVT	Arginine vasotocin
AVP	Arginine vasopressin
AUAP	Abridged universal amplification primer
BW	Body weight
cDNA	Complementary DNA
CLIP	Corticotropin-like intermediate lobe peptide
CPON	Carboxy-terminal peptide of neuropeptide Y
CRE	CAMP-responsive element
CRF	Corticotropin-releasing factor (corticolibérine)
CRF-BP	CRF binding protein
CYP11A1	Cholesterol side-chain cleavage or cholesterol desmolase
CYP11B1	11β-Hydroxylase
CYP17	17α-Hydroxylase/17,20-lyase
CYP21A2	21-Hydroxylase
GABA	Gamma-aminobutyric acid
GH	Growth hormone
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive element
GSP	Gene-specific primer
hnRNA	Heteronuclear RNA
HPA	Hypothalamic-pituitary adrenal
HPI	Hypothalamic-pituitary interrenal
hsp	Heat shock protein

JP	Joining peptide
LH	Luteinizing hormone
LPH	Lipotropin hormone
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MSH	Melanocyte-stimulating hormone
NE	Norepinephrine
NPO	Nucleus preopticus
NPY	Neuropeptide Y
ORF	Open reading frame
PBR	Peripheral benzodiazepine receptor
PCA	Principal components analysis
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PLC	Phospholipase C
POA	Preoptic area
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PVN	Paraventricular nucleus
PY	Fish pancreatic peptide
PYY	Peptide YY
RACE	Rapid amplification of cDNA ends
RIA	Radioimmunoassay
RPA	Ribonuclease protection assay
SE	Standard error of the mean
StAR	Steroidogenic acute regulatory protein
TSH	Thyrotropin
UI	Urotensin-I
UTR	Untranslated region

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"Fight for the highest attainable aim

But do not put up resistance in vain."

Dr. Hans Selye, 1973.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

1.1.1. Rationale

The relationship between stress and the levels of circulating cortisol is relatively well characterized in teleost fish. The exposure to different types of stress leads to a rapid increase in plasma cortisol (reviewed by Barton and Iwama, 1991; Wendelaar Bonga. 1997; Mommsen *et al.*, 1999). Our knowledge regarding the effects of stress on the hypothalamic neuropeptides involved in the control of cortisol release is more limited. Corticotropin-releasing factor (CRF) is a potent stimulator of adrenocorticotropic hormone (ACTH) secretion (Fryer *et al.*, 1983), which in turn stimulates the synthesis and release of cortisol (reviewed by Wendelaar Bonga. 1997). The sequence of the cDNA coding for the rainbow trout (*Oncorhynchus mykiss*) CRF precursor protein and the effects of stress on the levels of CRF mRNA were unknown until my work began.

Other neuroendocrine factors, including neuropeptide Y (NPY), could also be involved in the regulation of cortisol levels in response to stress. NPY is a potent stimulator of ACTH release in rats (Wahlestedt *et al.*, 1987) and several studies in rats support a stress-induced increase in NPY mRNA levels (Conrad and McEwen, 2000; Makino *et al.*, 1999, 2000; Sweerts *et al.*, 2001). There are synaptic connections between NPY nerve terminals and CRF-producing neurons in the rat brain (Liposits *et al.*, 1988; Mihaly *et al.*, 2002) and NPY stimulates CRF release *in vitro* (Haas and George, 1989; Tsagarakis *et al.*, 1989). These relationships have never

been examined in fish and the sequence of the cDNA coding for the rainbow trout NPY precursor protein was unknown until my work began.

Cortisol is thought to inhibit its own secretion by negative feedback loops. Cortisol would act at the hypothalamic and pituitary levels to inhibit further release of CRF and ACTH, respectively (Keller-Wood and Dallman, 1984). In human, glucocorticoids can have negative and positive effects on the synthesis of CRF mRNA in the paraventricular nucleus of the brain (Kovács *et al.*, 1986; Kovács and Mezey, 1987) and placenta (Robinson *et al.*, 1988), respectively. Bernier *et al.* (1999) suggested that glucocorticoids might also inhibit the synthesis of CRF mRNA in the goldfish brain. The potential effects of cortisol on CRF mRNA levels have never been examined in rainbow trout. NPY expression could also be modulated by glucocorticoids, as a glucocorticoid-responsive element (GRE) was reported in the promoter of the rat NPY gene (Misaki *et al.*, 1992).

The rainbow trout is a widely used teleost research model, in part due to its economic importance. In the aquaculture industry, fish are exposed to several stress factors that can potentially reduce financial benefits by inhibiting growth rates (reviewed by Barton and Iwama, 1991). Hence, it is important to acquire greater knowledge on the factors involved in the regulation of the stress response in fish. This knowledge is also important for developing assays to examine whether environmental contaminants disrupt the stress response. Also, the study of the neuroendocrine control of cortisol release in lower vertebrates provides an interesting evolutionary perspective. The comparison of fish neuroendocrine systems with those of mammalian models is likely to enhance our understanding of how the control of the stress response evolved.

1.1.2. Hypotheses

The studies presented in this thesis were designed to test the following hypotheses: 1) stress modifies the levels of CRF and NPY mRNA in the rainbow trout brain; and, 2) cortisol modifies the levels of CRF and NPY mRNA in the rainbow trout brain.

1.1.3. Objectives

The primary objective of this thesis is to improve our understanding of how CRF and NPY mRNA levels are modified in stressed rainbow trout. However, the initiation of this research project required the characterization of the cDNAs coding for the rainbow trout CRF and NPY precursor proteins. The studies presented were designed to address the following specific objectives: 1) clone and sequence the full length cDNAs of rainbow trout CRF and NPY; 2) determine the size and tissue distribution of CRF and NPY mRNAs; 3) determine the phylogenetic relationships of rainbow trout CRF and NPY sequences with previously characterized CRF and NPY sequences; 4) determine the effects of social interactions on CRF and NPY mRNA levels; 5) determine the effects of isolation on CRF and NPY mRNA levels; 6) determine the effects of physical disturbance to exhaustion on CRF and NPY mRNA levels; 7) determine the effects of food deprivation on CRF and NPY mRNA levels; and, 8) determine the effects of glucocorticoids on CRF and NPY mRNA levels. The selection of the brain tissue where mRNA levels were measured was based on the results of specific mRNA tissue distribution studies.

1.2. Stress

Pioneering studies by Walter Cannon led to the concept of critical stress levels, which represent the point inducing a breaking strain in homeostatic mechanisms (Cannon, 1935). However, the concept of stress was formulated and popularized by Professor Hans Selye. In his first paper, Selye proposed the term "general adaptation syndrome" to describe a non-specific response to virtually all noxious stimuli (Selye, 1936). Later, Selye defined stress as the non-specific response of the body to any demand made upon it (Selye, 1950, 1973). This definition has been a source of debate and criticism, particularly with regards to the non-specificity of the response (Mason, 1975a,b). Although this debate is beyond the scope of this thesis, it highlights the necessity to provide a definition of stress to the reader. In this thesis, stress is defined as a condition in which the homeostasis of an organism is threatened or disturbed as the result of the actions of stimuli or stressors (Chrousos and Gold, 1992).

The primary response to a stressor involves neuronal activation that leads to the release of the stress hormones, catecholamines and glucocorticoids (reviewed by Wendelaar Bonga, 1997). The release of these hormones is critical to mobilize the energy necessary for coping with the stress and returning the system to a homeostatic state. Catecholamines are released through the activation of the hypothalamic-sympathetic-chromaffin cell axis, whereas the synthesis and release of glucocorticoids depend on the activation of the hypothalamic-pituitary-interrenal (HPI) axis. This thesis will focus exclusively on the HPI axis. For an overview of the hypothalamic-sympathetic-chromaffin cell axis, refer to Reid *et al.* (1998) and references therein.

1.2.1. The hypothalamic-pituitary-interrenal (HPI) axis

The hypothalamic-pituitary-interrenal (HPI) axis is equivalent to the mammalian hypothalamic-pituitary-adrenal (HPA) axis (Fig. 1.1). The HPI axis, or stress axis, is a hormonal cascade that controls the synthesis and release of glucocorticoids (reviewed by Wendelaar Bonga, 1997). The initial step of this hormonal cascade is the release of corticotropin-releasing factor (CRF) from hypothalamic neurons. CRF-producing neurons primarily originate from the parvocellular and magnocellular divisions of the nucleus preopticus (NPO) in teleost fish (Olivereau and Olivereau, 1988). This area is equivalent to the mammalian paraventricular nucleus (PVN). After synthesis, CRF peptides are packaged to secretory vesicles that migrate along the axons to reach nerve terminals where the vesicles are released upon nerve stimulation. In teleosts, some of the CRF-containing nerve fibres terminate near ACTH-producing cells, *i.e.* corticotropes, of the anterior pituitary (Olivereau and Olivereau, 1988), whereas there is no direct contact between hypothalamic CRF neurons and anterior pituitary cells in terrestrial vertebrates (Asa *et al.*, 2002). Secreted CRF binds to specific membrane receptors and stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. Circulating ACTH acts on the interrenal cells to stimulate the synthesis and release of cortisol, the main glucocorticoid in teleost fish (reviewed by Wendelaar Bonga, 1997; Mommsen *et al.*, 1999).

The remainder of this section on the HPI will be primarily dedicated to the pituitary and interrenal components of the axis, with emphasis on teleosts where information is available. This will include information on ACTH, the control of ACTH release, the control of glucocorticoid synthesis and secretion, and the modulation of the HPI axis by feedback loops. As the primary focus of this thesis is CRF and NPY, these topics will be covered with greater emphasis in separate sections of the general introduction.

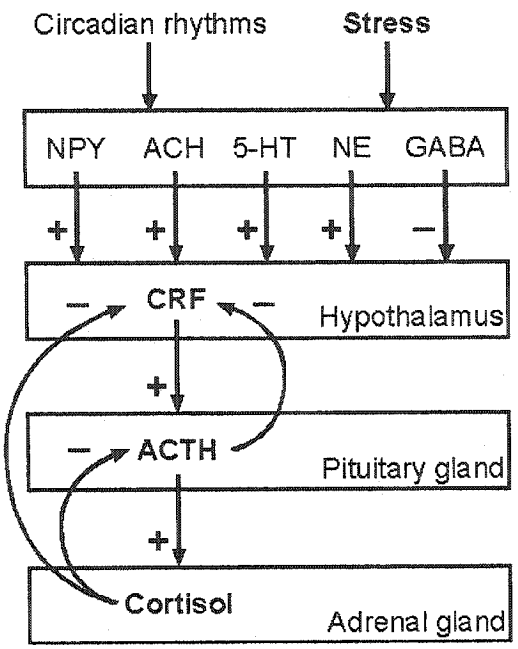

Figure 1.1 Schematic outline of the mammalian hypothalamic-pituitary-adrenal (HPA) axis. The activity of the HPA axis is modulated by circadian rhythms, stress, and neurotransmitters and neuropeptides including neuropeptide Y (NPY), acetylcholine (ACH), serotonin (5-HT), norepinephrine (NE), and gamma-aminobutyric acid (GABA). When secreted from the hypothalamus, corticotropin-releasing factor (CRF) stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH stimulates the synthesis and release of cortisol from the adrenal gland. Negative feedback loops limit the secretion of CRF and ACTH. Ultra-short feedbacks are not shown. Symbols: +, activation; -, inhibition. Adapted from Reichlin, 1993.

1.2.1.1. Adrenocorticotropic hormone (ACTH)

ACTH is a 39-amino acid peptide that is derived from the proteolytic cleavage of a large precursor peptide, pro-opiomelanocortin (POMC). The post-translational processing of this precursor can generate several biologically active peptides other than ACTH including α-melanocyte-stimulating hormone (α-MSH), β-endorphin and β-lipotropin (Roberts and Herbert, 1977a,b; Fig. 1.2). The nature of the peptides produced by a specific cell depends on the enzymatic cleavage of POMC. For example, the corticotropes present in the pars distalis of the

pituitary gland primarily produce ACTH, whereas ACTH is further cleaved into α-MSH and corticotropin-like intermediate lobe peptide (CLIP) in the melanotropes of the neurointermediate lobe (Rodrigues and Sumpter, 1983). Hence, the majority of ACTH is synthesized in the anterior pituitary corticotropes. In rainbow trout, two POMC cDNAs have been characterized (Salbert *et al.*, 1992; Chauveau *et al.*, 1993). Although the structure of POMC is highly conserved among vertebrates, fish POMC lacks the sequence for γ-MSH (Salbert *et al.*, 1992) and an unusual extension at the C-terminal of trout POMC-A generates two potentially bioactive decapeptides (Tollemer *et al.*, 1999).

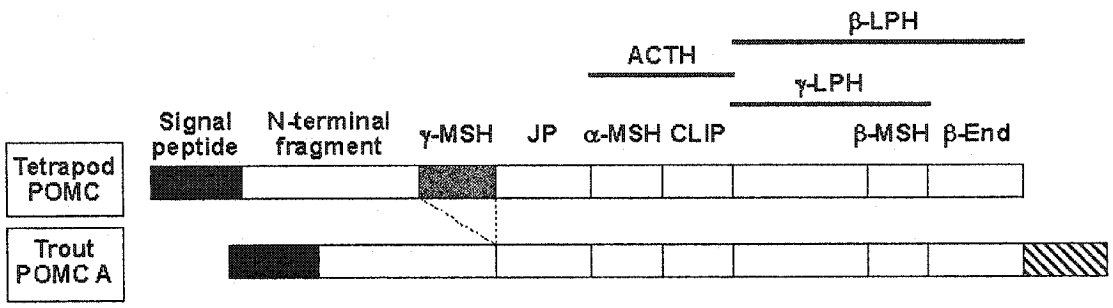

Figure 1.2 Schematic representation of the structure of tetrapod and trout POMC precursor proteins. Abbreviations are: ACTH, adrenocorticotropic hormone; End, endorphin; JP, joining peptide; LPH, lipotropin hormone; MSH, melanocyte-stimulating hormone. The C-terminal extension of trout POMC-A (hatched section) generates two potentially bioactive decapeptides. Adapted from Tollemer *et al.*, 1999.

1.2.1.2. Control of ACTH release

CRF is the most potent stimulator of ACTH secretion from the anterior pituitary of mammals (Rivier and Plotsky, 1986) and amphibians (Tonon *et al.*, 1986). In goldfish (*Carassius auratus*), a CRF-like peptide, urotensin-I (UI), is 2-3 times more potent than ovine CRF (Fryer *et al.*, 1983; Tran *et al.*, 1990). These studies were performed using CRF of

mammalian origin, whereas UI was of fish origin, so the potencies may not be correct. *In vitro* studies report several other factors may influence the release of ACTH. Arginine vasotocin (AVT), isotocin, arginine vasopressin (AVP), and angiotensins stimulate the release of ACTH from superfused goldfish anterior pituitary cells (Fryer *et al.*, 1985; Weld and Fryer, 1987). In salmonids, ACTH secretion is stimulated by AVT (Baker *et al.*, 1996) and somatostatin (Langhorne, 1986), but it is inhibited by melanin-concentrating hormone (MCH) (Baker *et al.*, 1986). AVT potentiates the effect of CRF on ACTH release in rainbow trout (Baker *et al.*, 1996). This contrasts with goldfish where AVT, isotocin and AVP had an additive effect but did not potentiate the actions of CRF or UI on ACTH release (Fryer *et al.*, 1985). AVP potentiates the stimulatory effect of CRF on ACTH secretion in isolated rat pituitaries (Gillies *et al.*, 1982). In rats, NPY also acts as a potent stimulator of ACTH release (Wahlestedt *et al.*, 1987), and high levels of NPY immunoreactivity are found in hypophysial portal blood (Danger *et al.*, 1990). Together, these findings suggest that NPY could modulate ACTH secretion *in vivo*, at least in rats.

Most fish studies on ACTH secretion have been performed *in vitro*. To achieve a better understanding of the neuroendocrine control of ACTH secretion, it is essential to determine which factors reach the anterior pituitary *in vivo*. In terrestrial vertebrates, there is no direct innervation of the anterior pituitary gland and neuropeptides reach ACTH cells through capillaries of the hypophysial portal system (Asa *et al.*, 2002). The direct innervation of the fish anterior pituitary corticotropes rather than capillary contact may limit the number of neuropeptides that can modulate the release of ACTH (Lovejoy and Balment, 1999).

CRF-immunoreactive fibres from the nucleus preopticus (NPO) terminate near ACTH cells within the pars distalis of the teleost pituitary (Olivereau *et al.*, 1984; Yulis *et al.*, 1986;

Olivereau and Olivereau, 1988; Matz and Hofeldt, 1999). The close proximity of CRF nerve terminals and ACTH cells suggests that CRF could be involved in the control of ACTH release *in vivo* (Yulis *et al.*, 1986). Although most UI-immunoreactive cells are located in the caudal neurosecretory system (urophysis), UI is also present in many locations within the central nervous system of the white sucker (*Catostomus commersoni*), including the nucleus lateralis tuberis of the hypothalamus (Yulis *et al.*, 1986). The absence of UI-immunoreactive fibres in the rostral pars distalis of the pituitary suggests that, if UI is involved in the control of ACTH release, this action is not based on a direct contact between UI fibres and corticotropes (Yulis *et al.*, 1986). AVT co-localizes with CRF in some neurons of the nucleus preopticus of white sucker (Yulis and Lederis, 1987), eels (Olivereau and Olivereau, 1988) and goldfish (Fryer and Lederis, 1988). It was suggested that AVT does not control ACTH cells directly in eels because few AVT-containing fibres reach corticotropes of the anterior pituitary (Olivereau and Olivereau, 1988). AVT-containing fibres were restricted to the large fibre bundles traversing the rostral pars distalis of the goldfish pituitary (Fryer and Lederis, 1988). NPY-containing fibres do not terminate within the rostral pars distalis of the rainbow trout pituitary but the NPO is densely innervated (Danger *et al.*, 1991). This suggests an indirect action of NPY on ACTH secretion through interaction with CRF-producing neurons. In rats, there are synaptic connections between NPY nerve terminals and CRF-producing neurons (Liposits *et al.*, 1988; Mihaly *et al.*, 2002) and NPY stimulates CRF release *in vitro* (Tsagarakis *et al.*, 1989) and *in vivo* (Haas and George, 1989).

As the pituitary gland is located outside the blood-brain-barrier, factors circulating in the bloodstream can also influence the release of ACTH. Glucocorticoids are the most widely studied peripheral inhibitors of ACTH release. The effects of glucocorticoids are not restricted to

the pituitary gland. Due to their lipophylic nature, glucocorticoids can cross the blood-brain-barrier and modulate the activity of the HPI axis at hypothalamic and possibly higher brain levels including the hippocampus of mammals (Sapolsky *et al.*, 1986).

1.2.1.3. Glucocorticoid synthesis and secretion

Cortisol is the main glucocorticoid in teleost fish (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999) and humans, whereas most rodents secrete primarily corticosterone (White, 2001). In mammals, glucocorticoids are produced in the cortex of the adrenal glands. Unlike mammals, fish do not possess a discrete adrenal gland and cortisol is produced in the interrenal cells, *i.e.* clusters of steroidogenic cells that are distributed in the head kidney (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). The biosynthesis of cortisol in fish is similar to that in mammals. It involves the modification of free cholesterol by steroidogenic enzymes of the mitochondrial membrane and the smooth endoplasmic reticulum (White, 2001; Fig. 1.3).

As there is no intracellular storage of cortisol, it must be synthesized *de novo* upon perception of a stressor. Resting plasma cortisol levels in rainbow trout are typically below 5 ng/ml and the stressed levels vary greatly depending on the type of stress but can achieve values greater than 250 ng/ml (Barton and Iwama, 1991). However, care must be taken when comparing absolute values of cortisol as these can vary greatly with different methods of analysis and sampling. I saw a two-fold difference in cortisol measurements obtained with two different radioimmunoassay kits (Fig. 1.4).

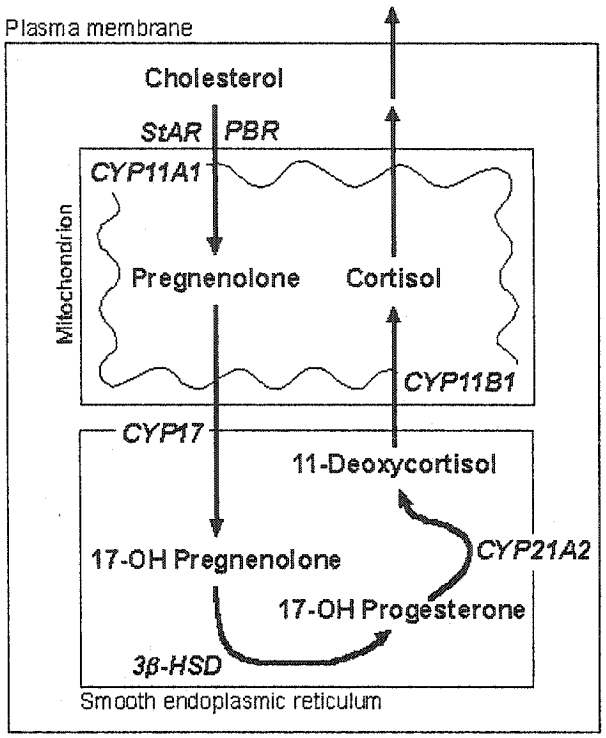

Figure 1.3 Biosynthesis of cortisol from cholesterol. The entry of cholesterol into the mitochondrion is facilitated by Steroidogenic Acute Regulatory protein (StAR) and Peripheral Benzodiazepine Receptor (PBR). Cholesterol is transformed by a series of steroidogenic enzymes including cholesterol side-chain cleavage (CYP11A1), 17α-hydroxylase/17,20-lyase (CYP17), 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4 isomerase (3β-HSD), 21-hydroxylase (CYP21A2), and 11β-hydroxylase (CYP11B1). Adapted from White, 2001.

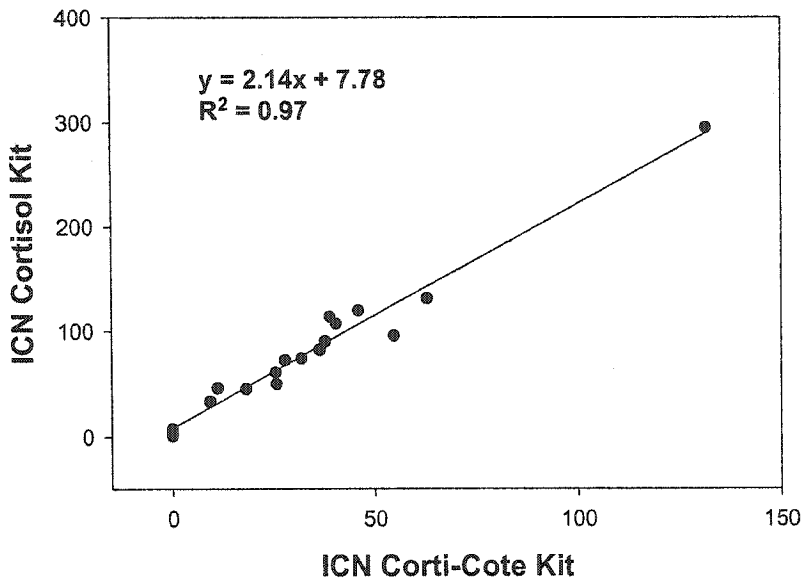

Figure 1.4 Comparison of two cortisol radioimmunoassay kits from ICN Diagnostics (Costa Mesa, CA) showing a two-fold difference in the cortisol (ng/ml) values obtained. Blood was collected from anaesthetized rainbow trout in heparinized syringes by caudal puncture and centrifuged. The plasma was removed and stored at –80°C for later analysis of cortisol levels using the Cortisol ^{125}I RIA Kit (#07-221102) and the Corti-Cote ^{125}I RIA Kit (#06B-256440). These two kits differ by their level of cross-reactivity with 11-deoxycortisol.

ACTH stimulates cortisol synthesis by binding to the melanocortin 2 receptor (MC2R) on adrenocortical cell membranes in mammals (Mountjoy *et al.*, 1992). The melanocortin receptors are members of the seven transmembrane domains, G-protein coupled family of receptors. So far, five melanocortin receptors have been identified in mammals. The binding of ACTH to MC2R activates adenylate cyclase, stimulates the production of cAMP, and activates protein kinase A, which phosphorylates proteins including transcriptional regulatory factors (Simpson and Waterman, 1988). The rate limiting step of cortisol synthesis is the transport of cholesterol to the matrix side of the mitochondrion inner membrane where the first enzyme of the steroidogenic pathway is located, *i.e.* the cytochrome P450-dependent cholesterol side-chain cleavage

(P450scc or CYP11A1) or cholesterol desmolase (Simpson, 1979; Stocco and Clark, 1996). The ACTH-induced accumulation of cAMP stimulates receptor-mediated lipoprotein uptake (Brown *et al.*, 1979) and the activity of StAR, a key protein for cholesterol transport into the mitochondria (Miller and Strauss, 1999). Also, ACTH has long-term effects on cortisol synthesis by increasing the transcription of genes encoding StAR and several enzymes of the steroidogenic pathway (Miller, 1988).

1.2.1.4. Feedback

The activity of the HPI axis is modulated through a series of negative feedback loops where released hormones inhibit the synthesis and release of hormones at higher levels of the axis. These feedback loops are thought to play an important role in terminating the activation of the HPI axis after stress. Depending on the components involved, these loops are referred to as long, short or ultra-short (Fig. 1.1). Short-loop feedback refers to the inhibitory effect of ACTH on the secretion of CRF (Seiden and Brodish, 1971; Upton *et al.*, 1973; Simpson *et al.*, 1980; Suda *et al.*, 1987), whereas long-loop feedback refers to the negative effect of glucocorticoids on ACTH and CRF secretion (Herman and Majzoub, 2002). The long-loop feedback is subdivided into fast and delayed. Fast feedback is a non-genomic process that occurs within seconds to minutes and inhibits CRF and ACTH secretion but not synthesis (Keller-Wood and Dallman, 1984; Hinz and Hirschelmann, 2000). Delayed feedback is further subdivided into intermediate and slow. Intermediate feedback occurs within hours and is due to the inhibition of ACTH secretion but not synthesis, and the inhibition of CRF synthesis and secretion (Keller-Wood and Dallman, 1984). Slow feedback occurs after more than 24 hours of exposure to glucocorticoids

and leads to the inhibition of POMC synthesis, the precursor of ACTH (Keller-Wood and Dallman, 1984).

Typically the actions of glucocorticoids are mediated through binding to a cytosolic glucocorticoid receptor. When it is not bound to its ligand, the glucocorticoid receptor forms a hetero-oligomer with two molecules of heat shock protein hsp90 and one molecule each of hsp70 and hsp56 (Pratt and Toft, 1997; Mommsen *et al.*, 1999). Binding of the ligand changes the conformation of the receptor, which dissociates from hsp90. This promotes the dimerisation of the receptor complex, which is transported to the nucleus where it binds to glucocorticoid-responsive elements (GRE) on promoter regions of target genes. The glucocorticoid receptor complex can also interact with other transcription factors to inhibit or activate the transcription of a series of genes (Diamond *et al.*, 1990; Truss and Beato, 1993; Bamberger *et al.*, 1996). Glucocorticoids inhibit the synthesis of CRF mRNA in the paraventricular nucleus of the mammalian brain (Kovács *et al.*, 1986; Kovács and Mezey, 1987) and a region of the human CRF promoter was identified as a potential negative GRE (Malkoski *et al.*, 1997). However, the effects of glucocorticoids on the synthesis of CRF are not always negative. In the placenta of higher primates, glucocorticoids stimulate CRF synthesis (Robinson *et al.*, 1988). This positive feedback does not depend on the binding of the glucocorticoid receptor to the DNA and requires a functional cAMP-responsive element (CRE) (Cheng *et al.*, 2000). Several studies also support a positive effect of glucocorticoids on CRF synthesis in the central nucleus of the amygdala in rats (Swanson and Simmonds, 1989; Makino *et al.*, 1994; Shepard *et al.*, 2000).

The possible presence of a GRE or a CRE in the CRF promoter of fish has not been investigated but the glucocorticoid receptor is expressed in the CRF-immunoreactive neurons of the rainbow trout preoptic nucleus (Teitsma *et al.*, 1997, 1998). Bernier *et al.* (1999) showed that

cortisol reduces the levels of CRF mRNA in the telencephalon-preoptic area of the goldfish brain. In mammals, although glucocorticoids reduce hypothalamic CRF mRNA levels, stress-induced increases in CRF mRNA cannot be prevented by changes in circulating glucocorticoids (Lightman and Harbuz, 1993).

The rapidity of the fast feedback suggests that this effect cannot be mediated by the classical mechanism involving the cytosolic glucocorticoid receptor (Hinz and Hirschelmann, 2000). There is increasing evidence that steroid hormones, including glucocorticoids, have non-genomic effects (reviewed by Wehling, 1997; Borski, 2000). Fast actions of glucocorticoids are not affected by actinomycin D or cycloheximide, which clearly indicates that these effects are independent of transcription and protein synthesis (Shih *et al.*, 1990; Baqué *et al.*, 1996). Some of the fast actions of glucocorticoids are believed to depend on stabilization of cell membranes and modification of calcium fluxes (Keller-Wood and Dallman, 1984). However, an effect on the physicochemical properties of membranes and the activity of membrane-associated proteins would require relatively high concentrations of glucocorticoids (Buttgereit *et al.*, 1998). It was suggested that some of the fast actions of glucocorticoids could be mediated by a membrane-bound steroid receptor that would activate second messenger pathways (Buttgereit *et al.*, 1998). For example, the synthetic glucocorticoid agonist dexamethasone suppresses cAMP production and ACTH secretion from mouse corticotrope cell lines, and evidence suggests that these effects were G protein-mediated (Iwasaki *et al.*, 1997). Although pharmacological evidence supports the presence of a membrane-bound glucocorticoid receptor (Wright and Paine, 1995), such a receptor remains to be structurally characterized. However, the first membrane-bound steroid receptor was recently cloned and characterized from spotted seatrout ovaries, and homologues were obtained from several species including human, mouse, pig, *Xenopus*, zebrafish and

pufferfish (Zhu *et al.*, 2003a,b). This seven transmembrane domains G protein-coupled receptor shows high affinity for progestins but low affinity for glucocorticoids.

In addition to short and long feedback loops, there is evidence for an ultra-short feedback where ACTH inhibits its own secretion (Boscaro *et al.*, 1988). This idea of a self-inhibition of ACTH secretion, however, is not supported by all studies (Cavagnini *et al.*, 1985). There is additional evidence for a positive ultra-short feedback loop where CRF stimulates the transcription of its own gene in the PVN of the rat brain (Ono *et al.*, 1985; Parkes *et al.*, 1993; Imaki *et al.*, 1996; Mansi *et al.*, 1996). Other investigators support a negative effect of CRF on its own activity (Cunningham *et al.*, 1988).

Negative feedback has received very little attention in fish compared with mammals. Cortisol inhibits CRF-induced ACTH secretion in superfused goldfish pituitary cells (Fryer *et al.*, 1984). Implantation of cortisol pellets near the nucleus preopticus or the nucleus lateralis tuberis of the goldfish brain implicates these CRF-rich brain areas as sites of glucocorticoid negative feedback (Fryer and Peter, 1977). Also, *in vitro* studies on the interrenal tissue of coho salmon provided evidence of a negative ultra-short feedback loop where cortisol inhibits its own secretion (Bradford *et al.*, 1992).

1.2.2. Sources of stress

Fish can be exposed to a great variety of stressors, whether they live in captivity or in their natural environment. In nature, fish may face major physiological challenges as they move to new environments with different physico-chemical characteristics. The necessity to adapt rapidly to new hydro-mineral conditions represents an important physiological stress.

Environmental changes in oxygen content (Maxime *et al.*, 1995) or water salinity (Nichols and Weisbart, 1985) are characterized by an initial elevation in plasma cortisol levels.

Human activities generate a large variety of stressors by physically and chemically modifying fish habitats. Several studies have shown that environmental contaminants can disrupt endocrine functions of the HPI axis (reviewed by Hontela, 1997). The activity of the HPI axis is increased by acute exposure to cadmium (Hontela *et al.*, 1996), mercury (Bleau *et al.*, 1996), and polychlorinated biphenyls (PCBs; Vijayan *et al.*, 1997; Stouthart *et al.*, 1998). However, prolonged exposure to heavy metals (Norris *et al.*, 1999), PCBs (Quabius *et al.*, 1997; Jørgensen *et al.*, 2002), or a mixture of contaminants (Hontela *et al.*, 1992) impairs the response of the HPI axis. Hontela *et al.* (1992) suggested that chronic exposures to chemical pollutants may cause an exhaustion of components of the HPI axis.

Stress can be particularly severe when humans interact directly with fish through activities such as angling. With catch and release angling, fish are injured by hooks, handled, confined in keepnets and air exposed before they are released. The stress associated with this practice leads to elevated plasma cortisol levels (Pankhurst and Sharples, 1992; Pottinger, 1998) and may impair survival (Wilkie *et al.*, 1996; Bettoli and Osborne, 1998; Dempson *et al.*, 2002).

Social interactions can represent an important stress when access to food resources, shelter or spawning grounds is limited. When held in captivity, salmonid fish establish a dominance hierarchy (reviewed by Sloman and Armstrong, 2002) in which subordinate fish have reduced access to food (McCarthy *et al.*, 1992), increased standard metabolic rates (Sloman *et al.*, 2000), decreased growth rates (Abbott and Dill, 1989; Ryer and Olla, 1996), and increased levels of plasma cortisol (Øverli *et al.*, 1999; Sloman *et al.*, 2001b) and ACTH (Höglund *et al.*, 2000). Similar dominance hierarchies occur in natural populations of salmonids (Bachman,

1984; Nakano, 1995), but social stress can be particularly important in captivity where environmental complexity is low (Sloman and Armstrong, 2002). In the aquaculture industry, high stocking densities combined with frequent netting, handling and transport represent important stress factors that can negatively affect growth and reproduction (reviewed by Barton and Iwama, 1991). In natural populations, predator-prey interaction may also represent an important source of physical and psychological stress.

1.2.3. Physiological consequences of stress

The acute response to stress involves catecholamine-induced modulation of cardiorespiratory systems and mobilization of stored energy (Reid *et al.*, 1998). Several of the long term physiological effects of stress can be directly associated with elevated levels of cortisol (reviewed by Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). Stress is generally associated with increased energy demand and a reallocation of energy for physiological processes that are necessary for coping with stress and returning to a homeostatic state. Several actions of cortisol are directed at increasing energy mobilization. For example, cortisol increases gluconeogenesis (Jones *et al.*, 1993), lipolysis (Sheridan, 1986), and proteolysis (Andersen *et al.*, 1991).

Consistent with the reallocation of energy, prolonged stress leads to a reduction in growth rate and a suppression of reproductive functions (Mommsen *et al.*, 1999). These effects are partly mediated by cortisol, but as discussed below, the inhibition of growth and reproduction is also a consequence of elevated CRF levels (Wendelaar Bonga, 1997). Stress-induced elevations in cortisol can also suppress the immune system, which leads to an increase in the susceptibility to infections (Graham and Tucker, 1984).

1.3. Corticotropin-releasing factor (CRF)

1.3.1. Structure and phylogeny

Geoffrey Harris first postulated in 1948 the existence of hypothalamic hormones that could trigger the release of hormones from the adenohypophysis (Harris, 1948). In 1955, two independent groups demonstrated the existence of a hypothalamic factor that could stimulate the secretion of ACTH from rat pituitary glands (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). This factor was named corticotropin-releasing factor (CRF). Although CRF was the first hypothalamic-releasing factor to be named, it took over 25 years before CRF was finally purified and characterized from ovine hypothalamic extracts (Vale *et al.*, 1981; Spiess *et al.*, 1981). At the same time, two peptides with closely related sequences were identified in non-mammalian vertebrates. Sauvagine was isolated from the skin of the South American frog *Phyllomedusa sauvagei* (Montecucchi *et al.*, 1980), whereas urotensin-I (UI) was isolated from the caudal neurosecretory system (urophysis) of two teleosts, the white sucker (Lederis *et al.*, 1982) and the common carp (*Cyprinus carpio*; Ichikawa *et al.*, 1982). These two peptides were first thought to be homologues of the mammalian CRF until the identification of peptides more closely related to CRF in white sucker (Okawara *et al.*, 1988) and *Xenopus laevis* (Stenzel-Poore *et al.*, 1992). Thus, CRF is highly conserved across vertebrates (Fig. 1.5). Later, a new mammalian CRF-related peptide (urocortin) was characterized from the rat brain (Vaughan *et al.*, 1995). Rat urocortin shares 65% and 43% sequence identity with white sucker UI and rat CRF, respectively.

It was suggested that UI, urocortin and sauvagine are orthologues, whereas CRF forms a paralogous lineage that has diverged as a result of gene duplication (Lovejoy and Balment, 1999). Recently, two urocortin-related peptides were identified from the human (Hsu and Hsueh,

2001) and mouse (Lewis *et al.*, 2001; Reyes *et al.*, 2001) genomes. These new peptides were named urocortin II (stresscopin-related peptide) and urocortin III (stresscopin). Although these new peptides share only 20-40% identity with urocortin, they bind with high affinity to the type 2 CRF receptor (Hsu and Hsueh, 2001; Lewis *et al.*, 2001; Reyes *et al.*, 2001). Urocortin-related peptides were also identified by database analysis in the pufferfish *Takifugu rubripes* and *Tetraodon nigroviridis* (Brunner *et al.*, 2000). The identification of these new peptides implies that there is still a lot to learn regarding the phylogeny of the CRF peptide family.

Human (NP_000747)	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII
Rat (P01143)	----------
Mouse (AAN07905)	----------
Porcine major (P06296)	----------
Porcine minor (1205369A)	----------NF
Sheep (P01142)	-Q----------TK-D----------LD-A
Bovine (AAK83231)	-Q----------TK-D-------N----LD-A
Dog (P49926)	----------PG----------
Xenopus laevis (P49188)	A----------I----------D--
Western spadefoot toad (AAP20883)	A----------I----------D--
Goldfish (AAF04625)	----------M---------M---F
Common carp (CAC84859)	---A----------M---------M---F
White sucker 1 (P13241)	----------M---F
White sucker 2 (P25308)	----------V--------M---F
Sockeye salmon	-DD----------M--QMM--S-----Q--------M---F
Arctic char (AAO89527)	-DD----------M--QMM--S-----Q--------M---F
Brown bullhead (AAP21785)	--D----------MM--S---------QN--RM--LF
Tilapia (CAB77056)	--D----------MM--S---------QN--RM--LF
Japanese pufferfish	--D----------MM--SK--------QN--IM--LV
Constant	**....PISLDLTFH.LR...EM..A.Q..QQA..NR.....**

Figure 1.5 Alignment of vertebrate CRF amino acid sequences. Only positions that differ from the human sequence are shown. GenBank accession numbers appear in parentheses. The sockeye salmon sequence was obtained from Ando *et al.* (1999) and the Japanese pufferfish sequence was obtained by sequence homology search of the genome database at http://www.ensembl.org.

CRF is encoded by a single gene that is localized on chromosome 8 in humans (Arbiser *et al.*, 1988) and chromosome 3 in mouse (Knapp *et al.*, 1993). Given that a single gene is present,

this implies that central and peripheral CRFs are transcribed from the same gene (Arbiser *et al.*, 1988). The structure of the mouse and human genes are similar; they both contain two exons separated by an intron of 686-800 bp, respectively (Shibahara *et al.*, 1983; Majzoub *et al.*, 1993). The second exon encodes the entire precursor protein of CRF (Shibahara *et al.*, 1983; Fig. 1.6). The 41-amino acid CRF mature peptide is generated by the proteolytic cleavage of the C-terminus of the precursor protein. This precursor also encodes a signal peptide at the N-terminus and a cryptic region for which there is no recognized function. Two distinct cDNAs encoding CRF precursor proteins have been reported in two tetraploid fish, the sockeye salmon (*Oncorhynchus nerka*; Ando *et al.*, 1999) and the white sucker (Morley *et al.*, 1991).

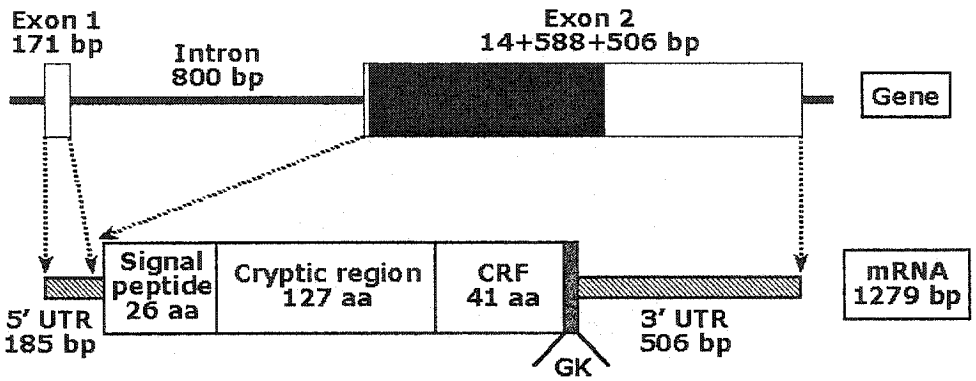

Figure 1.6 Schematic outline of human corticotropin-releasing factor (CRF) gene and mRNA. The coding sequence includes a signal peptide, a cryptic region, CRF, and a proteolytic processing site (GK). Based on Shibahara *et al.*, 1983.

1.3.2. Localization

Most CRF-containing fibres that reach the median eminence originate from the paraventricular nucleus (PVN) of the rat brain (Swanson *et al.*, 1983). Within the PVN, the majority of CRF-containing cells are concentrated in the parvocellular division and to a lesser

extent in the magnocellular division. CRF-containing neurons and fibres are also present in areas of the basal telencephalon, hypothalamus, and brain stem that play a role in the mediation of autonomic nervous system responses including the nucleus locus coeruleus. CRF-containing neurons are also found throughout most areas of the cerebral cortex. In mammals, CRF is expressed in peripheral tissues, particularly in the placenta where CRF expression rises exponentially during gestation (McLean *et al.*, 1995). CRF has also been localized in endocrine cells of the adrenal gland, testis, gut, spleen, thymus and skin (Vale *et al.*, 1997).

CRF-like immunoreactivity is mainly present in parvocellular and magnocellular divisions of the nucleus preopticus (NPO) of teleosts, an area equivalent to the mammalian PVN (Olivereau *et al.*, 1984; Yulis *et al.*, 1986; Olivereau and Olivereau, 1988; Matz and Hofeldt, 1999). CRF-immunoreactive fibres follow the NPO-pituitary pathway through the basal hypothalamus and terminate in the proximity of ACTH cells in the pars distalis of the pituitary (Olivereau and Olivereau, 1988; Fig. 1.7). To date, the presence of CRF in peripheral tissues has not been examined in fish.

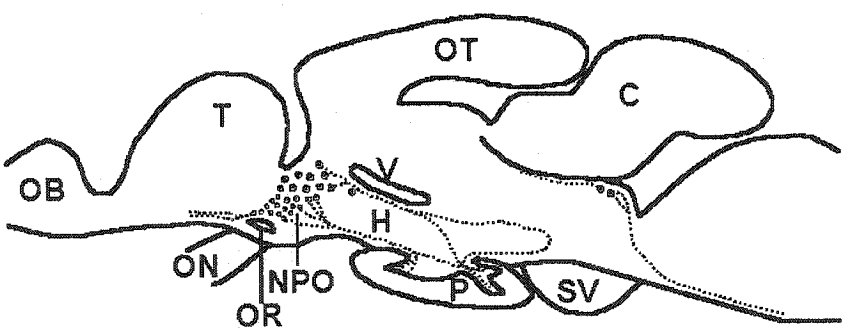

Figure 1.7 Schematic representation of a paramedian sagittal section of the brain of a European eel (*Anguilla anguilla*). Dots: CRF-like perikarya. Dashed lines: CRF-like fibers. Abbreviations are: olfactory bulbs (OB), telencephalon (T), optic nerve (ON), optic recess (OR), nucleus preopticus (NPO), hypothalamus (H), third ventricle (V), pituitary (P), saccus vasculosus (SV), optic tectum (OT), and cerebellum (C). Adapted from Olivereau and Olivereau, 1988.

1.3.3. Functions

Although CRF was first known for its role as a potent ACTH secretagogue (Vale *et al.*, 1981), it is also thought to mediate a wide array of endocrine, physiological, neurochemical and behavioural responses (reviewed by Dunn and Berridge, 1990). Evidences suggest that CRF can act as a neurotransmitter as well as a neurohormone (reviewed by Koob, 1999). CRF can act directly at specific sites of the central nervous system and modulate behavioural actions that appear to be independent of the HPA axis. For example, brain injections of CRF in rats can increase anxiety-like behaviour, decrease food intake, enhance learning, increase arousal, inhibit sexual behaviour, and alter locomotor activity (reviewed by Dunn and Berridge, 1990). Central administration of CRF increases locomotor activity in chinook salmon (*Oncorhynchus tshawytscha*), and this response is prevented by the CRF receptor antagonist α-helical CRF_{9-41} (Clements *et al.*, 2002).

CRF is thought to be one of the main mediators of stress-induced reduction in growth and reproduction (Wendelaar Bonga, 1997). The effect on reproduction is thought to be the result of CRF-induced inhibition of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretions in rats (Rivier and Vale, 1984; Ono *et al.*, 1984; Petraglia *et al.*, 1987). CRF could reduce growth by inhibiting the release of growth hormone (GH) (Ono *et al.*, 1984; Rivier and Vale, 1985). Part of the stress-induced reduction in growth could be attributed to CRF-mediated reduction in food intake; CRF suppresses food intake in both mammals (Glowa *et al.*, 1992) and teleosts (de Pedro *et al.*, 1993; Bernier and Peter, 2001). However, under normal conditions, CRF is not thought to be involved in the regulation of food intake, but may play a

role in the control of food intake during exposure to stressful conditions (de Pedro *et al.*, 1997). Intracerebroventricular administration of α-helical CRF_{9-41} in goldfish did not affect feeding on its own but prevented the CRF-induced reduction in food intake (de Pedro *et al.*, 1997).

As discussed above, CRF is a potent stimulator of ACTH release. CRF also stimulates the secretion of other POMC-derived peptides such as β-endorphin and α-MSH in mammals and fish (Chan *et al.*, 1982; Kraicer *et al.*, 1985; Fryer, 1989). The release of β-endorphin is thought to be responsible for the antinociceptive effects of CRF (Hargreaves *et al.*, 1990). Abnormal regulation of CRF expression and activity is linked to the development of psychiatric disorders including depression, anxiety and anorexia (reviewed by Behan *et al.*, 1996; Arborelius *et al.*, 1999).

CRF is also a potent stimulator of thyrotropin (TSH) release from the pituitary gland of coho salmon (*Oncorhynchus kisutch*; Larsen *et al.*, 1998). CRF was shown to stimulate the thyroid axis in representative species of each nonmammalian vertebrate class, *i.e.* fish, amphibians, reptiles and birds (reviewed by Denver, 1999). As a stimulator of thyroid activity, CRF accelerates amphibian metamorphosis (Denver, 1997).

1.3.4. Receptors

The effects of CRF-like peptides are mediated through specific membrane receptors on target cells. All CRF receptors identified to date are members of the class B subfamily of seven-transmembrane domains, G-protein-coupled receptors. CRF receptors in most cell types couple with a stimulatory G protein (Gs) (reviewed by Dautzenberg and Hauger, 2002). The binding of an agonist to most CRF receptors activates adenylyl cyclase and the cAMP second messenger pathway. However, in tissues such as the placenta, the type 1 CRF receptor is though to signal

through a Gq-mediated stimulation of phospholipase C (PLC) and formation of inositol phosphates (Grammatopoulos *et al.*, 2000).

Two CRF receptors have been identified in mammals, CRF-R1 (Chang *et al.*, 1993; Chen *et al.*, 1993; Perrin *et al.*, 1993; Vita *et al.*, 1993) and CRF-R2 (Kishimoto *et al.*, 1995; Lovenberg *et al.*, 1995; Perrin *et al.*, 1995; Stenzel *et al.*, 1995). Several alternatively spliced variants are reported for both receptor types. CRF-R1 has one functional and several non-functional variants (Dautzenberg and Hauger, 2002). Rodents have two functional variants of CRF-R2 (Lovenberg *et al.*, 1995), whereas a third variant (CRF-R2γ) was identified in humans (Kostich *et al.*, 1998). These variants differ at their N-terminal sequences and have different expression patterns. In rats, CRF-R2α is primarily expressed in the brain, whereas CRF-R2β is found largely in peripheral tissues (Chalmers *et al.*, 1996). CRF-R1 is expressed in anterior pituitary corticotropes, the intermediate lobe of the pituitary, and several sites within the central nervous system (Potter *et al.*, 1994).

The activation of a receptor depends largely on its ligand binding affinity. In rats, CRF-R1 has a high binding affinity for both CRF and urocortin, whereas the binding affinity of CRF-R2 for urocortin is greater than that for CRF (Vaughan *et al.*, 1995). This led to the hypothesis that urocortin might be the endogenous ligand of CRF-R2 (Vaughan *et al.*, 1995). However, a recent study found little overlap between urocortin-containing neurons and neurons expressing CRF-R2 in the rat brain (Bittencourt *et al.*, 1999).

cDNAs encoding CRF receptors have recently been cloned from two species of teleost fish. Three distinct CRF receptors were obtained from a diploid species of catfish, *Ameiurus nebulosus* (Arai *et al.*, 2001). The first two receptors (cfCRF-R1 and cfCRF-R2) share 93% and 88% identity to the mouse CRF-R1 and CRF-R2 receptors, respectively. The structure of the

third catfish receptor (cfCRF-R3) is closer to cfCRF-R1 (85%) than to cfCRF-R2 (80%). The expression pattern of cfCRF-R1 in the brain is similar to that of mammalian CRF-R1. However, cfCRF-R1 shows weak expression in the pituitary gland. High expression in the pituitary was only observed for cfCRF-R3, whereas cfCRF-R2 was expressed primarily in the heart.

Two cDNAs encoding CRF receptors have also been cloned from the chum salmon, *Oncorhynchus keta* (Pohl *et al.*, 2001). The expression of salmon CRF-R1 was widespread (brain, heart, skeletal muscle, gills, ovary and the lateral line system), whereas CRF-R2 expression was limited to the brain and heart. Both salmon receptors bind UI with approximately 8-fold higher affinity than rat/human CRF. However, the affinity for salmon CRF might be different from that of the rat/human form. The CRF sequence of a closely related salmonid, the sockeye salmon (*Oncorhynchus nerka*) has 10 substitutions relative to the rat and human forms (Ando *et al.*, 1999). Also, the binding assays were performed on transfected mammalian cells at 37°C. Because salmon are found in cold waters, the conformation of the receptors at 37°C might be different (Pohl *et al.*, 2001).

The regulation of CRF receptors can influence the biological response mediated by the release of CRF-like peptides. Levels of type 1 CRF receptor (CRF-R1) mRNA are increased in the paraventricular nucleus of stressed rats (Luo *et al.*, 1994; Makino *et al.*, 1995b; Rivest *et al.*, 1995; Imaki *et al.*, 1996; Bonaz and Rivest, 1998). Chronic stress decreases CRF receptors in the anterior pituitary of rats (Hauger *et al.*, 1988). In the anterior pituitary, sustained exposure to high concentrations of CRF desensitizes CRF-stimulated cAMP accumulation, decreases ACTH release, downregulates of CRF-R1, and decreases CRF-R1 mRNA (Aguilera *et al.*, 2001; Pozzoli *et al.*, 1996; Dautzenberg and Hauger, 2002). There is a rapid agonist-induced phosphorylation of the receptors in cells transfected with the human CRF-R1 (Hauger *et al.*, 2000).

1.3.5. Binding protein

CRF-binding protein (CRF-BP) can modulate the biological effects of CRF-like peptides. This 37 KDa glycoprotein was originally isolated from human plasma (Orth and Mount, 1987; Behan *et al.*, 1989) and the cDNA was cloned from human and rat (Potter *et al.*, 1991). CRF-BP have also been cloned from mouse, sheep and *Xenopus laevis* (Cortright *et al.*, 1995; Behan *et al.*, 1996; Brown *et al.*, 1996; Valverde *et al.*, 2001). Although no fish CRF-BP is reported, Seasholtz and colleagues presented evidence of CRF-BP activity in the sea lamprey (*Petrozymon marinus*) and tilapia (*Tilapia mossambicus*) (Seasholtz *et al.*, 2002).

The tissue localization of CRF-BP varies among species (reviewed by Kemp *et al.*, 1998). For example, CRF-BP is present in human plasma, but not in the plasma of rodents and sheep. In all species examined, CRF-BP is present in specific areas of the brain, the pituitary and peripheral tissues where it is thought to modulate the action of CRF-like peptides (Seasholtz *et al.*, 2002). The binding affinity of human CRF-BP for CRF and urocortin is greater or equal to that of CRF receptors (Vaughan *et al.*, 1995). The structural requirements for the binding of CRF-like peptides to CRF-BP, however, are different from those of CRF receptors (Sutton *et al.*, 1995). For example, human CRF-R1 binds ovine CRF with very high affinity, whereas the binding of ovine CRF to human CRF-BP is very weak.

The function(s) of CRF-BP is still unclear but numerous studies support an inhibitory role for CRF-BP on CRF action (see Seasholtz *et al.*, 2002). In humans, the levels of placental CRF rise exponentially during pregnancy (McLean *et al.*, 1995). Most plasma CRF is bound to its binding protein and therefore unable to bind to its receptor (Zhao *et al.*, 1997). The binding of CRF to plasma CRF-BP would prevent inappropriate stimulation of the stress axis by placental

CRF (Petraglia *et al.*, 1993). In a line of transgenic mice that overexpress CRF-BP in the pituitary only, the expression of hypothalamic CRF is elevated to maintain normal levels of free CRF and normal activity of the stress axis (Burrows *et al.*, 1998). In another line of transgenic mice where CRF-BP is overexpressed throughout the brain, mice show increased weight gain (Lovejoy *et al.*, 1998). Finally, in a CRF-BP-deficient mouse model, mice show increased anxiety-like behaviour and decreased weight gain (Karolyi *et al.*, 1999). These results are all consistent with an inhibitory role for CRF-BP on CRF action. The presence of CRF-BP in corticotropes of the anterior pituitary suggests that it could act to terminate the action of receptor-bound CRF (Seasholtz *et al.*, 2002). CRF-BP mRNA levels in the rat pituitary are increased by stress and decreased by adrenalectomy (McClennen *et al.*, 1998).

1.3.6. Control of CRF release

The release of CRF is stimulated by stress but the mechanism involved in the control of CRF secretion remains unclear. Catecholamines could be involved in the stress-induced activation of CRF release. Norepinephrine (NE) stimulates the release of CRF *in vitro* (Hillhouse and Milton, 1989b) and catecholaminergic axons innervate CRF-producing neurons in the rat PVN (Alonso *et al.*, 1986; Liposits *et al.*, 1986; Kitazawa *et al.*, 1987). In the brain, cells of the nucleus locus coeruleus, which is the major source of NE projections to the forebrain, are activated by stress (Valentino *et al.*, 1993). This activation stimulates the release of NE in the PVN, which in turn stimulates CRF release. CRF neurons also innervate the locus coeruleus and stimulate the activity of this brain region (Van Bockstaele *et al.*, 1998). These observations led to the hypothesis of a feed-forward system where forebrain CRF and brainstem NE activate each other (Koob, 1999).

Several other neurotransmitters can influence the secretion of CRF (Fig. 1.1). Both acetylcholine and serotonin (5-HT) stimulate CRF release (Hillhouse and Milton, 1989a), whereas gamma-aminobutyric acid (GABA) inhibits CRF secretion (Hillhouse and Milton, 1989b). Serotonin also stimulates CRF gene expression (Kageyama *et al.*, 1998), and there are synaptic connections between serotonergic axons and CRF-producing neurons in the rat PVN (Liposits *et al.*, 1987). GABAergic axons also innervate CRF-producing neurons in the rat PVN (Miklos and Kovács, 2002).

The release of CRF can be influenced by other neuroendocrine factors including NPY. Studies in the rat indicate that NPY increases CRF mRNA (Suda *et al.*, 1993), CRF immunoreactivity (Haas and George, 1987) and CRF release (Haas and George, 1989; Tsagarakis *et al.*, 1989). Several studies indicate that stress increases NPY mRNA in the arcuate nucleus of the rat brain (Conrad and McEwen, 2000; Makino *et al.*, 1999, 2000; Sweerts *et al.*, 2001), whereas only one study showed a stress-induced decrease in NPY mRNA in the same hypothalamic area (Krukoff *et al.*, 1999). Furthermore, NPY nerve terminals establish synaptic connections with parvocellular neurons producing CRF in the rat (Liposits *et al.*, 1988; Mihaly *et al.*, 2002).

Glucocorticoids have an inhibitory effect on CRF release in certain areas of the brain as noted above. Part of this inhibitory effect of glucocorticoids is mediated by actions at higher brain centers. Neurons of the hippocampus that are responsive to glucocorticoids project to the hypothalamus and affect the release of CRF to the anterior pituitary (Sapolsky *et al.*, 1986). In fact, under basal conditions, the hippocampus is the primary site for the glucocorticoid feedback inhibition of the HPA axis activity (Jacobson and Sapolsky, 1991). The mineralocorticoid receptor (MR) is highly expressed in the rat hippocampus but not in the PVN (Reul and de Kloet,

1985). The affinity of the mammalian MR for glucocorticoids is greater than that of GR, resulting in a predominant MR occupation at low glucocorticoid levels (Reul and de Kloet, 1985; Arriza *et al.*, 1988; Joels and de Kloet, 1994).

The release of CRF from the paraventricular nucleus is also under the influence of other brain areas including the suprachiasmatic nucleus. Basal CRF release is controlled by circadian rhythms, which are generated in the suprachiasmatic nucleus (Moore and Eichler, 1972). The diurnal changes in CRF release parallels the pattern of ACTH and cortisol secretion in humans (Watabe *et al.*, 1987).

1.3.7. Rainbow trout and CRF

The sequence of the cDNA coding for the rainbow trout CRF precursor protein was unknown before my work began. However, the sequence of a close relative, the sockeye salmon (*Oncorhynchus nerka*), was recently reported (Ando *et al.*, 1999). Ando *et al.* (1999) provided the first evidence that CRF mRNA levels are responsive to stress and that CRF may be involved in the stress response in rainbow trout; using a sockeye salmon probe, the number of CRF mRNA-positive perikarya was increased in the trout preoptic nucleus after 3 h of confinement stress. The fact that goldfish CRF mRNA levels are influenced by cortisol provides further support for CRF involvement in the stress response in fish (Bernier *et al.*, 1999). As noted above, the main objective of this thesis is to improve our understanding of how CRF mRNA levels are modified by stress and cortisol levels in rainbow trout, and this required the characterization of the cDNA coding for the trout CRF precursor protein.

1.4. Neuropeptide Y (NPY)

1.4.1. Structure

Neuropeptide Y (NPY) is a 36-amino acid peptide that was first isolated from the pig brain in the early 1980s (Tatemoto *et al.*, 1982). The name NPY derives from its neural origin and the presence of tyrosine residues (single letter code Y) at its amino- and carboxy-terminal ends. NPY is a member of a family of peptides that includes pancreatic polypeptide (PP), peptide YY (PYY) and fish pancreatic peptide (PY) (reviewed by Cerdá-Reverter and Larhammar, 2000). Because PP was the first member characterized (Kimmel *et al.*, 1975), this family of peptides was initially designated as the PP family. However, due to the higher degree of conservation of NPY, Larhammar *et al.* (1993b) suggested that the family should be renamed the NPY family. In fact, among neuropeptides of equal or larger size, NPY demonstrates the highest degree of conservation across vertebrates (Larhammar *et al.*, 1993a; Fig. 1.8).

The primary structure of the NPY precursor protein is also highly conserved (Cerdá-Reverter and Larhammar, 2000). It includes a hydrophobic signal peptide at the N-terminal end, the mature NPY peptide, an amidation-proteolytic site (GKR, GRR, or GKS), and a carboxy-terminal peptide of neuropeptide Y (CPON) (Fig. 1.9). The genomic sequence of human NPY is divided into four exons that are separated by three introns and most of the mature NPY sequence is encoded by the second exon (Minth *et al.*, 1986; Fig. 1.9). The exon-intron organization is conserved in all other vertebrate species examined, including rat (Larhammar *et al.*, 1987), chicken (Blomqvist *et al.*, 1992), *Xenopus laevis* (Griffin *et al.*, 1994) and zebrafish (Söderberg *et al.*, 2000).

To date, two distinct NPY paralogues have only been characterized in one species, the tetraploid frog *Xenopus laevis* (van Riel *et al.*, 1993; Griffin *et al.*, 1994).

Human (P01303)	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY
Rhesus monkey (AAD43583)	----------
Bovine (P79113)	----------L----------
Pig (P01304)	----------L----------
Guinea pig (B30485)	----------
Rabbit (A30485)	----------
Rat (P07808)	----------
Mouse (P57774)	----------
Sheep (P14765)	---------D------L----------
Chicken (P28673)	------S----------
Alligator (AAB27842)	----------
Rana ridibunda (P29949)	----------K----------
Rana temporaria (AAB21521)	----------K----------
Xenopus laevis 1 (AAA49913)	----------K----------
Xenopus laevis 2 (AAA49914)	---------D--------K----------
Rio Cauca caecilian (AAD48033)	----------K----------
European sea bass (CAB64932)	--V--E---------EL-K----------
Japanese pufferfish	--V--E---------EL-K----------
Japanese flounder (BAB62409)	--V--E---D-----EL-K----------
Cod (S27054)	--I--E--------DEL-K----------
Goldfish (P28672)	--T-------G----EL-K----------
Common carp (AAG00549)	--T----------EL-K----------
Zebrafish (AAF79941)	--T----------EL-K----------
Channel catfish (AAF71617)	--T--E-------V-EL-K----------
Rainbow trout (AAB25269)	--V--E-------T-EL-K--T----------
Marbled electric ray (P28674)	----------G-----L-K----------
European common dogfish (AAB23237)	----------G-----L-K----------
River lamprey (P48097)	F-N---S---------L---L--V----------
Constant	**.P.KP..PG..AP....A.Y..A.RHYINLITRQRY**

Figure 1.8 Alignment of vertebrate NPY amino acid sequences. Only positions that differ from the human sequence are shown. Adapted from Cerdá-Reverter and Larhammar, 2000. Genbank accession numbers appear in parentheses. The Japanese pufferfish sequence was obtained by sequence homology search of the genome database at http://www.ensembl.org.

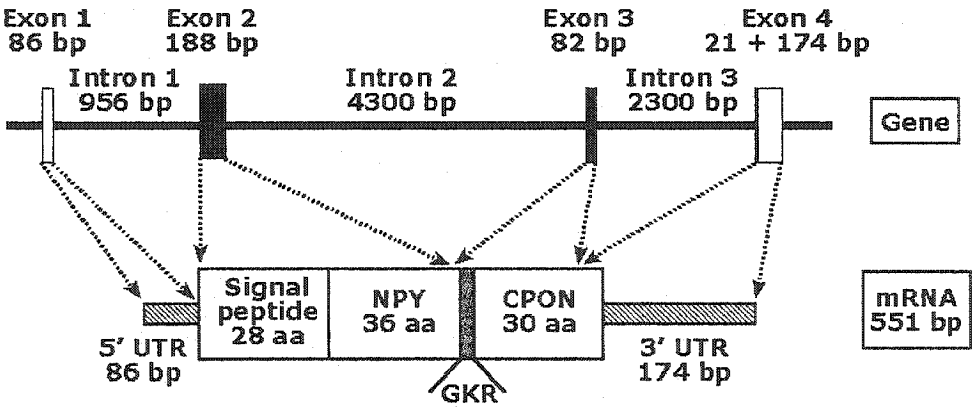

Figure 1.9 Schematic outline of the human neuropeptide Y (NPY) gene and mRNA. The coding sequence includes a signal peptide, NPY, a proteolytic processing site (GKR) and a carboxy-terminal peptide of neuropeptide Y (CPON). Adapted from Cerdá-Reverter and Larhammar, 2000.

1.4.2. Localization

NPY is expressed mainly in the central nervous system in mammals (Allen and Balbi, 1993). NPY is widely spread throughout the brain of all vertebrates but NPY expression is particularly high in the forebrain. In mammals, NPY levels are highest in hypothalamic nuclei including the paraventricular nucleus, arcuate nucleus, suprachiasmic nucleus, median eminence, and dorsomedial nucleus. In fish, NPY is most abundant in the nucleus endopeduncularis of the basal telencephalon (Danger *et al.*, 1991; Peng *et al.*, 1994).

1.4.3. Functions

The high degree of conservation of NPY and its abundance in the vertebrate brain suggest important roles for NPY. In fact, NPY is the most abundant peptide in the central and sympathetic nervous systems of mammals (Sundler *et al.*, 1993).

NPY is primarily known for its role as an orexigenic signal (Kalra *et al.*, 1991). In fact, NPY is considered to be the most potent appetite stimulator known to date (Dube *et al.*, 1994). For example, in mammals, NPY is the only known neurotransmitter able to induce obesity within a few days of continuous intracerebroventricular administration (Tomaszuk *et al.*, 1996). Fasting increases NPY mRNA levels in the goldfish brain (Narnaware and Peter, 2001a) and the preoptic area of the salmon hypothalamus (Silverstein *et al.*, 1998). Intracerebroventricular injections of NPY in goldfish increase food intake (López-Patiño *et al.*, 1999; Narnaware *et al.*, 2000; Narnaware and Peter, 2001b), and this response is completely abolished by the NPY receptor antagonist $NPY_{27\text{-}36}$ (López-Patiño *et al.*, 1999).

Several studies in mammals suggest that NPY could play a role in the stress-induced activation of the HPA axis. Stress increases NPY mRNA in the arcuate nucleus of rats (Conrad and McEwen, 2000; Makino *et al.*, 1999, 2000; Sweerts *et al.*, 2001), and NPY increases CRF mRNA (Suda *et al.*, 1993), CRF immunoreactivity (Haas and George, 1987) and CRF release (Haas and George, 1989; Tsagarakis *et al.*, 1989). Furthermore, NPY axons establish synaptic connections with parvocellular neurons producing CRF in rats (Liposits *et al.*, 1988; Mihaly *et al.*, 2002). NPY acts as a potent stimulator of ACTH release (Wahlestedt *et al.*, 1987), and high levels of NPY immunoreactivity are found in hypophysial portal blood (Danger *et al.*, 1990). Together, these findings suggest that NPY stimulates HPA activity at both the hypothalamic and pituitary levels.

NPY is also involved in the peripheral response to stress. NPY is found in the sympathetic nervous system where it co-localizes with the majority of catecholamine-producing neurons that innervate the heart and blood vessels (reviewed by Zukowska-Grojec, 1995;

Gibbins and Morris, 2000). NPY is co-released with norepinephrine and it independently stimulates vasoconstriction by interacting with a NPY receptor.

1.4.4. Receptors

The effects of the NPY family of peptides are mediated through seven-transmembrane domain, G-protein-coupled receptors. Four receptor subtypes have been cloned in mammals (Y_1, Y_2, Y_4, Y_5) and a fifth receptor (Y_3) pharmacologically characterized (Balasubramaniam, 1997; Blomqvist and Herzog, 1997). A sixth NPY receptor (Y_6) was cloned and identified under different names by several investigators (Gregor *et al.*, 1996a; Matsumoto *et al.*, 1996; Weinberg *et al.*, 1996), and pharmacologically characterized (Mullins *et al.*, 2000). This receptor is truncated in primates as a result of a single base deletion in the sixth transmembrane domain (Gregor *et al.*, 1996; Matsumoto *et al.*, 1996; Rose *et al.*, 1997).

The Y_1 receptor is found on post-synaptic membranes in the central nervous system, but also in blood vessels where it stimulates vasoconstriction. The Y_2 receptor is found mostly on pre-synaptic membranes where it inhibits transmitter release. The Y_3 receptor differs from Y_1 and Y_2 by its lack of affinity for PYY, whereas the Y_4 receptor has a greater affinity for pancreatic polypeptide (Lundell *et al.*, 1995, 1996; Gehlert *et al.*, 1996; Gregor *et al.*, 1996b). The Y_5 receptor is highly expressed in the brain. Both Y_1 and Y_5 receptors mediate NPY actions on food intake in rats (Marsh *et al.*, 1998; Kanatani *et al.*, 2000; Polidori *et al.*, 2000) and goldfish (Narnaware and Peter, 2001b).

Only three NPY receptor subtypes have been cloned in teleost fish: Ya (Starbäck *et al.*, 1999), Yb (Balasubramaniam, 1997; Lundell *et al.*, 1997; Sharma *et al.*, 1999), and Yc (Ringvall *et al.*, 1997). The pharmacological profile of the zebrafish Ya receptor is similar to that of Y_5,

although they share only 27% amino acid identity (Starbäck *et al.*, 1999). The Yb and Yc receptors are more closely related to the Y_1 receptor.

1.4.5. Rainbow trout and NPY

Although a sequence of the rainbow trout NPY mature peptide was reported (Jensen and Conlon, 1992), the primary structure of the cDNA coding for the trout NPY precursor protein was unknown before my work began. Until now, studies in fish had not addressed the issue of the possible regulation of NPY mRNA in response to stress. As noted above, the primary objective of this thesis is to improve our understanding of how NPY mRNA levels are modified by stress and cortisol levels in rainbow trout, and this required the characterization of the cDNA coding for the trout NPY precursor protein.

1.5. Summary

The studies described in the following chapters were designed to test the hypotheses and objectives that appear in sections 1.1.2 and 1.1.3. Chapter 2 describes the characterization of rainbow trout cDNA sequences coding for NPY and two CRF paralogues. Phylogenetic analyses were used to describe the relationship of these new sequences with other vertebrate sequences. Northern hybridizations were used to determine the size of CRF and NPY transcripts. A ribonuclease protection assay (RPA) was developed to assess CRF, NPY and β-actin mRNA levels. This RPA was used to describe the distribution of CRF and NPY mRNA in various brain regions and to determine the effects of social interactions on CRF1 and NPY mRNA levels. The levels of CRF1 and NPY mRNAs were elevated in the preoptic area of subordinate trout after 72 h of social interactions.

Chapter 3 looks at the effects of isolation and physical disturbance on CRF1 and NPY mRNA levels. Isolation in a large tank produced a transient increase in plasma cortisol, and a transient but delayed elevation in CRF1 mRNA levels. The magnitude and persistence of the elevation in CRF1 mRNA was greater in fish isolated in a confinement box. Physical disturbance increased both CRF1 and NPY mRNA levels but only after repeated chasing events.

Chapter 4 looks at the effects of cortisol on CRF1 and NPY mRNA levels. Fish were implanted with pellets containing cortisol, a glucocorticoid receptor antagonist (RU-486) or a cytochrome P450 antagonist that blocks cortisol synthesis (metyrapone). After the injection, trout were isolated in a large tank or confined. Cortisol prevented the increase in CRF1 and NPY mRNA in isolated fish, whereas RU-486 prevented the increase in CRF1 mRNA in both isolated and confined fish. Treatment with metyrapone increased the stress-induced elevation in CRF1 and NPY mRNA. The discussion focuses on whether or not my results support the presence of a glucocorticoid negative feedback loop affecting the levels of CRF and NPY mRNA in trout.

CHAPTER 2

CORTICOTROPIN-RELEASING FACTOR AND NEUROPEPTIDE Y mRNA LEVELS ARE ELEVATED IN THE PREOPTIC AREA OF SOCIALLY SUBORDINATE RAINBOW TROUT

Adapted from: Doyon, C., Gilmour, K.M., Trudeau, V.L., and Moon, T.W. 2003. *Gen. Comp. Endocrinol.* **133**: 260-271.

2.1. Introduction

Social stress can be detrimental to the growth and health of fish and other vertebrates. When salmonid fish are held in small groups, they establish a linear dominance hierarchy (reviewed by Sloman and Armstrong, 2002) in which subordinate fish have reduced access to food (McCarthy *et al.*, 1992), increased standard metabolic rates (Sloman *et al.*, 2000) and decreased growth rates (Abbott and Dill, 1989; Ryer and Olla, 1996). Similar dominance hierarchies occur in natural populations of stream-dwelling salmonids (Bachman, 1984). The reduction in growth rate may be attributed, at least in part, to the catabolic effects of cortisol (Pickering, 1990; Mommsen *et al.*, 1999). Characteristically, subordinate fish have sustained elevated levels of circulating cortisol (Øverli *et al.*, 1999; Sloman *et al.*, 2001b). Shortly after the onset of social interactions, cortisol levels are also elevated in dominant fish, but once the hierarchy is established, cortisol rapidly decreases to control levels. The chronically-elevated cortisol levels suggest that subordinate fish are under constant stress, which maintains the activation of the hypothalamic-pituitary-interrenal (HPI) axis.

The HPI axis is partly controlled by the 41 amino acid hypothalamic neuropeptide corticotropin-releasing factor (CRF), which stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Rivier and Plotsky, 1986; Fryer, 1989). In turn, ACTH stimulates the interrenal cells to produce cortisol, the main corticosteroid in fish (reviewed by Wendelaar Bonga, 1997). Subordinate trout have elevated plasma concentrations of ACTH (Höglund *et al.*, 2000) and pituitary levels of pro-opiomelanocortin (POMC) mRNA, the precursor of ACTH (Winberg and Lepage, 1998). In rats, exposure to both physical and psychological stressors increases the levels of CRF mRNA (Lightman and Harbuz, 1993). In the present study, the effect of social stress on the levels of CRF mRNA in rainbow trout (*Oncorhynchus mykiss*) was investigated.

The activation of the HPI axis in response to stress could also depend on other neuroendocrine factors, including neuropeptide Y (NPY). In mammals, although NPY is primarily known for its role as a potent appetite stimulator (Kalra *et al.*, 1991), stress increases NPY mRNA in specific areas of the brain (Conrad and McEwen, 2000), and NPY is a potent stimulator of ACTH release (Wahlestedt *et al.*, 1987). To date, studies in fish demonstrate effects of NPY on food intake similar to those in mammals (Lin *et al.*, 2000), but the response of NPY to stress and the potential involvement of NPY in the control of the HPI axis remain to be elucidated. Therefore, I also examined the effect of social stress on the levels of NPY mRNA in trout.

Thus, the primary objective of the present study was to determine the effect of chronic social stress on the levels of CRF and NPY mRNA in rainbow trout. The first step was to characterize the cDNA of trout CRF and NPY, and assess their mRNA levels in various tissues. After the cloning of trout CRF1 and NPY, I developed a ribonuclease protection assay (RPA) to

simultaneously measure the mRNA levels of these two neuropeptides in relation to β-actin. This RPA was used to investigate the impact of chronic social stress on CRF1 and NPY mRNA levels, using a model in which pairs of fish were confined together in tanks. A food deprivation experiment was also conducted to examine whether feeding modified NPY mRNA levels in rainbow trout.

2.2. Materials and Methods

2.2.1. Animals

Immature female rainbow trout, *Oncorhynchus mykiss*, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). They were acclimated to the laboratory for at least 6 weeks in 1275 L fiberglass tanks of well-aerated dechloraminated City of Ottawa tap water at 13±1°C with a constant 12h light/12h dark photoperiod. Fish were fed 3 times per week with commercial trout pellets (Classic Floating Trout Grower; Martin Mills Inc., Tavistock, ON).

2.2.2. Amplification of NPY-related cDNA fragments

NPY-related cDNA fragments were amplified by rapid amplification of cDNA ends (RACE; Invitrogen, Burlington, ON). For 3' RACE, poly(A)$^+$ RNA was extracted from the telencephalon and hypothalamus using the Straight A's mRNA Isolation System (Novagen, Madison, WI). First-strand cDNA was synthesized from 400 ng poly(A)$^+$ RNA with SUPERSCRIPT II and the 3'RACE adapter primer (Invitrogen). Gene-specific oligonucleotide primers (Synthaid Biotechnologies Inc., Ottawa, ON) were designed on the basis of high sequence identity between goldfish NPY and a preliminary sequence of salmon NPY (Dr. J. Silverstein, pers. comm.). NPY-related cDNA fragments were amplified using a first gene-

specific primer (GSP1; Table 2.1), the abridged universal amplification primer (AUAP; Invitrogen), *Taq* polymerase (Invitrogen), and a Mastercycler Gradient (Eppendorf, Mississauga, ON) with the following program: 3 min denaturing at 94°C, then 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. After the last cycle, further extension was performed at 72°C for 7 min. An amplified product of approximately 500 bp was extracted from a 1% agarose gel with the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). Semi-nested PCR was performed using GSP2 (Table 2.1) to confirm that the extracted product was NPY-related.

For 5' RACE, poly(A)$^+$ RNA was extracted from the preoptic area of the brain. The gene-specific primers (GSP3 to GSP6; Table 2.1) were designed based on sequencing results from the 3' RACE. First-strand cDNA was synthesized from 30 ng poly(A)$^+$ RNA with SUPERSCRIPT II and GSP3 (Table 2.1). The cDNA was purified after each step of the 5' RACE with the QIAquick PCR Purification Kit (Qiagen). Tailing of the 5' end of the cDNA was performed using dCTP and terminal transferase (Roche, Laval, QC). A first PCR was carried out using the program described above, GSP4 (Table 2.1) and the 5' RACE abridged anchor primer (Invitrogen). Two subsequent rounds of semi-nested PCR were performed using the AUAP and GSP5 or GSP6 (Table 2.1). Each PCR step yielded a single band of decreasing size, as the position of the GSP approached the 5' end of NPY.

Table 2.1 Sequences of the primers used for cloning and the amplification of template DNA for the ribonuclease protection assay (RPA).

	5' Primers	Position	3' Primers	Position
NPY GSP1 (M87297)	TGCGGAGGAGCTCGCCAAGTATTA	170–193		
NPY GSP2 (M87297)	TCTGCACTGAGACACTACATCAAC CTC	195–221		
NPY GSP3 (AF203902)			TCATCATATCTGGACTGTG	345–363
NPY GSP4 (AF203902)			TGTGCTCTCCTTCAGCAGCAATT	312–334
NPY GSP5 (AF203902)			TGTGTCCAGTGTGTCAGGGCTG	280–301
NPY GSP6 (AF203902)			CCTCTTCCCATACCTCTGCCTTG	255–277
CRF (AF098629)	CATGAAGCTCAATTTCCTCGT	90–110	ATGGAAAAGCAGCACTATGGTA	640–661
CRF GSP1 (AF296672)			TGTTCCCAACTTTGCCTTGT	414–433
CRF GSP2 (AF296672)			AGAGTTGCGGTTCCCGTTTC	305–324
CRF GSP3	AGCTCTCGACAAATACCTAAAATC TTC			
RPA CRF1 (AF296672)	*ATTTAGGTGACACTATAGAA* CGCGACATGGAGAGGAATAC	272–291	*TAATACGACTCACTATAGGGAGA* GGAGCATGTGGAACGTAAG	523–541
RPA CRF2 (AY156929)	*ATTTAGGTGACACTATAGAAG* GATTTGTTTTTACGTTTAG	577–595	*TAATACGACTCACTATAGGGAG* AATCTGAGAATTACACAAG	706–724
RPA NPY (AF203902)	*ATTTAGGTGACACTATAGAA* CCGCTCTCAGACATTACATCA	225–245	*TAATACGACTCACTATAGGG* TGGTGAGATCAGTTGCTCGT	423–442
RPA β-actin	*ATTTAGGTGACACTATAGAAGVG* CGAGCACGGCATCGTCACC	274–292	*TAATACGACTCACTATAGGGAGA* CGCGGCCGGCCAGATCCAG	593–611

Note: Primers were designed based on the sequences for which the GenBank accession numbers appear in parentheses. The underlined nucleotide differs from that of the sequences used in primer design. Nucleotides in italics represent the promoter sequences for T7 and SP6 RNA polymerases.

2.2.3. Amplification of CRF-related cDNA fragments

Poly(A)$^+$ RNA was extracted from the preoptic area of the trout brain using the Straight A's mRNA Isolation System (Novagen). Oligonucleotide primers (Synthaid Biotechnologies Inc.) were designed on the basis of high sequence identity between the goldfish (Bernier *et al.*, 1999) and the sockeye salmon (Ando *et al.*, 1999) CRF sequences (Table 2.1). First-strand cDNA was synthesized from 400 ng poly(A)$^+$ RNA with SUPERSCRIPT II (Invitrogen) and a CRF-specific antisense primer (Table 2.1). Amplification was carried out using the following program: 5 min denaturing at 94°C, then 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min. After the last cycle, further extension was performed at 72°C for 5 min. The PCR reaction contained 4 µl of cDNA in a 50 µl PCR reaction and goldfish cDNA was used as a positive control. The 3' and 5' ends of trout CRF were obtained by 3' and 5' RACE as described for NPY. For 3' RACE, only one gene-specific primer was used, *i.e.* the sense primer of the initial cloning procedure (Table 2.1). For 5' RACE, the cDNA synthesis and the first PCR reaction were performed using the anti-sense primer of the initial cloning procedure (Table 2.1). Two semi-nested PCR reactions were performed using gene-specific primers (GSP1 and GSP2; Table 2.1) that were based on initial sequencing results. The 5' RACE led to the amplification of two distinct products. An additional gene-specific primer (GSP3; Table 2.1) was designed based on what appeared to be a fragment of a second trout CRF and used for 3' RACE.

2.2.4. DNA sequencing

Amplified NPY and CRF products were extracted from a 1% agarose gel with the QIAquick Gel Extraction Kit (Qiagen), ligated into the pCR II-TOPO cloning vector (TOPO TA

cloning kit; Invitrogen), and transformed into *Escherichia coli* competent cells (One Shot TOP10 Electrocomp cell; Invitrogen). Single colonies were cultured and plasmids were recovered with Wizard Plus (Promega, Madison, WI). All procedures were carried out according to the manufacturer's instructions. For each new NPY and CRF form, both strands of at least three different cloned inserts were sequenced (Canadian Molecular Research Services, Ottawa, ON). Nucleotide sequences were submitted to BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi; Altschul *et al.*, 1997) for comparison with sequences present in GenBank.

2.2.5. Northern hybridization

Northern hybridization was used to determine the size of the NPY and CRF transcripts. Briefly, 10 μg of total RNA from the preoptic area for CRF and 1 μg of poly(A)$^+$ RNA from the telencephalon and hypothalamus for NPY were separated on denaturing 1.5% agarose gels. RNA was blotted to Hybond N$^+$ membranes (Amersham, Baie d'Urfé, QC) by capillary transfer with 20x SSC. The RNA was bound to the membranes by 5 min exposure to ultraviolet light. My trout sequences were used to prepare probes specific to CRF (position 144 to 692 on CRF1) and NPY (position 223 to 659). The probes were prepared using Rediprime II Random Prime Labelling System and 50 μCi of Redivue α^{32}P-dCTP at 3000 Ci mmol^{-1} (Amersham). Unincorporated radionucleotides were removed using a ProbeQuant G-50 Micro Column (Amersham). The probes were hybridized to the membranes at 65°C for 4 h using Rapid-hyb buffer (Amersham). The membranes were washed at 65°C with 0.1x SSC and 0.1% SDS. After 18 h of exposure to the membranes, an Image Screen-K (BIO-RAD, Mississauga, ON) was scanned using a Molecular Imager-FX (BIO-RAD). The size of the CRF and NPY transcripts was estimated in relation to the known size of 28S and 18S ribosomal RNA.

2.2.6. Phylogenetic analyses

CLUSTAL W (http://www2.ebi.ac.uk/clustalw; Thompson *et al.*, 1994) was used with default settings to align my new sequences with the equivalent regions of previously sequenced vertebrate genes obtained from GenBank and the Japanese pufferfish (*Takifugu rubripes*) genome project (http://www.ensembl.org). The phylogenetic analyses were performed on predicted amino acids for the complete coding sequences. Maximum-likelihood trees were calculated with PROML from the PHYLIP package version 3.6a2.1 (http://evolution.genetics.washington.edu/phylip.html; Felsenstein, 2001), with the JTT model of amino acid substitutions (Jones *et al.*, 1992), eight gamma-distributed rates of heterogeneity, and a fraction of invariable sites. The alpha value of the gamma distribution and the fraction of invariant sites were calculated using TREE-PUZZLE version 5.0 (http://www.tree-puzzle.de/; Schmidt *et al.*, 2002). The alpha values were 1.65 and 9.17, and the fractions of invariant sites were 0.08 and 0.28 for CRF and NPY, respectively. For NPY, the outgroup was the sequence of the marbled electric ray (*Torpedo marmorata*), a cartilaginous fish. For CRF, as sequences from a taxon ancestral to teleosts were not available, I used the rainbow trout sequence for urotensin-I, a gene closely related to CRF (Baršytė *et al.*, 1999; Lovejoy and Balment, 1999). Bootstrap values were obtained by PROML analysis of a data set containing100 replicates.

2.2.7. Ribonuclease Protection Assay (RPA)

The mRNA levels of CRF, NPY and β-actin were determined using a Ribonuclease Protection Assay Kit (RPA III; Ambion, Austin, TX). The DNA templates for the preparation of antisense RNA probes were generated by PCR using primers with incorporated SP6 and T7

phage polymerase promoters on the sense and antisense primers, respectively (Table 2.1). DNA templates were amplified using the following program: 3 min denaturing at 94°C, then 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. After the last cycle, further extension was performed at 72°C for 7 min. Amplified products were extracted from a 1% agarose gel with the QIAquick Gel Extraction Kit (Qiagen). Antisense RNA probes were prepared using the MAXIscript *In vitro* Transcription Kit (Ambion) and approximately 100 ng of template DNA. For CRF1 and NPY, high specific activity probes were prepared by incubating the DNA at 37°C for 12 min with 50 μCi of α^{32}P-CTP at 400 Ci mmol^{-1} (Amersham) as the limiting nucleotide. To limit the hybridization signal of β-actin, a highly expressed gene, I prepared a low specific activity probe by incubating at 37°C for 1 h with 40 μCi of α^{32}P-CTP at 400 Ci mmol^{-1} (Amersham) in the presence of unlabeled CTP. The transcription products were incubated for 30 min at 37°C with 5 units of RNase-free DNase 1 (Promega). Full-length probes were purified from a 5% acrylamide/bis-acrylamide (19:1), 8 M urea gel. Approximately 7.5 x 10^4 cpm of probe was hybridized overnight at 42°C to 10 μg of total RNA. Non-hybridized transcripts were digested at 37°C for 30 min with approximately 0.4 units of RNase A and 15 units of RNase T1 (Ambion). CRF2 was analyzed separately using the same method, except that non-hybridized transcripts were digested with RNase T1 only. The specificity of the RPA analyses of CRF1 and CRF2 was ensured by the use of probes within regions that differed by 24 and 23 nucleotides, respectively. Protected fragments were resolved on a 5% acrylamide/bis-acrylamide (19:1), 8 M urea gel. An Image Screen-K was exposed for 18 h to the dried gel and the optical density of the bands was analyzed with Quantity One version 4.1.1 (BIO-RAD). Results were expressed in counts per square millimeter, the arbitrary volume unit of Quantity One. The RPA was optimized by verifying the linearity of the relation between

quantified mRNA and the amount of total RNA (Fig. 2.1 and 2.2). The standard curves for the optimization of the RPA demonstrate that the levels of CRF1 (Fig. 2.2A), CRF2 (Fig. 2.2B), NPY (Fig. 2.2C), and β-actin (Fig. 2.2D) mRNA can be quantified with this technique.

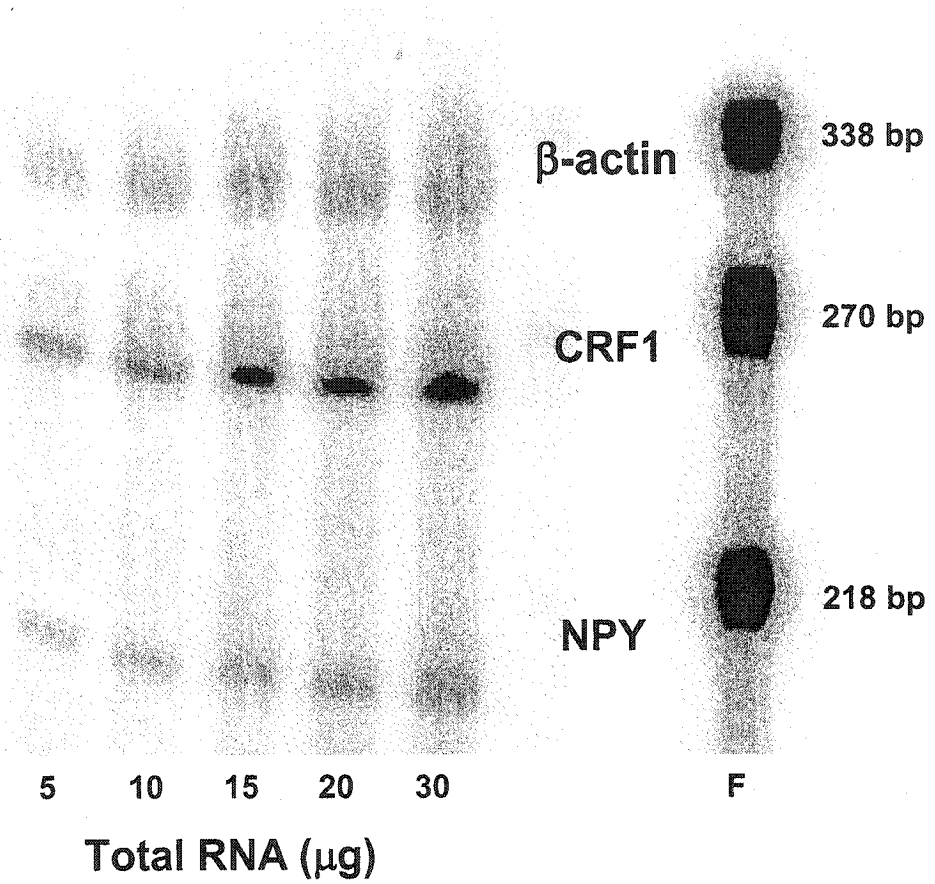

Figure 2.1 A representative ribonuclease protection assay (RPA) gel illustrating the increases in band density that occurred with increasing amounts of total RNA. The last lane (F) contains the full-length probes.

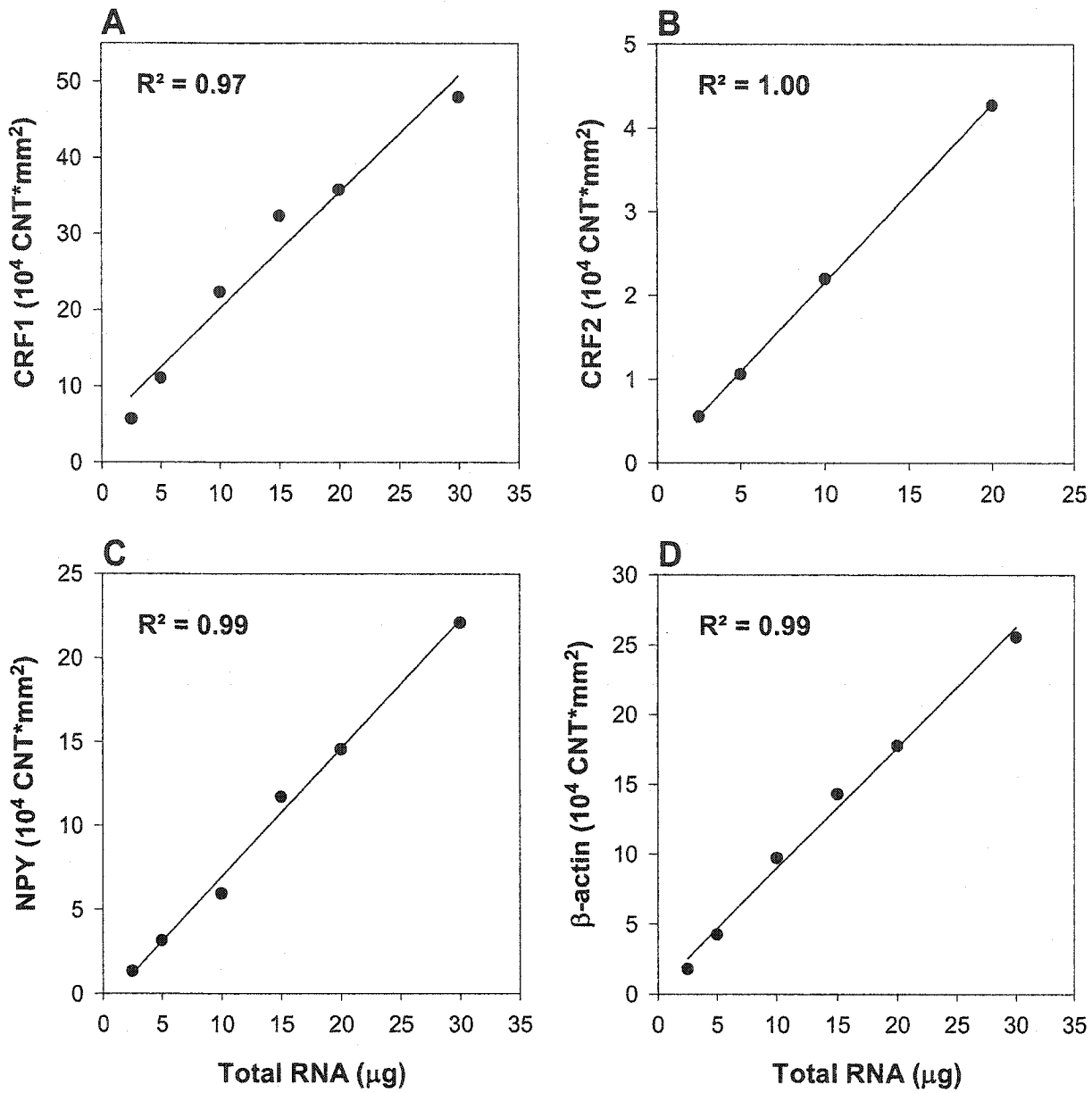

Figure 2.2 Representative standard curves demonstrate that the measured levels of (A) CRF1, (B) CRF2, (C) NPY, and (D) β-actin mRNA increase linearly with the amount of total RNA used in the ribonuclease protection assay (RPA). The correlation coefficients of the linear regressions appear on the graphs.

2.2.8. Tissue distribution

Sixteen rainbow trout were terminally anaesthetized in 150 mg L^{-1} benzocaine (ethyl p-aminobenzoate; Sigma-Aldrich, Oakville, ON) and tissues were pooled to obtain four groups of four fish. The following tissues were collected: pituitary (P), olfactory bulbs (OB), telencephalon without the preoptic area (T), preoptic area with part of the anterior hypothalamus (PO), basal hypothalamus (H), optic tectum (OT), cerebellum (C), mid-brain (MB), and hind-brain (HB) (see Fig. 2.6D). Total RNA was extracted with TRIZOL (Invitrogen), and CRF1, CRF2, NPY and β-actin mRNA levels were measured using RPA as described above.

2.2.9. Social stress

Social stress experiments were carried out in June 2001. Ten pairs of juvenile female trout (73.1 ± 2.5 g) were size matched on the basis of total length. The mean lengths of the dominant and subordinate fish were identical (19.9 ± 0.3 cm). The fish in each pair were placed in a 30 L flow-through plexiglass tank and separated by an opaque perforated divider for a 48 h acclimation period without feeding. Within each tank, fish were exposed to attenuated fluorescent lights with a total visible intensity ranging from 1 to 13 lux and lower attenuation at wavelengths corresponding to green light. After acclimation, the divider was removed and trout were allowed to interact for 72 h, during which time social ranks were determined by behavioural observations.

Dominance was measured by assigning points to specific behaviours, a method that has been used previously for determining social status among salmonid fish (*e.g.*, Metcalfe *et al.*, 1989; Johnsson *et al.*, 1996; Sloman *et al.*, 2000, 2001b). Within the first four hours of interaction, aggressive behaviour was recorded during three periods of five minutes. A fish that

chased and attacked another fish scored one point, while fish that did not attack or that were attacked received zero points. Behavioural observations of position in the tank and food acquisition were then carried out twice daily for the remainder of the experimental period. The position of each fish in the tank was scored as 0, 5, or 10 for swimming at the surface of the water, resting on the bottom of the tank, or holding its position in the water column, respectively. One pellet of food was presented to the fish twice daily, and the first fish to take the pellet received one point, while the other fish obtained zero points (following the second observation period, fish were fed until they refused further food). The social interactions, position and food acquisition scores were then processed with a principal components analysis (PCA; SYSTAT 9.01). This method combined the different measurements, weighting them according to the extent to which they correlated with the derived principal axis, to obtain an overall behaviour score for each fish, where higher values corresponded to more dominant behaviour. Thus, the fish within a pair with the higher behaviour score was assigned dominant social status, and the fish with the lower score was classified as the subordinate.

After 72 h of interaction, fish were quickly netted (both fish simultaneously) and terminally anaesthetized in 150 mg L^{-1} of benzocaine. Blood was collected in heparinized syringes by caudal puncture and centrifuged. The plasma was removed and stored at -80°C for later analysis of cortisol levels using a commercially available radioimmunoassay kit (Cortisol ^{125}I RIA Kit; ICN Diagnostics, Costa Mesa, CA). The preoptic area of the brain (which also included part of the anterior hypothalamus) was collected, and total RNA was extracted using TRIZOL (Invitrogen). CRF1, NPY and β-actin mRNA levels were analyzed by RPA as described above.

2.2.10. Food deprivation

Food deprivation experiments were carried out in December 2002 and April 2003. Six groups of 25 immature female rainbow trout were acclimated in 120 L holding tanks for one week before the start of the experiment. All groups were fed a daily ration of 2% body weight and after the acclimation period, feeding was stopped for three of the groups. A total of 84 trout with final weights ranging between 32 and 102 g (65.7 ± 1.7 g) was sampled throughout the experiment. Fourteen fed and unfed fish were sampled 5 and 10 days after feeding of the fasted groups was stopped. Tanks were visited once at each sampling day to avoid the stress of repeated sampling. Fish were quickly netted (4-5 fish/group) and immediately anaesthetized in 150 mg L^{-1} of benzocaine. Blood was collected by caudal puncture into syringes containing 0.5 M EDTA (Sigma-Aldrich) to obtain a final concentration of approximately 5-10 mM. After centrifugation of the blood, plasma was removed and stored at –80°C for later analysis of the levels of cortisol. The gut content of fed fish was verified to ensure that the fish were feeding. The preoptic area of the brain was collected, and total RNA was extracted using TRIZOL (Invitrogen). CRF1, NPY and β-actin mRNA levels were analyzed by RPA as described above.

2.2.11. Statistics

Results are presented as mean values ± one standard error of the mean (SE). For the optimization of the RPA, the linearity of the relation between quantified mRNA and the amount of total RNA was verified by linear regression. Statistical differences between social groups were determined by Student's *t*-test with a significance level of $P < 0.05$. The data on NPY mRNA levels did not meet the assumption of normality and were analyzed with the non-parametric Mann-Whitney Rank Sum Test. The relationship between CRF1 and NPY mRNA levels was

analyzed with the Pearson Product Moment Correlation. Statistical differences between fed and unfed groups were determined by two-way analysis of variance followed by Tukey's multiple comparison test, with the exception of plasma cortisol data that were analysed by a Mann-Whitney Rank Sum Test. All statistical analyses were carried out using SigmaStat (v2.0) software.

2.3. Results

2.3.1. Sequencing

Complete coding sequences were obtained for the cDNAs encoding rainbow trout NPY (GenBank accession AF203902), and two CRF paralogues, CRF1 (GenBank accession AF296672) and CRF2 (GenBank accession AY156929). The nucleotide sequence of CRF1 (1014 nt) is composed of a 123-bp 5'-untranslated region (UTR), a 501-bp open reading frame (ORF), and a 390-bp 3'-UTR that contains a polyadenylation signal (AATAAA). The sequence of CRF2 (994 nt) is composed of a 47-bp 5'-UTR, a 501-bp ORF, and a 446-bp 3'-UTR that contains two polyadenylation signals (AATAAA). The NPY sequence (659 nt) is composed of a 76-bp 5'-UTR, a 300-bp ORF, and a 283-bp 3'-UTR that contains a polyadenylation signal (ATTAAA). Here, I also report a partial nucleotide sequence coding for a second goldfish CRF (GenBank accession AY142110). This sequence was obtained as I was testing my CRF primers (Table 2.1) with goldfish cDNA using the method described above.

2.3.2. Phylogenetic analyses

The CRF tree is rooted with the rainbow trout sequence for urotensin-I, a gene closely related to CRF (Fig. 2.3). In this tree, both rainbow trout sequences group with the sequence of

the closely related sockeye salmon. However, trout CRF1 is closer to the salmon sequence than it is to trout CRF2. The grouping of both goldfish sequences with the common carp is consistent with both species belonging to the family Cyprinidae.

The NPY tree is rooted with the NPY sequence of the marbled electric ray, a cartilaginous fish (class Chondrichthyes; Fig. 2.4). The position of the trout sequence is not resolved as there are no sequences from closely related species in this phylogenetic analysis. The trout sequence is closer to the sequences from members of the Percomorpha (Japanese flounder, Japanese pufferfish and European sea bass) than it is to sequences from fish belonging to the Ostariophysi (channel catfish, zebrafish, goldfish and common carp).

2.3.3. Northern hybridization

The Northern hybridization revealed that both CRF and NPY probes detected single size gene transcripts of approximately 1.1 and 0.7 kb, respectively (Fig. 2.5).

2.3.4. Tissue distribution

The tissue distribution clearly indicated that the mRNAs of both CRF paralogues are predominantly present in the preoptic area of the rainbow trout brain (Fig. 2.6A). NPY transcripts were primarily detected in the telencephalon, but the levels of NPY mRNA were also relatively high in the preoptic area and the optic tectum (Fig. 2.6B). Using ribonuclease protection assay, CRF and NPY mRNAs were not detected in the pituitary gland (Fig. 2.6A, B). β-actin was not used to correct the results for CRF and NPY because its mRNA levels were lower in the pituitary (Fig. 2.6C).

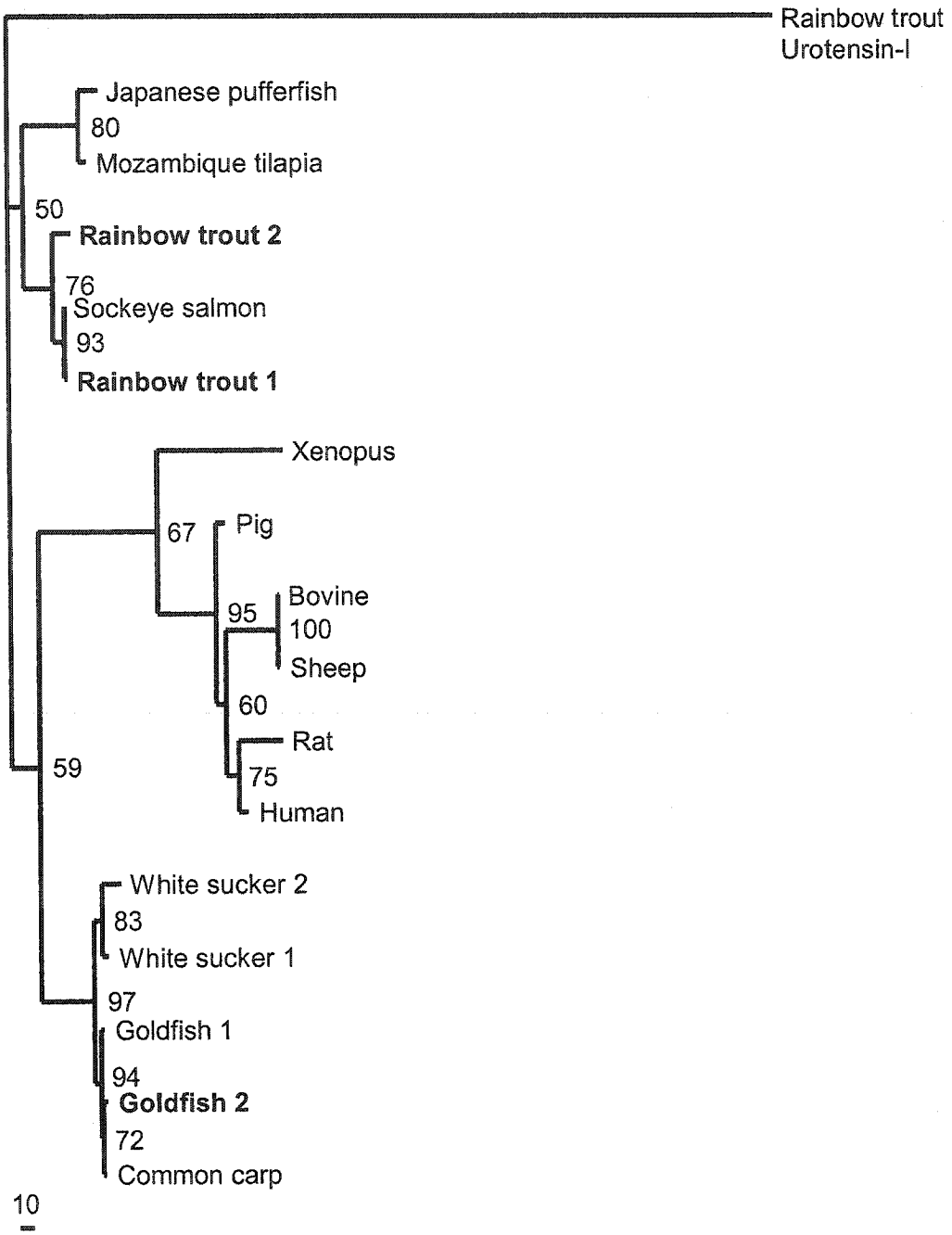

Figure 2.3 A phylogenetic tree for the complete predicted amino acid sequences of vertebrate corticotropin-releasing factor (CRF). Maximum-likelihood analyses were performed with PROML from the PHYLIP package version 3.6a2.1, the JTT model of amino acid substitutions, eight gamma-distributed rates of heterogeneity, and a fraction of invariant sites. Support values are shown next to their nodes. The scale bar represents the number of amino acid substitutions per amino acid site. This tree was rooted with the rainbow trout urotensin-I sequence. Sequences from species that appear in bold were obtained in this study.

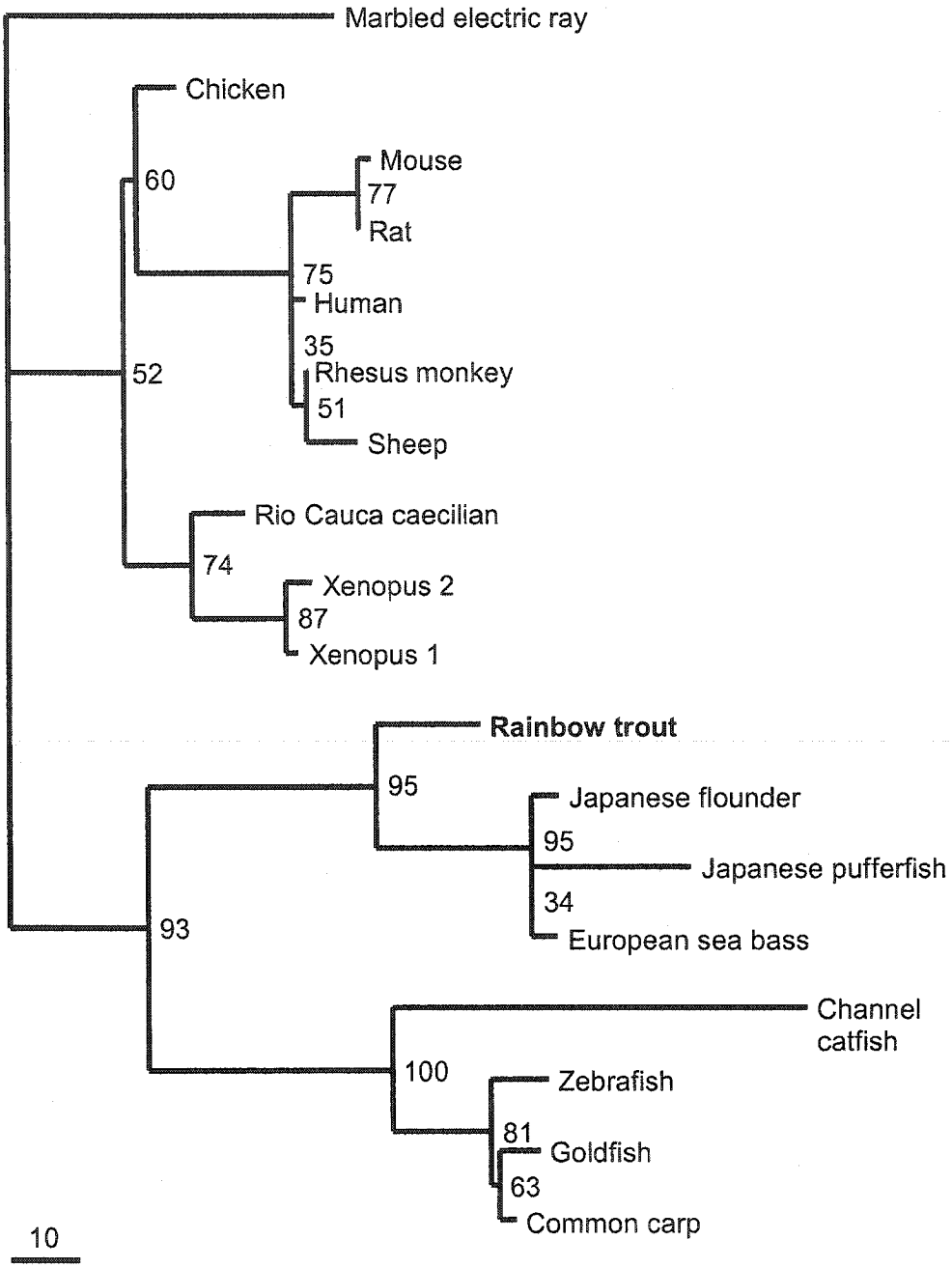

Figure 2.4 A phylogenetic tree for the complete predicted amino acid sequences of vertebrate neuropeptide Y (NPY). Maximum-likelihood analyses were performed with PROML from the PHYLIP package version 3.6a2.1, the JTT model of amino acid substitutions, eight gamma-distributed rates of heterogeneity, and a fraction of invariant sites. Support values are shown next to their nodes. The scale bar represents the number of amino acid substitutions per amino acid site. This tree was rooted with the sequence of the marbled electric ray. The sequence from the species that appears in bold was obtained in this study.

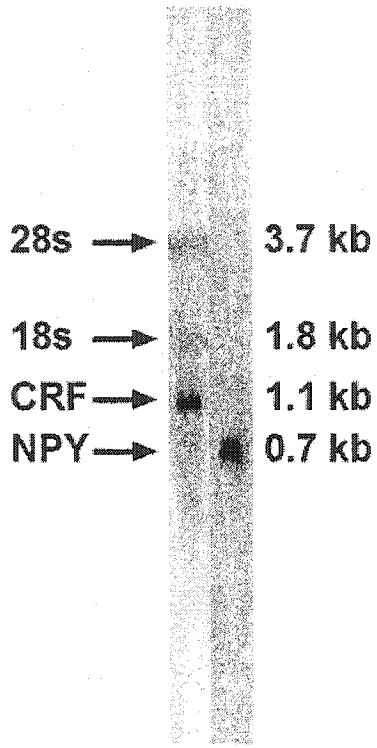

Figure 2.5 Northern blots showing single hybridization signals for both CRF and NPY mRNAs. The size of CRF and NPY mRNAs were determined relative to the size of 28S and 18S ribosomal RNA.

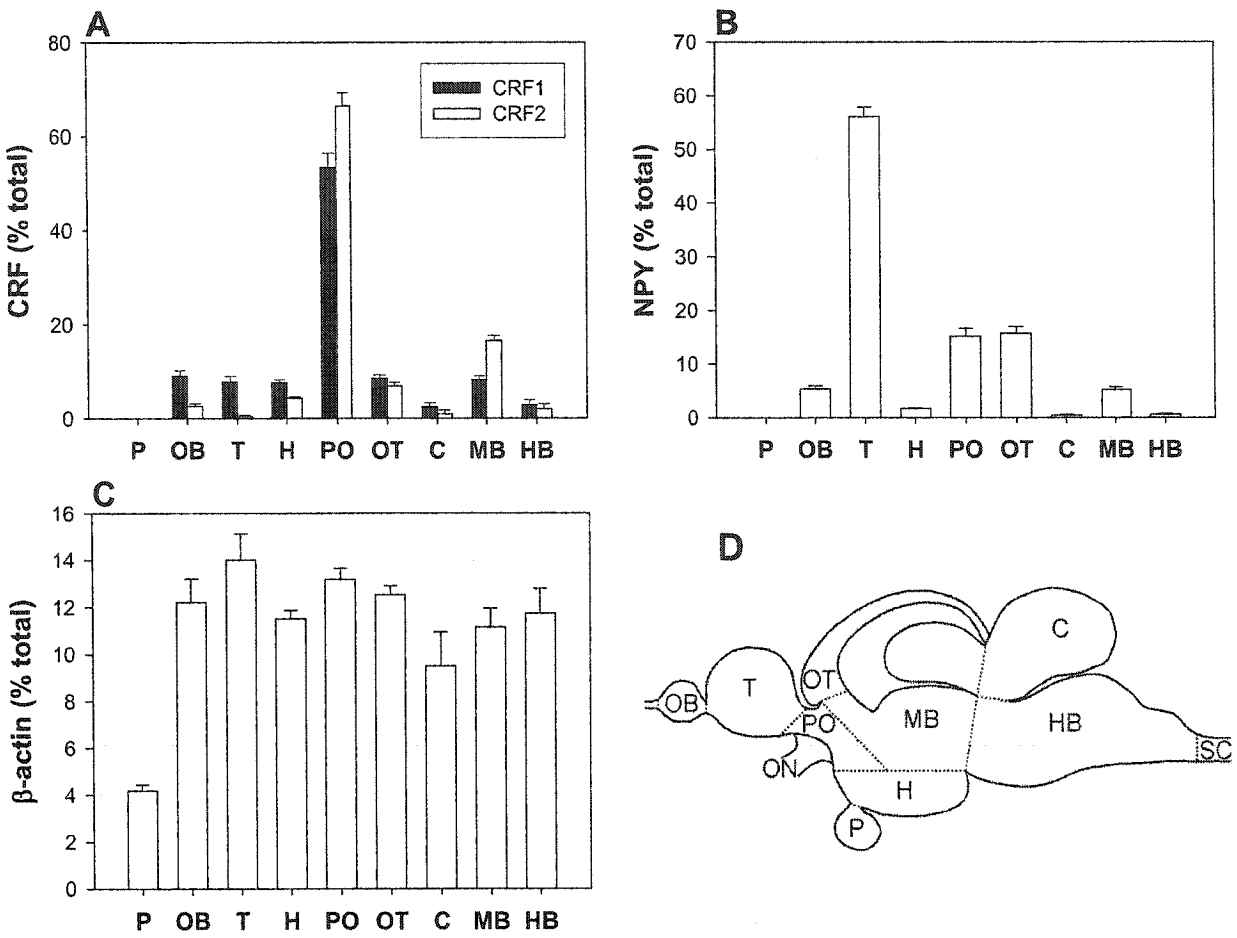

Figure 2.6 The distribution of (A) CRF, (B) NPY, and (C) β-actin mRNA levels in various tissues (D) of the juvenile female rainbow trout brain. The tissues are: pituitary gland (P), olfactory bulb (OB), telencephalon without the preoptic area (T), preoptic area with part of the anterior hypothalamus (PO), basal hypothalamus (H), optic tectum (OT), cerebellum (C), mid-brain (MB), and hind-brain (HB). The optic nerve (ON) and the spinal cord (SC) were not analyzed. Bars represent the average percentage of the total hybridization signal for four different pools of tissue, each pool representing four fish. These results were obtained using a ribonuclease protection assay (RPA).

2.3.5. Social stress

The behavioural observations and scoring system allowed a clear distinction between the social rank of each fish within a pair. The mean behaviour scores were 0.92 ± 0.16 and –0.93 ± 0.10 for dominant and subordinate fish, respectively. After 72 h of interaction, mean plasma cortisol levels were 13-fold higher in subordinate than in dominant fish ($P < 0.001$; Fig. 2.7A). As measured by RPA, CRF1 and NPY mRNA levels were 51% ($P = 0.002$; Fig. 2.7B) and 32% ($P = 0.038$; Fig. 2.7C) higher in the preoptic area of subordinates, respectively. There was a positive correlation between levels of CRF1 and NPY mRNA ($R^2 = 0.44$, $P = 0.001$; Fig. 2.7D).

2.3.6. Food deprivation

The levels of CRF1 (Fig. 2.8A) and NPY (Fig. 2.8B) mRNA were respectively 26% and 37% ($P < 0.01$) higher in fasted fish compared with fed controls after 5 days, but this difference disappeared at 10 days of food deprivation. There was no significant difference in the levels of plasma cortisol between fasted and fed fish after 5 days of food deprivation (Fig. 2.8C).

2.4. Discussion

2.4.1. Sequence characterization and phylogenies

I report the complete coding sequences for the cDNAs coding for the precursor proteins of rainbow trout NPY and two CRF paralogues. The deduced amino acid sequences of both trout CRFs have a similar structure to that of other members of the CRF family (Bernier *et al.*, 1999). They include a signal peptide, a cryptic region that contains a characteristic sequence of conserved amino acids (residues 51 to 63), and at the C-terminus, a 41-amino acid CRF. Using the program SignalP v2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/), the predicted lengths of

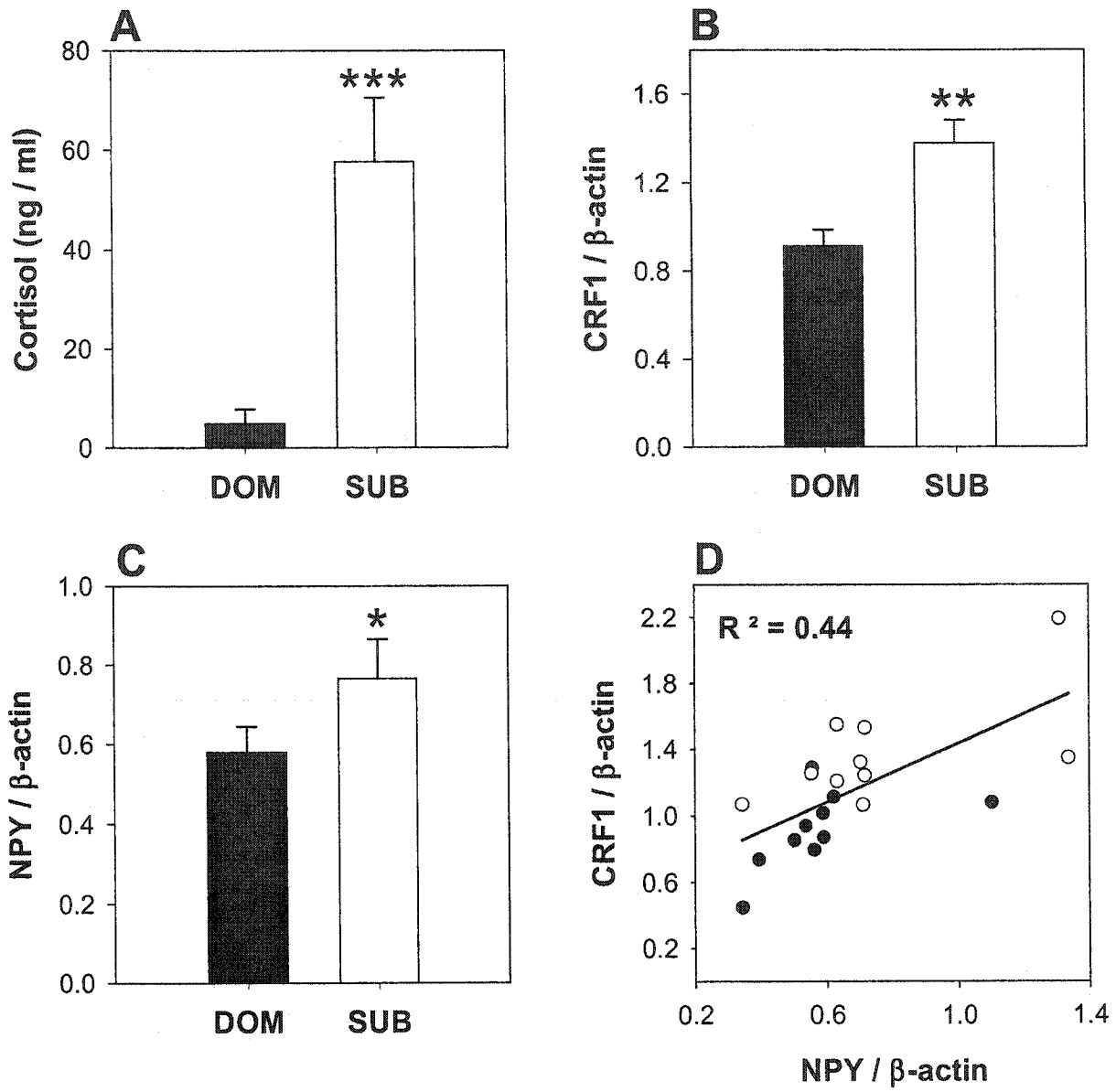

Figure 2.7 Comparisons of the levels of (A) plasma cortisol ($P < 0.001$), (B) CRF1 mRNA ($P = 0.002$), and (C) NPY mRNA ($P = 0.038$) between dominant (DOM) and subordinate (SUB) rainbow trout, as well as (D) the relationship between NPY and CRF1 mRNA levels ($P = 0.001$). Measurements were carried out after ten pairs of size-matched juvenile female rainbow trout were allowed to interact for 72 h. β-actin was used as a control to correct for the amount of total RNA used in the ribonuclease protection assay (RPA). In panel D, filled and unfilled symbols represent dominant and subordinate trout, respectively.

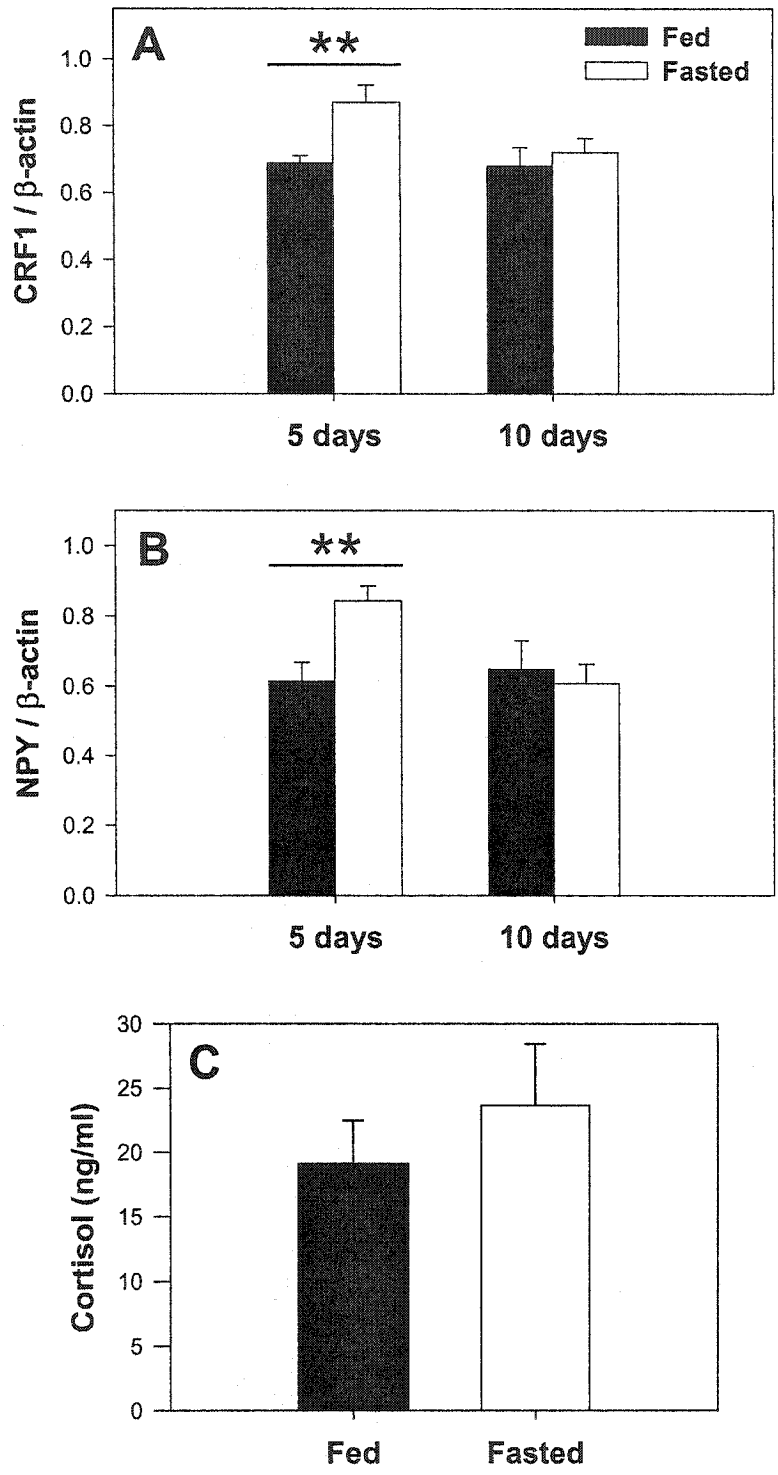

Figure 2.8 Time course effects of food deprivation on the levels of (A) CRF1 mRNA and (B) NPY mRNA, as well as (C) plasma cortisol levels after 5 days of food deprivation in juvenile female rainbow trout. Stars show statistical difference compared with fed control ($P < 0.01$).

the putative signal peptides of both trout CRFs are 22 and 24 amino acids when based on neural networks (Nielsen *et al.*, 1997) and the hidden Markov model (Nielsen and Krogh, 1998), respectively. The predicted amino acid sequences of the two trout CRFs diverge at 19 sites that are all located within the signal peptide or the cryptic region of the CRF precursor protein. The identical predicted amino acid sequences of CRF mature peptides are both flanked by a potential proteolytic processing signal at the N-terminus (RGRR), and at the C-terminus, by a potential amidation-proteolytic site (GK).

The predicted amino acid sequence of the trout CRF1 precursor protein shares 99% identity with that of sockeye salmon (Ando *et al.*, 1999), whereas it shares only 89% identity with the trout CRF2 precursor protein. In the CRF phylogenetic tree, the grouping of the trout CRF1 with the sockeye salmon sequence is consistent with this result. This suggests that the duplication that gave rise to the two trout CRF paralogues appeared in a common ancestor of these two members of the genus *Oncorhynchus*. In fact, it was suggested that all salmonids are tetraploid as a result of a gene duplication event in a common ancestor (reviewed by Allendorf and Thorgaard, 1984).

Given the tetraploid nature of rainbow trout, I expected to isolate more than one CRF paralogue. Two distinct cDNAs coding for CRF precursor proteins have been reported in other tetraploid fish, *i.e.* the sockeye salmon (*Oncorhynchus nerka*; Ando *et al.*, 1999) and the white sucker (*Catostomus commersoni*; Okawara *et al.*, 1988; Morley *et al.*, 1991). Here, I also report the presence of a cDNA coding for a second CRF precursor protein in another tetraploid species, the goldfish (*Carassius auratus*). Within the coding sequence of the predicted CRF precursor protein, this second form is different at 13 nucleotide positions from that reported by Bernier *et*

al. (1999). However, the predicted amino acid sequences of the two goldfish CRF mature peptides are not different.

The deduced amino acid sequence for trout NPY has the same structure as other members of the NPY gene family (Cerdá-Reverter and Larhammar, 2000). It contains a 28-amino acid signal peptide, a 36-amino acid NPY mature peptide, a potential amidation-proteolytic site (GKR), and a 33-amino acid carboxy-terminal peptide of neuropeptide Y (CPON). However, the sequence of my trout NPY mature peptide differs from those reported by Jensen and Conlon (1992) and Barton *et al.* (1992) at one and three amino acid positions, respectively. The sequence reported by Barton *et al.* (1992) is identical to the peptide YY sequence of coho salmon (*Oncorhynchus kisutch*; Kimmel *et al.*, 1986), and it shares a greater identity with other peptide YY genes (Jensen and Conlon, 1992; Cerdá-Reverter and Larhammar, 2000).

The difference between my NPY sequence and that of Jensen and Conlon (1992) is restricted to position 22, where a serine residue replaces a threonine. I have confidence in my nucleotide sequence, as the six clones overlapping this region of NPY have identical sequences. The observed difference is subtle and could be the result of a single point mutation in different strains of rainbow trout. In my nucleotide sequence, the codon for serine (UCC) requires the change of a single nucleotide to be converted into threonine (ACC).

Alternatively, the difference between my NPY sequence and that of Jensen and Conlon (1992) could result from the sequencing of products from different loci. As Jensen and Conlon (1992) only reported the amino acid sequence of the mature peptide, further sequencing information is required to determine if this hypothesis is valid. However, as the rainbow trout is a tetraploid species, it could have more than one copy of NPY. So far, the only organism where two NPY paralogues have been characterized is *Xenopus laevis*, another tetraploid species (van

Riel *et al.*, 1993; Griffin *et al.*, 1994). Larhammar and Risinger (1994) suggested that, in tetraploid species, duplicated genes might accumulate mutations that lead to differences in the function of peptides. However, the difference between the two trout NPYs is unlikely to have a functional significance. The substitution of a threonine for a serine is conservative, as both amino acids are hydrophilic. Out of 23 vertebrate NPY sequences, the sequence reported by Jensen and Conlon (1992) is the only sequence that does not have a serine at position 22 (Cerdá-Reverter and Larhammar, 2000).

2.4.2. Expression pattern

Northern hybridization analyses revealed single size gene transcripts of approximately 1.1 kb for CRF and 0.7 kb for NPY mRNAs. These estimates are very similar to the full-length sequences of the cDNAs reported here. A similar analysis in goldfish also revealed a single size CRF transcript of approximately 1.3 kb (Bernier *et al.*, 1999). In white sucker, both CRF paralogues have the same size but Northern hybridization revealed bands of two different sizes, 1.3 and 1.8 kb, due to the presence of multiple polyadenylation signals (Morley *et al.*, 1991). For NPY, Peng *et al.* (1994) reported that the goldfish brain contains a single size transcript with a molecular weight slightly over 1 kb. The length of the trout NPY mRNA is closer to that of the marbled electric ray (*Torpedo marmota*), which is reported to be 641 bp (Blomqvist *et al.*, 1992).

The similarity in the distribution pattern of the two trout CRF paralogues suggests that they might be regulated in a similar fashion. The high levels of CRF mRNA in the dissected preoptic area of trout are consistent with results from *in situ* hybridization that show CRF-positive neurons in the magnocellular portion of the preoptic nucleus (Ando *et al.*, 1999). Also, CRF-like immunoreactive perikarya were mainly present in parvocellular and magnocellular

nuclei of the preoptic area of salmonids (Olivereau and Olivereau, 1988; Matz and Hofeldt, 1999). In goldfish, Northern hybridization revealed equally strong signals in the telencephalon-preoptic area, and in the olfactory bulb (Bernier *et al.*, 1999). The latter result contrasts with the situation in trout, where the hybridization signal for CRF in the olfactory bulb was relatively weak compared with that of the preoptic area.

In trout, NPY mRNA was primarily detected in the telencephalon. Both the *in situ* distribution of NPY mRNA in goldfish (Peng *et al.*, 1994) and NPY-like immunoreactivity in trout (Danger *et al.*, 1991) indicate that NPY is most abundant in the nucleus endopeduncularis of the basal telencephalon. My results, showing high RPA hybridization signals in the preoptic area and the optic tectum, are consistent with studies that previously showed intermediate levels of NPY-like immunoreactivity and NPY mRNA in these tissues (Danger *et al.*, 1991; Peng *et al.*, 1994). However, NPY mRNA levels in the basal hypothalamus of trout were low compared to those of goldfish (Peng *et al.*, 1994).

2.4.3. Social stress

The confinement of rainbow trout in pairs results in significant physiological differences between the fish within the pair (reviewed by Schreck, 1981; Pickering, 1992; Sloman and Armstrong, 2002). Shortly after the onset of social interaction, a dominance hierarchy is established where subordinate fish have elevated levels of plasma ACTH (Höglund *et al.*, 2000) and cortisol (Øverli *et al.*, 1999; Sloman *et al.*, 2001b). Similarly, the levels of circulating cortisol were significantly elevated in subordinate compared to dominant fish in the present study. The levels of cortisol obtained in this study are similar to those of previous studies where pairs of trout interacted for 24, 48, and 168 h (Øverli *et al.*, 1999; Sloman *et al.*, 2001b). Cortisol

levels of socially stressed trout are within the range reported with other types of stressors such as handling plus confinement (Sumpter *et al.*, 1986; for more references, see Barton and Iwama, 1991).

The levels of CRF1 mRNA were also significantly elevated in subordinate trout. This result suggests that CRF may be involved in maintaining the activation of the HPI axis in chronically stressed subordinate trout. Chronic social stress increased the levels of CRF mRNA in subordinate rats (Albeck *et al.*, 1997). However, the effect of an increase in CRF may be attenuated by a decrease in CRF receptors in the anterior pituitary of chronically stressed rats (Hauger *et al.*, 1988).

Other neural pathways could also be involved in maintaining the activation of the HPI axis in subordinate fish. For example, brain serotonergic activity is elevated in subordinate rainbow trout (Winberg and Lepage, 1998), and it was suggested that the monoamine neurotransmitter serotonin could be involved in the activation of the HPI axis (Winberg *et al.*, 1997). Also, neuropeptides such as urotensin-I, arginine vasotocin, isotocin and arginine vasopressin are shown to stimulate ACTH release *in vitro* (Fryer *et al.*, 1983, 1985). The role of such neuropeptides in activating the HPI axis *in vivo* remains uncertain; however, the finding that arginine vasotocin mRNA levels in the preoptic area of the rainbow trout brain were increased by acute but not chronic stress (Gilchriest *et al.*, 2000) suggests that such a role is possible. In rats, NPY also acts as a potent stimulator of ACTH release (Wahlestedt *et al.*, 1987).

NPY mRNA levels were significantly elevated in subordinate trout in the present study, and CRF1 and NPY mRNA levels were positively correlated. These results suggest that NPY could be involved in the activation of the HPI axis, possibly through interactions with CRF. Precedents for this possibility can be found in the mammalian literature. For example, several

studies on rats indicate that stress increases NPY mRNA in the arcuate nucleus (Conrad and McEwen, 2000; Makino *et al.*, 1999, 2000; Sweerts *et al.*, 2001), whereas only one study showed a stress-induced decrease in NPY mRNA in the same hypothalamic area (Krukoff *et al.*, 1999). Although NPY and CRF have opposing actions on feeding behaviour in fish (Lin *et al.*, 2000) and mammals (Inui, 1999), studies in rats indicate that NPY increases CRF mRNA (Suda *et al.*, 1993), CRF immunoreactivity (Haas and George, 1987) and CRF release (Haas and George, 1989; Tsagarakis *et al.*, 1989). Furthermore, NPY axons establish synaptic connections with parvocellular neurons producing CRF in rats (Liposits *et al.*, 1988; Mihaly *et al.*, 2002). There is no direct evidence for such interactions in the trout brain. However, dense immunoreactive NPY fibres are found throughout the preoptic area (Danger *et al.*, 1991), in the vicinity of CRF-containing cell bodies (Olivereau and Olivereau, 1988). In light of these reports and my results, the possibility of connections between NPY- and CRF-producing neurons in the preoptic area of the fish brain should be investigated.

The elevated levels of NPY mRNA observed in subordinate trout may also be related to feeding behaviour. With *in situ* hybridization, Silverstein *et al.* (1998) demonstrated that NPY mRNA was increased specifically in the preoptic area of the brain in fasted salmon. The subordinate fish in the present study did not feed, whereas half of the dominant fish fed regularly (data not shown). This reduction in food intake may be the result of an increase in CRF release. CRF is though to be an important mediator of the stress-induced decrease in food intake (de Pedro *et al.*, 1997).

2.4.4. Food deprivation

A food deprivation expertiment was conducted to examine whether feeding modified NPY mRNA levels in the preoptic area of the rainbow trout brain. Fish were fasted for 5 days to mimic the food deprivation of subordinates in the social stress experiment. Five days of fasting increased NPY mRNA levels in the preoptic area of the trout brain. This result suggests that the difference in the levels of NPY mRNA between dominant and subordinate fish may be due to feeding status. However, the difference between dominants and subordinates is similar to that between fed and fasted despite the fact that only half of the dominant fish were feeding. Also, if feeding status was responsible for the differences in NPY mRNA levels, these differences should persist with time. The difference between fed and fasted fish disappeared at 10 days of fasting. This may imply an acclimation of fasted fish to their new feeding regime. My results contrast with salmon where elevation in NPY-like mRNA was observed only after 2-3 weeks of food deprivation (Silverstein *et al.*, 1998). In goldfish, NPY mRNA levels keep increasing until fish are refed (Narnaware *et al.*, 2000, 2001). Alternatively, fasting may induce a transient stress response, possibly due to increased social interactions. Although the levels of plasma cortisol after 5 days of food deprivation did not differ between fed and fasted fish, the fact that the levels of CRF1 mRNA follow the same pattern as NPY mRNA supports this second hypothesis. The elevation of both CRF and NPY mRNA levels is consistent with reported effects of stress on these neuropeptides in rats (Makino *et al.*, 1999). CRF mRNA levels in the PVN of rodents are increased, unaltered or reduced by food deprivation (Brady *et al.*, 1990; Pesonen *et al.*, 1992; Mercer *et al.*, 1996, 1997; Timofeeva and Richard, 1997). Considering the complexity of the interactions between the neuroendocrine systems involved in the stress response and the regulation of food intake (Glowa *et al.*, 1992; Bernier and Peter, 2001; Richard *et al.*, 2002),

further studies are clearly required to determine whether changes in preoptic levels of NPY mRNA are related to stress, feeding or both.

In conclusion, I have characterized the cDNAs coding for the precursor proteins of rainbow trout NPY and two CRF paralogues, as well as their mRNA distribution patterns within the trout brain. In subordinate fish, chronic social stress increased the levels of CRF1 and NPY mRNA in the preoptic area, and CRF1 and NPY mRNA levels in the preoptic area were positively correlated. These results support CRF being involved in maintaining the activation of the HPI axis during chronic social stress, and that NPY could also participate in this activation, possibly through interactions with CRF.

CHAPTER 3

ISOLATION AND PHYSICAL DISTURBANCE INCREASE CORTICOTROPIN-RELEASING FACTOR mRNA LEVELS IN RAINBOW TROUT

3.1. Introduction

Salmonid fish establish dominance hierarchies in captivity (reviewed by Sloman and Armstrong, 2002) and in natural populations (Bachman, 1984). When held in small groups, subordinate fish have elevated levels of circulating cortisol (Øverli *et al.*, 1999; Sloman *et al.*, 2001b) and ACTH (Höglund *et al.*, 2000), and pituitary pro-opiomelanocortin (POMC) mRNA, the precursor of ACTH (Winberg and Lepage, 1998). Recently, I demonstrated that mRNAs for corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) are elevated in the preoptic area of the brains of socially subordinate rainbow trout (Doyon *et al.*, 2003). Although social interactions are stressful to trout, isolation can also be detrimental. I observed that isolated rainbow trout require several days to acclimate to their new status and resume feeding. Isolation can be particularly stressful when combined with other sources of stress including confinement. For example, the confinement of cannulated trout leads to a transient increase in plasma cortisol levels that can last several days (Gamperl *et al.*, 1994). This study examines whether the levels of CRF1 and NPY mRNA parallel the changes in plasma cortisol levels in isolated rainbow trout.

Trout frequently resort to acute bursts of exercise to escape predation or aggression from socially dominant fish. In confined pairs of trout, subordinate fish are frequently chased to exhaustion by dominants. Several studies indicate that physically disturbing rainbow trout to exhaustion increases plasma cortisol levels (Milligan and Wood, 1986; Pagnotta *et al.*, 1994;

Kakizawa *et al.*, 1995, 1996). This study examines whether physical disturbance to exhaustion modifies the levels of CRF1 and NPY mRNA in the trout brain.

Thus, the objectives of this study were to examine the effects of isolation and physical disturbance on the levels of CRF1 and NPY mRNA in the preoptic area of the rainbow trout brain. The preoptic area was selected as it contains the highest levels of brain CRF mRNA and relatively high levels of NPY mRNA (Doyon *et al.*, 2003). Trout were isolated in large tanks or small confinement boxes for 4, 24 and 72 h. The effect of physical disturbance was examined with single or repeated chasing events. The levels of CRF1 and NPY mRNA were measured by ribonuclease protection assay (Doyon *et al.*, 2003).

3.2. Materials and Methods

3.2.1. Animals

Immature female rainbow trout, *Oncorhynchus mykiss*, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). They were acclimated to the laboratory for at least 6 weeks in 1275 L fiberglass tanks of well-aerated dechloraminated City of Ottawa tap water at 13±1°C with a constant 12h light/12h dark photoperiod. Fish were fed 3 times per week with commercial trout pellets (Classic Floating Trout Grower; Martin Mills Inc., Tavistock, ON).

3.2.2. Isolation protocol

Isolation experiments were carried out in August and September 2002, and used a total of 86 trout weighing between 57 and 136 g (96.1 ± 2.2 g). Fourteen trout were quickly netted from a 1275 L holding tank and immediately anaesthetized for use as unstressed controls. Seventy-two trout were individually isolated in either 120 L fiberglass tanks or 1.5 L black plexiglass

confinement boxes. For each treatment, twelve fish were anaesthetized after 4, 24 and 72 h of isolation in total darkness.

3.2.3. Physical disturbance protocol

Physical disturbance experiments were carried out from December 2002 to March 2003 and used a total of 40 trout weighing between 35 and 97 g (63.2 ± 2.3 g). Sixteen trout were quickly netted from a 1275 L holding tank and immediately anaesthetized for use as unstressed controls. Twelve trout were physically disturbed by continuous chasing until exhaustion (approximately 5 min) and anaesthetized after 2 h of recovery in individual 120 L isolation tanks. An additional 12 trout were subjected to three consecutive periods of chasing and 2 h recoveries. Hence, fish exposed to repeated disturbances were anaesthetized 6 h after the beginning of the first chasing event.

3.2.4. Hormone measurements and ribonuclease protection assay (RPA)

Fish were quickly netted and terminally anaesthetized in 150 mg L^{-1} of benzocaine (ethyl *p*-aminobenzoate; Sigma-Aldrich, Oakville, ON). Blood was collected in heparinized syringes by caudal puncture and centrifuged. The plasma was removed and stored at –80°C for later analysis of cortisol levels using a commercially available radioimmunoassay (Cortisol ^{125}I RIA Kit; ICN Diagnostics, Costa Mesa, CA). The preoptic area of the brain (which also included part of the anterior hypothalamus) was collected, and total RNA was extracted using TRIZOL (Invitrogen). Levels of CRF1, NPY and β-actin mRNA were analyzed using the ribonuclease protection assay (RPA III, Ambion, Austin, TX) described previously (Doyon *et al.*, 2003; see section 2.2.7).

3.2.5. Statistics

Results are presented as mean values ± one standard error of the mean (SE). For the isolation experiment, statistical differences between treatments were determined by two-way analysis of variance with a significance level of $P < 0.05$. For each treatment, statistical differences between time points and control were determined by one-way analysis of variance. CRF1 mRNA values for the confined fish were transformed using a natural logarithm to meet the assumption of normality. For the physical disturbance experiment, statistical differences between treatments were determined by one-way analysis of variance. All parametric analysis of variance were followed by Tukey's multiple comparison tests. For both experiments, data on cortisol levels did not meet the assumption of normality and differences between treatments were determined by Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's multiple comparison test. All statistical analyses were carried out using SigmaStat (v2.0) software.

3.3. Results

3.3.1. Isolation

The levels of plasma cortisol in both treatments were significantly elevated compared with control after 4 and 24 h of isolation but were back to control values after 72 h (Fig. 3.1A). The elevation in plasma cortisol at 4 h was greater in confined trout compared with fish isolated in a large tank. Although cortisol levels were highest after 4 h of isolation, CRF1 mRNA levels were different from control values in the confined fish only (Fig. 3.1B). After 24h of isolation, both groups had elevated levels of CRF1 mRNA compared with both the control and 4 h values. CRF1 mRNA levels remained elevated at 72 h in confined fish despite low cortisol values,

whereas levels of CRF mRNA were back to control values in isolated fish. Although NPY mRNA levels showed similar trends, the differences were not statistically significant (Fig. 3.1C).

3.3.2. Physical disturbance

One period of physical disturbance led to a significant increase in plasma cortisol levels, and levels after three consecutive chasing events were further increased (Fig. 3.2A). One chasing event was not sufficient to increase the levels of CRF1 mRNA despite elevated cortisol levels (Fig. 3.2B). However, three chasing events significantly increased the levels of CRF1 mRNA compared with both control and a single chasing event (Fig. 3.2B). The levels of NPY mRNA after three periods of physical disturbance were significantly elevated compared with a single chasing episode, but not to control (Fig. 3.2C).

3.4. Discussion

Isolation led to a transient increase in the levels of plasma cortisol despite continuous exposure to the stressor. Plasma cortisol levels were elevated in both treatments after 4 and 24 h of isolation but were back to control values after 72 h. Several studies reported transient increases in plasma cortisol with the continuous confinement of rainbow trout (Pottinger and Moran, 1993; Gamperl *et al.*, 1994) but the time necessary for cortisol to return to resting levels varies as a result of experimental conditions and the magnitude of stress. For example, plasma cortisol levels of cannulated trout returned to resting levels after 6 days of confinement in black boxes (Gamperl *et al.*, 1994). The levels of plasma ACTH also show a transient increase with confinement (Balm and Pottinger, 1995). Here, I also report that isolation led to a transient increase in CRF1 mRNA levels in the preoptic area of the trout brain. Although part of this

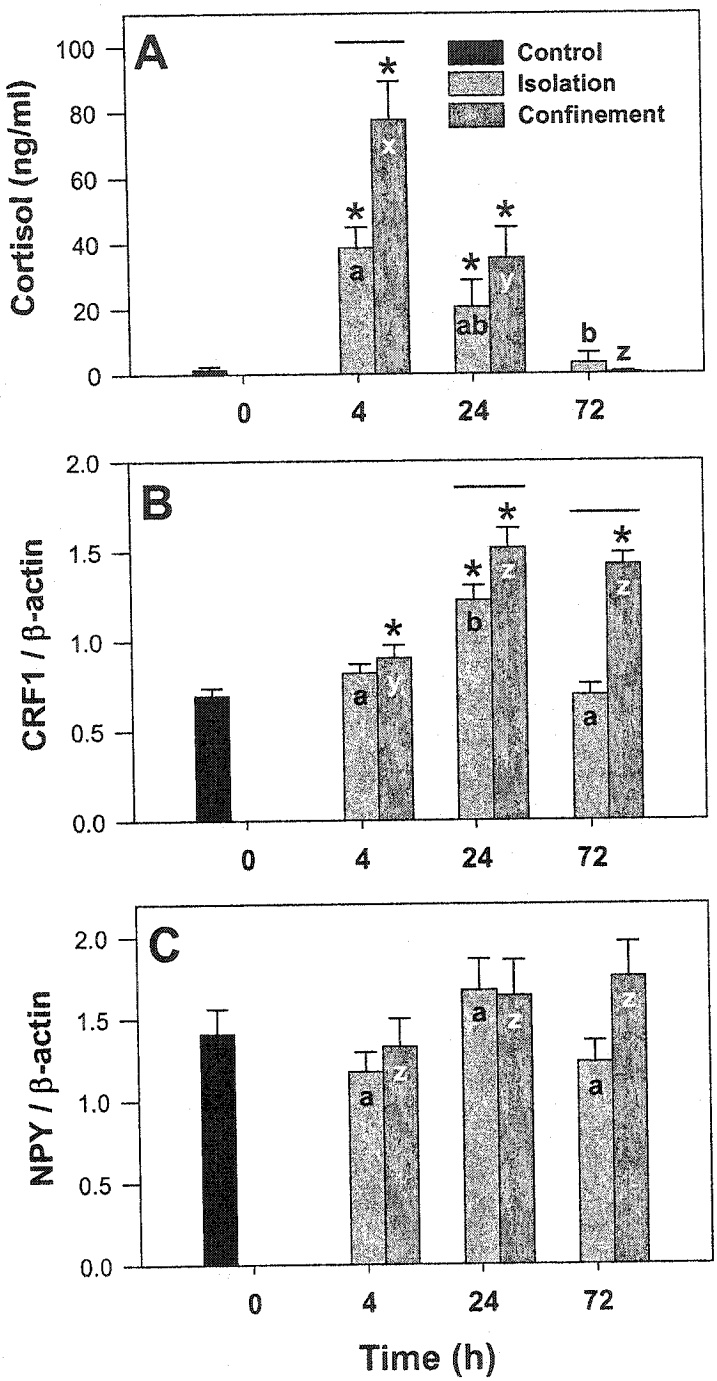

Figure 3.1 Time course of changes in the levels of (A) plasma cortisol, (B) CRF1 mRNA, and (C) NPY mRNA in juvenile female rainbow trout that were isolated in large tanks (Isolation) or small confinement boxes (Confinement). β-actin was used as a control to correct for the amount of total RNA used in the ribonuclease protection assay (RPA). Treatments that do not share a common letter within isolation or confinement are significantly different from each other ($P < 0.05$). Stars show statistical difference compared with control, whereas bars show difference between isolation and confinement ($P < 0.05$).

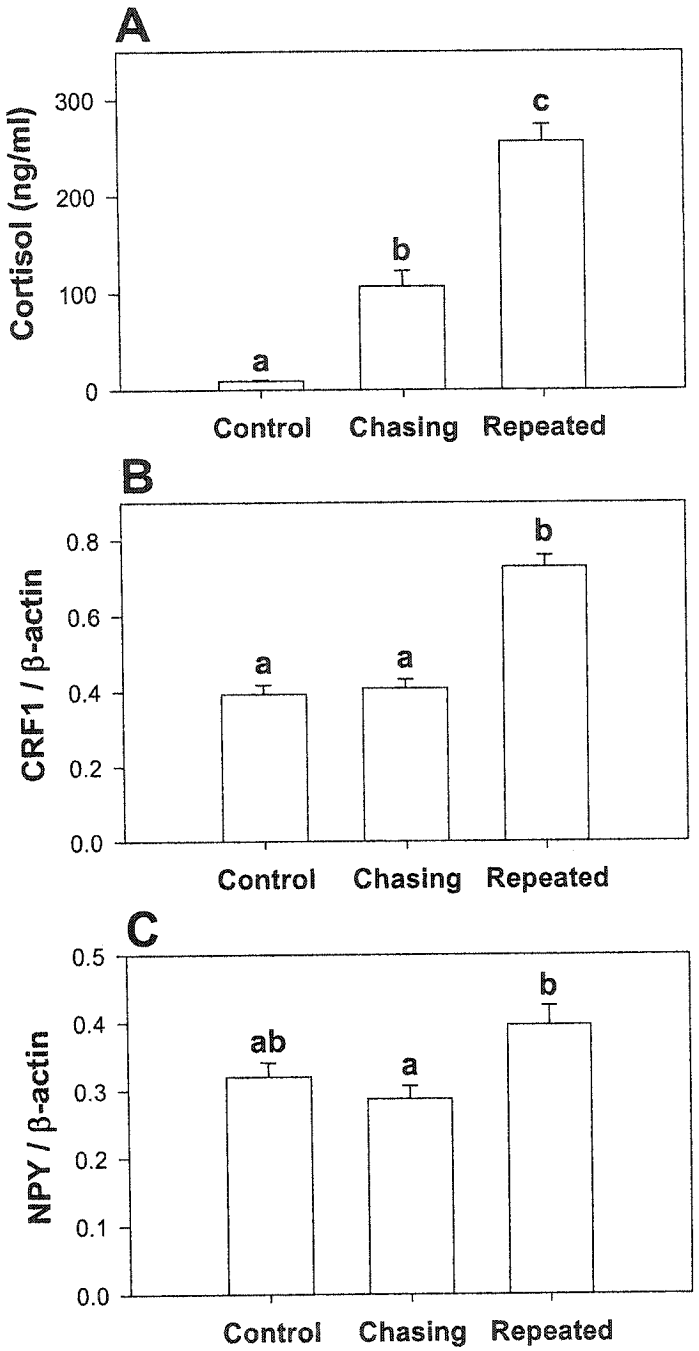

Figure 3.2 Effects of single and repeated periods of physical disturbance on the levels of (A) plasma cortisol, (B) CRF1 mRNA, and (C) NPY mRNA in juvenile female rainbow trout. Measurements were carried out after a single (Chasing) or three consecutive (Repeated) periods of physical disturbance followed by 2 h recovery in isolation. β-actin was used as a control to correct for the amount of total RNA used in the ribonuclease protection assay (RPA). Treatments that do not share a common letter are significantly different from each other ($P < 0.05$).

increase in the levels CRF1 mRNA may be due to the initial netting and handling stress, this result implies an adaptation to the isolation stress. This may contribute to maintain the responsiveness of the HPI axis to a novel stressor in situations of chronic stress. The responsiveness of the HPA axis is maintained in chronically stressed mammals (Aguilera, 1994).

Plasma cortisol levels were higher in confined fish compared with fish isolated in a large volume of water. This indicates that confinement was more stressful than isolation, and this greater stress intensity was reflected in the levels of CRF1 mRNA. The magnitude and persistence of the elevation in CRF1 mRNA was greater in confined fish. After 4 h, a mild elevation in CRF1 mRNA was only observed in confined fish, and the levels of CRF1 mRNA in confined fish were higher than those of isolated fish at both 24 and 72 h. CRF1 mRNA levels were back to control values in isolated fish after 72 h but remained elevated in confined fish. These results suggest that the magnitude and duration of the elevation in CRF mRNA levels is dependent on the intensity of stress in trout.

The results from the experiment on physical disturbance provide further evidence that the intensity and duration of stress are determining factors in elevating of CRF mRNA levels. Although one chasing event significantly increased plasma cortisol levels, there was no detectable effect on CRF1 mRNA. Three consecutive periods of physical disturbance and isolation led to a greater elevation in plasma cortisol and a highly significant increase in CRF1 mRNA levels. These results are consistent with studies in rats showing that exposure to repeated footshock stress increases CRF mRNA levels in the paraventricular nucleus (PVN), whereas a single footshock is not sufficient to produce this effect (Deutch *et al.*, 1987; Imaki *et al.*, 1991; Rivest and Rivier, 1994). However, 5 min of immobilization stress is sufficient to increase the levels of CRF heteronuclear RNA (hnRNA) in the rat PVN, which indicates activation of CRF

transcription (Imaki *et al.*, 1995, 1996). Despite a rapid increase in CRF hnRNA, the elevation in CRF mRNA levels is only detectable 60 to 120 min after the beginning of stress (Imaki *et al.*, 1992, 1995).

A study on rainbow trout arginine vasotocin (AVT) also suggests that the intensity of stress is an important factor in stress-induced elevation of mRNA (Gilchriest *et al.*, 2000). Brief low-water exposure did not affect AVT mRNA, whereas acute confinement, which elicited a greater cortisol response, significantly increased the levels of AVT transcripts in parvocellular neurons of the preoptic nucleus of the trout brain (Gilchriest *et al.*, 2000). AVT co-localizes with CRF in some neurons of the preoptic nucleus in teleost fish (Yulis and Lederis, 1987; Fryer and Lederis, 1988; Olivereau and Olivereau, 1988) and it potentiates the effect of CRF on ACTH release in rainbow trout (Baker *et al.*, 1996).

The stress-induced elevation in the levels CRF1 mRNA was delayed compared with plasma cortisol. CRF1 mRNA levels peaked at 24 h, whereas maximum plasma cortisol levels were observed after 4 h of isolation. Cortisol levels were already declining when CRF1 mRNA reached maximum values. Although plasma cortisol returned to control values at 72 h, CRF1 mRNA levels were still elevated in confined fish. These results suggest that cortisol may not be directly involved in mediating the post-stress reduction in CRF mRNA levels. However, this does not exclude a direct effect of cortisol on CRF transcription. As there is a time lag between the activation of CRF transcription and a detectable elevation in CRF mRNA levels (Herman *et al.*, 1992; Imaki *et al.*, 1995), the presence of a delay between a potential cortisol-induced reduction in CRF transcription and a detectable reduction in CRF mRNA levels is plausible. In the rat brain, the effects of glucocorticoids on CRF mRNA synthesis are negative in the hypothalamic paraventricular nucleus (Kovács *et al.*, 1986; Kovács and Mezey, 1987) and

positive in the central nucleus of the amygdala (Swanson and Simmonds, 1989; Makino *et al.*, 1994; Shepard *et al.*, 2000). Glucocorticoids also stimulate CRF gene expression in human placenta (Robinson *et al.*, 1988). Bernier *et al.* (1999) recently suggested that cortisol might inhibit CRF mRNA synthesis in the goldfish brain. Cortisol implantation reduced CRF mRNA levels, whereas the glucocorticoid receptor antagonist RU-486 increased the levels of CRF mRNA in the telencephalon-preoptic area of the goldfish brain (Bernier *et al.*, 1999). Clearly, further studies are necessary to determine how glucocorticoids influence the levels and the rate of synthesis of CRF mRNA in fish.

The persistent elevation in CRF mRNA in confined fish raises the possibility of an autoregulation mechanism at the level of CRF-producing neurons. Greater stress presumably leads to a greater release of CRF peptides and a greater need to replenish depleted CRF stores. As mRNAs represent a potential for peptide synthesis, this potential needs to remain elevated for a longer period of time when stress intensity is greater. It was suggested that CRF has an autoregulatory effect on CRF-producing neurons in rats (Ono *et al.*, 1985; Parkes *et al.*, 1993). Central administration of CRF increases both CRF and type 1 CRF receptor (CRF-R1) mRNA in the rat PVN (Parkes *et al.*, 1993; Imaki *et al.*, 1996; Mansi *et al.*, 1996). The levels of CRF-R1 mRNA in the rat PVN increase in response to various types of stressors (Luo *et al.*, 1994; Makino *et al.*, 1995b; Rivest *et al.*, 1995; Imaki *et al.*, 1996; Bonaz and Rivest, 1998).

In both experiments, NPY mRNA levels followed trends that were similar to the changes in CRF1 mRNA, but with a lower magnitude. A statistically significant difference in NPY mRNA levels was only observed between one and three chasing events. Despite weak changes in NPY mRNA, CRF1 and NPY mRNA levels were positively correlated in both experiments (data not shown). This result is consistent with my previous observation that CRF1 and NPY mRNA

levels are positively correlation in the preoptic area of the rainbow trout brain (Doyon *et al.*, 2003). This supports a role for NPY in stress-induced activation of the HPI axis, possibly through interaction with CRF-producing neurons. Studies in rats indicate that NPY increases CRF mRNA (Suda *et al.*, 1993), CRF immunoreactivity (Haas and George, 1987) and CRF release (Haas and George, 1989; Tsagarakis *et al.*, 1989). Furthermore, NPY axons establish synaptic connections with parvocellular neurons producing CRF in rats (Liposits *et al.*, 1988; Mihaly *et al.*, 2002).

In conclusion, my results suggest that the intensity and duration of stress are important factors determining the magnitude and persistence of the elevation in CRF mRNA levels. The fact that plasma cortisol levels decrease before CRF mRNA suggests that cortisol may not be directly involved in reducing post-stress CRF mRNA levels in the trout preoptic area. Several mechanisms other than glucocorticoid negative feedback could be involved in terminating the HPI axis activation after stress, including downregulation of CRF receptors and upregulation CRF binding protein (CRF-BP) in the pituitary gland. The present study provides a good experimental framework to examine the effects of acute and chronic stress on several aspects of the HPI axis in fish, including the regulation of CRF receptors and CRF-BP.

CHAPTER 4

CORTICOTROPIN RELEASING FACTOR AND NEUROPEPTIDE Y mRNA LEVELS ARE MODIFIED BY GLUCOCORTICOIDS IN RAINBOW TROUT

4.1. Introduction

The primary response to stress involves neuronal activation that leads to the release of stress hormones including glucocorticoids into the circulatory system (reviewed by Wendelaar Bonga, 1997). Stress-activated hypothalamic neurons release corticotropin-releasing factor (CRF), which stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In turn, circulating ACTH stimulates the synthesis and release of glucocorticoids from the interrenal tissue of fish (Mommsen *et al.*, 1999) or the adrenal cortex in higher vertebrates (Orth and Kovács 1998). Circulating glucocorticoids are thought to influence their own synthesis and release through a negative feedback mechanism that inhibits the activity of the hypothalamic and pituitary components of the stress axis (Keller-Wood and Dallman, 1984). The effects of glucocorticoids on the synthesis and release of CRF and ACTH are relatively well established in mammals (Keller-Wood and Dallman, 1984). The reduction of circulating glucocorticoid levels by removal of the adrenal glands, *i.e.* adrenalectomy, increases CRF mRNA levels and CRF-immunoreactivity in the rat hypothalamus (Jingami *et al.*, 1985; Wolfson *et al.*, 1985; Young *et al.*, 1986). In adrenalectomized rats, the increase in CRF mRNA levels is prevented by treatment with dexamethasone, a synthetic glucocorticoid (Jingami *et al.*, 1985).

Few studies have addressed the issue of glucocorticoid negative feedback in fish. The removal of the interrenal tissue, *i.e.* interrenalectomy, activates CRF-immunoreactive neurons in

the nucleus preopticus of the eel brain (Olivereau and Olivereau, 1991). Cortisol inhibits CRF-induced ACTH secretion in superfused goldfish pituitary cells (Fryer *et al.*, 1984). In addition, the implantation of cortisol pellets near the nucleus preopticus or the nucleus lateralis tuberis of the goldfish brain implicate these CRF-rich brain areas as sites of glucocorticoid negative feedback (Fryer and Peter, 1977). Studies in goldfish also suggest that glucocorticoids reduce the levels of CRF mRNA (Bernier *et al.*, 1999; Bernier and Peter, 2001).

I previously showed that the transient stress-induced elevation in plasma cortisol levels is followed by a transient but delayed elevation in CRF mRNA levels in the preoptic area of the rainbow trout brain (Chapter 3). The timing of the elevation in plasma cortisol and CRF mRNA did not support a role of cortisol in the termination of the activation of CRF neurons. The present study examines whether a modification in the levels or the action of cortisol can influence the levels of CRF mRNA.

NPY expression could also be modulated by glucocorticoids. Previously, I demonstrated that NPY mRNA levels are elevated in socially subordinate trout (Doyon *et al.*, 2003). Several studies also support a stress-induced elevation of NPY mRNA in the rat PVN (Conrad and McEwen, 2000; Makino *et al.*, 1999, 2000; Sweerts *et al.*, 2001). A glucocorticoid-responsive element (GRE) was reported upstream of the rat NPY gene (Misaki *et al.*, 1992). Also, adrenalectomy reduces NPY gene expression in the arcuate nucleus and medial basal hypothalamus of rats (White *et al.*, 1990; Savontaus *et al.*, 2002), whereas dexamethasone increases NPY gene expression *in vitro* (Higuchi *et al.*, 1988) and in the paraventricular and arcuate nuclei of the rat hypothalamus (McKibbin *et al.*, 1992; Wilding *et al.*, 1993). To date, studies in fish have not addressed the potential effects of glucocorticoids on NPY gene expression.

Thus, the objective of this study was to evaluate the potential effects of glucocorticoids on CRF and NPY mRNA levels in rainbow trout brain. Intraperitoneal implants of cortisol, RU-486 or metyrapone were given to modify the circulating levels or the function of glucocorticoids. RU-486 is a competitive antagonist of the progesterone and glucocorticoid receptors that blocks the receptor-mediated actions of glucocorticoids in mammals (Gaillard *et al.*, 1984; Healy *et al.*, 1985; Baulieu, 1997). Metyrapone is a general cytochrome P450 inhibitor that blocks the last step of glucocorticoid synthesis by inhibiting 11β-hydroxylase (Meikle *et al.*, 1975; Schoneshofer and Claus, 1985) and hence, reduces plasma glucocorticoid levels in mammals (Conte-Devolx *et al.*, 1992; Herman *et al.*, 1992).

4.2. Materials and Methods

4.2.1. Animals

Immature female rainbow trout, *Oncorhynchus mykiss*, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). They were acclimated to the laboratory for at least 6 weeks in 1275 L fiberglass tanks of well-aerated dechloraminated City of Ottawa tap water at 13±1°C with a constant 12h light/12h dark photoperiod. Fish were fed 3 times per week with commercial trout pellets (Classic Floating Trout Grower; Martin Mills Inc., Tavistock, ON).

4.2.2. Chemical implants

RU-486 (RU36486, mifepristone) was kindly donated by Roussel UCLAF, France. Cortisol (hydrocortisone; Sigma-Aldrich, Oakville, ON), RU-486 and metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone; Sigma-Aldrich) were individually mixed with melted coconut oil (Sigma-Aldrich) to obtain concentrations of 100, 100 and 200 mg/ml, respectively. Fish were

anaesthetized in 40 mg L^{-1} benzocaine (ethyl *p*-aminobenzoate; Sigma-Aldrich, Oakville, ON) and chemical solutions were injected intraperitoneally (1 ml/kg body weight) at 30°C with a 250-µl 20-gauge microsyringe.

4.2.3. Effects of RU-486 and metyrapone - pilot studies

The pilot studies were used to verify whether RU-486 increased and metyrapone reduced plasma cortisol levels. The pilot study on the effects of RU-486 was carried out in April and May 2002, and used a total of 49 trout weighing between 76 and 172 g (115.6 ± 3.3 g). Twenty-four trout were injected with coconut oil only (sham treatment), whereas 25 trout received a dose of RU-486 of 100 mg/kg body weight (BW). After the injection, fish were individually isolated in 120 L tanks for 24 h. Two hours before the termination of the experiment, approximately half of the fish that received sham (n = 10) and RU-486 (n = 12) implants were physically disturbed by continuous chasing until exhaustion (approximately 5 min).

The pilot study on the effects of metyrapone was carried out in October 2002 and used a total of 37 trout weighing between 70 and 280 g (154.2 ± 8.8 g). Eighteen trout were injected with coconut oil alone (sham treatment), whereas 19 trout received a dose of metyrapone of 200 mg/kg BW. After the injection, fish were individually isolated in 120 L tanks for 24 h. Two hours before the termination of the experiment, approximately half of the fish that received sham (n = 9) and metyrapone (n = 9) implants were chased to exhaustion (approximately 5 min).

4.2.4. Effects of cortisol, RU-486 and metyrapone

These experiments were carried out from December 2002 to March 2003 and used a total of 136 trout weighing between 35 and 182 g (105.2 ± 2.5 g). Sixteen trout were quickly netted

from a 1275 L tank and immediately anaesthetized for use as unstressed controls. One hundred and twenty trout received a single intraperitoneal injection of coconut oil alone (n = 32) or coconut oil containing cortisol (100 mg/kg BW; n = 32), RU-486 (100 mg/kg BW; n = 32) or metyrapone (200 mg/kg BW; n = 24). After the injection, half of the fish (isolated fish) from each treatment were individually isolated in 120 L tanks for 96 h, whereas a more stressful protocol was used with the other half (confined fish). After 72 h of isolation in 120 L tanks, confined fish were maintained for 24 h in 1.5 L confinement boxes.

4.2.5. Hormone measurements and ribonuclease protection assay (RPA)

Fish were quickly netted and terminally anaesthetized in 150 mg L^{-1} of benzocaine. For the pilot studies with RU-486 and metyrapone, blood was collected by caudal puncture into heparinized syringes. For the remaining experiments, heparin was substituted by 0.5 M EDTA (Sigma-Aldrich) to obtain a final concentration of approximately 5-10 mM. After centrifugation of the blood, plasma was removed and stored at -80°C for later analysis of the levels of cortisol, ACTH and 11-deoxycortisol using commercially available radioimmunoassay kits (Cortisol ^{125}I RIA Kit; ACTH ^{125}I RIA Kit; 11-Desoxycortisol ^{125}I RIA Kit; ICN Diagnostics, Costa Mesa, CA). The cross-reactivity of RU-486 with the antibody of the cortisol RIA is negligible (Bernier *et al.*, 1999). Levels of ACTH were not measured in the plasma from the pilot studies due to the reported interference of heparin with the radioimmunoassay. Validation of the RIA for measuring ACTH and 11-deoxycortisol in rainbow trout plasma was provided by the observation that the dilution curves of immunoreactive ACTH and 11-deoxycortisol in trout plasma were parallel to the standard curves (Fig. 4.1A,B). The preoptic area of the brain (which also included part of the anterior hypothalamus) was collected, and total RNA was extracted using TRIZOL

(Invitrogen). Levels of CRF1, NPY and β-actin mRNA were analyzed by the ribonuclease protection assay (RPA III, Ambion, Austin, TX) described previously (Doyon *et al.*, 2003).

4.2.6. Statistics

Results are presented as mean values ± one standard error of the mean (SE). For the first RU-486 experiment, statistical differences between treatments were determined by two-way analysis of variance. The data from the first metyrapone experiment did not meet the assumption of normality and statistical differences between treatments were determined by two-way analysis of variance on ranks. For the second series of experiments, statistical differences between sham and control, as well as differences between sham and each treatment for a given stress protocol were determined by one-way analysis of variance. Statistical differences between isolation and confinement within each treatment were determined by two-way analysis of variance. The data on plasma levels of cortisol and 11-deoxycortisol did not meet the assumption of normality and were analyzed by two-way analysis of variance on ranks or Kruskal-Wallis one-way analysis of variance on ranks. Comparisons of the levels of plasma ACTH within each stress protocol and comparisons of the levels of CRF1 mRNA between sham and control were also analyzed by Kruskal-Wallis one-way analysis of variance on ranks. Multiple comparisons were performed using Dunn's test after Kruskal-Wallis one-way analysis of variance on ranks and Tukey's test after all other tests. The relationship between CRF1 and NPY mRNA levels was analyzed with the Pearson Product Moment Correlation. The significance level for all statistical tests was $P < 0.05$. All statistical analyses were carried out using SigmaStat (v2.0) software.

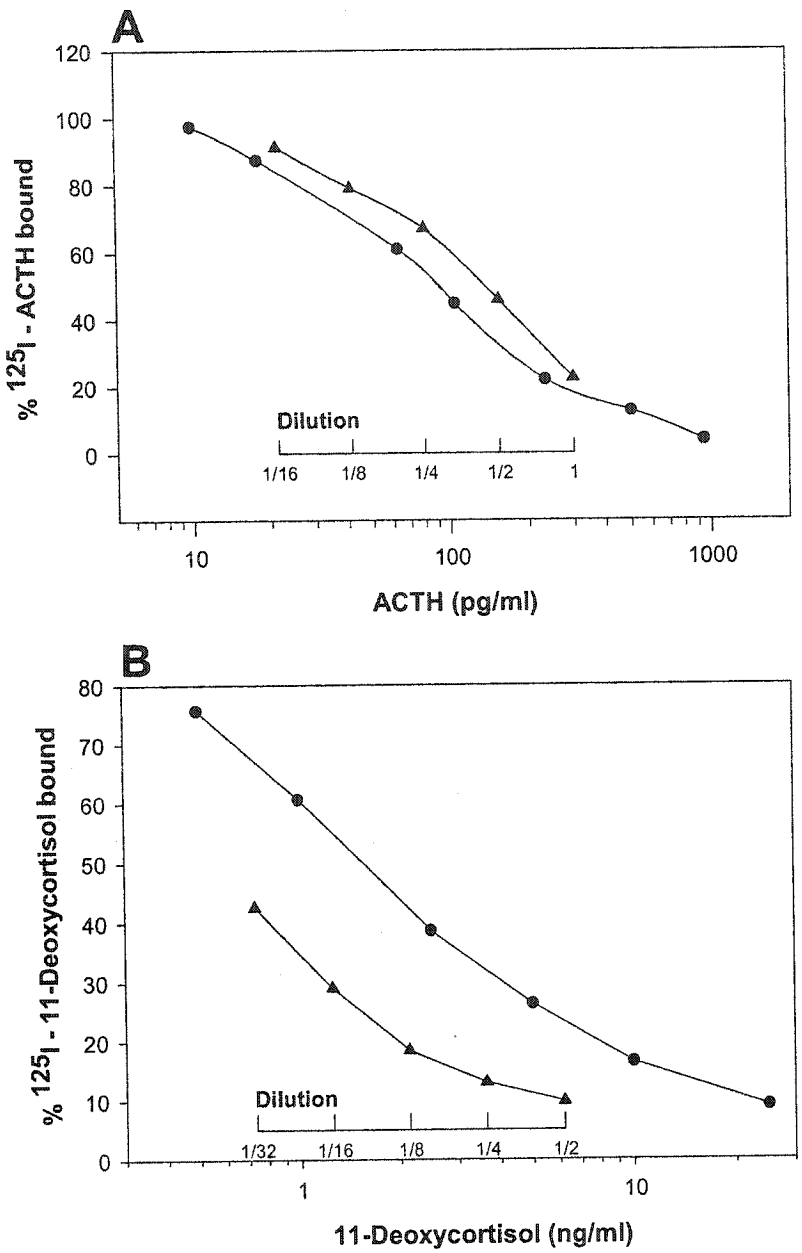

Figure 4.1 Standard curves for (A) ACTH and (B) 11-deoxycortisol radioimmunoassay (circle), and serial dilutions of rainbow trout plasma (triangle).

4.3. Results

4.3.1. Effects of RU-486 and metyrapone – pilot studies

4.3.1.1. RU-486

Implantation with RU-486 increased plasma cortisol levels (Fig. 4.2A) but reduced CRF1 mRNA levels after both isolation and physical disturbance (Fig. 4.2B). Although physical disturbance increased the levels of plasma cortisol in both sham and RU-486 treatments (Fig. 4.2A), it did not influence the levels of CRF1 mRNA (Fig. 4.2B). There was no difference in the levels of NPY mRNA between treatments (Fig. 4.2C).

4.3.1.2. Metyrapone

Plasma cortisol levels in fish treated with metyrapone were elevated compared with sham treatment after 24 h isolation (Fig. 4.3A). Physical disturbance significantly increased plasma cortisol levels in the sham treatment but not in metyrapone-treated fish (Fig. 4.3A). Although there was a tendency for lower plasma cortisol levels in metyrapone-treated fish after chasing, the difference was not statistically significant (Fig. 4.3A). Physical disturbance significantly increased the levels of plasma 11-deoxycortisol in both sham and metyrapone treatments (Fig. 4.3B). Metyrapone treatment significantly increased the levels of plasma 11-deoxycortisol in both isolation and physical disturbance (Fig. 4.3B).

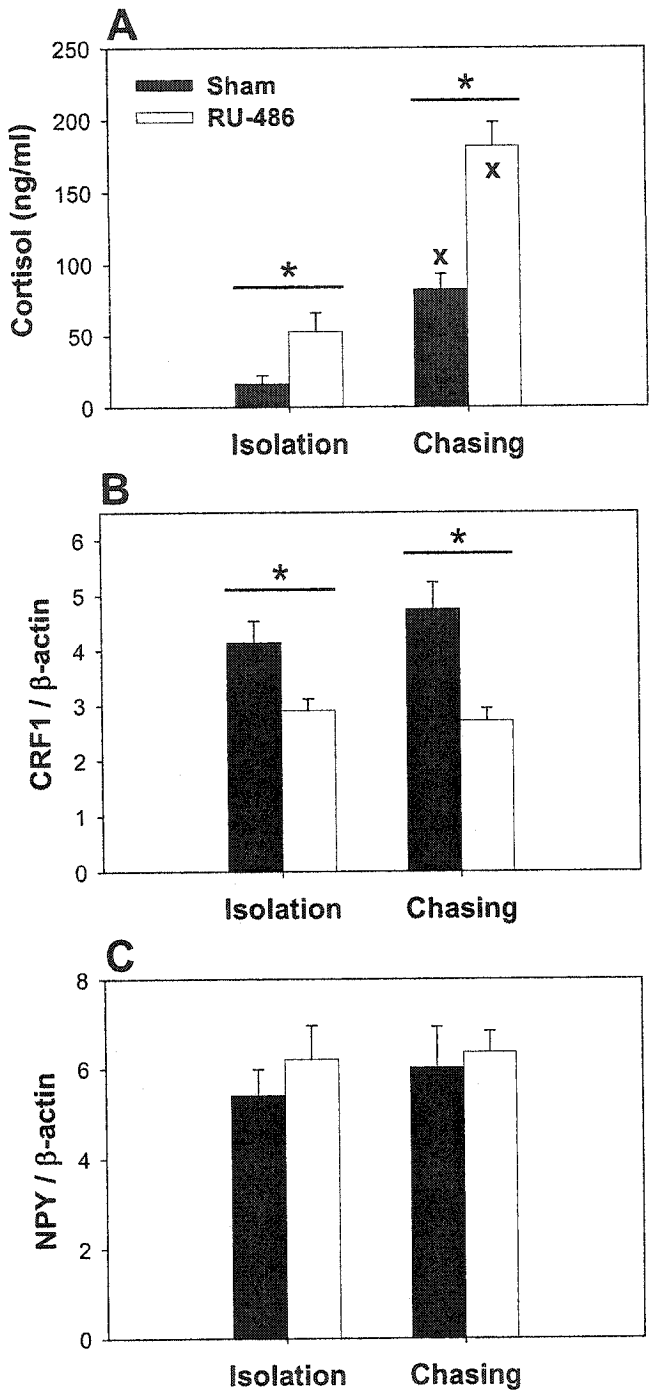

Figure 4.2 Effects of RU-486 on the levels of (A) plasma cortisol, (B) CRF1 mRNA, and (C) NPY mRNA in juvenile female rainbow trout after 24 h of isolation with (Chasing) or without (Isolation) a period of physical disturbance followed by 2 h of recovery in isolation. β-actin was used as a control to correct for the amount of total RNA used in the ribonuclease protection assay (RPA). Stars show statistical difference between sham and RU-486 for a given stress protocol, and X shows difference between isolation and chasing for a given treatment ($P < 0.05$).

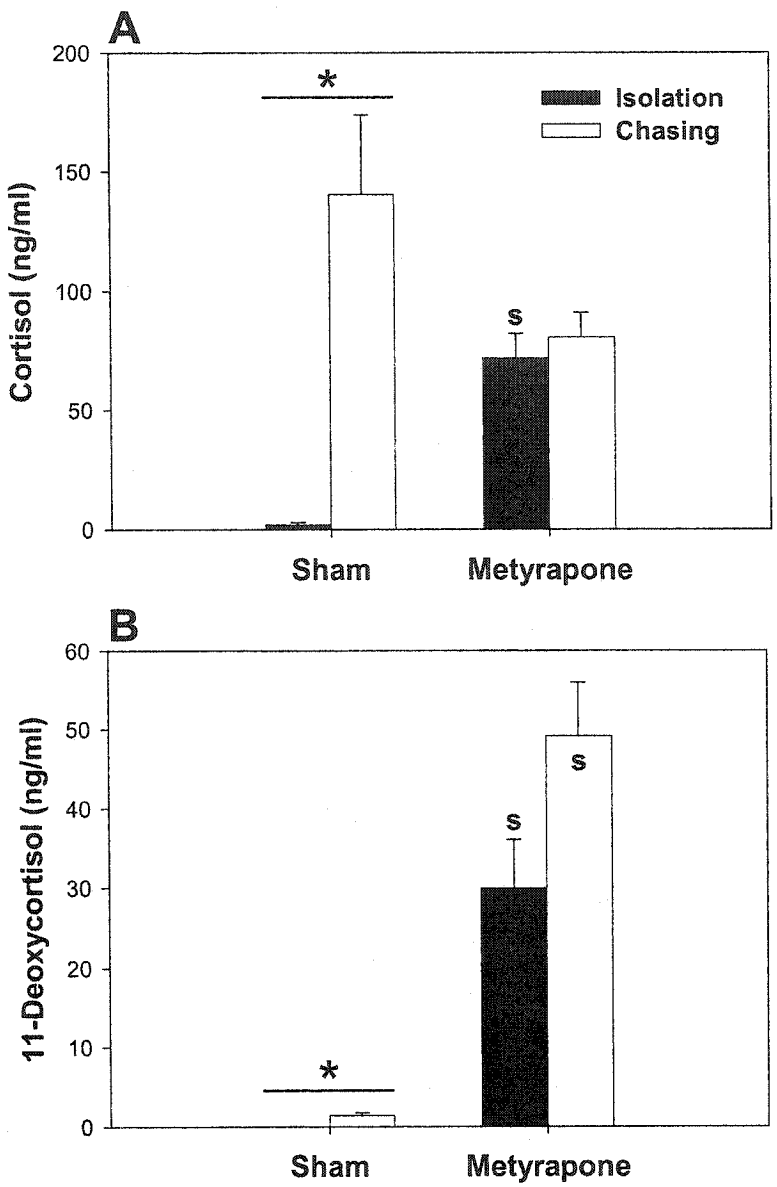

Figure 4.3 Effects of metyrapone on the levels of plasma (A) cortisol and (B) 11-deoxycortisol in juvenile female rainbow trout after 24 h of isolation with (Chasing) or without (Isolation) a period of physical disturbance followed by 2 h of recovery in isolation. Stars show statistical difference between isolation and chasing for a given treatment, and S shows difference between metyrapone and sham for a given stress protocol ($P < 0.05$).

4.3.2. Effects of cortisol, RU-486 and metyrapone

4.3.2.1. Sham

The levels of plasma cortisol (Fig. 4.4A) and ACTH (Fig. 4.4B) were not different from control values after 96 h of isolation but they were significantly elevated after 24 h of confinement. The levels of CRF1 (Fig. 4.5A) and NPY (Fig. 4.5B) mRNA were elevated compared with control after both isolation and confinement. There was no difference in the levels of CRF1 mRNA between isolated and confined fish (Fig. 4.5A) but the levels of NPY mRNA were significantly lower after confinement (Fig. 4.5B).

4.3.2.2. Cortisol

Cortisol implantation increased, as expected, the levels of plasma cortisol relative to sham treatment in isolated fish (Fig. 4.4A). In confined fish implanted with cortisol, plasma cortisol levels were not different from sham but they were significantly elevated compared with isolated fish (Fig. 4.4A). Treatment with cortisol increased the levels of plasma ACTH compared with sham after both isolation and confinement (Fig. 4.4B). The levels of CRF1 (Fig. 4.5A) and NPY (Fig. 4.5B) mRNA in isolated fish were significantly lower compared to sham and confined fish. Cortisol implantation did not influence the levels of CRF1 (Fig. 4.5A) and NPY (Fig. 4.5B) mRNA in confined fish.

4.3.2.3. RU-486

RU-486 implantation did not significantly influence plasma cortisol levels in isolated fish (Fig. 4.4A). However, the cortisol response to confinement was increased in RU-486-treated fish (Fig. 4.4A). Treatment with RU-486 increased plasma ACTH levels compared with sham after

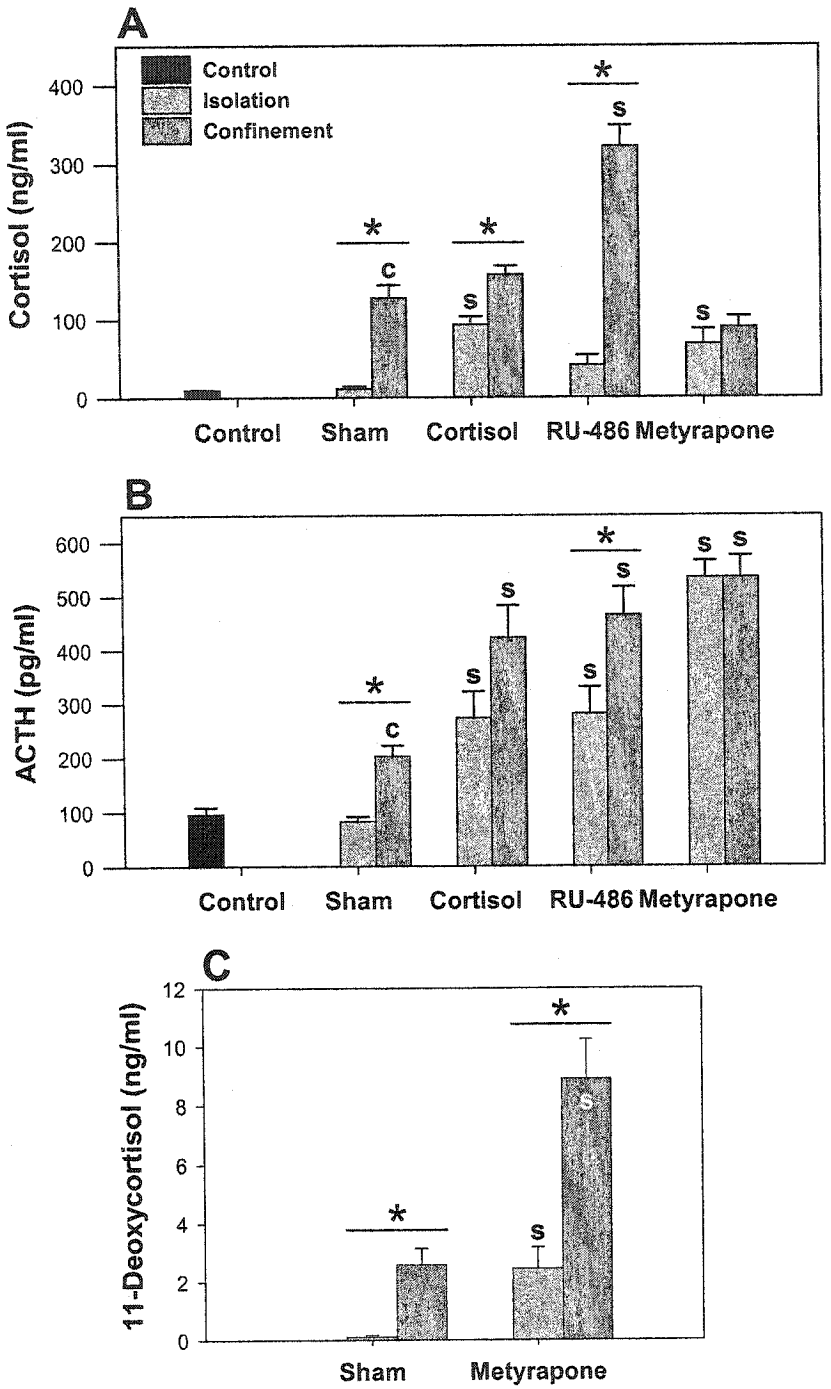

Figure 4.4 Effects of cortisol, RU-486 and metyrapone on the levels of plasma (A) cortisol and (B) ACTH after juvenile female rainbow trout were isolated for 96 h (Isolation) or isolated for 72 h and confined for 24 h (Confinement), as well as (C) the effects of metyrapone on the levels of plasma 11-deoxycortisol. Stars show statistical difference between isolation and confinement for a given treatment, C shows difference between sham and control, and S shows difference between treatments and their respective sham ($P < 0.05$).

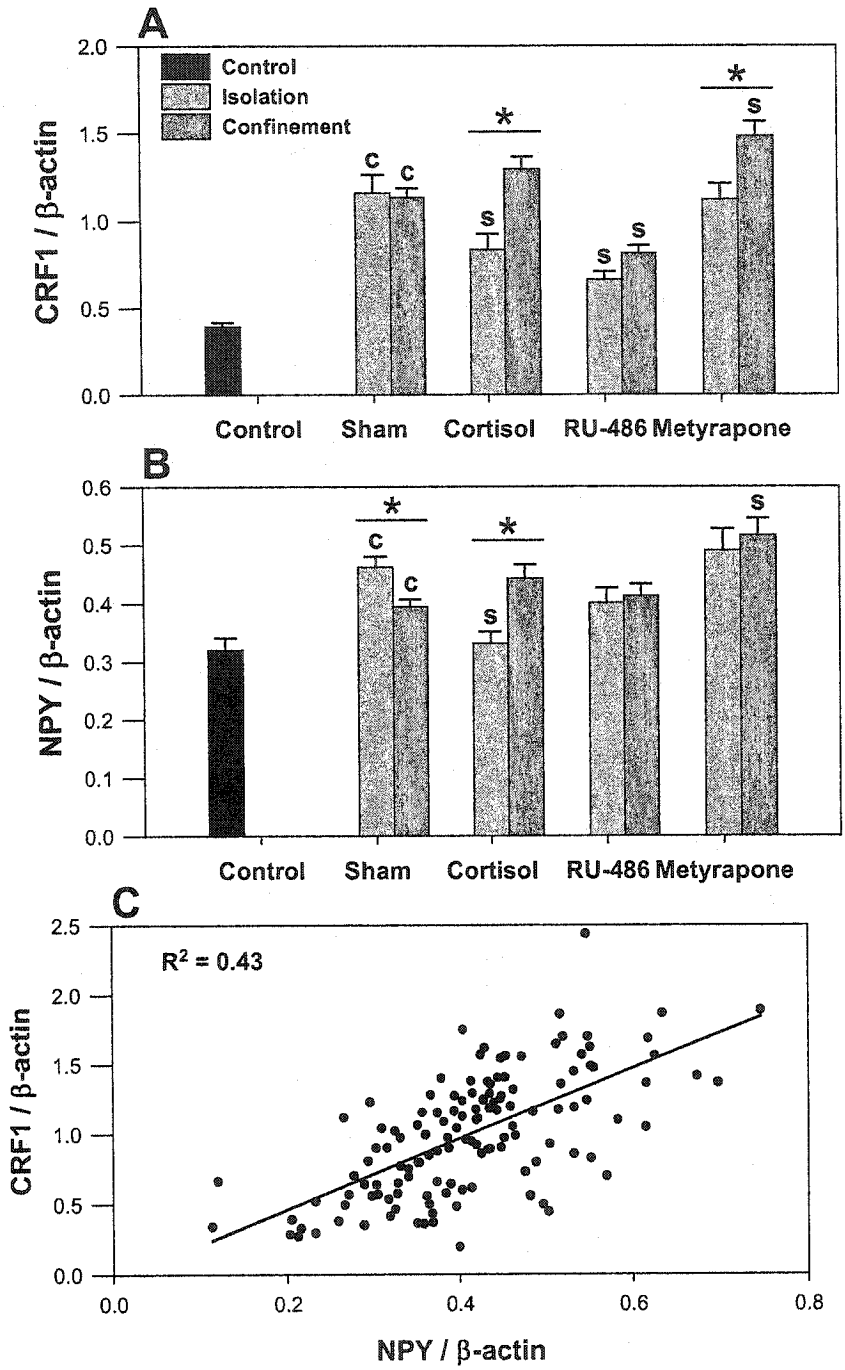

Figure 4.5 Effects of cortisol, RU-486 and metyrapone on the levels of (A) CRF1 mRNA and (B) NPY mRNA after juvenile female rainbow trout were isolated for 96 h (Isolation) or isolated for 72 h and confined for 24 h (Confinement), as well as (C) the relationship between NPY and CRF1 mRNA levels ($P < 0.001$). β-actin was used as a control to correct for the amount of total RNA used in the ribonuclease protection assay (RPA). Stars show statistical difference between isolation and confinement for a given treatment, C shows difference between sham and control, and S shows difference between treatments and their respective sham ($P < 0.05$).

both isolation and confinement (Fig. 4.4B). The levels of CRF1 mRNA in both isolated and confined fish were significantly lower than those of sham (Fig. 4.5A), but there was no difference in the levels of NPY mRNA (Fig. 4.5B).

4.3.2.4. Metyrapone

Metyrapone implantation significantly increased plasma cortisol levels in isolated fish (Fig. 4.4A). The levels of plasma ACTH (Fig. 4.4B) and 11-deoxycortisol (Fig. 4.4C) in isolated and confined fish were significantly elevated compared to sham. Confinement increased plasma 11-deoxycortisol levels in sham and the response was further enhanced in metyrapone-treated fish (Fig. 4.4C). The levels of CRF1 (Fig. 4.5A) and NPY (Fig. 4.5B) mRNA in confined fish were elevated compared with sham, and confinement increased CRF1 mRNA levels in metyrapone-treated fish.

4.3.2.5. Relationship between CRF1 and NPY mRNA

The data of all treatments from the isolation and confinement experiments were combined to examine the relationship between CRF1 and NPY mRNA levels. There was a significant positive correlation ($R^2 = 0.43$; $P < 0.001$) between the levels of CRF1 and NPY mRNA (Fig.4.5C).

4.4. Discussion

This study was designed to examine the effects of cortisol on the levels of CRF1 and NPY mRNA in the preoptic area of the rainbow trout brain. The pilot studies were essentially used to verify whether RU-486 increased and metyrapone reduced plasma cortisol levels.

Implanted fish of the pilot studies were isolated for 24 h with or without a single period of physical disturbance. The experimental design for the second series of experiments was modified based on the results of isolation and physical disturbance experiments (Chapter 3). CRF1 mRNA levels were elevated after 24 h of isolation but they were back to control values after 72 h, and a single chasing event had no effect on CRF1 mRNA levels (see Fig. 3.1 and 3.2, Chapter 3). As it is impossible to inject a trout without stimulating the HPI axis (Pickering *et al.*, 1987), implanted fish in the second series of experiments were either isolated for 96 h to recover from the injection stress or confined for 24 h to further stress the fish after 72 h of recovery. This discussion will focus primarily on the results of the second series of experiments.

4.4.1. Sham

The levels of CRF1 and NPY mRNA after 96 h of isolation were elevated compared with those of control despite low levels of plasma cortisol and ACTH. In a similar experiment, I showed that after 72 h of isolation, CRF1 mRNA was back to control values (Chapter 3). The main difference between these two experiments is that fish from the present study received an intraperitoneal injection of coconut oil. Thus, the stress associated with the injection procedure had a relatively long lasting effect on CRF1 and NPY mRNA levels. I previously showed that greater stress intensities lead to longer lasting elevations in CRF1 mRNA despite low plasma cortisol values (Chapter 3). However, the elevated levels of CRF1 and NPY mRNA could also be attributed to the feeding status of isolated fish. I previously demonstrated that 5 days of food deprivation increased levels of CRF1 and NPY mRNA in the preoptic area of the trout brain (Chapter 2). In the present experiment, all fish except the controls were unfed for 96 h. For this reason, all results are compared with the sham treatment rather than control. The higher levels of

plasma cortisol and ACTH in confined fish compared with isolated fish confirm that the confinement protocol was more stressful. The fact that confinement did not further elevate the levels of CRF1 mRNA indicates that isolated fish had stressed levels of CRF1 mRNA. The reduction in NPY mRNA levels in confined fish compared with isolated fish was unexpected. This result is not consistent with my previous observation that social stress increased NPY mRNA levels in the preoptic area of the trout brain (Doyon *et al.*, 2003). Several studies support a stress-induced elevation of NPY mRNA in the rat PVN (Conrad and McEwen, 2000; Makino *et al.*, 1999, 2000; Sweerts *et al.*, 2001).

4.4.2. Cortisol

Plasma cortisol levels in cortisol-implanted fish were similar to those of vehicle-treated fish that were stressed by confinement. These physiological levels of plasma cortisol were associated with a 28% reduction in the levels of both CRF1 and NPY mRNA in the preoptic area of isolated fish compared with sham. This result supports the idea of a glucocorticoid-mediated negative feedback affecting both CRF1 and NPY mRNA levels. This is consistent with a previous study that showed a cortisol-induced reduction in CRF mRNA levels in the telencephalon-preoptic area of the goldfish brain (Bernier *et al.*, 1999). The presence of heavy hybridization for glucocorticoid receptor (GR) mRNA in the nucleus preopticus and the colocalization of GR-immunoreactivity in all CRF-immunoreactive neurons of the rainbow trout nucleus preopticus support glucocorticoids regulating the activity of CRF-producing neurons (Teitsma *et al.*, 1997, 1998). The synthetic glucocorticoid agonist dexamethasone reduced CRF mRNA levels in the rat PVN but not corticosterone (Kovács and Mezey, 1987). However, different populations of neurons in the rat brain show differences in their responsiveness to

glucocorticoids (Swanson and Simmons, 1989). Corticosterone reduced CRF mRNA levels in parvocellular neurons, but increased these levels in PVN neurons with descending projections, in certain magnocellular neurosecretory neurons, and in a part of the central nucleus of the amygdala (Swanson and Simmons, 1989).

My result on the effect of cortisol on NPY mRNA levels was unexpected. Dexamethasone increased NPY gene expression *in vitro* (Higuchi *et al.*, 1988) and in the paraventricular and arcuate nuclei of the rat hypothalamus (McKibbin *et al.*, 1992; Wilding *et al.*, 1993). However, I previously demonstrated that CRF1 and NPY mRNA levels are positively correlated in the preoptic area of the trout brain (Doyon *et al.*, 2003) and this result was further supported by these studies (Fig. 4.5C). CRF and NPY mRNAs are perhaps co-regulated by glucocorticoids in the preoptic area of the trout brain.

Cortisol implantation did not prevent the stress-induced elevation in the levels of plasma cortisol, or CRF1 and NPY mRNA during confinement. Glucocorticoids reduced but did not totally prevent the stress-induced increase in CRF mRNA in the rat paraventricular nucleus (Harbuz and Lightman, 1989; Imaki *et al.*, 1995; Lightman and Harbuz, 1993). In the present experiment, chronically elevated levels of plasma cortisol may have downregulated glucocorticoid receptors (GR), attenuating the glucocorticoid-mediated negative feedback. Chronic confinement stress and glucocorticoid treatment reduced glucocorticoid binding capacity in the rainbow trout liver and brain (Pottinger, 1990; Lee *et al.*, 1992). Also, cortisol treatment significantly decreased GR content in trout liver *in vitro* (Sathiyaa and Vijayan, 2003) and *in vivo* (Vijayan *et al.*, 2003). This may represent an essential adaptive mechanism to maintain the responsiveness of the HPI axis in situations of chronic stress. In chronically stressed

mammals, the responsiveness of the HPA axis is maintained and in some situations, the HPA axis shows hyper-responsiveness to a novel stressor (reviewed by Aguilera, 1994).

Cortisol-implantation increased the levels of plasma ACTH in both isolated and confined fish compared with their respective sham group. Although this result was unexpected, precedents for this can be found in the mammalian literature. Glucocorticoids stimulated ACTH secretion from pituitaries of chronically stressed rats *in vitro* (Young and Akil, 1988). The authors suggested that under some conditions of chronic stress, the feedback effect of glucocorticoids at the pituitary level is positive rather than negative. It was suggested that chronic stress modifies the functional interaction of GR with genomic regulatory elements (Aguilera, 1994).

4.4.3. RU-486

RU-486 was initially developed as an antagonist of the progesterone receptor but it is also a potent antagonist of GR in mammals (Gaillard *et al.*, 1984; Healy *et al.*, 1985; Baulieu, 1997). RU-486 also binds with high affinity to GR in fish (Lee and Bols, 1989). By blocking GR, and essentially those in the brain, RU-486 is thought to prevent glucocorticoid-induced inhibition of the HPI axis activity. This should lead to increased hormone synthesis and release at all levels of the HPI axis.

RU-486 implantation increased, as expected, the levels of plasma cortisol after both isolation and physical disturbance in the pilot study. This result is consistent with previous studies showing that RU-486 increases plasma cortisol levels after chasing in trout (Eros and Milligan, 1996) and 24 h after implantation in goldfish (Bernier *et al.*, 1999; Bernier and Peter, 2001). RU-486 did not significantly elevate plasma cortisol levels in isolated fish 96 h after implantation. Similarly, RU-486 did not elevate plasma cortisol levels 3 and 7 days after

implantation in trout (Vijayan *et al.*, 1994; Reddy *et al.*, 1995; Sloman *et al.*, 2001a). However, RU-486 treatment combined with confinement stress significantly increased plasma cortisol levels compared with sham. Plasma ACTH levels were also elevated in RU-486-treated fish after both isolation and confinement. Together, these results support the activation of the HPI axis in the absence of glucocorticoid negative feedback. However, I also report a reduction of CRF1 mRNA levels in the preoptic area of trout treated with RU-486. This unexpected result was consistent throughout all experiments. This contrasts with the goldfish where RU-486 treatment increased CRF mRNA levels in the telencephalon-preoptic area of the brain (Bernier *et al.*, 1999; Bernier and Peter, 2001).

Several hypotheses can be presented to explain why the levels of CRF1 mRNA are reduced by RU-486 in trout. First, the levels of plasma ACTH was increased in RU-486-treated fish and several studies suggest the presence of a short-loop feedback where ACTH inhibits CRF release in mammals (Seiden and Brodish, 1971; Upton *et al.*, 1973; Simpson *et al.*, 1980; Suda *et al.*, 1987). This represents a mechanism by which ACTH could regulate its own secretion in the absence of glucocorticoid feedback (Suda *et al.*, 1987). The second hypothesis is that RU-486 could affect other systems that interact with the HPI axis. For example, RU-486 is a potent antagonist of the progesterone receptor and progesterone increases CRF mRNA levels in the bed nucleus of the stria terminalis in sheep (Broad *et al.*, 1995). A third hypothesis is that RU-486 may not always act as an antagonist in trout. Although RU-486 is known as a glucocorticoid receptor antagonist, its action as a partial agonist in humans is also reported (Laue *et al.*, 1988). It is interesting to note that the levels of plasma ACTH in fish implanted with RU-486 are similar to those in cortisol-treated fish. The fact that cortisol and RU-486 have similar long-term effects on the levels of both CRF1 mRNA and plasma ACTH raises the possibility that RU-486 may act

as an agonist at both hypothalamic and pituitary levels in trout. A fourth hypothesis is that elevated levels of plasma cortisol and ACTH in RU-486-treated fish may not be due to increased CRF release. It was suggested that glucocorticoid feedback selectively inhibits the transcription of arginine vasopressin (AVP) rather than CRF in mammals (Makino *et al.*, 1995a; Kovács *et al.*, 2000). Finally, the negative feedback of glucocorticoids could be mediated through a receptor other than GR. A mineralocorticoid-like receptor was recently cloned and characterized from the rainbow trout testis (Colombe *et al.*, 2000), but the presence of this receptor in the brain has not been investigated. Physiological evidence suggests that some of the actions of glucocorticoids are mediated through a mineralocorticoid-like receptor (Sloman *et al.*, 2001a). RU-486 is a selective antagonist of GR in mammals, but not the mineralocorticoid receptor. Therefore, in my experiment, I did not block potential mineralocorticoid-mediated effects of elevated cortisol in stressed animals. The fact that RU-486 implantation had no effects on NPY mRNA levels suggests that the effects of glucocorticoids on NPY gene expression may not be mediated through GR. The affinity of the mammalian MR for glucocorticoids is greater than that of GR, resulting in a predominant MR occupation at low glucocorticoid levels (Reul and de Kloet, 1985; Arriza *et al.*, 1988; Joels and de Kloet, 1994).

4.4.4. Metyrapone

Adrenalectomy is often used in mammals to reduce circulating levels of glucocorticoids (Jingami *et al.*, 1985; Wolfson *et al.*, 1985; Young *et al.*, 1986). This surgical procedure is very difficult in teleost fish due to the dispersed nature of the interrenal tissue within the head kidney (Pickering *et al.*, 1987; Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). To my knowledge, surgical interrenalectomy has been performed only in eels. This procedure reduced the levels of

plasma cortisol (Butler *et al.*, 1969; Chan *et al.*, 1969) and activated CRF-immunoreactive neurons in the nucleus preopticus of the eel brain (Olivereau and Olivereau, 1991). Alternatively, circulating glucocorticoids can be reduced by metyrapone treatment, also referred to as pharmacological adrenalectomy (Jacobson *et al.*, 1989; Conte-Devolx *et al.*, 1992). Metyrapone reduces plasma glucocorticoid levels by inhibiting the activity of the last steroidogenic enzyme involved in glucocorticoid synthesis, the cytochrome P450-dependent 11β-hydroxylase or CYP11B1. Treatment with metyrapone is expected to reduce resting plasma glucocorticoid levels and prevent the stress-induced elevation in plasma glucocorticoids. These changes should attenuate the negative effects of glucocorticoids on the activity of the HPI axis, and lead to increased synthesis and release of CRF and ACTH.

Metyrapone implantation increased plasma cortisol levels in isolated fish compared with sham in both experiments. This result suggests that metyrapone induced a stress response rather than simply blocking cortisol synthesis. It was previously suggested that treatment with metyrapone is stressful to trout (Fagerlund *et al.*, 1968; Freeman and Idler, 1973) and rats (Rotllant *et al.*, 2002). In fact, several studies in fish reported increased mortality following metyrapone treatment (Olivereau, 1965; Fagerlund *et al.*, 1968; Leach and Taylor, 1980). In the present study, metyrapone-treated fish were slow to recover from anaesthesia and 2 out of 41 fish died (data not shown), which suggests that metyrapone at the doses used may be toxic to trout. Although metyrapone is primarily used to block cortisol synthesis through inhibition of 11β-hydroxylation, it is a non-specific inhibitor of cytochrome P450 activity (Schoneshofer and Claus, 1985; Miranda *et al.*, 1998). Hence, the toxic effects of metyrapone may be mediated through inhibition of other cytochrome P450-dependent reactions. Elevated plasma cortisol levels in isolated trout may be due to the inhibition of the cytochrome P450-dependent

hydroxylation reactions involved in the first steps of cortisol catabolism. Also, it was suggested that metyrapone blocks the conversion of cortisol to cortisone (Freeman and Idler, 1973). Nevertheless, metyrapone treatment prevented a further stress-induced elevation of plasma cortisol in both experiments. This result is consistent with previous studies in trout (Fagerlund *et al.*, 1968; Pagnotta *et al.*, 1994; Eros and Milligan, 1996). Elevated levels of plasma 11-deoxycortisol, the precursor to cortisol, also imply a metyrapone blockade of cortisol synthesis, at least to some extent. Treatment with metyrapone increased the levels of 11-deoxycortisol in mammals (Conte-Devolx *et al.*, 1992). However, the plasma levels of 11-deoxycortisol in trout are very low compared with those of humans, which range between 100 and 300 ng/ml after a metyrapone test (Loriaux, 2001). The maximum levels of plasma 11-deoxycortisol in the pilot study were approximately five-fold higher than those of the second experiment. This suggests that metyrapone was less efficient at blocking cortisol synthesis in the second experiment, possibly due to reduced levels or effectiveness of metyrapone 96 h after the implantation.

Metyrapone implantation increased plasma ACTH levels in both isolated and confined fish compared to their respective sham. The levels of CRF1 mRNA in confined fish were elevated compared to sham treatment. Previous studies in fish showed that metyrapone increased the activity of parvocellular neurons of the nucleus preopticus (Fryer and Boudreault-Châteauvert, 1981) and the levels of CRF mRNA in the telencephalon-preoptic area of goldfish (Bernier and Peter, 2001). Immunoreactive studies in the eel suggested that metyrapone treatment increased CRF synthesis, axonal transport and release into the rostral neurohypophysis (Olivereau and Olivereau, 1990). Although my results are consistent with these studies, the elevated levels of cortisol raise the possibility of a different mechanism of action. Here, chronically elevated levels of plasma cortisol may have downregulated GR, attenuating the

inhibitory effect of cortisol on the activity of the HPI axis. Alternatively, metyrapone may act directly on the central nervous system, although I do not know whether metyrapone crosses the blood-brain-barrier. Intracerebral administration of metyrapone increased the activity of the CRF-ACTH neuroendocrine system in fish (Jain *et al.*, 1994). This effect is possibly mediated through the inhibition of neurosteroids synthesis. Stress increased the brain concentration of allopregnanolone, a neurosteroid that attenuates the stress-induced increase in plasma corticosterone and ACTH by attenuating the increase in PVN vasopressin levels (reviewed by Mellon and Griffin, 2002).

4.4.5. Relationship between CRF1 and NPY mRNA

All the data from the second series of experiments were pooled to examine the relationship between CRF1 and NPY mRNA levels. The positive correlation between CRF1 and NPY mRNA in the preoptic area of the trout brain is consistent with my previous observations (Doyon *et al.*, 2003). This result suggests that CRF and NPY gene expression in the preoptic area may be regulated by similar factors and/or that there may be interactions between CRF and NPY producing neurons. Although NPY and CRF have opposing actions on feeding behaviour in fish (Lin *et al.*, 2000) and mammals (Inui, 1999), studies in rats indicate that NPY increases CRF mRNA (Suda *et al.*, 1993), CRF immunoreactivity (Haas and George, 1987) and CRF release (Haas and George, 1989; Tsagarakis *et al.*, 1989). Furthermore, NPY axons establish synaptic connections with parvocellular neurons producing CRF in rats (Liposits *et al.*, 1988; Mihaly *et al.*, 2002).

In conclusion, I demonstrated that glucocorticoids modify the levels of CRF and NPY mRNA in the preoptic area of the rainbow trout brain. Some of my results support the idea of a

glucocorticoid-induced negative feedback while other results do not. My results highlight the difficulty of using chemicals for which the mode of action in fish is not well characterized. Chemicals like RU-486 and metyrapone have multiple sites of action and their effects in fish are not well understood. The regulation of the HPI axis activity is complex and involves several factors that were not considered in this study, including urotensin-I, arginine vasotocin, cathecholamines, GABA and others. Several other studies will be necessary to understand how CRF and NPY mRNA levels are regulated in fish, and how glucocorticoids modulate the expression of these neurohormones.

CHAPTER 5

GENERAL CONCLUSIONS AND PERSPECTIVES

The objective of this thesis was to examine the impact of stress and glucocorticoids on the levels of CRF and NPY mRNA in the rainbow trout brain. This study required the cloning and characterization of the cDNAs coding for trout CRF and NPY. Full-length cDNAs were obtained for rainbow trout NPY and two different CRF paralogues (Chapter 2). Based on phylogenetic analyses, it is proposed that these two CRF paralogues resulted from a recent gene duplication that took place in a common ancestor of salmonids. A single NPY form was obtained although preliminary Southern hybridization experiments suggest the possible presence of more than one NPY gene in trout (data not shown). Using a ribonuclease protection assay (RPA), the two CRF paralogues demonstrated a similar distribution pattern with the highest expression being in the preoptic area of the trout brain. NPY was most highly expressed in the telencephalon, but the expression of NPY was also relatively high in the preoptic area and the optic tectum.

The first hypothesis of this thesis was that stress modifies CRF and NPY mRNA levels in the rainbow trout brain. The results presented support a role for stress in modifying CRF mRNA levels. Levels of CRF1 mRNA were elevated in the preoptic area of socially subordinate trout (Chapter 2). Isolation, confinement and repeated physical disturbances also increased CRF1 mRNA levels (Chapter 3). Increased CRF1 mRNA levels in isolated trout were transient despite continuous exposure to the isolation stress. This result implies an adaptation to the stressor that may contribute to maintain the responsiveness of the HPI axis to a novel stressor in situations of

chronic stress. The responsiveness of the stress axis to a secondary stressor is maintained in chronically stressed rats (Aguilera, 1994), but this remains to be determined for fish.

Increased CRF1 mRNA levels in isolated trout were delayed compared with plasma cortisol levels (Chapter 3). This timing in the elevations of plasma cortisol and CRF1 mRNA implies a potential stimulatory effect of cortisol on CRF1 mRNA synthesis (Fig. 5.1). However, levels of CRF mRNA do not necessarily represent an indicator of transcriptional activity. Several studies in rats demonstrated that there is a temporal lag between the activation of CRF transcription and detectable changes in CRF mRNA levels (Herman *et al.*, 1992; Imaki *et al.*, 1995). CRF heteronuclear RNA (hnRNA) levels in the rat PVN are increased after 5 min of immobilization stress (Imaki *et al.*, 1995, 1996), whereas the elevation in CRF mRNA levels are detected only 60 to 120 min after the beginning of stress (Imaki *et al.*, 1992, 1995). Nevertheless, the fact that elevated CRF1 mRNA levels in confined fish persist after the decrease in plasma cortisol levels is evidence against a direct role for cortisol in reducing post-stress CRF mRNA levels. However, considering that the half-life of mRNAs can vary from minutes to months (Ross, 1988), it is reasonable to propose that CRF mRNA levels remain elevated long after the presumed post-stress inhibition of CRF transcription. Hence, my results do not yet exclude an inhibitory effect of cortisol on CRF transcription. The temporal lag between the decrease in cortisol and CRF mRNA may be essential to maintain CRF peptide synthesis and replenish depleted stores of CRF peptides after stress. Cortisol may also influence the levels of CRF mRNA by inhibiting the release of CRF peptides and hence, reducing the need to synthesize new peptides.

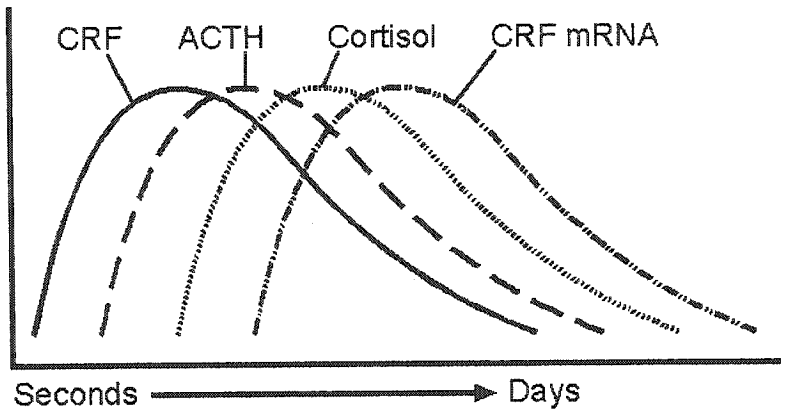

Figure 5.1 Hypothetical representation of the stress-induced release of CRF, ACTH and cortisol in relation to CRF mRNA levels. CRF stimulates the release of ACTH, which stimulates the release of cortisol (reviewed by Wendelaar Bonga. 1997). The elevation in CRF mRNA levels is delayed compared with plasma cortisol (see Fig. 3.1). The shapes of the curves do not necessarily correspond to reality and may vary with different stress protocols.

The levels of plasma cortisol in isolated trout suggested that confinement was more stressful than isolation in a large tank (Chapter 3). The elevation in CRF1 mRNA levels in confined fish was detectable earlier, greater in magnitude, and more persistent than that in isolated fish. CRF1 mRNA levels were also increased in trout physically disturbed to exhaustion but only after repeated periods of chasing (Chapter 3). Together, these results suggest that the intensity and duration of a stress are important factors determining the magnitude and persistence of the elevation in CRF mRNA levels in the trout preoptic area. Stress-induced neuronal activation triggers the release of CRF peptides that are stored in secretory vesicles near synaptic membranes of nerve terminals (Reichlin, 1998). The amount of CRF secreted likely depends on the intensity and the duration of the stress. Greater stress intensity and longer lasting stress would lead to a greater depletion of stored CRF peptides, and hence, a greater need to replenish these stocks. As the levels of CRF mRNA represent a potential for CRF peptide synthesis at a given

time, this potential would need to remain elevated for a longer period of time if more CRF peptides were secreted. This suggests that the levels of CRF mRNA may depend on an autoregulatory mechanism where released CRF determines the need to synthesize new peptides. Evidence from studies in rats support an autoregulatory effect of CRF on the activity of CRF-producing neurons (Ono *et al.*, 1985; Parkes *et al.*, 1993). Central administration of CRF increased both CRF and type 1 CRF receptor (CRF-R1) mRNA in the rat PVN (Parkes *et al.*, 1993; Imaki *et al.*, 1996; Mansi *et al.*, 1996). The levels of CRF-R1 mRNA in the rat PVN are also increased in response to various types of stressors (Luo *et al.*, 1994; Makino *et al.*, 1995b; Rivest *et al.*, 1995; Imaki *et al.*, 1996; Bonaz and Rivest, 1998). The possible autoregulatory effect of CRF on the activity of CRF neurons has not been examined in fish.

The first hypothesis of this thesis led to the prediction that stress would also increase NPY mRNA levels in the rainbow trout brain. Here, I demonstrated for the first time in a fish that certain stress conditions increased NPY mRNA levels. Levels of NPY mRNA were increased in the preoptic area of subordinate trout (Chapter 2). A mild increase in NPY mRNA levels was also detected after repeated physical disturbances (Chapter 3). Isolation, however, did not significantly modify NPY mRNA levels (Chapter 3) although there was a significant positive correlation between the levels of NPY and CRF mRNA (data not shown). In fact, the magnitude of the stress-induced elevation in NPY mRNA levels was lower than that of CRF mRNA in all experiments. This difference in responsiveness could be explained partly by the fact that the dissection of the preoptic area was targeting specifically CRF-producing neurons. Although some of my results support the presence of stress-responsive NPY neurons in the trout brain, the response of these neurons could partly be masked by the presence of non-responsive neurons in the dissected preoptic area. The use of *in situ* hybridization would facilitate the localization of

putative stress-responsive populations of NPY-producing neurons. Initially, my decision to examine NPY mRNA levels in the preoptic area was based on the responsiveness of this brain area to food deprivation in salmon (Silverstein *et al.*, 1998). The hypothalamus was not considered due to the low levels of expression in this tissue, and in preliminary experiments, I could not detect changes in NPY mRNA levels in the telencephalon. Nevertheless, the levels of CRF1 and NPY mRNA in the preoptic area were positively correlated in several studies using different stress protocols (Chapters 2 and 4). This suggests that CRF and NPY gene expression in the preoptic area may be regulated by similar factors and/or that there is an interaction between CRF- and NPY-producing neurons. It is proposed that NPY participates in the activation of the HPI axis, possibly through interactions with CRF. Both peripheral and central NPY injections activate CRF neurons in rats. For example, NPY increases CRF mRNA (Suda *et al.*, 1993), CRF immunoreactivity (Haas and George, 1987) and CRF release (Haas and George, 1989; Tsagarakis *et al.*, 1989), and NPY nerve terminals establish synaptic connections with parvocellular neurons producing CRF (Liposits *et al.*, 1988; Mihaly *et al.*, 2002). Possible synaptic connections between NPY- and CRF-producing neurons in fish should be investigated using, for example, double immunostaining combined with electronic microscopy. Potential interactions between NPY and CRF in fish could also be examined by looking for the presence of NPY receptors on CRF-producing neurons, and whether these receptors are coupled with stimulatory or inhibitory second messenger pathways.

Elevated levels of NPY mRNA in socially subordinate trout may also be related to feeding behaviour. My results demonstrated that 5 days of food deprivation increased NPY mRNA levels in the preoptic area of the trout brain, but the difference between fed and fasted disappeared after 10 days of food deprivation (Chapter 2). If feeding status were responsible for

the differences in NPY mRNA levels, these differences should persist with time. Also, the fact that the levels of CRF1 mRNA follow the same pattern as NPY mRNA suggests that fasting may induce a transient stress response, possibly due to increased social interactions. Immobilization stress increased the levels of CRF mRNA in the PVN and NPY mRNA in the arcuate nucleus of rats (Makino *et al.*, 1999). The reduction in food intake associated with the immobilization stress was thought to be mediated through the actions of CRF and increased NPY mRNA levels suggested a compensatory response to retain appetite (Makino *et al.*, 1999). Considering the complexity of the interactions between the neuroendocrine systems involved in the stress response and the regulation of food intake (Glowa *et al.*, 1992; Bernier and Peter, 2001; Richard *et al.*, 2002), further studies are clearly required to determine whether changes in preoptic levels of NPY mRNA are related to stress, feeding or both.

Finding appropriate controls is a constant challenge that all stress physiologists are facing. In my opinion, every stress experiments should include a control where the level of stress is minimized. In rainbow trout, the lowest levels of plasma cortisol are normally obtained by quickly netting fish from a large holding tank containing a large number of fish to minimize social interactions. However, this type of control makes it very difficult to isolate the effect of a single source of stress. For example, the effects observed in the isolation experiments (Chapter 3) represent the combined effects of isolation and the initial netting and handling of the fish. However, the objective of that experiment was not to determine the effect of a single source of stress but to examine the difference between the effects of different intensities of stress, no matter what the source of stress was. The separation of the potential effect of isolation and that of netting and handling would have required the use of an additional control, *i.e.* fish that are netted, handled and returned to the large holding tank. In the experiments on the effect of

glucocorticoids (Chapter 4), the best controls are the sham treated fish because the treatment of these fish only differs by the absence of the tested chemical.

The second hypothesis of this thesis was that glucocorticoids modify the levels of CRF and NPY mRNA in the rainbow trout brain. Although my results support this hypothesis, only part of my results support the presence of a glucocorticoid-induced negative feedback loop affecting CRF and NPY mRNA levels. Cortisol implantation prevented the increase in CRF1 mRNA levels in isolated fish but not in fish exposed to the more stressful confinement protocol (Chapter 4). This result is consistent with the prediction that glucocorticoids inhibit the activity of the HPI axis. RU-486 implantation prevented the increase in CRF1 mRNA levels in both isolated and confined fish (Chapter 4). This result does not agree with the prediction that by blocking the glucocorticoid receptor (GR), RU-486 should remove the inhibitory effect of glucocorticoids on the HPI axis and increase the activity of the HPI axis. Both cortisol and RU-486 increased the levels of plasma ACTH (Chapter 4). The similarity between the effects of cortisol and RU-486 at both the hypothalamic and pituitary levels suggests that RU-486 may act as an agonist in chronically stressed trout (Fig. 5.2). It was suggested that RU-486 acts a partial agonist in humans (Laue *et al.*, 1988).

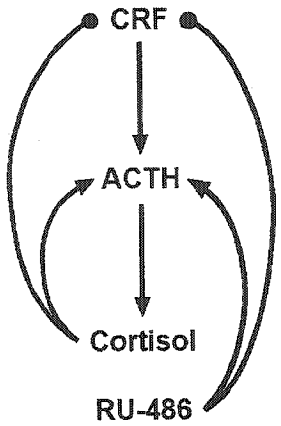

Figure 5.2 Summary of the results of cortisol and RU-486 implantations suggesting that RU-486 acts as an agonist in chronically stressed rainbow trout. Arrows indicate stimulation and dots indicate inhibition.

Metyrapone treatment is expected to reduce plasma glucocorticoid levels by inhibiting the synthesis of glucocorticoids. This should attenuate the negative effects of glucocorticoids on the activity of the HPI axis, and lead to increased synthesis and release of CRF and ACTH. Metyrapone implantation increased, as expected, the levels of CRF1 mRNA in the preoptic area of confined fish (Chapter 4). However, in light of my observations that metyrapone implantation increased the levels of plasma cortisol, my results should be interpreted with caution. In fact, based on my observations and previous reports on the toxicity of metyrapone in fish (Olivereau, 1965; Fagerlund *et al.*, 1968; Leach and Taylor, 1980), the utility of metyrapone for studies on the effects of cortisol in trout should be viewed with scepticism.

Cortisol implantation prevented the increase in NPY mRNA levels in isolated but not in confined fish (Chapter 4). RU-486 implantation had no effects on NPY mRNA levels, which implies that the effects of cortisol on NPY mRNA levels may not be mediated through GR. To date, the impacts of glucocorticoids on NPY have received very little attention. Adrenalectomy

reduced NPY gene expression in the arcuate nucleus and medial basal hypothalamus of rats (White *et al.*, 1990; Savontaus *et al.*, 2002), whereas dexamethasone increased NPY mRNA levels in rodent neural cell lines (Higuchi *et al.*, 1988) and in the paraventricular and arcuate nuclei of the rat hypothalamus (McKibbin *et al.*, 1992; Wilding *et al.*, 1993). Also, a glucocorticoid-responsive element (GRE) was identified upstream of the rat NPY gene (Misaki *et al.*, 1992), implying a glucocorticoid-NPY interaction.

Although the inhibitory effects of glucocorticoids on CRF mRNA synthesis are relatively well established in mammals, the situation is not simple. Glucocorticoids have both inhibitory and stimulatory effects on CRF gene expression, and different populations of neurons in the rat brain show differences in their responsiveness to glucocorticoids (Swanson and Simmons, 1989). Corticosterone reduced CRF mRNA levels in parvocellular neurons, but increased these levels in PVN neurons with descending projections, in certain magnocellular neurosecretory neurons, and in a part of the central nucleus of the amygdala (Swanson and Simmons, 1989). Also, glucocorticoids stimulate CRF synthesis in the placenta of higher primates (Robinson *et al.*, 1988). There is no obvious GRE in the human CRF promoter but a region of the human CRF promoter was identified as a potential negative GRE (Malkoski *et al.*, 1997). The positive feedback of glucocorticoids in placenta requires a functional cAMP-responsive element (CRE) (Cheng *et al.*, 2000). In order to better understand the regulation of CRF gene expression in trout and the involvement of glucocorticoids, it is essential to characterize the CRF promoter and look for the presence of possible GRE and CRE. Regions of the CRF promoter involved in the binding of the activated GR could also be identified by using electrophoretic mobility shift assay.

Most of the research aimed at understanding how the HPI axis activation is terminated after a stressful event has focussed on negative feedback by glucocorticoids. Several other

components of the HPI axis could play a very important role in this process. Termination of HPI axis activation may be mediated in part by a reduction in CRF activity caused by changes in the levels of CRF receptors and CRF binding protein (CRF-BP). Chronic stress reduced the levels of CRF receptor mRNA in the pituitary gland (Hauger *et al.*, 1988) and led to a rapid desensitization of CRF receptors in rats. The inhibitory effects of glucocorticoids on ACTH secretion may be in part mediated through a glucocorticoid-induced reduction in the number of CRF receptors in the pituitary gland (Hauger *et al.*, 1987). Acute restraint stress increased the levels of CRF-BP mRNA in the rat pituitary (McClennen *et al.*, 1998). Increased CRF-BP is thought to reduce the activity of CRF by segregating the CRF peptide from its receptor (Seasholtz *et al.*, 2002). To date, studies in fish have not examined the potential involvement of CRF receptors and CRF-BP in the control of the HPI axis activity.

A better understanding of the regulation of the HPI axis would necessitate an integrative approach where all known components of the system are examined simultaneously. For example, obvious targets for studies on the control of ACTH release would include CRF, CRF receptors, CRF-BP, GR, ACTH and other potential modulators such as arginine vasotocin, urotensin-I and neuropeptide Y. The simultaneous analysis of all of these components of the HPI axis at both the mRNA and peptide levels would provide a better perspective on the regulation of this system under various types and degrees of stress.

The measurement GR content is particularly important when studying the effects of glucocorticoids. Cortisol treatment decreased GR content in rainbow trout hepatocytes (Sathiyaa and Vijayan, 2003; Vijayan *et al.*, 2003). Also, chronic confinement stress and glucocorticoid treatment reduced glucocorticoid binding capacity in the trout liver and brain (Pottinger, 1990; Lee *et al.*, 1992). Data on GR content in the preoptic area of the trout brain would have

facilitated the interpretation of the results of my implantation experiments. Chronically elevated levels of plasma cortisol in fish implanted with cortisol and metyrapone possibly down regulated GR (Chapter 4).

Receptors other than GR could also be involved in mediating glucocorticoid feedback effects. A homologue of the mammalian mineralocorticoid receptor (MR) with high affinity for glucocorticoids was recently cloned and characterized from rainbow trout testis (Colombe *et al.*, 2000), and physiological evidence suggests that some of the actions of glucocorticoids are mediated through a mineralocorticoid-like receptor (Sloman *et al.*, 2001a). Future studies should examine whether a trout MR is localized to the brain, particularly in CRF-producing neurons of the nucleus preopticus. The possible involvement of MR in mediating glucocorticoid feedback should be investigated using spironolactone (RU28318), a known MR antagonist in amphibians and mammals (Delyani *et al.*, 2000). There is also growing evidence that membrane-bound steroid receptors mediate some of the actions of glucocorticoids (Wright and Paine, 1995; Wehling, 1997; Borski, 2000). For example, the rapidity of the fast feedback suggests that this effect cannot be mediated by the classical mechanism involving the cytosolic GR (Hinz and Hirschelmann, 2000). The first membrane-bound steroid receptor was recently cloned and characterized from spotted seatrout ovaries, and homologues were obtained from several species including human, mouse, pig, *Xenopus*, zebrafish and pufferfish (Zhu *et al.*, 2003a,b). This receptor shows high affinity for progestins but low affinity for glucocorticoids. The search for a membrane-bound glucocorticoid receptor should be a research priority.

The focus of this thesis was on mRNA. Levels of mRNA depend on the balance between synthesis and degradation. Future studies on the neuroendocrine control of the stress axis in fish should not only focus on mRNA levels but also estimate mRNA synthesis. Intronic probes have

been used in several studies to measure the levels of CRF hnRNA, which provide an estimate of transcriptional activity (Herman *et al.*, 1992; Imaki *et al.*, 1995, 1996; Kovács and Sawchenko, 1996; Chen *et al.*, 2001). Because of their transitory nature, levels of hnRNA are typically low and necessitate the use of *in situ* hybridization.

In conclusion, my first contribution to the field of stress neuroendocrinology is the characterization of full-length cDNAs coding for rainbow trout NPY and two CRF paralogues. Findings on the modification of CRF1 and NPY mRNA levels in response to different types of stress and glucocorticoids also represent important data. Increased levels of NPY mRNA in the preoptic area of stressed trout raises the possibility of NPY involvement in the control of the stress axis in fish. The finding that CRF1 and NPY mRNA levels are positively correlated in the preoptic area of the trout brain is a unique contribution that highlights the necessity to investigate the possibility of interactions between these CRF- and NPY-producing neurons in fish. This finding is very important because of the fundamental role of NPY as a neuromodulator in several important physiological processes including reproduction, feeding and growth. Finally, this thesis provides the necessary basis for further investigations on the neuroendocrine control of the stress axis in rainbow trout and possibly other fish species.

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APPENDIX

My thesis was focused initially on the mechanisms involved in PCB-induced wasting syndrome. The lipophilic nature of PCBs suggested that wasting could be associated with disruptions occurring in adipose tissues. An obvious candidate for such disruption was the hormone leptin, which is mainly produced in adipose tissues. Based on the similarity between the effects of PCBs and leptin on appetite and lipid metabolism, I hypothesized that PCB-induced wasting is in part mediated by a disruption in leptin expression. Despite several months of efforts at trying to clone a trout homologue of the leptin gene, I was not successful. At least a dozen other laboratories throughout the world have also failed at cloning a fish leptin and now, the recent sequencing of the Japanese pufferfish (*Takifugu rubripes*) genome suggests that there might not be a fish leptin. Nevertheless, my efforts were not entirely wasted. As I was testing my cloning technique with mammalian tissues, I cloned several novel leptin sequences. These new sequences were used in phylogenetic analyses and this led to a publication in *General and Comparative Endocrinology*. Later, I cloned leptin from other mammals where tissues were collected on road-kill and game hunting samples. The objective of this study was to show that mRNA could be used to obtain gene-sequence information from tissues collected several hours after the death of an animal. This study led to a publication in *Canadian Journal of Zoology*. Below, I included versions of these two publications.

APPENDIX I
Molecular Evolution of Leptin

Adapted from: Doyon, C., Drouin, G., Trudeau, V.L., and Moon, T.W. 2001. *Gen. Comp. Endocrinol.* **124**: 188-198.

Abstract

Leptin, a hormone mainly produced by adipocytes, is involved in the regulation of food intake, metabolism and reproduction. The objective of this study was to determine the evolutionary relationships of leptin genes. Partial nucleotide sequences of leptin were cloned and sequenced from six mammalian species: large hairy armadillo (*Chaetophractus villosus*), rabbit (*Oryctolagus cuniculus*), big brown bat (*Eptesicus fuscus*), striped skunk (*Mephitis mephitis*), raccoon (*Procyon lotor*) and beluga whale (*Delphinapterus leucas*). The PUZZLE program was used to construct maximum-likelihood trees. Our phylogenetic analysis shows that the grouping of these new mammalian sequences with those currently available in GenBank respect the evolutionary relationships generally accepted for mammals. However, when leptin sequences for chicken and turkey are included in the analysis, these are found to group with mouse and rat leptins. Chicken and mouse leptins are 95 % identical. However, when mouse is compared with closer relatives, such as rabbit or bat, identities are approximately 80 %. A comparison of extant and ancestral leptin sequences suggests that convergent or parallel evolution is the most plausible hypothesis to explain the similarity between bird and rodent leptins.

Introduction

Molecular phylogeny uses molecular sequence information to reconstruct evolutionary relationships between taxa and to estimate the divergence time of organisms (Li, 1997). The molecular clock hypothesis suggests that the rate of substitution is constant for any given protein. Hence, the differences in the sequences of two organisms should reflect their evolutionary distance. Phylogenetic trees based on molecular information or morphological observations are often congruent. However, as we show below, leptin may be an exception to this general rule.

Leptin was first discovered in adipose tissue of the obese ob/ob mouse in 1994 (Zhang *et al.*, 1994). The full coding sequence contains 167 amino acids (a.a.) and is composed of a 21-amino acid signal peptide and a 146-amino acid circulating, bioactive hormone. In mammals, this cytokine-like peptide is produced mainly in adipose tissues, although recent RT-PCR studies demonstrated leptin mRNA in the placenta (Masuzaki *et al.*, 1997; Hoggard *et al.*, 1997; Senaris *et al.*, 1997), the stomach (Bado *et al.*, 1998; Sobhani *et al.*, 2000), and the brain (Jin *et al.*, 1999; Morash *et al.*, 1999) of rodents and humans. Leptin was first described as an adiposity factor that circulates in the blood in proportion to energy stored as fat (Frederich *et al.*, 1995). When fat reserves increase, leptin acts on hypothalamic neuropeptidergic systems to reduce appetite and to increase energy expenditure (Caro *et al.*, 1996). Leptin is also recognized as an important reproductive factor that is necessary for the onset of puberty (reviewed in Chehab, 2000).

In 1998, a putative chicken homologue of leptin was sequenced (Taouis *et al.*, 1998). This was the first report of a nonmammalian leptin. The coding sequence of chicken leptin showed minor differences from the mammalian form, including the presence of only 163 amino acids and an unpaired cysteine at position 3 of the mature peptide (Dridi *et al.*, 2000). Chicken leptin mRNA appears to be most abundant in the liver (Ashwell *et al.*, 1999), which is the major site of fat synthesis in birds (Saadoun and Leclercq, 1983). Since the publication of the chicken

sequence, there has been some doubt concerning the true nature and origin of this sequence (Friedman-Einat *et al.*, 1999). Despite the fact that an independent group has confirmed the chicken sequence (Ashwell *et al.*, 1999), controversy persists (Dunn *et al.*, 2001). The reason for this controversy is the abnormally high sequence similarity between chicken and mouse leptins. The identity of the full coding sequence is 95% at both nucleotide and amino acid levels. This identity is higher than that found between mammalian leptins (Table A1.1). For example, the identity between human and rhesus monkey leptons is 90%.

Table A1.1 Percentage of amino acid identity for known leptin sequences of chicken, mouse and human compared with mammalian sequences. Only animals with available full coding sequence (167 amino acids) are presented. GenBank accession numbers are given in Table A1.2.

Species	Chicken	Mouse	Human
Mouse	94.6	100	83.2
Rat	91.6	96.4	82.0
Human	79.0	83.2	100
Cow	78.4	83.2	84.4
Pig	77.8	82.0	85.0
Rhesus monkey	77.2	81.4	89.8
Cat	76.0	80.8	84.4
Dog	73.1	77.8	80.2
Dunnart	64.1	67.1	67.7

The aim of this study was to determine the evolutionary relationships between leptin genes. We constructed phylogenetic trees using leptin sequences available in GenBank and six new mammalian sequences.

Methods

Animals and tissue collection

Pablo Carmanchahi and Dr. Gustavo Somoza (Fundacion Pablo Cassará, Buenos Aires, Argentina) provided adipose tissue from large hairy armadillo (*Chaetophractus villosus*). Dr. Shannon Reidy (Department of Biology, University of Ottawa, Canada) provided retroperitoneal white adipose tissue from rabbit (*Oryctolagus cuniculus*). Dr. Brian Hickey (St. Lawrence Institute, Cornwall, Canada) provided the big brown bat (*Eptesicus fuscus*). Lesley Howes and Rick Rosette (Ontario Ministry of Natural Resources, Canada) provided the striped skunk (*Mephitis mephitis*) and the raccoon (*Procyon lotor*). After dissection, white adipose tissue from these animals was frozen in liquid nitrogen or on dry ice, and stored at -80°C. Genomic DNA from beluga whale (*Delphinapterus leucas*) was provided by Dr. Bradley N. White (Director of Natural Resources, DNA Profiling and Forensics, Trent University, Canada).

RNA extraction, RT-PCR, and DNA sequencing

Total RNA was extracted from armadillo adipose tissue with Trizol (Gibco BRL, Burlington, ON) in the laboratory of Dr. Somoza. Extraction of poly(A)$^+$ RNA from armadillo

total RNA and from adipose tissue of other species was performed with the Straight A's mRNA Isolation System (Novagen, Madison, WI). Oligonucleotide primers (Synthaid Biotechnologies Inc., Ottawa, ON) were designed on the basis of high sequence identity between known leptin sequences. The sense primer was 5'-AGGATCAATGACATTTCACACACG-3' and the antisense primer was 5'-CCACCACCTCTGTGGAGTAGA-3'. First-strand cDNA was synthesized from 100–500 ng poly(A)$^+$ RNA with SUPERSCRIPT II (Gibco BRL) and an oligo(dT) primer (Gibco BRL) or our leptin-specific antisense primer. The cDNA was stored at -20°C until used. Leptin-related cDNA fragments were amplified with *Taq* polymerase (Gibco BRL) and an Eppendorf Mastercycler Gradient with the following program: 5 min denaturing at 94°C and then 35 cycles of denaturation at 94°C for 30 s, annealing at 56.5°C for 30 s, and extension at 72°C for 1 min. After the last cycle, further extension was performed at 72° for 5 min. PCRs contained 4 µl of cDNA or 30 ng of genomic DNA (for beluga whale) in a 50-µl PCR. The expected size of amplified products was 316 bp, except for beluga whale, for which we expected to amplify part of exon 3 and the entire intron preceding it. Amplified products were extracted from a 1% agarose gel with the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON), ligated into the pCR II-TOPO cloning vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* competent cells (One Shot TOP10 Electrocomp cell; Invitrogen). Single colonies were cultured and plasmids were recovered with the Plasmid Mini Kit (Qiagen) or Wizard Plus (Promega, Madison, WI). All procedures were done according to the manufacturer's instructions. For each new leptin form, both strands of three different cloned inserts were sequenced by Canadian Molecular Research Services Inc. (Ottawa, ON). Nucleotide sequences were submitted to BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi; Altschul *et al.*, 1997) for comparison with sequences present in GenBank.

Phylogenetic analyses

Leptin sequences were aligned with CLUSTAL W (http://www2.ebi.ac.uk/clustalw; Thompson *et al.*, 1994). Our partial leptin sequences were aligned with the equivalent regions of previously sequenced vertebrate genes obtained from GenBank. Accession numbers of leptin sequences for all species used in our phylogenetic analyses are shown in Table A1.2. The phylogenetic analyses were performed on predicted amino acid sequences (89–90 a.a.) encoded by the cDNA fragments. Maximum-likelihood trees were calculated with PUZZLE version 4.0.2 (http://www.tree-puzzle.de; Strimmer and von Haeseler, 1996), with the chicken or the fat-tailed dunnart sequences as outgroups, the JTT model of amino acid substitutions (Jones *et al.*, 1992), and one fixed and eight gamma-distributed rates estimated from the data set. Relative-rate tests were performed with the program RRTree version 1.1.10 (http://pbil.univ-lyon1.fr/software/rrtree.html; Robinson-Rechavi and Huchon, 2000) with the chicken or the fat-tailed dunnart as outgroups. These tests were performed with only the species for which the full coding sequence of leptin was available. The rate of leptin evolution of rodents (mouse and rat) was compared with that of the other mammals (rhesus monkey, human, cat, dog, pig, and cow) grouped together.

Table A1.2 Systematic arrangement of the taxa considered in this study and GenBank accession number for leptin sequences.

	Scientific name	Common name	Accession
Class Aves			
Order Galliformes			
Family Phasianidae	*Gallus gallus*	Chicken	O42164
Family Meleagrididae	*Meleagris gallopavo*	Turkey	AAC32381
Class Mammalia			
Infraclass Metatheria (= Marsupialia)			
Order Dasyuromorphia			
Family Dasyuridae	*Sminthopsis crassicaudata*	Fat-tailed dunnart	AAD44337
Infraclass Eutheria (= Placentalia)			
Order Edentata			
Family Dasypodidae	*Chaetophractus villosus*	Large hairy armadillo	AF203904
Order Lagomorpha			
Family Leporidae	*Oryctolagus cuniculus*	Rabbit	AF203903
Order Rodentia			
Suborder Sciurognathi			
Family Muridae	*Mus musculus*	Mouse	P41160
	Rattus norvegicus	Rat	AAC52514
Order Primates			
Suborder Catarrhini			
Family Cercopithecidae	*Macaca mulatta*	Rhesus monkey	Q28504
Family Hominidae	*Pongo pygmaeus*	Orangutan	Q95234
	Gorilla gorilla	Gorilla	Q95189
	Pan troglodytes	Chimpanzee	O02750
	Homo sapiens	Human	AAA60470
Order Chiroptera			
Suborder Microchiroptera			
Family Vespertilionidae	*Eptesicus fuscus*	Big brown bat	AF296670
Order Carnivora			
Suborder Feliformia			
Family Felidae	*Felis catus*	Cat	BAA95481
Suborder Caniformia			
Family Canidae	*Canis familiaris*	Dog	BAA35129
Family Procyonidae	*Procyon lotor*	Raccoon	AF296669
Family Mephitidae	*Mephitis mephitis*	Striped skunk	AF296668
Order Artiodactyla			
Suborder Suiformes			
Family Suidae	*Sus scrofa*	Pig	Q29406
Suborder Ruminantia			
Family Bovidae	*Bos taurus*	Cow	CAA72197
	Ovis aries	Sheep	Q28603
Order Cetacea			
Suborder Odontoceti			
Family Delphinidae	*Delphinapterus leucas*	Beluga whale	AF296671

Note: The classification essentially follows Montgelard *et al.* (1997) and the NCBI taxonomy server (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/).

Convergent evolution

Convergent evolution was examined by inference of ancestral mammalian leptin sequences with the ANCESTOR program (http://mep.bio.psu.edu/ancseq.html; Zhang and Nei, 1997). All mammalian sequences shown in Fig. A1.1, except for the big brown bat sequence, were used, because the exact relationship of bats with other mammals is still uncertain (Allard *et al.*, 1996). Ancestral sequences were inferred under the assumption of the phylogenetic tree topology shown in Fig. A1.4, but the branch lengths of the phylogenetic tree shown in Fig. A1.4 were inferred by the ANCESTOR program with the JTT model of amino acid substitutions (Jones *et al.*, 1992).

Results

Phylogenetic trees

A partial genomic sequence of beluga whale leptin that contained part of exon 3 (271 nt) and the intron preceding it (1531 nt) was cloned and sequenced. Partial nucleotide sequences (268–271 nt) of leptin genes were also obtained from five mammalian species: large hairy armadillo, rabbit, big brown bat, striped skunk, and raccoon. These new leptin sequences represent more than half of the total length of the coding sequence. Deduced amino acid sequences were compared to the equivalent region of leptin sequences available in GenBank (Fig. A1.1), and the alignments were used to construct maximum-likelihood phylogenetic trees. The first tree was based only on mammalian sequences and rooted with the fat-tailed dunnart, a carnivorous marsupial from Australia (Fig. A1.2). Here, the most primitive mammals are at the base of the tree and the various groupings of species are consistent with mammalian orders (Table A1.2). The primates all group together with humans closer to other members of the family Hominidae (chimpanzee, gorilla, and orangutan) than to Old World monkeys (rhesus monkey). The carnivores all group together with strong support at the skunk and raccoon node. The grouping of beluga whale with species from the order Artiodactyla respects the genealogical ties between these organisms (Montgelard *et al.*, 1997). In this tree, beluga whale leptin is closer to that of pig than cow or sheep. The grouping of cow and sheep, both members of the suborder Ruminantia, is very well supported. Rodents group together with the *big* brown bat as their closest relative. We did not obtain support values for most interordinal relationships. Our phylogenetic analysis is not based on a sufficient number of species and sufficient molecular information to obtain a detailed resolution within and between mammalian orders. When bird sequences are included in the analysis (Fig. A1.3), the accepted evolutionary relationships between the species analyzed are not respected. This tree, with the chicken sequence as the outgroup, shows rodents grouping with birds. All the other groupings are preserved, but the most primitive mammals, the marsupial and the armadillo, end up in the tree with the other mammals.

Relative-rate tests

When chicken is used as the outgroup, there is a highly significant difference in the number of substitutions per site for synonymous substitutions (Ks), nonsynonymous substitutions (Ka), and amino acids in rodents and other mammals (Table A1.3). However, when the fat-tailed dunnart is used as the outgroup, there is no difference in the relative rate of leptin evolution in rodents and other mammals.

```
Chicken     -SVSAKQRVTGLDFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLQKPESLDGVLEAS
Turkey      -......S.....................................................................H..........L
Mouse       Q...........................................................................................
Rat         Q....R.....................................I.............H.....................R..............
Bat         R...S.....................V.....................I......G.....S.....................S.N..PF.R.RS.KTL.G..D.....
Pig         Q...S.....................V.....I...I......R..I..S.....................S....P...ARA.ETL...G.......
Beluga      Q...S.....T.V.....I...I......R..I..S.....................S....P...ARA.ETL...G.......
Cow         Q...S.....L.....I...I......R..V..S.....................A....P...VRA.ESL...GV.....
Sheep       Q...S.....L.....I...I.A....R..I..S.....................A....P...VRA.ESL...GV.....
Gorilla     Q...S..K.....T.....I...M..R.MI..S.....V.....H..WA...ETLD..G.....
Chimpanzee  Q...S..K.....T.....I...M..R.MI..S.....V.....H..WA...ETLD..G.....
Orangutan   Q...S..K.....T.....I...M..R..I..S.....V.....H..WA...ETLDR.G.....
Human       Q...S..K.....T.....I...M..R..I..S.....V.....H..EA...ETLD..G.....
Monkey      Q...S.....V.T..Q.....I...I.IN...R..I..S.....H..LA...ETL...GD.....
Rabbit      Q...SR...V.....N....T.....I...I.A....R..I.....S....P..RA...ETL.G.G.....
Raccoon     -A..S....A.....V....R.....I...I....H.R..V..S.....R...S....P..RAR..ESF...G.....
Skunk       -A..S....A.....V....R.....I...I....H.R..V..S.....S....P..RAR..ESF...G.....
Dog         Q...S....A.....Q.V....R.....I...I.N..H.R..V..S.....S....P..RAR..ETF...G.....
Cat         Q...S....A.....V.....I...I..G...R..V..S.....S..N.P..RAR..ETL...G.A....
Armadillo   QFIPS.....I..V.....I...I....S.R..I..V.....S....P..RAG..ETL...G.I....
Dunnart     Y.I.....FQ...D.....I...I.SN.S.R.MV..S.....GSL...PFDEAG..SALGN.E..M...
```

Figure A1.1 Alignment of deduced amino acid sequences of vertebrate leptins. Partial amino acid sequences were aligned using CLUSTAL W. Dots indicate amino acid identities with chicken leptin; sequence gaps are indicated by dashes. The first amino acid corresponds to position 49 and to the first amino acid of exon 3 in the mammalian genomic sequences.

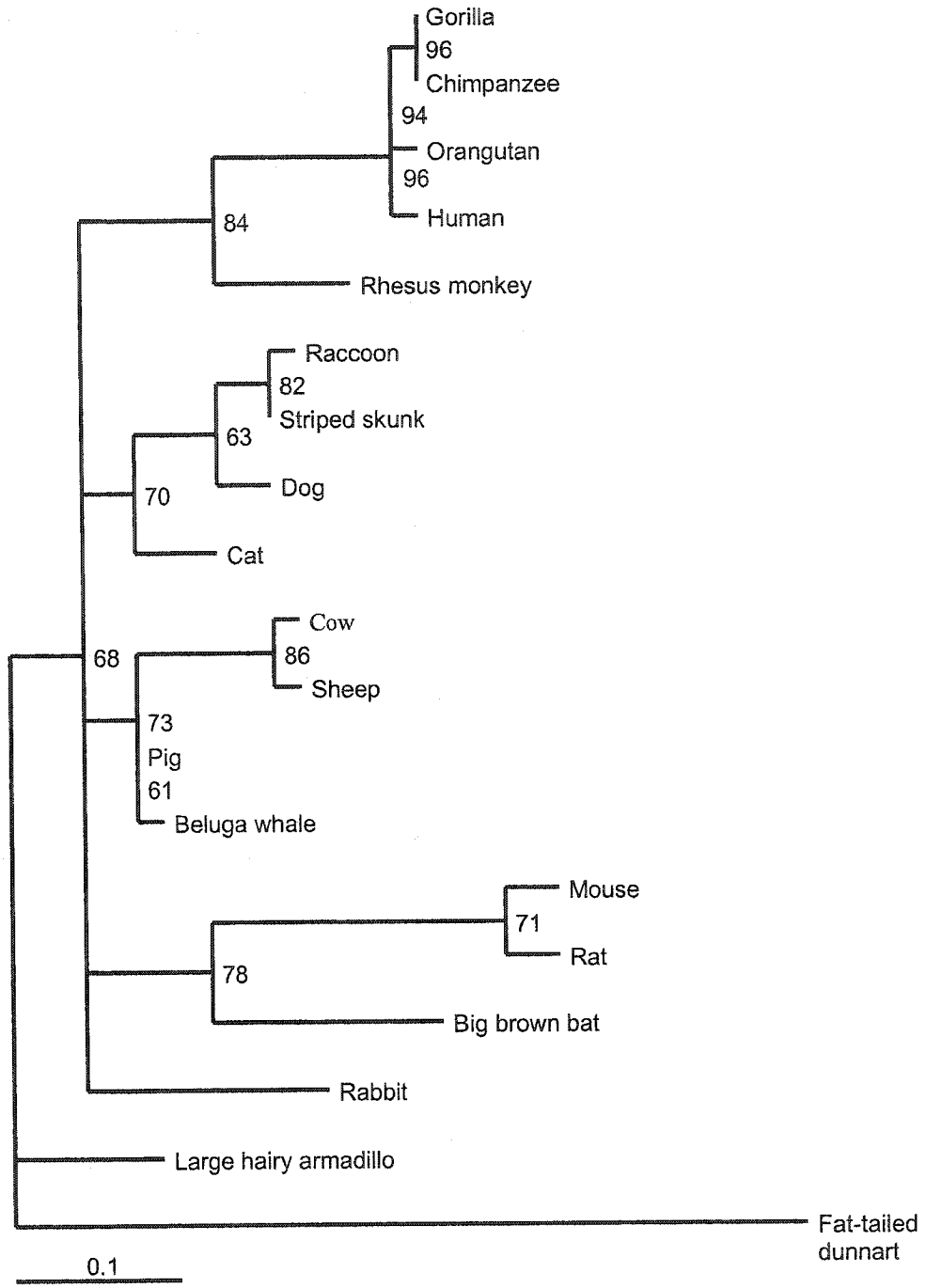

Figure A1.2 Phylogenetic tree for mammalian leptin sequences. This tree was based on aligned sequences presented in Figure A1.1. Maximum-likelihood analyses were performed with PUZZLE version 4.0.2, the JTT model of amino acid substitutions, and one fixed and eight gamma-distributed rates estimated from the data set. The fat-tailed dunnart sequence was used as the outgroup. Support values are shown next to their nodes. The scale bar represents 0.1 amino acid substitution per amino acid site.

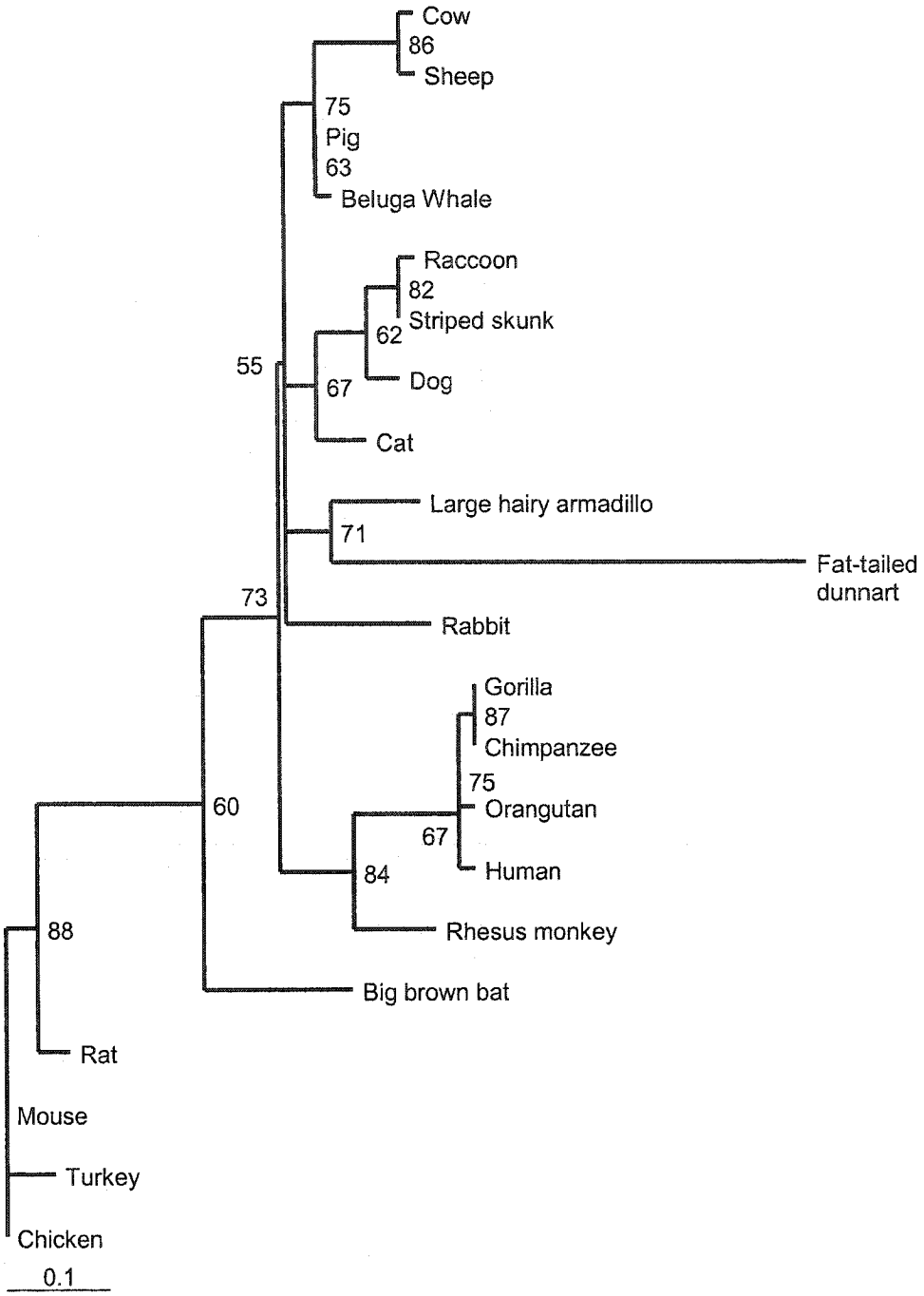

Figure A1.3 Phylogenetic tree for vertebrate leptin sequences. This tree was based on aligned sequences presented in Figure A1.1. Maximum-likelihood analyses were performed with PUZZLE version 4.0.2, the JTT model of amino acid substitutions, and one fixed and eight gamma-distributed rates estimated from the data set. The chicken sequence was used as the outgroup. Support values are shown next to their nodes. The scale bar represents 0.1 amino acid substitution per amino acid site.

Table A1.3 Comparison of the relative rate of leptin evolution between rodents (mouse and rat) and other mammals (rhesus monkey, human, cat, dog, pig and cow). Only animals with available full coding sequence (167 amino acids) were used for this analysis. Ks represents the number of synonymous substitution per synonymous site, Ka represents the number of non-synonymous substitution per non-synonymous site, a.a. represents the number of amino acid substitution per amino acid site, dK/sd represents the difference in the number of substitutions between rodents and mammals divided by the standard deviation, and *P* represents the probability associated with dK/sd.

Outgroup	Type of substitution	Rodents	Mammals	dK/sd	*P*
Chicken	Ks	0.131	0.512	-5.97	$1\ e^{-7}$
Chicken	Ka	0.026	0.124	-6.15	$1\ e^{-7}$
Chicken	a.a.	0.047	0.240	-6.11	$1\ e^{-7}$
Dunnart	Ks	1.030	0.836	1.30	0.192
Dunnart	Ka	0.221	0.193	1.56	0.118
Dunnart	a.a.	0.404	0.361	1.23	0.217

Convergent evolution

Ancestral sequences were inferred by the method of Zhang and Nei (1997) with the assumption of the tree topology shown in Fig. A1.4. The comparisons of some of the extant and ancestral leptin amino acid sequences are shown in Fig. A1.5. The goal of this analysis was to determine whether the high similarity between the bird (chicken and turkey) and the rodent (mouse and rat) sequences was due to the fact that the rodent sequences had conserved the same amino acids as those of bird sequences or whether the rodent sequences independently converged to the same amino acids as those of bird leptin sequences. This was examined by inference of ancestral amino acid sequences of mammalian leptins with only extant mammalian sequences (*i.e.*, the chicken and turkey sequences were not included as outgroups for this analysis). Figure A1.5A shows a comparison of chicken and turkey leptin sequences with mouse and rat sequences and with the sequences ancestral to the mouse and rat. Of 22 variable sites, 7 are not informative and 15 are informative. Here, informative sites refer to sites that show a clear pattern of convergent or divergent evolution. Of the 15 informative sites, 13 show a clear pattern of rodent sequences converging to the same amino acids as those of birds. The other 2 informative sites are only partially (half) convergent because either the two bird or the two rodent sequences are different. As a "control," Fig. A1.5B shows a comparison of chicken and turkey leptin sequences with human and chimpanzee sequences and with the sequences ancestral to human and chimpanzee. Of 23 variable sites, 3 are not informative and 20 are informative. Of the 20 informative sites, only 3 show a clear pattern of human and chimpanzee sequences converging to the same amino acids as those of birds. The other 17 informative sites are clearly divergent; *i.e.*, the human and chimpanzee sequences have evolved toward amino acids that are different from those of bird leptin sequences. The amino acid substitutions that occurred in the mouse–rat lineage are clearly different from those that occurred in the human–chimpanzee lineage. The fraction of convergent informative amino acid sites is significantly larger in the mouse–rat lineage (13/15) than in the human–chimpanzee lineage (3/20; χ^2 test, $P < 0.001$).

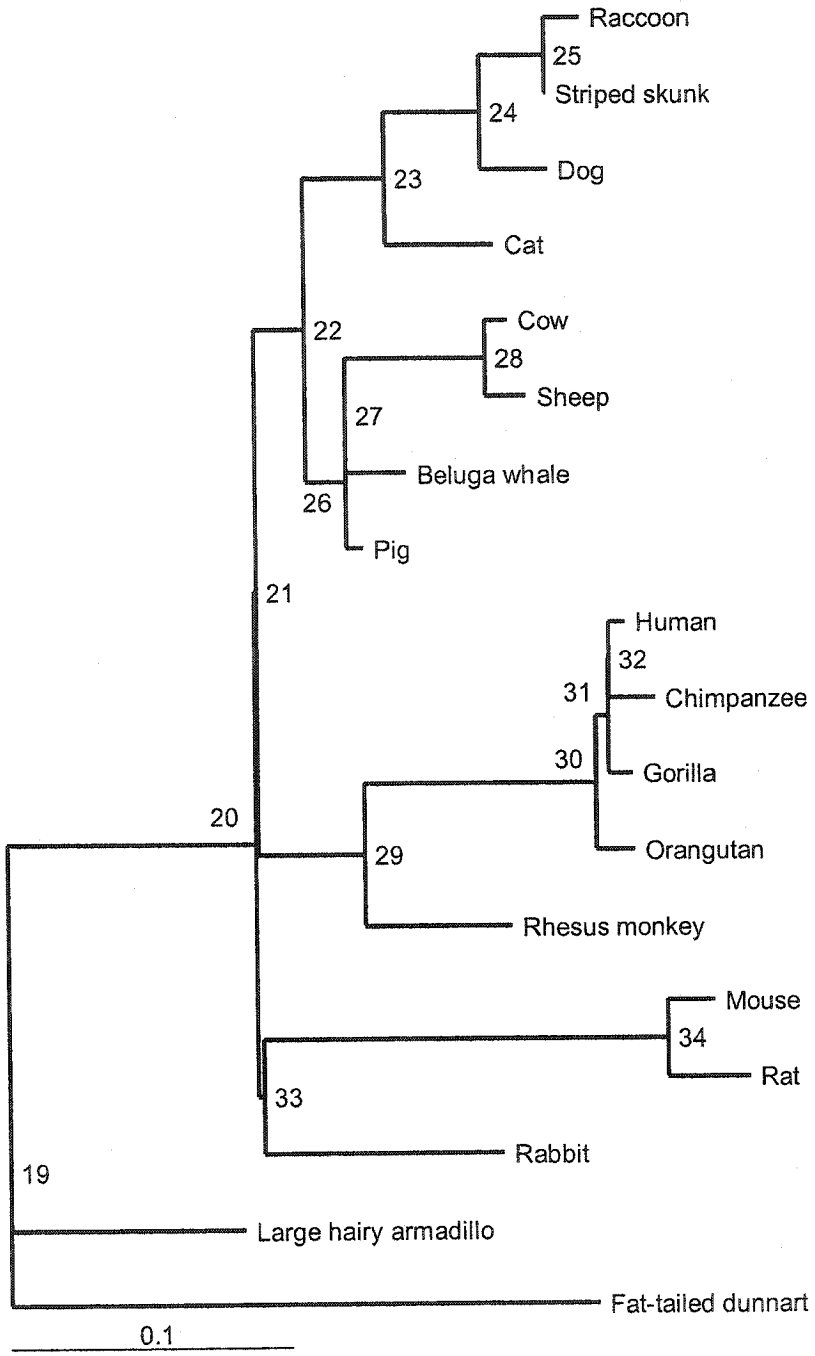

Figure A1.4 Phylogenetic tree used by the ANCESTOR program to infer ancestral leptin sequences. The number written at the nodes represent the numbering of the ancestral sequences inferred. The scale bar represents 0.1 amino acid substitution per amino acid site.

A

```
Chicken SVSAKQRVTGLDFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLQKPESLDGVLEAS
Turkey  SVSAKQSVTGLDFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLHKPESLDGVLEAL

node 19 SISSKQRVTGLDFIPGLHPVLSLSKMDQTLAIYQQILTSLSSRNVIQISNDLENLRDLLHLLASSKSCPLPRAGGLETLESLGGVLEAS
node 20 SVSSKQRVTGLDFIPGLHPVLSLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLPRASGLETLESLGGVLEAS
node 33 SVSSKQRVTGLDFIPGLHPVLSLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLPRASGLETLESLGGVLEAS

node 34 SVSAKQRVTGLDFIPGLHPILSLSKMDQTLAVYQQILTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLQKPESLDGVLEAS
Mouse   SVSAKQRVTGLDFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLQKPESLDGVLEAS
Rat     SVSARQRVTGLDFIPGLHPILSLSKMDQTLAVYQQILTSLPSQNVLQIAHDLENLRDLLHLLAFSKSCSLPQTRGLQKPESLDGVLEAS
         - c- -            c          c  ½   - c  c  c-            c    c  cc-  ½cc   c    -
```

B

```
Chicken SVSAKQRVTGLDFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLQKPESLDGVLEAS
Turkey  SVSAKQSVTGLDFIPGLHPILSLSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQTSGLHKPESLDGVLEAL

node 19 SISSKQRVTGLDFIPGLHPVLSLSKMDQTLAIYQQILTSLSSRNVIQISNDLENLRDLLHLLASSKSCPLPRAGGLETLESLGGVLEAS
node 20 SVSSKQRVTGLDFIPGLHPVLSLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLPRASGLETLESLGGVLEAS
node 21 SVSSKQRVTGLDFIPGLHPVLSLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLPRASGLETLESLGGVLEAS

node 29 SVSSKQRVTGLDFIPGLHPVLTLSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLAFSKSCHLPRASGLETLESLGGVLEAS
node 30 SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLPWASGLETLDSLGGVLEAS
node 31 SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLPWASGLETLDSLGGVLEAS

node 32 SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLPWASGLETLDSLGGVLEAS
Chimp   SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNMIQISNDLENLRDLLHVLAFSKSCHLPWASGLETLDSLGGVLEAS
Human   SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLPEASGLETLDSLGGVLEAS
         -   d           c d        c   d   d  d -d  d          d  c   d   dd-  dddd d     d
```

Figure A1.5 Comparisons of extant and ancestral leptin sequences. A) Comparison of chicken and turkey leptin sequences with mouse and rat sequences. B) Comparison of chicken and turkey leptin sequences with human and chimpanzee sequences. The numbering of the internal nodes correspond to the numbering shown in Figure A1.4. Symbols are as follow: -, uninformative site; c, convergent site; d, divergent site; ½, partially convergent site.

Data deposition

The sequences reported in this paper have been submitted to GenBank. Accession numbers appear in Table A1.2.

Discussion

The topology of the phylogenetic tree that we obtained is strongly affected by the inclusion of leptin sequences from birds. When bird sequences are not included, groupings of species show evolutionary relationships that are consistent with mammalian orders (Table A1.2, Fig. A1.2). For example, all primates group together, with human closer to other members of the family Hominidae (chimpanzee, gorilla, and orangutan) than to Old World monkeys (rhesus monkey). However, the relationships within the Hominidae do not reflect current views suggesting that the chimpanzee is human's closest relative (Li, 1997). The six mammalian leptin sequences reported here group well with those available in GenBank. The large hairy armadillo is part of the order Edentata, which is believed to be the most basal extant order of the infraclass

Eutheria (Allard *et al.*, 1996). Here, the armadillo stands at the base of the tree together with the fat-tailed dunnart, an Australian carnivorous marsupial. The grouping of raccoon and skunk is consistent with the inclusion of their respective families, Procyonidae and Mephitidae, in the superfamily Musteloidea (Ledje and Arnason, 1996a,b). As members of the suborder Caniformia (dog-like carnivores), raccoon and skunk are closer to dog than to cat, a member of the suborder Feliformia (cat-like carnivores). The grouping of beluga whale with species from the order Artiodactyla (cow, sheep, and pig) is consistent with recent molecular evidence suggesting that the orders Cetacea (whales) and Artiodactyla should be united under Cetartiodactyla to form a monophyletic group (Montgelard *et al.*, 1997). In this tree, the beluga whale sequence is closer to pig but the support value for this relationship is relatively low. Graur and Higgins (1994) suggested that whales and ruminants (cow and sheep) are more closely related to each other than either of them are to other suborders included in the order Artiodactyla, such as Suiformes (pig). However, the combined analysis of cytochrome b and 12S rRNA mitochondrial sequences suggests that the whales'closest relative is the hippopotamus, which was traditionally included in the suborder Suiformes (Montgelard *et al.*, 1997). Ideally, our analysis would need to include at least two species from each artiodactyl suborder to clarify their relationship with whales. The rabbit, a member of the order Lagomorpha, does not group with species from other mammalian orders (Fig. A1.2). Although some trees based on molecular information support the monophyletic nature of Lagomorpha, the superorder Glires that includes the orders Lagomorpha and Rodentia is largely supported by morphological and some molecular data (Wyss *et al.*, 1987; Allard *et al.*, 1996). Finally, the *big* brown bat groups with rodents (Fig. A1.2). Based on morphological data, bats were originally included in the superorder Archonta along with primates, tree shrews, and flying lemurs (Miyamoto, 1996). However, molecular evidence suggests that these latter groups have a recent common ancestor but that bats are genealogically distant (Adkins and Honeycutt, 1991). Recent morphological evidence suggests that bats are more closely related to the order Dermoptera (flying lemurs) than to other orders traditionally included in Archonta (Lemelin, 2000). In our analysis, the grouping of the big brown bat with rodents can probably be attributed to the lack of leptin sequence data from more closely related groups such as flying lemurs. Overall, our phylogenetic analysis is not based on a sufficient number of species and sufficient molecular information to obtain a detailed resolution within and between mammalian orders. The presence of more than one species per order and suborder would reduce the chances of long-branch attraction. For example, the grouping of bats and rodents might not persist with more than one bat species. Also, our analysis is based on part of a single gene that does not provide enough informative sites to provide a clear view of mammalian classification.

When bird sequences are included in our analysis, the groupings are not consistent with expected tree topologies (Fig. A1.3). The grouping of bird and rodent leptins is attributable to the unusually high sequence similarity shared by these genes (Table A1.1). To our knowledge, this is the first description of a mouse gene sequence having a greater similarity with a chicken sequence than with those of other mammals. This high sequence similarity has provoked strong reaction from some investigators who believe that the reported chicken sequence is not a chicken leptin (Friedman-Einat *et al.*, 1999). It has been suggested that this chicken sequence is the result of a cloning artifact and that the true chicken leptin has yet to be found (Dunn *et al.*, 2001). However, a year after the first publication of the chicken sequence (Taouis *et al.*, 1998), this sequence was independently confirmed by Ashwell *et al.* (1999). We believe that it is very unlikely that two independent research groups could obtain the same PCR artifact. In our view,

the chicken sequence cannot result from a contamination by mouse material. One would assume that contamination would lead to a higher sequence similarity than the reported 95% for chicken and mouse. Moreover, the deposition in GenBank of a turkey leptin sequence also suggests that bird and rodent leptins shares high similarity. The partial amino acid sequence of the turkey leptin shares 98% identity with the chicken leptin. The recent cloning of a leptin receptor gene in chicken (Horev *et al.*, 2000; Ohkubo *et al.*, 2000) also supports the existence of a leptin-like molecule in this species. The chicken receptor gene, however, shares only 60% identity with the long isoform of mammalian leptin receptors, including the mouse receptor (Horev *et al.*, 2000; Ohkubo *et al.*, 2000).

According to Dunn and colleagues (2001), horizontal transmission of genomic DNA between mouse and chicken is the only hypothesis, other than a cloning artifact, that could explain such strong similarity between mouse and chicken leptins. However, they immediately reject this very unlikely hypothesis. We have examined other hypotheses as possible explanations of this unexpectedly high homology between bird and rodent leptin sequences.

Our first hypothesis is that there have been differential rates of evolution for leptin genes in different species. Rodent leptins could have evolved at a slower rate than other mammalian leptins, leading to a sequence more similar to the presumptive ancestral form of leptin. However, this hypothesis is contrary to current evidence showing rodent genes evolving at a higher rate than those of other mammalian species (Li, 1997). Using the relative-rate test we compared the rate of leptin evolution between rodents and other mammals grouped together. The results obtained are ambiguous. With the chicken sequence as the outgroup, rodent leptins appear to evolve at a much slower rate than do those of other mammals. However, with a marsupial species as the outgroup, there is no significant difference between the rate of evolution of rodent and that of other mammalian leptins (Table A1.3). If a molecular clock exists, the difference in the rate of evolution should be similar no matter which outgroup is used.

The second hypothesis that we propose is that of convergent evolution of the bird and rodent leptins. Convergent evolution was estimated by comparison of some extant leptin amino acid sequences and some of the ancestral leptin amino acid sequences inferred by the method of Zhang and Nei (1997). Our results clearly indicate the convergence of rodent and bird amino acid sequences. The fraction of informative amino acid sites that have converged to the chicken and turkey sequences is significantly larger in the mouse–rat lineage than in the human–chimpanzee lineage. This large number of convergent changes cannot be explained by random chance under any known substitution model. Note that the convergence analysis that we performed cannot discriminate between convergent and parallel amino acid substitutions. Convergent substitutions convert two different amino acids to the same amino acid, whereas parallel substitutions convert two identical amino acids to another identical amino acid (Zhang and Kumar, 1997). To discriminate between these two types of substitutions we would have needed to infer the ancestral state of the amino acids in the bird lineage. Unfortunately, leptin sequences from species ancestral to birds are not available (see below). On the other hand, we could infer, using the new mammalian sequences presented here, the ancestral state of the amino acids in the mammalian lineage. Our approach can therefore show only that the rodent sequences have evolved in such a way as to become similar to bird sequences. This observed similarity could have been the result of either convergent or parallel changes.

The term convergent evolution has been widely used for molecules or characters that share common functions but have emerged independently during evolution (Beschin *et al.*, 1999). For example, *E. coli* thioredoxin reductase and glutathione reductase are structurally

divergent enzymes that acquired their disulfide reductase activities independently (Kuriyan *et al.*, 1991). In our case, bird and rodent leptins are likely to have evolved from a common ancestor but the amino acid sequences have converged. A classical example of convergent evolution in protein is the stomach lysozymes of foregut-fermenting animals (Stewart *et al.*, 1987). Lysozymes of animals such as cows and langurs have evolved to gain sequence similarity, and they share 75% identity at the amino acid level. This identity is low compared with that shared by bird and rodent leptins. The convergence of lysozymes of foregut-fermenting animals is attributed to selective pressures that are associated with this mode of digestion (Jollès *et al.*, 1989). It seems that stronger selective pressures would be required to lead to the high sequence similarity observed in bird and rodent leptins. These sequences even show a high similarity at synonymous sites, which contradicts current beliefs that selective pressure against mutations is low at these sites (Li, 1997). Dunn and colleagues (2001) estimated at less than one in one million the probability of having such a low rate of synonymous substitution between mouse and chicken leptins. Also, unlike stomach lysozymes, there seems to be no physiological reason that selective pressure would have favored a high similarity between bird and rodent leptins.

In conclusion, partial leptin sequences of six new mammalian species are presented. The grouping of these sequences with other mammals is consistent with the evolutionary relationships that are generally accepted for mammals. However, when bird sequences were included in the analysis, accepted tree topologies are not respected. Our understanding of the phylogenetic relationships of leptin genes would be enhanced with the addition of sequences from other birds and ectothermic animals. Using a degenerate set of primers (not shown) and the primers employed here, we failed to clone leptin from adipose tissue and liver of rainbow trout, American eel, goldfish, leopard frog, and snapping turtle (data not shown). Although our phylogenetic analysis does not resolve the controversy surrounding the chicken sequence, we cannot support the hypothesis of a cloning artifact (Dunn *et al.*, 2001). As an alternative hypothesis to explain the high similarity of rodent and bird leptin sequences, we propose convergent or parallel evolution. To test this hypothesis, one would need to obtain leptin sequences from animals that have diverged prior to birds. Leptin sequences from other bird and rodent species would also help in testing the hypothesis of convergent evolution.

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APPENDIX II
mRNA Analysis in Flattened Fauna: Obtaining Gene-Sequence Information from Road-Kill and Game Hunting Samples

Adapted from: Doyon, C., Trudeau, V.L., Hibbert, B.H., Howes, L.A., and Moon, T.W. 2003. *Can J. Zool.* **81**: 692-698.

Abstract

Whether gene-sequence information could be obtained using mRNA from road-kill and hunting samples was investigated. Adipose tissue was used to clone cDNA fragments of the hormone leptin and brain tissue was used for the enzyme glutamic acid decarboxylase (GAD). Tissues collected from road-killed animals were used to clone leptin from RNA samples of raccoon (*Procyon lotor*) and woodchuck (*Marmota monax*). We were able to extract RNA and clone GAD67 from samples of masked shrew (*Sorex cinereus*), although the time of death was unknown. We collaborated with hunters who provided tissues from which we cloned leptin and GAD isoforms from beaver (*Castor canadensis*), red squirrel (*Tamiasciurus hudsonicus*), black bear (*Ursus americanus*), and moose (*Alces alces americana*). Molecular phylogenetic analyses confirmed that the sequences obtained did not result from contamination. A time-course experiment showed that even 24 h after the death of rats, sufficient mRNA remains to amplify leptin from adipose tissue. These results suggest that road-kill and hunting samples could be used as a valuable source of gene-sequence information.

Introduction

Road traffic is a major cause of wildlife mortality. Road mortality has been identified as a contributing factor in the decline of amphibian populations (Fahrig *et al.*, 1995) and it can have an impact on species of interest to conservationists (Rosen and Lowe, 1994). While amphibians and reptiles are the most common road-kill on roads adjacent to wetlands (Ashley and Robinson, 1996), informal surveys suggest that it is the mammals which are most readily visible and identifiable by the travelling public (Edutel Communications, 2003). In a preliminary survey of road-kill that covered 873 km through diverse habitats (urban, rural, agricultural, forest, wetlands), we observed a total of 166 vertebrates, an average of one specimen every 5.3 km. The majority of the observed specimens were mammals (73%), and of those, half were easily identifiable. The most common road-kills were woodchucks and squirrels (Table A2.1). Road-kills remain largely an underexploited source of information for biologists. Here we demonstrate that road-kill and hunting samples can provide valuable information, even for applications such as RNA-based gene-cloning strategies. There is a general belief that in order to work with mRNA, precautions, including quick-freezing of tissues, must be taken to ensure the integrity of the samples. However, considering that the half-life of mRNAs can vary from minutes to months (Ross, 1988), it is reasonable to suggest that some intact mRNA should remain even hours after the death of road-kill specimens. This study investigates whether gene-sequence information could be obtained using mRNA from road-kill and hunting samples.

We collaborated with hunters and collected samples from mammalian road-kills. Standard cloning techniques were used to obtain sequence information on two genes that are expressed in different tissues and are likely to have different RNA-degradation rates. The first gene encodes the hormone leptin, which is primarily produced in adipose tissue. Leptin is released in the circulatory system of mammals in proportion to body-fat content and plays

important roles in a variety of physiological processes, including the regulation of reproduction, food intake, and energy expenditure (Magni *et al.*, 2000). The second gene encodes the enzyme glutamic acid decarboxylase (GAD). In the brain, GAD participates in the regulation of two very important neurotransmitters by catalyzing the decarboxylation of glutamate to produce γ-aminobutyric acid (GABA), the rate-limiting step in GABA production (Martin and Rimvall, 1993). In mammals, there are two GAD isoforms that are products of independent genes on different chromosomes and named after their respective molecular weights, GAD65 and GAD67 (Bosma *et al.*, 1999; Martin and Rimvall, 1993).

Table A2.1 Summary of identifiable mammalian species recorded during road-kill surveys on 873 km of road near Ottawa, ON, Canada (June-July, 2002).

Scientific Name	Common Name	Number recorded
Marmota monax	Woodchuck	18
*Sciurus carolinensis**	Gray squirrel	15
*Tamiasciurus hudsonicus**	Red squirrel	
Erethizon dorsatum	North American porcupine	11
Procyon lotor	Raccoon	7
Mephitis mephitis	Striped skunk	4
Sylvilagus floridanus	Eastern cottontail	3
Odocoileus virginianus	White-tailed deer	2
Vulpes vulpes	Red fox	1
Ondatra zibethicus	Muskrat	1

* Note: The two squirrel species were not distinguished during the survey.

Methods

Animals and tissue collection

Samples of brain and adipose tissue were collected from road-killed raccoon (*Procyon lotor*) and woodchuck (*Marmota monax*) and preserved in RNAlater (Ambion, Austin, Tex.). Brain tissue from masked shrews (*Sorex cinereus*) was frozen approximately 3 h after collection. For raccoon and shrews, the time of death is unknown, whereas woodchuck tissues were collected 1.5 h after the death of the animal. Tissues from moose (*Alces alces americana*), black bear (*Ursus americanus*), beaver (*Castor canadensis*), and red squirrel (*Tamiasciurus hudsonicus*) were obtained with the collaboration of hunters. Brain and adipose tissues were sampled from a moose 4–5 h after it was killed, whereas approximately 1 h had elapsed before bear tissues could be collected. These tissues were preserved in RNAlater and kept at -20°C until they were shipped to Ottawa. Beaver and squirrel tissues were frozen on dry ice less than 30 min after capture and stored at –80°C.

RNA and DNA extraction, reverse transcription, PCR, and DNA sequencing

Poly(A)$^+$ RNA was extracted from adipose tissue of raccoon, woodchuck, and squirrel and from brain tissue of shrew and beaver, using the Straight A's mRNA Isolation System (Novagen, Madison, Wis.). Total RNA was extracted from adipose tissue of bear and brain tissue of bear and moose, using the GITC method (Chomczynski and Sacchi, 1987). Leptin-related

cDNA fragments were amplified using a previously described method (Doyon *et al.*, 2001). Briefly, first-strand cDNA was synthesized from 100–500 ng poly(A)$^+$ RNA with SUPERSCRIPT II (Invitrogen, Burlington, Ont.) and a leptin-specific antisense primer (Doyon *et al.*, 2001). Amplification was carried out using the following program: 3 min denaturing at 94°C, then 35 cycles of denaturation at 94°C for 30 s, annealing at 56.5°C for 30 s, and extension at 72°C for 1 min. After the last cycle, further extension was performed at 72°C for 7 min. GAD-related cDNA fragments were amplified from brain tissue by means of a previously described method (Bosma *et al.*, 1999). First-strand cDNA was synthesized with oligo(dT) (Invitrogen) for shrew and beaver and a GAD-specific reverse primer (Bosma *et al.*, 1999) for bear and moose. Amplification was carried out using the following program: 5 min denaturation at 94°C, then 10 cycles of denaturation at 94°C for 30 s, annealing at 43°C for 30 s, and extension at 72°C for 1 min, followed by 35 cycles at 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min. After the last cycle, further extension was performed at 72°C for 5 min. Polymerase chain reactions (PCR) contained 4 μL of cDNA or 30 ng of genomic DNA in 50 μL. Amplified products were extracted from a 1% agarose gel with the QIAquick gel-extraction kit (Qiagen, Mississauga, Ont.), ligated into the pCR II-TOPO cloning vector (TOPO TA cloning kit; Invitrogen), and transformed into *E. coli*-competent cells (One Shot TOP10 Electrocomp cell; Invitrogen). Single colonies were cultured and plasmids were recovered with Wizard Plus (Promega, Madison, Wis.). All procedures were undertaken according to the manufacturers' instructions. For each new leptin and GAD form, both strands of three different cloned inserts were sequenced by Canadian Molecular Research Services Inc. (Ottawa, Ont.). Nucleotide sequences were submitted to BLAST (Altschul *et al.*, 1997) for comparison with sequences present in GenBank.

Time course, semi-quantitative RT–PCR, and Northern hybridization

We were concerned with the fact that the time of death of most road-kill samples was not known, therefore it was difficult to estimate whether intact mRNA could be isolated. In an attempt to determine the time limits for taking samples in the wild, we performed a laboratory experiment on recently sacrificed laboratory rats. Two male Sprague–Dawley rats (460 and 500 g) were euthanized with CO_2 . Samples of retroperitoneal white adipose tissue were collected at sacrifice and 2, 4, 8, 18, and 24 h following euthanasia. The body temperature of the carcasses decreased rapidly from 37°C to reach room temperature (20°C) after 4 h. Total RNA was extracted using the GITC method (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized using 2.5 μg of total RNA and a leptin-specific reverse primer (Doyon *et al.*, 2001). Semi-quantitative PCR was performed using 2 μL of cDNA in a 50-μL PCR reaction. The PCR program was as described above but amplification was carried out for 22 and 25 cycles. For Northern hybridization, 5 mg of total RNA was separated on a denaturing 1% agarose gel and blotted on a Hybond N$^+$ membrane (Amersham, Baie d'Urfé, Qué.) by capillary transfer. The RNA was cross-linked to the membrane by 5 min UV exposure. A rat leptin-specific probe (position 82–397, GenBank accession No. U48849) was prepared using Rediprime II Random Prime Labelling System and 50 μCi of Redivue a ^{32}P-dCTP at 3000 Ci/mmol (Amersham). Unincorporated radionucleotides were removed using a ProbeQuant G–50 Micro Column (Amersham). The probe was hybridized to the membrane at 65°C for 4 h using Rapid-hyb buffer (Amersham). The membrane was washed at 65°C with 0.1× SSC and 0.1% SDS. After 18 h of exposure to the membrane, an Image Screen-K (Bio-Rad, Mississauga, Ont.) was scanned using a Molecular Imager-FX (Bio-Rad).

Phylogenetic analyses

New partial sequences were aligned with the equivalent regions of previously sequenced vertebrate genes using CLUSTAL W (Thompson *et al.*, 1994) with default settings. The phylogenetic analyses were performed on the predicted amino-acid sequences (182 a.a.) for both GAD isoforms. The leptin analyses were performed on the nucleotide sequences (268–271 nt) because the amino acid sequence was too short and did not provide enough informative sites. For leptin, a maximum-likelihood tree was calculated with DNAML from the PHYLIP package version 3.6a2.1 (Felsenstein, 2001), eight gamma-distributed rates of heterogeneity, and a fraction of invariant sites. For GAD65 and GAD67, maximum-likelihood trees were calculated with PROML from PHYLIP, the JTT model of amino-acid substitutions (Jones *et al.*, 1992), eight gamma-distributed rates of heterogeneity, and a fraction of invariant sites. The a value of the gamma distributions and the fraction of invariant sites were calculated using TREE-PUZZLE version 5.0 (Schmidt *et al.*, 2002). The outgroups were fat-tailed dunnart, an Australian marsupial, for leptin and spotted ratfish, a cartilaginous fish, for GAD65 and GAD67.

Results

Sequencing and phylogenetic trees

Partial nucleotide sequences (268–271 nt) of leptin were obtained from adipose tissue of four mammalian species: raccoon, woodchuck, red squirrel, and black bear. From brain tissue we obtained partial nucleotide sequences (547nt) for GAD65 from beaver and for GAD67 from beaver, moose, masked shrew, and black bear. The partial leptin sequence obtained from the raccoon road-kill was identical with the one previously obtained from fresh tissue (Doyon *et al.*, 2001). Phylogenetic analyses were used to confirm that the sequences obtained were not the result of contamination from other species. The leptin tree is based only on mammalian species and it is rooted with the fat-tailed dunnart (Fig. A2.1). The bear sequence groups with those of other carnivores. The woodchuck and squirrel, two members of the family Sciuridae, group together within the rodent branch. The GAD trees were based on representative species of various vertebrate classes and were rooted with the sequences of the spotted ratfish (Fig. A2.2, A2.3). In both GAD trees, our new GAD sequences group with other mammalian sequences, but the support values at the nodes are relatively weak (Fig. A2.2, A2.3). In the GAD65 tree, the beaver sequence groups with those of other rodents (Fig. A2.2). In the GAD67 tree, the beaver sequence is also closer to those of rodents and the bear sequence groups with that of the cat, the only other carnivore in the analysis (Fig. A2.3).

The new sequences reported in this paper were submitted to GenBank and the accession numbers appear on the phylogenetic trees.

Time course

Results for the time-course experiments are presented for only one rat, as the two rats yielded identical results. The ethidium bromide-stained RNA gel shows that RNA degradation increases with time after death (Fig. A2.4A). Hybridization with a probe specific for rat leptin produced a single band in lanes 1–4, but failed to reveal the presence of transcripts 18 and 24 h after the death of the rats (Fig. A2.4B). Results from the semi-quantitative reverse transcriptase – PCR (RT–PCR) show that after 22 PCR cycles, there is a decrease in leptin mRNA for tissues collected 18 and 24 h after the death of the rats (Fig. A2.5A). However, after 25 PCR cycles, most reactions seem to have reached their plateau (Fig. A2.5B). The specific leptin cDNA fragment was still detectable up to 24 h following the sacrifice of the rat (Fig. A2.5B).

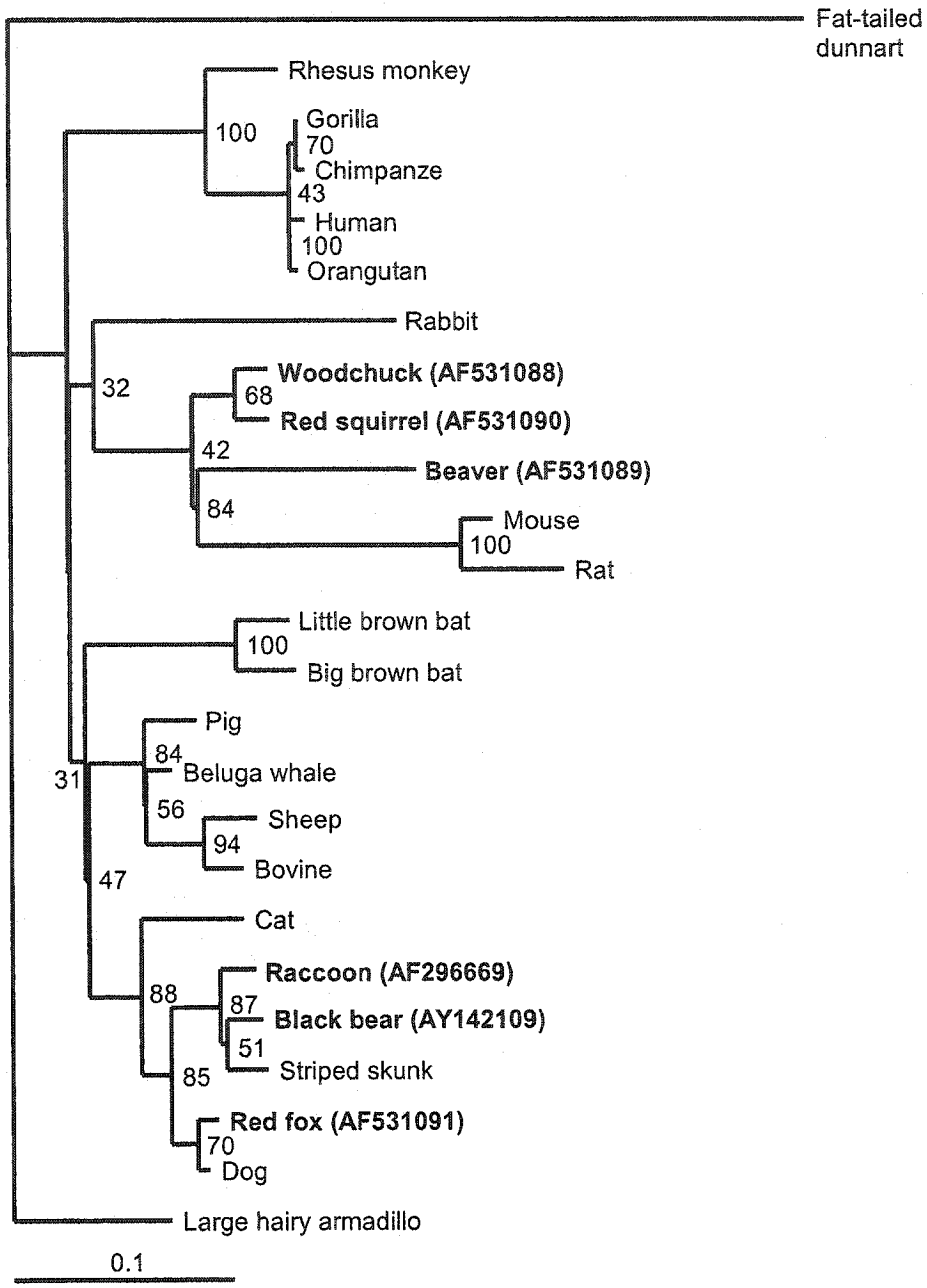

Figure A2.1 Phylogenetic tree for partial nucleotide sequences (271 nt) of mammalian leptins. Maximum-likelihood analyses were performed with DNAML from the PHYLIP package version 3.6a2.1, eight gamma-distributed rates, and a fraction of invariant sites. Support values are shown next to their nodes. The scale bar represents the number of nucleotide substitutions per nucleotide site. The tree was rooted with the fat-tailed dunnart. Sequences from species that appear in bold were obtained in this study and GenBank accession numbers appear in parentheses.

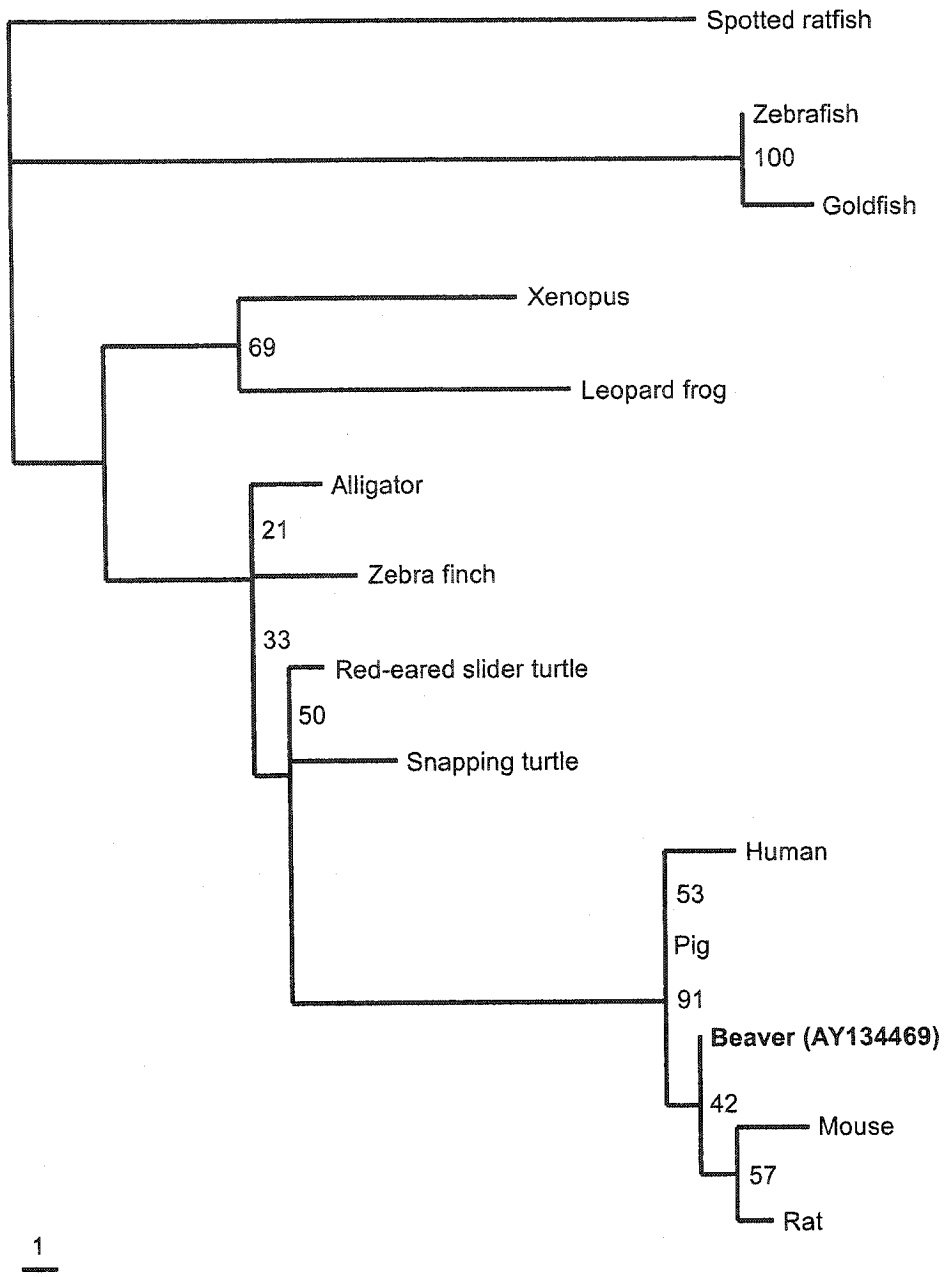

Figure A2.2 Phylogenetic tree for partial predicted amino acid sequences (182 a.a.) of vertebrate GAD65. Maximum-likelihood analyses were performed with PROML from the PHYLIP package version 3.6a2.1, the JTT model of amino acid substitutions, eight gamma-distributed rates, and a fraction of invariant sites. Support values are shown next to their nodes. The scale bar represents the number of amino acid substitutions per amino acid site. The tree was rooted with the spotted ratfish sequence. Sequences from species that appear in bold were obtained in this study and GenBank accession numbers appear in parentheses.

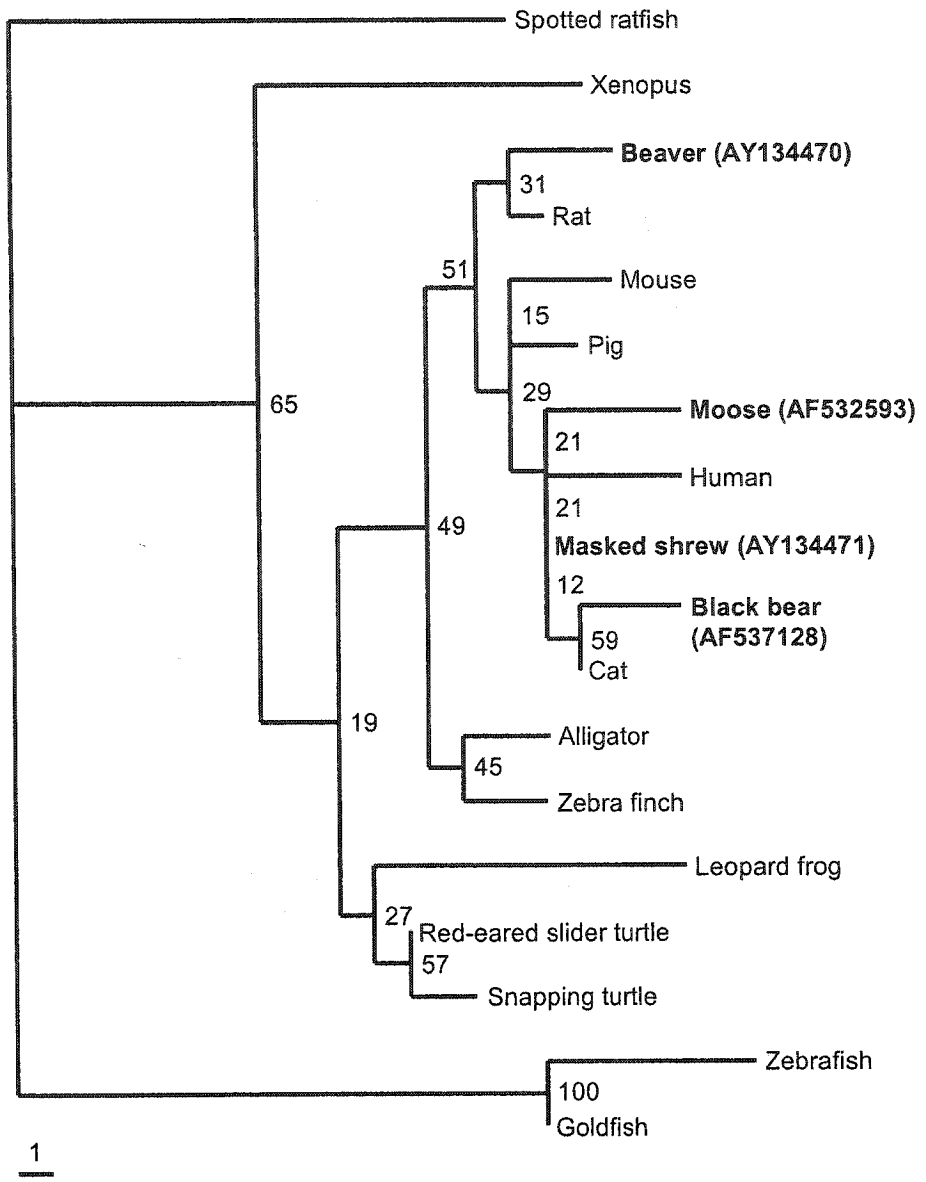

Figure A2.3 Phylogenetic tree for partial predicted amino acid sequences (182 a.a.) of vertebrate GAD67. Maximum-likelihood analyses were performed with PROML from the PHYLIP package version 3.6a2.1, the JTT model of amino acid substitutions, eight gamma-distributed rates, and a fraction of invariant sites. Support values are shown next to their nodes. The scale bar represents the number of amino acid substitutions per amino acid site. The tree was rooted with the spotted ratfish sequence. Sequences from species that appear in bold were obtained in this study and GenBank accession numbers appear in parentheses.

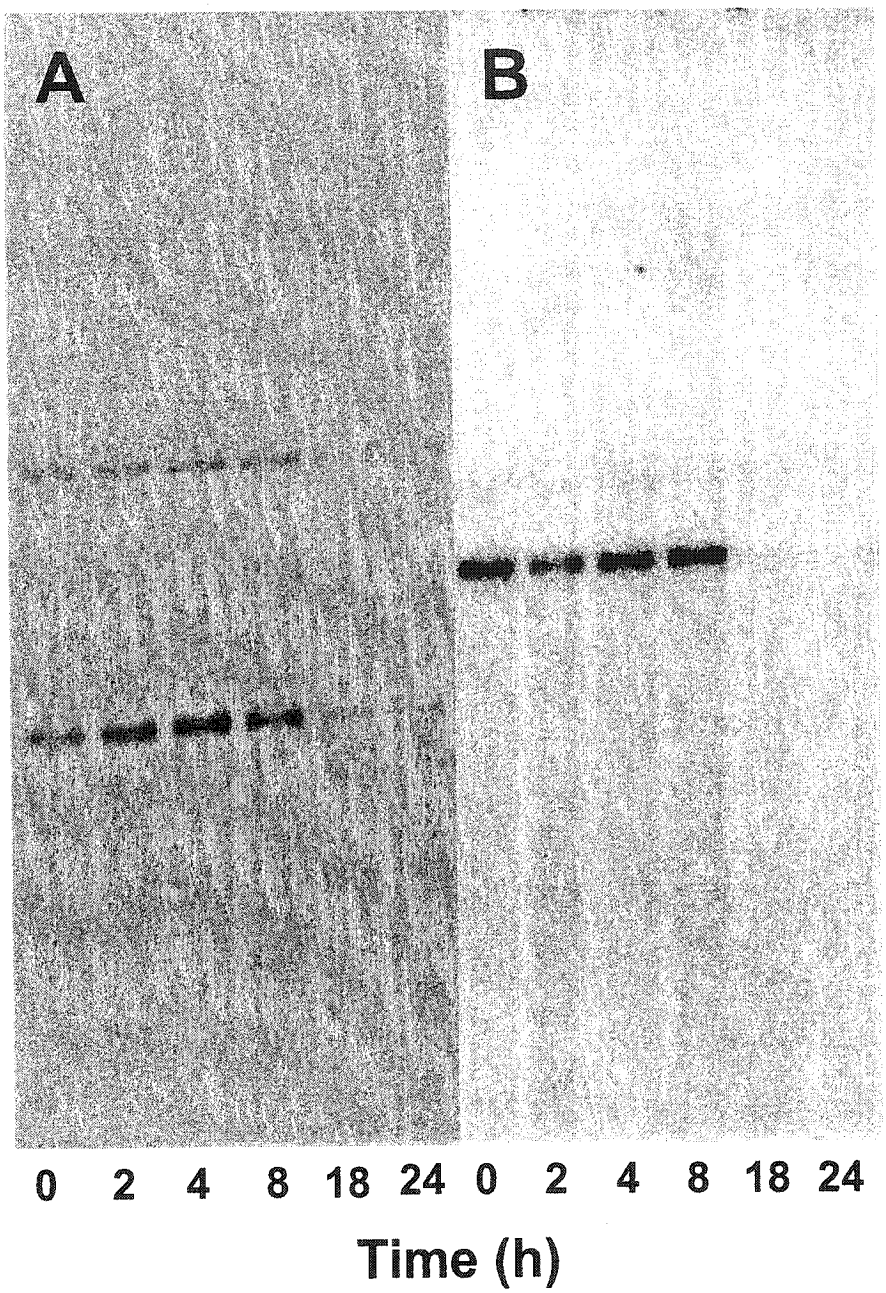

Figure A2.4 RNA gel showing A) ethidium bromide staining, and B) Northern hybridization with a random-prime labeled rat leptin-specific probe. Lanes contain 5 μg of total RNA from rat adipose tissues collected at various times after death of the animal.

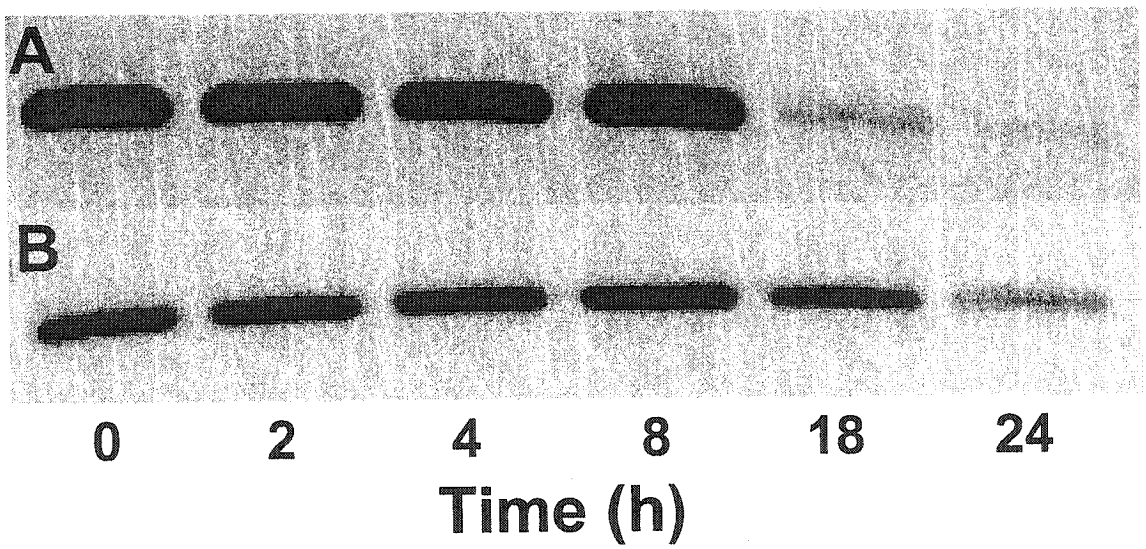

Figure A2.5 Semi-quantitative RT-PCR showing the ethidium bromide staining of a 316 bp rat leptin cDNA amplification product after A) 22, and B) 25 cycles of PCR. Bands were obtained from rat adipose tissues collected at various times after death of the animal.

Discussion

We have demonstrated that it is possible to obtain cDNA sequence information using mRNA from road-kill and hunting samples. The size of the amplified cDNA fragments confirms that none the sequences presented in this paper resulted from genomic DNA contamination. For both leptin and GAD, the use of the PCR primers (Bosma *et al.*, 1999; Doyon *et al.*, 2001) on genomic DNA would result in the amplification of introns and the production of much larger DNA fragments. When working with partially degraded samples, the chances of success are greater if genomic DNA is used because it is more stable than mRNA. However, the use of genomic DNA is not always possible or desirable. Using our cloning strategy, GAD could not be amplified from genomic DNA, owing to the complexity of its gene structure. For example, the coding sequence of mouse GAD65 is divided into 16 exons that cover a region of more than 75 kilobases (kb) of genomic DNA (Makinae *et al.*, 2000). The use of the GAD primers (Bosma *et al.*, 1999) on genomic DNA would result in amplified fragments of approximately 9 kb. However, leptin primers (Doyon *et al.*, 2001) amplify DNA fragments of less than 2 kb that correspond to part of exon 3 and the intron preceding it. Here we also report leptin sequences from beaver and road-killed red fox (*Vulpes vulpes*). These sequences were obtained from genomic DNA, as the amount of adipose tissue we could obtain from these two animals was not sufficient for RNA analysis. In the leptin phylogenetic tree, the fox and the beaver both group with their closest relatives (Fig. A2.1).

Our raccoon leptin sequence provides the first evidence that we can obtain accurate information from the mRNA of road-kill samples. The sequence we obtained from road-kill tissues is identical with the one we previously obtained from fresh material (Doyon *et al.*, 2001). Unlike the raccoon, for which the time of death is not known, we know that the woodchuck had been dead for 1.5 h before tissues could be sampled. In the leptin tree, the woodchuck groups with its closest relative, the red squirrel, and the bear groups with other carnivores. These results confirm that the sequences obtained are not the result of contamination from other species. Overall, the leptin tree topology obtained here is similar to that from our other study on the molecular evolution of leptin (Doyon *et al.*, 2001).

A GAD sequence was obtained from brain mRNA of moose, although the animal had been dead for at least 4 h before tissues were preserved in RNAlater. Also, we obtained a GAD sequence from brain mRNA of a shrew for which the time of death is unknown. Our phylogenetic analyses show that within the trees of each GAD isoform, all the mammalian GAD sequences group together. This result confirms that all the new GAD sequences we obtained are of mammalian origin. However, GAD is highly conserved among vertebrates and our PCR target fragments do not provide sufficient informative sites to reconstruct phylogenies with resolution to the level of order within the class Mammalia. The GAD tree topologies obtained here are similar to those from previous studies (Bosma *et al.*, 1999; Larivière *et al.*, 2002).

Our time-course experiment shows that despite substantial RNA degradation with time after death, sufficient mRNA remains for cDNA synthesis and PCR amplification of leptin gene fragments even 24 h after death. This result is not surprising if we consider that the theoretical limit for PCR amplification is a single RNA transcript. For rat tissues collected 18 and 24 h post mortem, the amount of full-length leptin mRNA remaining was not sufficient to be visualized by Northern hybridization, but was sufficient for RT–PCR amplification. Our RT–PCR strategy increased our chances of amplifying leptin. For first-strand cDNA synthesis, we used a leptin-specific reverse primer (Doyon *et al.*, 2001) rather than oligo(dT). The initial step of most mRNA-degradation processes is a gradual shortening of the poly(A) tail (van Hoof and Parker,

2002). Hence, the efficiency of priming the reverse transcription with oligo(dT) would be reduced when mRNA is partly degraded. Deadenylation can be followed by a rapid 3' to 5' exosome-mediated degradation, or by decapping and subsequent 5' to 3' exonuclease-mediated degradation (van Hoof and Parker, 2002). With either of these processes, the use of PCR primers that are well within the coding region of mRNAs increases the chance of cloning genes from partially degraded material. However, the drawback of reducing the length of the target mRNA region is that it also reduces the amount of information available for subsequent phylogenetic analyses. For applications that require the use of random primers, partial degradation of mRNA ends might reduce the number of amplified transcripts.

In well-fed animals, such as the rats in the time-course experiment, leptin is relatively highly expressed. Amplification from partially degraded tissues might not be possible with low-expression genes. Also, the mRNA turnover times in eukaryotic cells can vary from minutes to months depending on the mRNA structure, the local environment of the cell, and its state of differentiation (Ross, 1988). For example, mRNAs that contain adenosine- and uridine-rich (AU-rich) elements such as those encoding growth factors and cytokines are very unstable (van Hoof and Parker, 2002). Unfortunately, we have no data on the turnover rates of leptin and GAD mRNAs. However, we can assume that the death of the animal will accelerate the mRNA-degradation process. In view of this, it is possible that some cells remain alive and maintain RNA transcription many hours after the death of a road-kill specimen.

Finally, our time-course experiment was performed in a controlled environment at 20°C. The rate of mRNA degradation could vary greatly as a function of ambient temperature and humidity, such as the conditions in which road-kill specimens are recovered. Also, the presence of an open wound on the animal would facilitate the intrusion of maggots and microorganisms, and hence accelerate the rate of degradation. One danger of working with road-kill samples is the possibility of contamination by other organisms. This type of contamination was not an issue for leptin because this gene has only been found in mammals and birds (Doyon *et al.*, 2001). Insect (Jackson *et al.*, 1990), nematode (Jin *et al.*, 1999) and bacterial (Omura *et al.*, 2001) GADs have been identified but they are highly divergent and not similar to the mammalian GADs we have presented. Our road-kill samples did not contain obvious maggots and the phylogenies confirm that the sequences presented here are of mammalian origin. Our time-course experiment shows that if a road-kill specimen is in reasonable condition, sequences can be obtained from its mRNA several hours after its death.

In conclusion, we have shown that cDNA sequence information can be obtained using mRNA from road-kill and hunting samples. Our time-course experiment demonstrated that even 24 h after the death of an animal, enough mRNA remains to amplify gene fragments. However, considering all the factors that could affect mRNA degradation in a road-kill specimen, much more research on RNA stability must be conducted to establish standardized cloning strategies based on mRNA-derived cDNA templates. Such approaches may have applications in conservation biology and forensic sciences. For example, mRNA from species for which fresh material is limited or absent could be used to construct cDNA libraries. Although the quality of these preserved transcriptomes would depend on the degree of degradation of the starting material, this approach could generate new sequence information from rare species.

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