CLONING AND EXPRESSION ANALYSIS OF LEPTIN AND ITS RECEPTOR IN THE AXOLOTL (*Ambystoma mexicanum*)

A THESIS SUBMITTED TO THE FACULTY OF SAGE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Abstract

Since its discovery in 1994, the adipose tissue hormone leptin has been well established as a key regulator of energy balance in mammals. However, little is known about the molecular evolution of the hormone and its function in non-mammalian vertebrates. This project builds on the recent identification of leptin in an amphibian, the tiger salamander, to investigate the leptin signalling system in a laboratory salamander, the axolotl. The overall aim of the project was to obtain cDNA sequences of the axolotl leptin and leptin receptor (LEPR) genes, to analyse their expression and to study their expression due to nutritional state. Cloning the axolotl LEPR was a key component of the work because no sequence information was previously available. Semi-degenerate primers were used to clone a 248 bp fragment of the LEPR, which shared 62% identity with human leptin at the amino acid level. Attempts to obtain the full-length cDNA sequence were unsuccessful. However, the sequence grouped in proximity to a *Xenopus* LEPR in a phylogenetic tree, and Northern hybridization revealed a transcript size of approximately 3 kb, which corresponded with that of other vertebrate LEPRs. To establish the expression pattern of leptin and the LEPR between tissues, quantitative real-time PCR was performed in two different age groups of animals. In adults, the highest expression of leptin was observed in the fat, brain and heart whereas in juveniles leptin expression was significantly higher in the fat body compared to all other tissues. The highest expression of LEPR was found in the brain and skeletal muscle. These findings agree with the main sites of leptin and LEPR expression in mammals, *Xenopus*, and fish providing further evidence that the gene fragments cloned represents the axolotl leptin and LEPR. In order to understand the possible role(s) of leptin in the regulation of food intake and energy metabolism in amphibians, changes in leptin and LEPR expression due to nutritional state were investigated. Short-term fasting did not result in any significant changes in leptin expression in the fasted animals, nevertheless it showed a tendency towards a lower leptin and LEPR expression of fasted axolotls. These findings indicate that the regulation of leptin expression by nutritional state more closely resemble the situation in other ectotherms such as teleost fish. This work provides the opportunity to explore how the physiological functions of leptin have changed during evolutionary history.

For Luke and his endless patience

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Chapter 1. Introduction

1.1 Introduction

The leptin gene was discovered using positional cloning in the mouse in 1994 and its product was described as an adiposity factor that circulates in the blood in proportion to energy stored as fat (Zhang et al., 1994). Before this, several different theories had been postulated for how the control of energy balance is controlled in mammals. One theory proposed that temperature controls food intake (Brobeck, 1948) while the glucostatic theory claimed that energy stores are regulated by the plasma glucose level (Mayer, 1955) A third theory was called the lipostatic theory. This proposed that the amount of energy stored as body fat depot is regulated by the central nervous system, with a product of fat metabolism circulating in plasma and affecting food intake and energy expenditure to maintain a constant body weight, by interacting with the hypothalamus (Kennedy, 1953). The possibility that one of the components of the signalling system circulates in bloodstream was shown by Hervey (1959) in experiments on rats where the circulatory systems of lean and obese animals were surgically joined (parabiosis).
The gene that was later identified as the leptin gene was discovered in 1950 as a genetic defect which led to mice becoming obese when homozygous for the mutation (Ingalls et al., 1950). This mouse mutant was termed the *obese* or *ob/ob* mouse. A link with the lipostatic theory was made from experiments by Coleman (1973) on the *ob/ob* mouse, and a related mutant, the *db/db* mouse. Both disorders are characterized by hyperphagia, obesity, hyperglycemia and hyperinsulinemia, associated with pancreatic changes. When adult *ob/ob* mice were parabiosed to normal mice, the *ob/ob* mice lost weight. This finding showed that a weight-regulating factor from the blood of the normal mice could modify the obesity. The result suggested that the *ob/ob* mouse did not produce sufficient satiety factor to regulate food intake and energy expenditure. A similar investigation on paired normal mice with *db/db* mice showed that normal mice rejected food and died of starvation. This suggested that *db/db* mice produced a satiety factor, but they did not respond to it. The *db/db* phenotype appears to reflect a defect in the action of a receptor. Consistent with the results of these experiments, *ob/ob* mice paired with *db/db* mice reduced their food intake and lost weight. So the *ob/ob* mice appeared to respond to the putative excess of ob protein produced by their *db/db* partners

(Coleman, 1973). It was evident that a satiety factor produced by adipose tissue had yet to be discovered.

1.2 Leptin in mammals

1.2.1 *Discovery of leptin*

The leptin gene was discovered by Friedman and colleagues in 1994 by cloning and sequencing of the mouse *ob/ob* gene using positional cloning techniques (Zhang et al., 1994). In this important paper, they determined that the *ob/ob* gene is expressed in adipose tissue and encodes a 167 amino acid protein that has the characteristics of a secreted hormone. The *obese* mutation in mice, was shown to be a result of a nonsense mutation associated with an absence of RNA encoding the hormone. Southern hybridization of a mouse *obese* gene probe to genomic DNA from mammals (mouse, rat, rabbit, vole, cat, cow, sheep, pig and human) and non-mammalian vertebrates (chicken and eels) showed that at moderate stringency, there were detectable signals in all vertebrate DNAs tested. A human orthologue of the obese gene was also identified and alignment of the predicted human and mouse amino-acid sequences showed 84% overall identity. The conservation of the *obese* gene among vertebrates suggested that the function of its encoded protein is highly conserved.

After the original paper describing the cloning of the *obese* gene was published, the hypothesis was tested that the Ob protein is involved in regulation of energy balance by observing the effects of administering it in *ob/ob* mice. Several studies showed that intraperitoneal injection of normal and *ob/ob* mice with recombinant Ob protein decreased their body weight, percent body fat, food intake, and serum concentrations of glucose and insulin. In addition, metabolic rate, body temperature, and activity levels were increased by this treatment (Campfield et al., 1995; Pelleymounter et al., 1995; Halaas et al., 1995; Stephens et al., 1995). Central administration of Ob protein into the lateral or third brain ventricle lowered food intake and body weight of *ob/ob* and diet-induced obese mice but not in *db/db* obese mice. These results suggest that Ob protein can act directly on neuronal networks that control feeding and energy balance (Campfield et al., 1995; Stephens et al., 1995). Because administration of the Ob protein reversed obesity in *ob/ob* mice, Halaas et al. (1995) proposed that it should be given the

name leptin, derived from the Greek *leptos*, meaning thin. Stephens et al. (1995) showed that one mechanism by which this protein regulated food intake was inhibition of neuropeptide Y expression, a neuropeptide which stimulates food intake, decreases thermogenesis and increases plasma insulin and corticosterone levels.

Other studies performed soon after the discovery of leptin showed that obese humans and rodents are still able to produce leptin RNA and the level of leptin protein is higher than in lean individuals. These data suggest that obesity may be a consequence of leptin resistance, rather than insufficient amounts of leptin itself (Maffei et al., 1995; Considine et al., 1995; Lönnqvist et al., 1995; Hamilton et al., 1995).

1.2.2 *Structure of the leptin gene and leptin protein*

The mouse leptin gene and its human homologue encodes a 4.5 kb adipose tissue mRNA with a highly conserved 167-amino acid open reading frame (Chmurzynska et al., 2003). The leptin gene consists of three exons separated by two introns with the coding sequence in exons 2 and 3, and a minor fraction of the leptin mRNA contains an extra, untranslated, exon between exons 1 and 2 (He et al., 1995, Figure 1). The first exon and the first intron arise in the 5'-untranslated region (UTR) (Chmurzynska et al., 2003). The first exon is located ~7.5 kb upstream of the 175-bp exon 2. Intron 2 is ~1.7 kb long and codes for 48 amino acids. Exon 3 is at least 2.5kb in size and consists of the coding region (codes for 118 or alternatively 119 amino acids) and 3'UTR (Isse et al., 1995). Exon 2 is more conserved then exon 3. It codes for the amino acids of the □□helix responsible for binding leptin to its receptor (Chmurzynska et al., 2003).

The promoter contains a TATA motif occurs upstream of exon 1 at 229 to 234 nucleotide position (He et al., 1995). The Sp1 consensus sequence (GGGCGG) was found at 295 to 2100. Between 249 and 258 is a short palindrome CCAAT/enhancer that is predicted to bind C/EBP motifs (a transcription factor important in adipose cell differentiation). Co-transfection with the C/EBP motif caused a significant increase in leptin reporter expression, which suggests that C/EBP can activate the promoter of leptin and that transcription of the obese gene may be sensitive to lipid status (He et al., 1995).

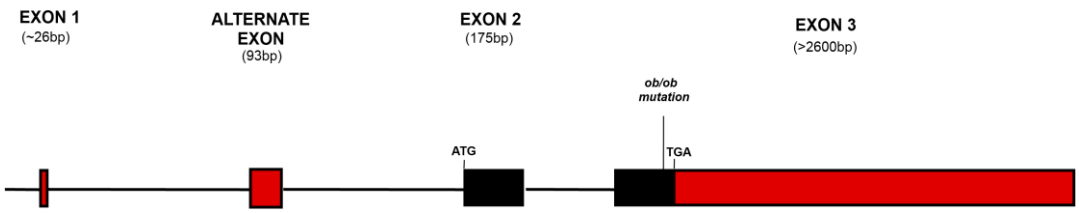


Figure 1 Structure of the mouse leptin gene (modified from He et al., 1995). Diagram showing the intron (thin line)/ exon (thick line) structure. Exons are shown in red and their coding regions in black. The arginine codon, which is mutated in *ob/ob* mice, is marked at aa position 105.

The leptin protein is approximately ~16kDa in mass and belongs to the class-I helical cytokine family, a large group of signalling molecules (Huising et al., 2006)**.** The leptin protein structure (Figure 2) consists of four antiparallel □-helices (A, B, C and D) and is similar to that of the long-chain helical cytokine family, which includes granulocyte colony-stimulating factor (G-CSF), leukaemia inhibitory factor (LIF) and ciliary neurotropic factor (CNTF). The extra-cellular domain of the leptin receptor shows homology to receptors of the G-CSF, LIF and CNTF, which belongs to the same group of class-I helical cytokines (Huising et al., 2006).

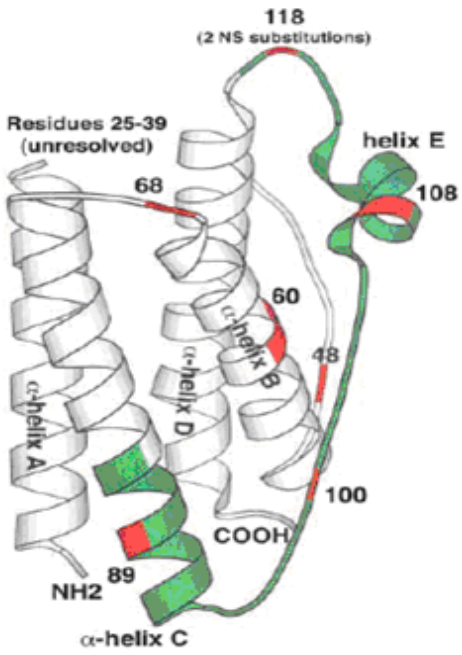


Figure 2 Tertiary structure of human leptin, PDB accession 1AX8. Segment 85-119, responsible for appetite suppression and weight loss in obese mice, is shown in green, the nonsense substitution associated with segment 85-119 in red (Gaucher et al., 2003).

1.2.3 *Leptin receptor*

The *db/db*, or *diabetes*, strain of obese mouse referred to above was, like the *ob/ob* mutation, discovered at the Jackson Laboratory (Hummel et al., 1966). Coleman's parabiosis studies on normal mice paired with *db/db* mice showed that normal mice rejected food and died of starvation. This suggested that *db/db* mice produce a satiety factor, but do not respond to it. Therefore, the *db/db* phenotype appeared to reflect a defect in the action of a receptor (Coleman, 1973).

The leptin receptor (LEPR, also known as the obese receptor or ObR) was identified by Tartaglia et al. (1995) shortly after the discovery of leptin. To search for a LEPR, leptin-alkaline phosphatase (AP) fusion proteins were generated and used to screen mouse tissues and cell lines. Leptin binding was identified in the choroid plexus, which was used to prepare a cDNA expression library. The library was screened with a leptin-AP fusion protein to identify a LEPR, consisting of 5.1 kb with an 894-amino acid open reading frame. The mouse sequence was used to identify a human orthologue that shared 78% amino acid identity. The mature protein consisted of an extracellular domain which is 816 amino acids long, followed by a transmembrane domain (23 amino acids) and a short cytoplasmic domain (34 amino acids) (Tartaglia et al., 1995). Soon after the discovery of the LEPR, it was discovered that the *db/db* mutation consists of a single substitution in the LEPR (Chen et al., 1996). This provided the link between the *db/db* mutation and the LEPR that had been suggested by Coleman's experiments.

The LEPR is a cell surface receptor belonging to the cytokine receptor superfamily which plays an important role in mammalian body weight homeostasis and energy balance (Huising et al., 2006). A variety of LEPR isoforms have been discovered, which are products of alternative splicing at the 3'-end of gene transcript. They are divided into three groups: the complete protein – long form; short forms; and a soluble binding protein consisting of the extra-cellular domain. The full length LEPR isoform containing the extracellular and transmembrane domains together with intracellular motifs is considered to be the fully functional receptor. In additional, shorter, isoforms the intracellular domain is truncated or absent (Richards and Poch, 2003). In the mouse, the splice variants of the receptor consist of six different isoforms, commonly referred to in the literature as LEPRa-f (Cioffi et al., 1996) (Figure 3). Of these, the Re form is the soluble binding protein, while forms Ra, Rc, Rd and Rf share the same intracellular

and transmembrane domains but have an intracellular domain of different lengths. The Rb isoform is the long form in mice and the Ra isoform, the predominant short form of the receptor.

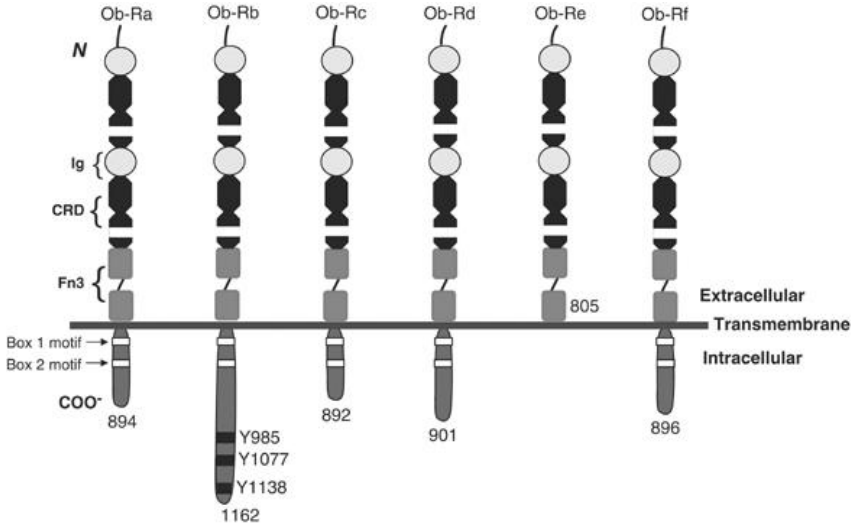


Figure 3 Mouse leptin receptor isoforms (Ceddia, 2005). All six receptors share identical extracellular ligand-bind domains but are differentially spliced at the C terminus resulting in proteins with different cytoplasmic domains. Only Ob-Rb is the functional receptor. The intra-cellular domain includes conserved motifs (boxes 1-2), which take part in binding of Janus kinase (JAK), as part of the signal transduction pathway. The cytoplasmic domain contains unique tyrosine phosphorylation sites (Y985, Y1077, Y1138). Ig=immunoglobulin domain; CRD=cytokine receptor domain; Fn3=fibronectin III domain (Ceddia, 2005).

The LEPR long form consists of three regions: an extracellular domain, a transmembrane domain and an intra-cellular domain (Huising et al., 2006). The extracellular region contains the putative leptin binding site and a pair of repeated tryptophan/serine motifs (WSXWS), which have been shown to be required for receptor folding, but not involved in ligand binding. The intracellular domain includes three conserved motifs (boxes 1-3), which take part in binding of Janus kinase (JAK) as part of the signal transduction pathway, as well as unique tyrosine phosphorylation sites (Tartaglia, 1997). The LEPR exists constitutively as a dimer in the cell membrane, which is required for intracellular signalling. Each receptor in the pair is bound to a leptin molecule (Devos et al., 1997). The binding of the ligand to the receptor, which requires the presence of an intact intracellular domain, induces intracellular signalling by the Janus kinase and signal transducer and activator of transcription (JAK-STAT)

pathway. JAKs phosphorylate tyrosine residues on the receptor, which interact with STATs, and are themselves tyrosine-phosphorylated by JAKs. These phosphorylated tyrosines create docking sites for other STATs, mediating their dimerisation. Activated STAT dimers activate transcription of their target genes in the cell nucleus (Myers, 2004).

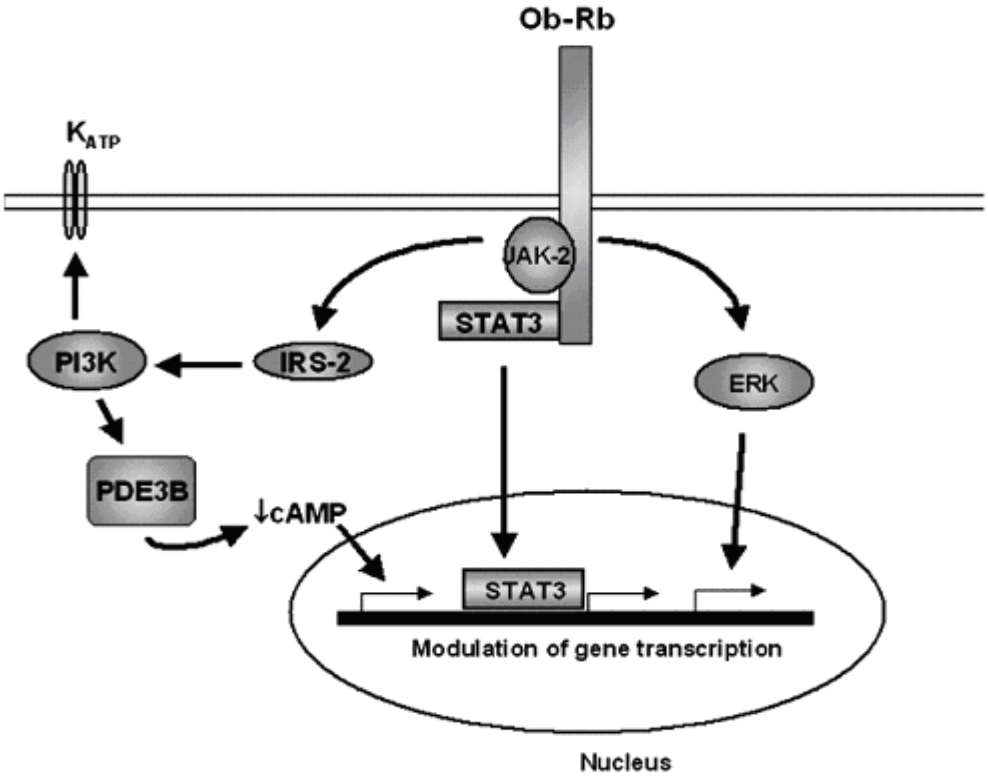


Figure 4 Leptin receptor signalling pathway in hypothalamus (Rahmouni and Haynes, 2004). Leptin modulates gene transcription via activation of signal transducer and activator of transcription (STAT) proteins, phosphoinositol 3 kinase (PI3-K), and extracellular factor-regulated kinase (ERK) (Rahmouni and Haynes, 2004).

In addition to generating transcripts with cytoplasmic domains of different length, alternative splicing of the LEPR also generates variants with different 5' untranslated regions. In one of these, an alternative AUG initiation codon starts a distinct open reading frame encoding a putative protein named leptin receptor gene-related protein (OB-RGRP), also known as leptin receptor overlapping transcript (LEPROT) (Bailleul et al., 1997; Huang et al., 2001). The protein was first identified in humans by analysis of a large expressed sequence tag database. Genomic organization and cDNA sequence comparisons indicate that the LEPROT gene shares its promoter and two exons with the LEPR gene, however the protein does not share amino acid sequence similarity in the

open reading frame to the LEPR itself (Bailleul et al., 1997). A related gene identified in humans, LEPROT1, has 70% amino acid sequence similarity with LEPROT (Huang et al., 2001). Using in situ hybridisation the distribution of LEPROT mRNA overlapped closely with LEPR mRNA in the mouse brain (Mercer, et al., 2000). However, a different pattern of expression was observed in the placenta, suggesting a difference in promoter activity.

A link between LEPROT and LEPR expression has been suggested by experiments where LEPROT has been overexpressed or silenced in cell culture (Couturier et al., 2007). These demonstrated that LEPROT negatively regulates the cell-surface expression of the LEPR. Moreover, in vivo silencing of LEPROT in the mouse prevented the onset of diet-induced obesity (Couturier et al., 2007).

1.2.4 *Functions of leptin - introduction*

The initial conception of the physiological role of leptin was the regulation of energy balance in mammals (Zhang et al., 1994). The physiological role of leptin was seen as rising with increasing adiposity to generate a signal that limits further weight gain. A greater amount of hormone is produced and secreted as fat storage increases. Leptin's effects on body weight are mediated through effects on hypothalamic centres that control feeding behaviour and hunger, body temperature and energy expenditure. It is actively transported into the brain where it acts on the hypothalamus to reduce food intake and increase energy expenditure. The initial view, that leptin functions primarily as an anti-obesity hormone, required revision as a result of new data which showed that leptin has a wider range of biological effects (Figure 5).

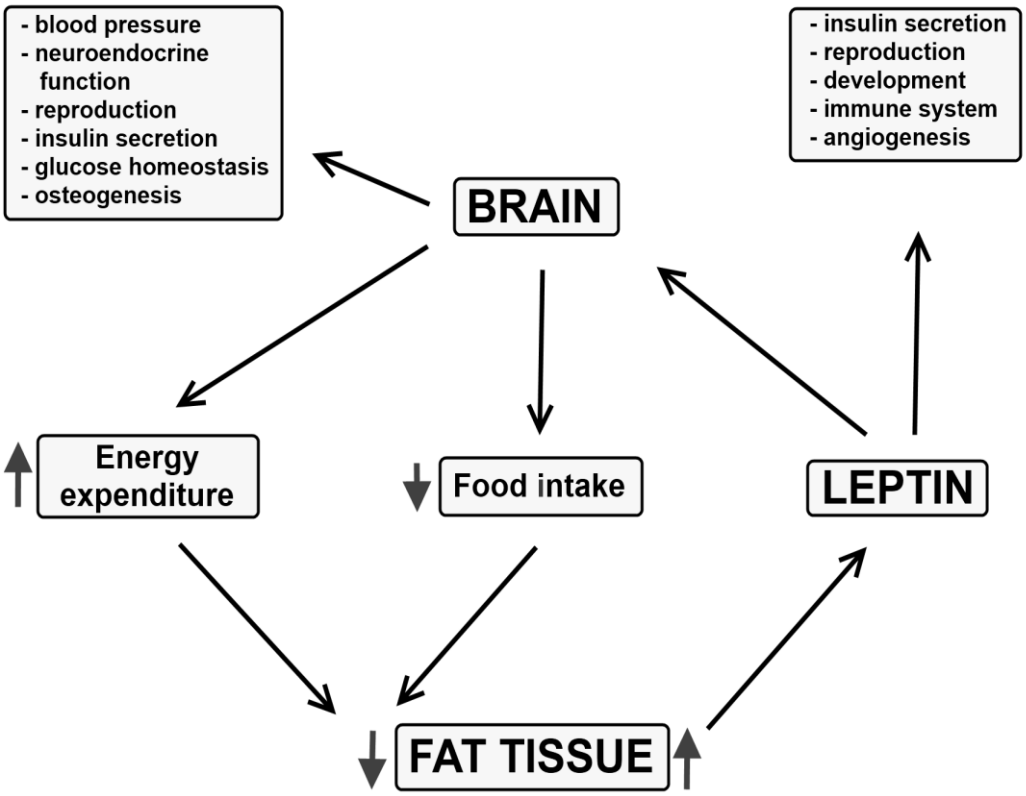


Figure 5 Role of leptin in the regulation of body weight and other functions (modified from Rahmouni and Haynes, 2004)

1.2.5 *Functions of leptin - energy balance*

As discussed in 2.1 above, experiments soon after the discovery of leptin established that leptin acts directly on neuronal networks that control feeding and energy balance, indicating that it is a signal to the brain of body fat content. However, it was apparent from a study by Ahima et al. (1996) that it is falling, rather than rising, blood concentrations of leptin that are the most physiologically relevant physiological signal. Leptin gene expression and blood leptin concentrations are reduced by fasting (Fredrich et al., 1995; Ahima et al., 1996; Grinspoon et al., 1997; Andersen et al., 1997). Leptin deficient *ob/ob* mice show a physiological state characteristic of starvation and this can be generally reversed by administering leptin (Halaas et al., 1995). Also, in normal mice, physiological changes associated with starvation can be reduced by providing exogenous leptin to prevent leptin concentrations from falling (Ahima et al., 1996).

Studies reviewed above indicated that leptin decreases food intake when injected either peripherally or centrally, and a primary site of action within the brain was suggested by the demonstration that the LEPR is expressed in the hypothalamus (Tartaglia et al., 1995). In order to reach the brain circulating leptin must cross the blood-brain barrier (BBB). To investigate how this occurs, administration of leptin labelled with ^{125}I was performed (Banks et al., 1996). The results were visualised using autoradiography, which showed localization of leptin *in vivo* in the choroid plexus and arcuate nuclei of the hypothalamus after injection. This study indicated that circulating leptin reaches the central nervous system via a saturable active transport process across the BBB. The system was inhibited by unlabeled leptin, however unlabeled tyrosine and insulin, proteins also known to have saturable transport systems, did not affect the influx of leptin. This indicates that the saturable transport system for leptin is different and specific (Banks et al., 1996). Similar results were demonstrated by Golden et al. (1997), where an *in vitro* experiment was performed using a model of human blood-brain barrier. The study showed binding of mouse recombinant ^{125}I- leptin in isolated human brain capillaries (Golden et al., 1997). Within the hypothalamus leptin activates the central melanocortin signalling pathway through the arcuate nucleus of the hypothalamus (ARC) by modulating the activity of neuropeptide Y and proopiomelanocortin neurons (Fan et al., 1997, Huszar et al., 1997, Lin et al., 2000). Within the arcuate nucleus, signalling from the LEPR acts on two groups of neurons: the anorexigenic peptide Cocaine and Amphetamine Related Transcript (CART) and the large precursor peptide proopiomelanocortin (POMC), which reduce food intake, while the other the orexigenic peptides neuropeptide Y (NPY) and agouti related protein (AgRP), which increase food intake (Lin et al., 2000). Leptin decreases NPY/AgRP expression (Lewis et al., 1993; Mizuno and Mobbs, 1999; Stephens et al., 1995) and in contrast, stimulates POMC neurons and expression of this protein (Cowley et al., 2001). Moreover, the synaptic density onto NPY and POMC neurons in arcuate nuclei differs between *ob/ob* and wild type mice (Pinto et al., 2004). In the *ob/ob* animals, excitatory synapses on NPY neurons are more numerous compared to wild-type mice, where they have significantly more inhibitory synapses. The amount of synapses onto the POMC neurons is lower in *ob/ob* mice. Pinto and colleagues (2004) provide evidence, that leptin changes neuronal connections in the arcuate nucleus: it was shown in *ob/ob* mice that there was a significant decrease in the total number of synapses onto NPY neurons and increase in those onto POMC neurons after leptin injection (Pinto et al., 2004). These findings suggest that leptin action in hypothalamus involves altered and co-

ordinated expression of key neuropeptide genes, and implicate leptin in the hypothalamic response to fasting.

1.2.6 *Functions of leptin - leptin and human obesity*

The physiological role of leptin in the regulation of body weight makes it relevant to the pathogenesis of human obesity (Zhang et al., 1994; Flier 1995; Rink 1994). Circulating leptin concentrations were seen as rising with increasing adiposity to generate a signal that limits further weight gain (Zhang et al., 1994). Therefore, this hormone has been considered as a new pharmacological approach to the treatment of human obesity (Thorburn et al., 2000; Sinha and Caro 1998; Lee et al., 2002). However, clinical trials based on leptin administration to obese patients, have not shown significant weight loss in the subjects (Heymsfield et al., 1999). These studies demonstrate that obese people are insensitive to leptin rather than being leptin deficient. Although autosomal recessive mutations in the leptin gene (*ob/ob; db/db*) are responsible for obesity in mouse models (Zhang et al. 1994; Friedman and Halaas 1998), leptin or its receptor gene defects are rare in human obesity (Maffei et al., 1996, Carlsson et al., 1997). It is been demonstrated that obese people have much higher expression level of leptin in adipose tissue than non-obese subjects in the absence of leptin gene mutation (Lönnqvist et al., 1995; Hamilton et al., 1995). This finding suggests that obese people are insensitive to the function of the obese gene product and excess leptin does not reduce food intake or increase energy expenditure. This state has been termed leptin resistance (Hamilton et al., 1995). Several mechanisms underlying leptin resistance have been identified. These mechanisms can be divided into three steps: the transport of leptin across the blood-brain barrier (BBB), defect of the LEPR, and disturbance of receptor signalling pathway. In order to reach the brain circulating leptin must cross the blood-brain barrier (BBB) (Banks et al., 1996). The short form of the LEPR mediates this transport however in obese people the level of this receptor is lower and it contributes to the leptin resistance (Shimizu et al., 2002). It has been shown that leptin level in hypothalamus compared to the plasma level is lower in obese subjects (Schwartz et al., 1996; Dötsch et al., 1997). Another mechanism involved in leptin resistance is a negative control of LEPR signalling pathway (Yasukawa et al., 2000). Molecules like SOCS-3 (member of the suppressors of cytokine signalling family) (Bjorbaek et al., 1998), SHP-2 (downregulates Jak2/STAT3 activation by leptin in the hypothalamus)

(Carpenter et al., 1998; Zhang et al., 2004) and (PTP)-1B (protein tyrosine phosphatase) (Cheng et al., 2002) act as inhibitors of leptin signalling. Several mechanisms underlying leptin resistance have been discovered however the cascade of the events is still unknown. Although the initial idea of leptin as an anti-obesity drug failed, there is still interest in manipulating the leptin signalling system in order to manage body weight in obese patients. New approaches to enhance leptin signalling and increase leptin sensitivity include reduction of SOCS3 activity, inhibition of PTP-1B or manipulation of POMC and activation of melanocortin receptors (Foster-Schubert et al., 2006). Another idea to overcome the effects of leptin resistance is to combine leptin with potential leptin sensitizers like pramlintide, an amylin analogue. It has been demonstrated that this combination causes significantly more weight loss than either treatment alone (Ravussin et al., 2009). Further research is needed to reveal whether leptin has a role in weight loss maintenance.

1.2.7 *Functions of leptin - leptin and seasonal fattening cycles*

One area of research into leptin's effects on energy balance in mammals has focused on species which show natural seasonal cycles of adiposity, food intake and energy balance. Studies on Siberian and Djungarian hamsters (*Phodopus sungorus and Phodopus campbelli*) (Klingenspor et al., 1996; Mercer 1998), sheep *(Ovis aries*) (Adam and Mercer 2004), blue fox (*Alopex lagopus*) (Mustonen et al., 2005), Iberian red deer (*Cervus elaphus hispanicus*) (Gaspar-Lopez et al., 2009), woodchuck (*Marmota monax*) (Concannon et al., 2001), European brown bear (*Ursus arctos arctos*) (Hissa et al., 1998), and raccoon dog (*Nyctereutes procyonoides*) (Nieminen et al., 2001) demonstrate that leptin concentrations increase in long days (summer) which is associated with weight gain and high food intake, and that leptin levels decrease in short days (winter) when food intake and animal weight is reduced. This appears paradoxical in relation to what is known about leptin in laboratory rodents, because high levels of leptin might be expected to be associated with the lean state. However, seasonal body weight cycles are associated with seasonal changes in sensitivity to leptin. Effects of leptin administration in short days are greater than in long days, when the animals show insensitivity to leptin (Mercer et al., 2001, Adam and Mercer 2001; Rousseau et al., 2003). These results suggest that the animals show a phenomenon of seasonal leptin resistance regulated by photoperiod (Adam and Mercer, 2001).

Therefore seasonal fattening in mammals appears to involve the regulation of hypothalamic pathways independent of leptin.

1.2.8 *Functions of leptin - leptin and metabolism*

In addition to leptin's role in energy homeostasis, it can regulate glucose and insulin homeostasis via the central nervous system (Pelleymounter et al., 1995). Inhibitory effects on hepatic glucose production (Pocai et al., 2005; van den Hoek et al., 2008) and stimulation of glucose uptake in skeletal muscle (Cusin et al., 1998; Haque et al., 1999; Kamohara et al., 1997; Minokoshi et al., 1999) were observed after intracerebroventricular injection of murine leptin. Moreover, leptin dramatically improves insulin sensitivity in human lipodystrophy (Oral et al., 2002; Petersen et al., 2002; Shimomura et al., 1999). The signalling effects of insulin and leptin on glucose homeostasis are linked because both hormones activate the enzyme phosphatidylinositol- 3-OH kinase (PI3K) in the hypothalamus (Niswender et al., 2001, Morton et al., 2005, Minokoshi et al., 2004). To investigate leptin and insulin activation of PI3K, intracerebroventricular (i.c.v.) injections of leptin and histochemical and biochemical methods were performed (Niswender et al., 2001, Niswender et al., 2003). The studies have shown an increase in hypothalamic PI3K activity connected with the insulin receptor substrate IRS, which activates cell-surface receptors of the tyrosine-kinase type (Niswender et al., 2001). Insulin stimulates tyrosine phosphorylation of IRS, which binds to PI3K and activates another protein kinase (Niswender et al., 2003, Morton et al., 2005). The results suggest that PI3K takes part in the signal transduction pathway which leads to reduced appetite. Moreover, these findings indicate that both insulin and leptin play an important role in the food intake regulation by hypothalamic activity (Nisweder et al., 2001)

1.2.9 *Functions of leptin - reproduction*

Leptin is an important signal in the regulation of neuroendocrine function and fertility, interacting with the reproductive axis at multiple sites. The lack of leptin in *ob*/*ob* mice results in infertility (Coleman, 1982); however exogenous leptin injections to *ob/ob* mice restore fertility (Chebab et al., 1996; Rosenboum and Leibel 1998; Ahima et al.,

1997). Leptin has been found as a hormone that plays a role in reproductive organs, such as the gonads (Karlsson et al., 1997; Caprio et al., 1999), endometrium (Kitawaki et al., 2000), placenta (Hoggard et al., 1997; Masuzaki et al., 1997), and mammary gland (Smith-Kirwin et al., 1998), with related influences on important physiological processes such as menstruation (Ludwig et al., 2000), pregnancy, and lactation (Mounzih et al., 1998). It has been shown that leptin is involved in hypothalamic and pituitary regulation of gonadotropin secretion by stimulation of GnRH (gonadotropin-releasing hormone) release. Leptin stimulates directly luteinizing hormone (LH) and follicle-stimulating hormone (FSH), release by the pituitary via nitric oxide (NO) synthase activation in gonadotropes (Yu et al., 1997).

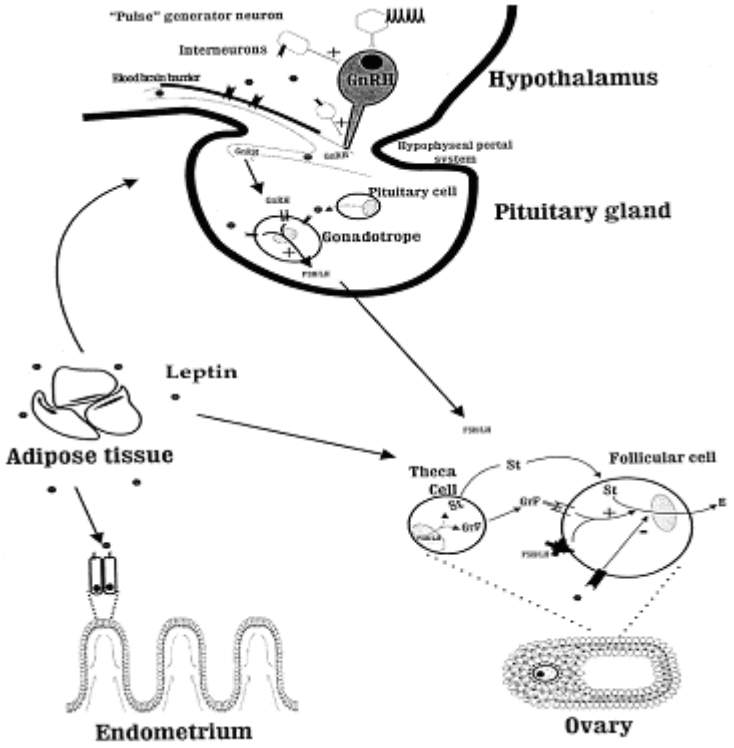


Figure 6 Schematic diagram illustrating the interaction of leptin with the hypothalamic-pituitary-gonadal axis and endometrium (Machos et al., 2002)

A number of studies have shown that leptin administration advances the time of puberty. An increase in leptin levels may be the signal of the initiation of puberty (Chehab et al., 1997). Animal experiments and observations have revealed significant variation in leptin levels throughout the menstrual cycle, with higher levels in the midluteal rather than follicular phase. This finding suggests the action of ovarian steroids on production of the leptin in adipose tissue (Ludwig et al., 2000). Leptin is

also involved in regulating maternal nutrition and the metabolic adaptation of nutrient partitioning during pregnancy and lactation. Pregnancy, as an energy-consuming process, appears to be a state of leptin resistance (Mounzih et al., 1998). Leptin may also be important in regulation of the male reproductive axis. Recent studies show that leptin is able to act at different levels of the hypothalamic-pituitary-testicular axis. It inhibits directly the signal for testicular steroidogenesis, which may be relevant to observations of decreased testosterone secretion in obese men (Tena-Sempere et al., 2001). In conclusion, leptin may act as a link between adipose tissue and the reproductive system, showing that sufficient energy reserves are required for normal reproductive function.

1.2.10 *Functions of leptin - development*

Several studies have implicated leptin in the growth and development of the fetus, both through placental and fetal expression of the leptin and LEPR genes. The leptin gene and mature leptin protein are produced in a number of tissues in the fetal mouse, where leptin may be multifunctional and have both paracrine and endocrine effects (Hoggard et al., 1997). It may act as a fetal growth factor or a signal to the fetus of maternal energy status. Other possible roles of leptin in the placenta may be stimulation of placental angiogenesis and a local autocrine immunomodulatory or anti-inflammatory role (Takahashi et al., 1999). A number of studies have shown that umbilical cord blood leptin levels are positively correlated with fetal insulin, birth weight, length and head circumference (Schubring et al., 1996). These findings suggest a potential function of leptin in fetal growth. Several studies have reported that leptin is involved in the modulation of bone mass during skeletal development (Heaney et al., 1996). The hormone is an important stimulator of cortical bone formation in obese mice. In growing *ob/ob* mice, administration of leptin results in a dramatic increase of bone formation (Steppan et al., 2000). Both body weight and fat mass have been correlated to bone mineral density (Felson et al., 1993). The long form of LEPR has been found in chondrocytes and osteoblasts (Steppan et al., 2000), which suggests that leptin may play a role as a physiological signal between bone and fat mass: it may serve as a signal to bone to remodel in response to changes in body mass.

Leptin also plays a role in lung development. It is produced by lipofibroblasts (Torday et al., 2002), cells located in the alveolar walls (Rehan et al., 2006), which are involved in lung protection against oxygen free-radicals (Torday et al., 2001), and in regulation of pulmonary surfactant production (Torday and Rehan, 2002). Furthermore, leptin induces an increase of air space diameter by stimulation of lung epithelial cell surfactant phospholipid synthesis (Torday and Rehan, 2002). The ability to raise the surfactant production induced by stretch is especially vital in diving animals (Hall et al., 2009). In addition, leptin signalling activates TACE (Tumor Necrosis Activating Factor), an enzyme important in the function of epidermal growth factor receptor (EGF-R) involved in development and regulation of role of the alveolar blood gas barrier (Nielsen et al., 2009). Leptin is involved in the stretch-induced surfactant production pathway, which is essential for diving animals to increase ability of the lungs to stretch under hydrostatic pressure and to prevent collapse of the lungs (Miller et al., 2006). Cloning and sequencing of the seal leptin genes (grey *Halichoerus grypus* and harbour *Phoca vitulina* seals) have shown non-synonymous substitutions in regions of the leptin molecule that are conserved in other vertebrate groups (Hall et al., 2009). It has been hypothesised that the unusual positive selection of leptin in seals is associated with a change in leptin function to meet the increased demand for pulmonary surfactant in these species (Hall et al., 2009; Torday et al., 2010). Neural development is also influenced by leptin (Ahima et al., 1999; Steppan and Swick 1999, Udagawa et al., 2006; Bouret et al., 2004). The brains of mutant mice (*ob/ob*; *db/db*) differ from the wild type controls (Bereiter and Jeanrenaud, 1979). The structural abnormalities in obese mice include reduced volume and weight of brain, cell density and proliferation activity, as well as alterations in the dendritic orientation of hypothalamic neurons and immature pattern of expression of synaptic and glial proteins (Ahima et al., 1999; Steppan and Swick 1999). Exogenous administration of leptin can increase total cell number in brain and repair these impairments (Ahima et al., 1999). These results imply that leptin raises proliferation activity in neural stem/progenitor cells, and induces neuronal differentiation and migration (Udagawa *et al*. 2006). Moreover, LEPR is expressed in the cingulate cortex (Diano et al., 1998), a part of the brain responsible for motor and cognitive processes (Vogt et al., 1992). This finding agrees with observations on mutant mice (*ob/ob*, *db/db*), which showed reduced locomotor activity and changed cognitive functions (Pelleymounter *et al*. 1995, Campfield *et al*. 1995; Halaas *et al*. 1995). In addition, leptin stimulates formation and neural projections of neurons in the arcuate nucleus associated with feeding circuits (Bouret et al., 2004). The above

evidence demonstrates leptin's importance for controlling structural and functional brain development.

1.2.11 *Functions of leptin - immune response*

Leptin production dramatically increases during infection and inflammation, suggesting that leptin, as a long-chain helical cytokine, plays a role in inflammatory-immune response and the host defence mechanism (Grunfeld et al., 1996; Sarraf et al., 1997; Faggioni et al., 1998). Leptin stimulates the production of pro-inflammatory cytokines from cultured monocytes and enhances the production of Th1 type cytokines from stimulated lymphocytes (Otero et al., 2006). Leptin also plays a role in inflammatory processes involving T cells and has been reported to modulate T-helper cell activity in the cellular immune response (Lord et al., 1998; Martin-Romero et al., 2000). Leptin deficient (*ob/ob*) mice, show increased susceptibility to infections (Meade et al., 1979; Chandra 1980), and are resistant to TH 1-mediated experimental autoimmune diseases including encephalomyelitis, arthritis, glomerulonephritis, colitis and hepatitis (La Cava and Matarese 2004). Also, several studies have implicated leptin in the pathogenesis of autoimmune inflammatory conditions, such as experimental autoimmune encephalomyelitis, type 1 diabetes, rheumatoid arthritis, and intestinal inflammation (Otero et al., 2005). These findings provide evidence that leptin links the neuroendocrine and the immune system because of its dual nature as a hormone and cytokine. Leptin appears to have a dual effect of stimulating immunity against infection, while promoting the development of autoimmunity.

It has also been shown that leptin and LEPR are involved in the production of multiple blood cell lineages and hematopoiesis (Bennet et al., 1996; Faggioni et al., 2000; Umemoto et al., 1997; Cioffi et al., 1996; Hirose et al., 1998). Alterations in normal and *db/db* mutant mice demonstrate that leptin and its receptor play an important role in hematopoietic differentiation. *db/db* mice have a deficit in lymphopoietic progenitors and faulty erythrocyte production in the spleen, however the level of erythrocytes in blood is normal (Bennet et al., 1996). These findings suggest that leptin might act at the level of the hematopoietic progenitor cell. Moreover, a decrease in the concentration of lymphocytes and an increase in monocytes have been reported in *ob*/*ob* mice (Faggioni et al., 2000). Studies on colony forming assays in the culture of bone morrow cells have shown that leptin activates generation of granulocyte-macrophage in both normal and

db/db mice; however the effect in *db/db* mice is significantly reduced (Umemoto et al., 1997). In addition, it is been observed that leptin enhances the activity of stem cell factor and erythropoietin (Umemoto et al., 1997). These results agree with the LEPR expression pattern, showing that the long form of LEPR is expressed in hematopoietic stem cells and a variety of hematopoietic cell lnes (Cioffi et al., 1996). Furthermore, leptin has a proliferative effect on BAF-3 cells, which leads to an increase in the proliferation of hematopoietic stem cell populations (Bennet et al., 1996). The above studies demonstrate that LEPR signalling stimulates the proliferation of hematopoietic progenitors.

1.2.12 *Functions of leptin - cardiovascular system*

Leptin can contribute to different cardiovascular actions, although sympathoactivation is probably the most important. The hormone causes a significant increase in overall sympathetic nervous activity, which is correlated with increased expression of neuropeptides such as POMC and corticotropin-releasing hormone (Rahmouni *et al*., 2003). A selective leptin resistance may explain how leptin is involved in obesity-related hypertension, despite loss of its metabolic effects (Rahmouni et al., 2004). These observations suggest that the cardiovascular actions of leptin may help explain the link between excess fat mass and cardiovascular diseases.

It has been demonstrated both in vitro and in vivo, that leptin is involved in angiogenesis (Bouloumié et al., 1998, Fukuda et al., 2003, Sierra-Honigmann et al., 1998, Cao et al., 2001, Anagnostoulis et al., 2008). Experiments performed on cultured human umbilical venous endothelial cells (HUVECs) showed that leptin induces cell proliferation, development of capillary-like tubes and neovascularisation (Bouloumié et al., 1998). Moreover, leptin stimulates the secretion of vascular permeability factor/vascular endothelial growth factor (VEGF) which promotes angiogenic processes (Bouloumié et al., 1998, Anagnostoulis et al., 2008, Cao et al., 2001). This finding suggests that leptin signalling, generated by LEPR expressed in human vasculature and endothelial cells, enhances the formation of new blood vessels.

1.3 Leptin in invertebrates

It is currently uncertain whether leptin signalling systems are present in invertebrates. Jiang et al. (2010) reported the cloning of a leptin receptor-like sequence in the Chinese Mitten Crab (*Eriocheir sinensis*) that shared sequence identity with invertebrate sequences deposited in databases including sea squirt (*Ciona intestinalis*) (XP_002128678), parasitic wasp (*Nasonia vitripennis*) (XP_001605479), red flour beetle (*Tribolium castaneum*) (XP_973202), pea aphid (*Acyrthosiphon pisum*) (BAH70994), triatomid bug (*Rhodnius prolixus*) (AAQ20841) and sea lice (*Caligus clemensi; Caligus rogercresseyi*) (ACO14858; ACO11244). The sequence also appears to share similarities and conserved amino acids with other amino acid LEPR sequences from vertebrates including the Vps domain and three cysteine residues, critical for fundamental structure and function of the LEPR. RT-PCR analysis revealed expression of the LEPR-like sequence in crab tissues linked to nutrition and reproduction, including the intestine and hepatopancreas, and in the gonad and accessory gonad (Jiang et al. 2010). However the existence of LEPR-like molecules in invertebrates should be interpreted with caution. Liongue and Ward (2007) point out that the evolutionary divergence of Class I cytokine receptors means that phylogenetic trees and alignments are sometimes unreliable. For example, Kurokawa et al. (2009) state that the XP_002128678 sea squirt sequence that Jiang et al classified as a LEPR, is actually a LEPROT and that the LEPR is absent in sea squirts, suggesting that the LEPROT arose earlier in evolution. The sequencing of the sea squirt genome has allowed comparison of this invertebrate chordate with vertebrate genomes. Orthologues of JAK, STAT and SOCS are present in the sea squirt genome, suggesting that cytokine signalling pre-dates vertebrates (Hino et al., 2003). Liongue and Ward (2007) searched for Class I cytokine receptors in the seq squirt using a variety of bioinformatics approaches including receptor topology and conservation of synteny. They identified only two Class I receptors, one resembling the GP-130 receptor, and the other with similarity to the CLF-3 receptor. No orthologues of the LEPR were identified. This suggests that diversification of the Class I cytokine receptor family, including the appearance of the leptin receptor, occurred after the divergence of urochordates and vertebrates. The fact that no leptin-like sequences have been reported in invertebrates, including the Chinese mitten crab, supports this.

1.4 Leptin in non-mammalian vertebrates - birds

The first evidence to suggest that the leptin gene has been conserved in non-mammalian vertebrates was based on Southern hybridization of a mouse leptin probe to genomic DNA from chicken and eels (Zhang et al., 1994). Afterwards, two independent laboratories reported the cloning of a chicken leptin cDNA, using primers based on the mouse leptin sequence (Taouis et al., 1998, Ashwell et al., 1999) and indicated that the main site of leptin gene expression was in the liver, which is the major site of fat synthesis in birds. The cDNA identified shares 97% identity with mouse leptin at the amino acid level in both cases. This percentage is greater than for sequence identities found between mammalian leptin sequences (Table 1). This high level of sequence similarity is not repeated for leptin genes between mammalian species (Doyon et al., 2001). The close similarity between mouse and chicken leptin induces doubt concerning the nature and origin of this sequence. Several independent laboratories (Friedman-Einat et al., 1999, Dunn et al., 2001) have argued the improbability of the existence in nature of the published chicken leptin cDNA sequence. This evidence is outlined below.

1.4.1 *Evidence against the existence of the published chicken leptin cDNA sequences*

A basic problem has been the inability of several independent laboratories to repeat the amplification with primers and conditions specified by Taouis et al., (1998), and using other appropriate primers, of chicken leptin cDNA (Friedman-Einat et al. 1999; Pitel et al., 2000; Amills et al., 2003, Carre et al., 2006). For example, Friedman-Einat et al. performed PCR using fourteen primers based on the mouse leptin sequence. No PCR products sharing close similarity to the mouse leptin sequence were obtained from any avian templates.

A second piece of evidence comes from attempts to hybridize mouse leptin probes to chicken mRNA or genomic DNA using Northern and Southern blotting. If the sequence similarity between the mouse and chicken genes is as high as suggested, they should hybridize easily. However, no signal was obtained when Northern hybridization was performed using a mouse leptin probe against chicken fat and liver mRNA (Friedman-

Einat et al., 1999), whereas a strong signal was obtained from control mouse fat total RNA.

Southern hybridization under low stringency washing conditions showed a weak hybridization signal of chicken genomic DNA to a mouse leptin probe (Friedman-Einat et al., 1999, Dunn et al., 2001). This supports the suggestion of Zhang et al. (1994) that a leptin gene orthologue may be present in the chicken. However, the hybridization signal between chicken and mouse DNA was lost after washing under higher stringency conditions, while a signal between the mouse probe and sheep DNA (which shares 83% sequence identity with the mouse) remained. This therefore suggests that the sequence similarity between the chicken and the mouse leptin genes is not as high as reported (Taouis et al., 1998).

A third piece of evidence inducing doubt about the nature and origin of the published chicken leptin cDNA sequences comes from consideration of the high sequence identity between the mouse and chicken leptin genes. The improbability that the high amino acid sequence identity of 95 % between the mouse and chicken sequences would have arisen during molecular evolution was indicated by analysis of the rate of synonymous substitutions between these genes (Dunn et al., 2001). The frequency of synonymous substitutions (nucleotide changes in codons that do not change the encoded amino acids) was calculated between randomly selected mouse and chicken genes and the distribution was compared statistically with the minimal number of synonymous substitutions present between the mouse and chicken leptin sequences. The results showed that the chicken leptin sequence lies at the extreme of the estimated distribution of synonymous substitutions, with a statistical probability of less than 1 in 1 million (Dunn et al., 2001) (Figure 7).

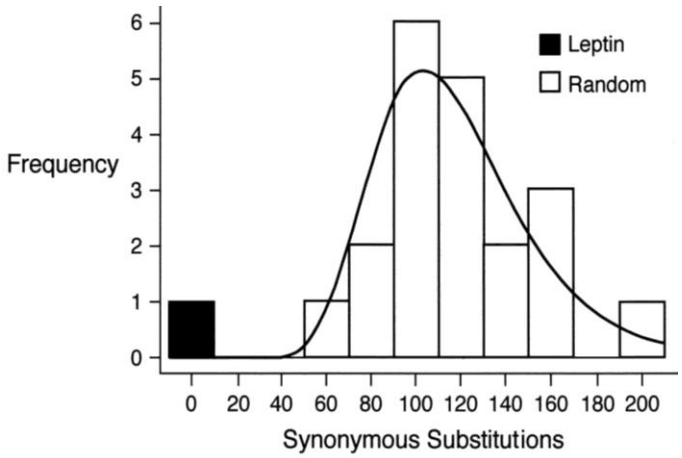


Figure 7 Distribution of values for the rate of synonymous substitutions for 20 randomly-selected genes and for leptin. Using randomly generated numbers 20 genes which had mouse homologues in GenbEmbl and contained alignable protein coding regions were selected from a list of 1073 chicken genes. The chicken sequence accession number is listed followed by the gene name and the mouse sequence accession number; 1) D45416 Neuropilin D50086, 2) L13234 Jun-binding protein. X75312, 3) AF131057 Substance P Receptor X62934, 4) X89507 AMPA Receptor AB022913 5) M74057 Growth Hormone Receptor M33324 6) U37273 CWH-2 Y08222 7) AF041799 Insulin Receptor-related tyrosine kinase AF056187 8) M26810 NGF V00836 9) AF082666 Interleukin receptor 10-2 U53696 10) X65458 Stathmin X94915 11) X04810 Carbonic anhydrase II K00811 12) L21719 C-eyk L11625 13) AF036942 Photoreceptor guanylate cyclase I L41933 14) AF085248 Calmodulin X14836 15) U20216 Inward Rectifying K channel AF021136 16) L12695 En-1 Y00201 17) U62143 Hoxb-1 X53063 18) AF071026 Truncated testis-specific box1 BPRCR X73372 19) L18784 TGF-b type II receptor D32072 20) AB002410 17-β hydroxy steroid dehydrogenase X89627 21) AF012727 Leptin U18812 (Dunn et al., 2001)

The rate of synonymous substitutions has been used to construct phylogenetic trees for leptin and for prolactin (another cytokine hormone) to show the relationship between members of gene families and the taxonomic relationship between vertebrate classes. The tree for prolactin indicates early divergence for the avian and mammalian lineages following the accepted model for vertebrate evolution. However, the tree derived from leptin shows divergence of birds from rodents in the relatively recent past. This research suggests that, contrary to expectation, there is higher sequence identity between chicken and mouse leptin sequences than between the mouse and other mammals, further demonstrating the unlikelihood that the published chicken sequence is correct.

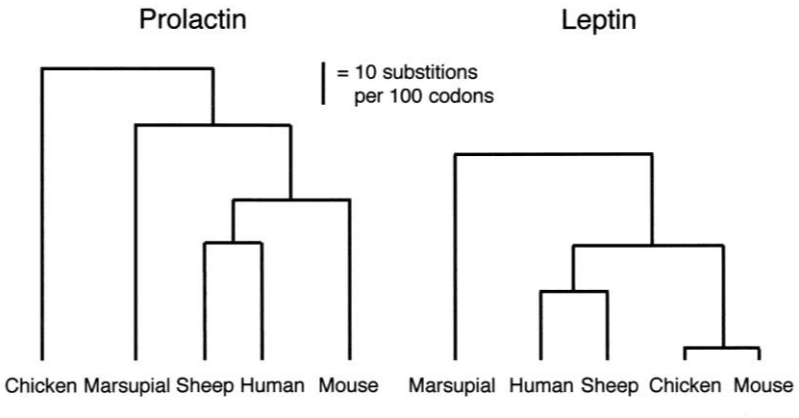


Figure 8 Phylogenetic trees constructed using synonomous substitutions for leptin and prolactin in marsupial, sheep, human, chicken and mouse sequences. Comparisons were produced in the same way as for Figure 7. The rate of synonomous substitution was determined with multiple alignments of human, sheep, mouse, marsupial and chicken genes. Sequences used were for prolactin; human (V00566), sheep (M27057), marsupial (AF067726), mouse (NM011164), and chicken (J04614) and for leptin; human (NM000230), sheep (U84247), marsupial (AF159713), mouse (U18812), and chicken (AF012727). The regions aligned were equivalent to base 120-557 of GenEmbl leptin sequence U18812 and 118-687 of GenEmbl prolactin sequence J04614 (Dunn et al., 2001).

A key fourth piece of evidence against the existence of the published chicken leptin cDNAs is that no evidence has been provided that the sequence is present in avian genomes. Thus, the available information has been based solely on identification of cDNA sequences and no evidence of a genomic sequence corresponding to the cDNA, including intronic sequence, has been shown. The sequencing of the chicken genome in 2004 (International Chicken Genome Sequencing Consortium, 2004) and zebra finch genome in 2010 (Warren et al. 2010) have not helped to resolve the issue because the published cDNA sequences cannot be aligned to them, and the leptin gene is missing from the chromosomal region where it would be expected to be located on the basis of conservation of synteny (Pitel et al., 2010). Additionally, there is no evidence for the published chicken leptin cDNA sequences in the available chicken EST clones, of which there are approximately 0.5 million from a variety of tissues and developmental stages (Pitel et al., 2010).

Taken together, this evidence based on experimental and evolutionary analysis reveals how unlikely it is that the published chicken leptin sequence exists in the chicken genome. The best explanation for the published cDNA sequences is that they represent cloning artefacts.

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

Table 1 Percentage of amino acid identity for known leptin sequences of chicken, mouse and human compared with mammalian sequences (Doyon et al., 2001).

1.4.2 *The avian leptin receptor*

Although the evidence for a chicken leptin gene is uncertain, there is evidence that a leptin-like signaling system is present in birds because receptor sequences have been cloned in the chicken (chLEPR) and turkey that share greater than 90% sequence identity at both the nucleotide and amino acid level (Horev et al., 2000; Ohkubo et al., 2000; Richards and Poch, 2003). The chicken and turkey LEPR gene (long form) encodes a protein of 1147 amino acids that has features similar to other LEPRs including: a signal peptide, a single transmembrane domain, and specific conserved motifs defining putative leptin-binding and signal transduction regions of the protein. The identity between chicken and mouse LEPRs is 60%, indicating a relatively low similarity (Horev et al., 2000). Sequences among the mammalian LEPR genes show a much higher similarity; 80–92% identical nucleotides, 74–91% identical amino acids. This level of sequence similarity is consistent with the estimated evolutionary divergence time of about 300 million years between birds and mammals (Ohkubo et al., 2000). It also provides further evidence against the existence of the published chicken

leptin sequences because, as the leptin sequence identity is so close between the chicken and mammals, a greater sequence similarity between chicken and mammalian leptin receptors would have been expected.

Sequence analysis provides evidence that the cloned avian receptors show sequence conservation of motifs with mammalian LEPRs. Thus far, comparisons between the predicted protein sequences have shown a conservation of key LEPR motifs, predicted exon boundaries and essential tyrosine residues. Exons 9 and 10, involved in ligand binding, are conserved in the avian receptor, with a sequence identity in this region between chicken and human of 75% (Ohkubo et al., 2000).

The characterized chLEPR consists of the putative signal peptide, a single transmembrane domain and the conserved box 1, 2 and 3 motifs in the cytoplasmic region, strongly suggestive of functional conservation. In the extracellular region of chLEPR the Trp-Ser-X-Trp-Ser motif implicated in ligand binding and signal transduction of the cytokine receptor gene family is present. This motif is conserved in terms of sequence and positions. Similarly conserved are the box 1 motif and the tyrosine Y-986, Y-1079, and Y-1141, implicated in the JAK/STAT signaling of the mammalian LEPR genes (Tartaglia et al., 1995). In the predicted transmembrane domain, all amino acid changes are conservative, thereby keeping its hydrophobic characteristic.

In 2000, the leptin receptor gene was mapped to the chicken chromosome 8 in the equivalent syntenic position to the leptin receptor in the human genome. This finding provides additional evidence, along with the preservation of sequence motifs, that the chicken gene cloned is a leptin receptor (Dunn et al., 2000). High levels of chLEPR mRNA expression were observed in ovary and brain and this pattern of mRNA expression is similar to the mammalian LEPR genes (Horev et al., 2000, Richards and Poch, 2003). The expression of leptin receptor mRNA was identified in granulose and theca cells in the ovary (Cassy et al., 2004). This finding suggests that the level of expression of the leptin receptor regulates the action of its ligand in the ovary.

If the leptin signalling system has been conserved between birds and mammals, conservation of the mammalian pattern of splice variation in the receptor might be expected in the avian genes. Northern analysis revealed two transcripts of the LEPR

mRNA, about 5 and 7 kb (higher intensity) in size (Horev et al., 2000). This study suggested that two splicing variants are present in the chicken, with higher expression of the long form of the chLEPR. However, another study (Ohkubo et al., 2000) only found evidence for a single transcript. More recently, (Liu et al., 2007), an alternatively spliced short form of the chLEPR was identified. Alternative splicing of the chLEPR has been predicted on the basis of sequence conservation between birds and mammals at the junction between exons 19 and 20 (Richards and Poch, 2003). The results suggest that the short form of chLEPR is not directly comparable with the mammalian LEPR short form, in that its expression appears to be lower and could not be detected in the choroid plexus, a major site of expression of the short form of the LEPR in mammals. The expression of the chLEPR short form was highest in the pituitary gland and ovary (Liu et al., 2007), but it remains to be determined whether the mRNA identified is translated and has any functional significance.

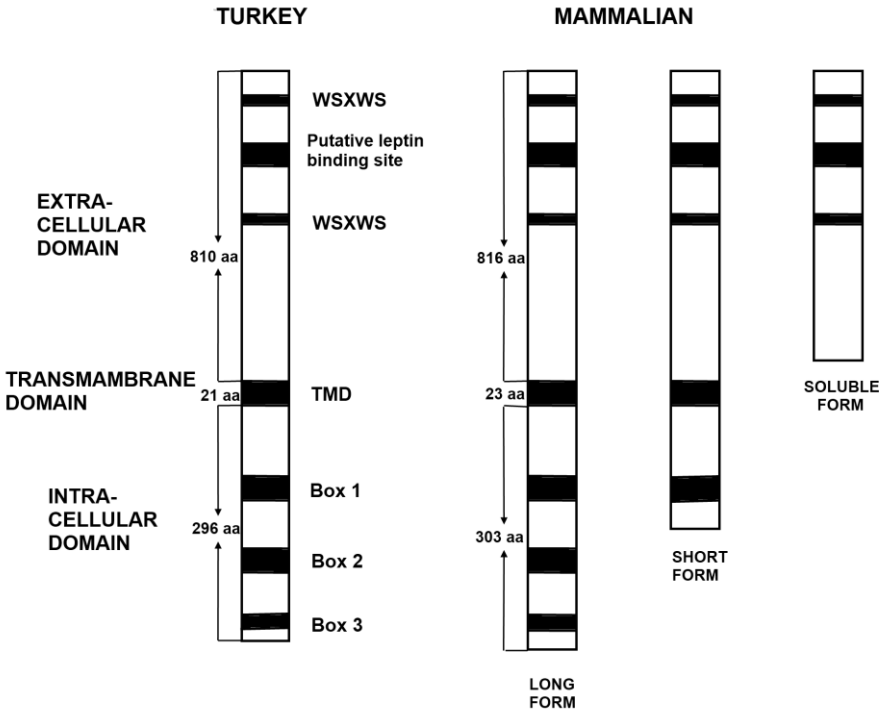


Figure 9 A scheme of turkey and mammalian LEPRs (long form and other splice variants that have been identified for the mammalian receptor); positioning of conserved motifs (Richards and Poch, 2003).

1.4.3 *Effects of leptin administration*

To investigate different function of leptin in birds, injections of recombinant mouse or chicken (97% identical to mouse) leptin have been performed. Feeding behaviour in domestic chicks was studied after intracerebroventricular administration of mouse leptin (Bungo et al., 1999). Central administration of mouse leptin did not influence food intake relative to saline controls in the time periods examined. The effect does not agree with the result of leptin injection in mammals, where leptin rapidly lowers food intake (Mistry et al., 1997). This study suggests that either mouse leptin does not bind to the chicken leptin receptor or that leptin may be absent in the chicken. This is evidence against the existence of the published chicken leptin cDNA sequences. It seems unlikely that amino acid sequence of chicken leptin shares with mouse 97% identity if there are differences in effect of administration between mammalian and avian species. Later studies demonstrated an inhibitory effect of mouse or chicken leptin on food intake in birds when administered centrally or peripherally (Denbow et al., 2000; Dridi et al., 2005).

Previous research in mammals has shown that leptin is involved in regulating the secretion and expression of several neurotransmitters and neuropeptides expressed in the hypothalamus. A study in chickens indicated some similarity with mammalian systems in that reduced food intake induced by central injection of recombinant chicken leptin was associated with reduced hypothalamic gene expression of neuropeptide Y (NPY) an orexigenic neuropeptide that stimulates appetite and inhibit energy expenditure (Dridi et al., 2005). An inhibitory effect of leptin on NPY neurones, which express the leptin receptor, is well established (Schwartz et al., 1996).However leptin administration did not have an effect on other hypothalamic neuropeptides that have been demonstrated to be responsive to leptin in mammals such as agouti-related protein (AgRP – an orexigenic/anabolic neuropeptide) and proopiomelanocortin (POMC) and corticotropin (CRH) (anorexigenic/ catabolic neuropeptides). This finding does not correlate with the results obtained in mammals. AgRP, that stimulates food intake, is a negative regulator of leptin action and leptin decreases hypothalamic AgRP production (Mizuno et al., 1998, Ebihara et al., 1999). POMC and CRH are anorexigenic hormones, which are involved in inhibition of appetite and stimulation of energy expenditure and treatment mice with leptin stimulates hypothalamic POMC and CRH mRNA (Mizuno et al., 1998). The contrast between the mammalian and avian findings

is consistent with the possibility that the sequence similarity of mouse and chicken leptins with a native chicken leptin is not close.

Other findings from studies of leptin administration demonstrate the existence of a leptin signalling pathway in birds. For example, a stimulatory effect of mouse leptin has been found on cell proliferation and protein synthesis in muscle and liver cells from chicken embryos (Lamosova and Zeman, 2001) and mouse leptin administration during embryonic development of birds revealed permanent changes of endocrine and metabolic parameters regulating growth and development (Lamosova et al., 2003). Thus, leptin administration to eggs affected thyroid hormone (TH) levels that regulate growth and development and increase metabolism. Treated chickens had higher body weight compared to the control group consistent with the observed alterations in thyroid status. The most prominent changes in triiodothyronine (T3), and thyroxine (T4) appeared immediately after hatching and before sexual maturity. The finding suggested that leptin may act as a general signal of low energy status to neuroendocrine systems in birds. Previous studies on administration of leptin to mice have revealed also changes of the thyroid axes (Ahima *et al*. 1996).

The possibility that leptin may act as a signal of body fat stores in birds is also suggested by the effect of leptin injections on prepubertal development and the timing of reproductive maturity in chickens (Lamosova et al., 2003, Paczoska-Eliasiewicz et al., 2006). Leptin treatment during embryonic development precipitated the onset of puberty in comparison to controls, evidenced by age at first oviposition and increased testicular weight in males. Injection of mouse leptin shows in males higher weight of the testes and in females earlier sexual maturnity than the controls (Lamosova et al., 2003). Similarly, treatment of prepubertal female chickens with systemic injections of mouse leptin advanced the onset of puberty (laying of the first egg) and abolished the delay caused by food restriction. Analysis of the ovaries revealed that leptin injections advanced follicular development, particularly in birds fed ad libitum, and significantly reduced follicular apoptosis both in full-fed and feed-restricted birds. Moreover, the increases of luteinizing hormone, estradiol and progesterone in blood plasma were also advanced by leptin treatment (Paczoska-Eliasiewicz et al., 2006). These findings agree with studies in mammals (Ahima et al., 1997; Cheung et al., 2001).

In addition to effects on energy balance and reproduction, administration of mammalian leptin to birds has also been observed to influence the immune system. For example, positive effects on T-cell proliferation in birds have been revealed during leptin injection with mitogen used to stimulate lymphocytes and assess immune function (concavalin A, phytohemagglutinin). The response to mitogens was greater in leptin treated birds during the leptin administration (Lõhmus et al., 2004). The study provides that leptin enhances mitogen stimulated T-cell proliferation in birds. The results correspond with previous reports on mammals (Lord et al., 1998), suggesting that leptin is an important modulator of the immune response regarding the T-cell response.

Overall, studies involving administration of recombinant mammalian leptin in birds show some similarity with mammalian systems. These findings suggest that a leptin-like signaling system is present in birds, however the differences in results suggest that the sequence similarity between the chicken and the mouse leptin genes is not as high as reported (Taouis et al., 1998).

1.4.4 *Leptin receptor signalling*

To assess the functionality of the chicken receptor (chLEPR), leptin bioassays based on the activation of chLEPR in cultured cells, were performed. The experiments showed that chickens response to exogenous administration of human and frog leptins *in vitro* and the chLEPR can mediate the leptin signal (Hen et al., 2008; Adachi et al., 2008). Exogenous leptin binds specifically to the chLEPR and activates luciferase which leads to phosphorylation of signal transducer, activators of transcription 3 (STAT3) and Janus kinase-2 (JAK-2) (Adachi et al., 2008). These findings indicate that the LEPR in chicken tissues is functional and capable of binding leptin, leading to activation of the JAK-STAT signal transduction pathway and inducing physiological processes (Hen et al., 2008; Adachi et al., 2008). However, although the bioassay was able to detect leptin in human serum samples, no signalling activity was detected in samples from fat and lean chickens, or from turkeys (Hen et al. 2008). A similar lack of leptin bioactivity was shown in blood samples collected from wild Adelie penguins and bar-tailed godwits (Yosefi et al., 2010). These species show natural seasonal fattening cycles linked to incubation and migration and blood samples from these species were taken and compared for leptin signalling activity using the chicken receptor assay with human

samples taken over a similar range of body fat content. Again, the human samples activated the receptor but the bird samples did not. The data from the bioassays suggest that leptin is either absent in birds, or circulates in the blood at much lower levels than it does in mammals.

Overall the available information on leptin in birds supports the existence of a leptin signalling system but the identity of the ligand remains uncertain: it is possible that the receptor has evolved to interact with another ligand, or may be constitutively active. If leptin is present, it may play a paracrine, rather than endocrine role. The possible absence of leptin in birds may be related to the different way, that birds regulate glucose metabolism, connected with the evolution of flight (Pitel et al., 2010).

1.5 Leptin in non-mammalian vertebrates – reptiles

Very recent studies have provided the evidence for the existence of leptin-like molecules in reptiles. Evidence for the presence of leptin-like proteins has been presented for two species of reptiles; *Sceloporus undulates* and *Podarcis sicula* (Niewiarowski et al., 2000, Paolucci et al., 2001; Paolucci et al., 2006; Sciarrillo et al., 2005, Spanovich et al., 2005).

1.5.1 *Detection of leptin-like immunoreactivity*

The evidence for the presence of leptin protein in reptiles was provided by immunolabelling of tissues from two lizards (Newiarowski et al., 2000, Paolucci et al., 2001). The presence of the hormone was detected using anti-mouse leptin antibodies. These recognized bands of the appropriate size for leptin (16kDa) in the brain of *Sceloporus undulates* (Niewiarowski et al., 2000) and in the plasma, liver and fat bodies of the female of *Podarcis sicula* (Paolucci et al., 2001). It was suggested that the presence of leptin in brain was caused by binding of hormone by its receptor rather than leptin being synthesized in the brain itself. Leptin levels in plasma, liver and fat bodies fluctuated during the reproductive cycle, in a way consistent with its possible role in reproduction (Paolucci et al., 2001). The amount of fat increases in this lizard during the

winter months and decreases as soon as sexual activity resumes at the beginning of the spring (Paolucci et al., 2001). It is correlated to the fact that reproduction is related with energy metabolism and requires sufficient nutrition. These findings agree with studies in mammals where the first evidence to suggest that leptin is involved in reproduction was based on the observation that obese (*ob/ob* and *db/db*) mice, later found to be lacking functional leptin or leptin receptor, are unable to undergo sexual maturation, and are infertile (Swerdloff et al., 1976).

1.5.2 *Identification of leptin-like genes*

There is evidence from analysis of the *Anolis carolinensis* lizard genome (http://genome.ucsc.edu/cgi-bin/hgGateway) for a leptin-like sequence that shares approximately 44% and 53% amino acid sequence identity with human and mouse leptins, respectively. Importantly, the gene is present in the syntenic chromosomal position predicted for leptin (Boswell 2011). However, the gene remains to be characterised.

1.5.3 *Leptin receptor*

LEPR-like immunoreactivity was detected in both the A and B cells of the endocrine pancreas of *Podarcis sicula*. This was the first report of LEPR immunoreactivity on A cells (Paolucci et al., 2006). This supports the observation of increased circulating insulin and glucagon concentrations after leptin administration, indicating a direct effect of leptin on pancreactic cells (Paolucci et al., 2006). This study supported the involvement of leptin in glucose metabolism in reptiles, although regulation of this system appears quite different from mammals (Paolucci et al., 2006).

LEPR-like immunoreactity was also found in the thyroid gland (Sciarrillo et al., 2005) and testis (Putti et al., 2009) of *Podarcis sicula*.

1.5.4 *Effects of leptin administration*

To investigate the function of leptin in reptiles, injections of recombinant murine leptin have been performed. Systemic injection of recombinant murine leptin in lizards (*Sceloporus undulatus*) produces phenotypic effects similar to those observed when leptin injections are given to mice, such as higher body temperature and reduction of food intake (Niewiarowski et al., 2000). However, administration of leptin did not cause a decrease in body mass relative to saline controls in the time periods examined. The effect does not agree with the result of leptin injection in mammals, where leptin rapidly lowers food intake and reduce body mass (Mistry et al., 1997). It is possible that leptin has different functions in reptiles and mammals and this may be related to the differences in energy metabolism between endotherms and ectotherms (Niewiarowski et al., 2000).

Data on the effect of leptin treatment on circulating levels of insulin and glucagon show significant increases in the concentrations of both hormones (Paolucci et al., 2006). These findings do not agree with studies in mammals, where leptin plays a role as an antiobesity hormone and inhibits insulin secretion (Kulkarni et al., 1997; Kieffer and Habener, 2000). This study shows also that leptin is involved in glucose metabolism as it is in mammals (Mizuno et al., 1996). However, the effects of leptin administration suggest that leptin regulation of glucose metabolism is different from mammals and leptin may have a wider range of functions in reptiles (Paolucci et al., 2006)**.**

Leptin administration stimulates the thyroid gland in reptiles, increasing levels of circulating thyroid hormones (T3 and T4) that regulate growth and development and raise metabolism (Sciarrillo et al., 2005). Previous studies on administration of leptin to mice have also revealed effects on the thyroid axes (Ahima *et al*. 1996). In addition, administration of leptin in the lizard (*Podarcis sicula*) increased sex steroid concentrations and and stimulated the epididymis epithelium (Putti et al., 2009). Moreover, leptin injections delay a testis regression (Putti et al., 2009), which normally takes place in summer when lizards do not respond to hormonal and environmental stimuli (Angelini and Botte, 1992). These findings suggest that leptin is involved in testicular function and reproduction in reptiles, in agreement with mammalian studies (Karlsson et al., 1997; Caprio et al., 1999).

Overall, the evidence of the presence of leptin signalling proteins in reptiles, suggests that leptin plays a regulatory role in the energy metabolism of these ectothermic vertebrates (Niewiarowski et al., 2000).

1.6 Leptin in non-mammalian vertebrates - fish

The first evidence to suggest that the leptin gene has been conserved in fish was based on Southern hybridization of a mouse leptin probe to genomic DNA from eels (Zhang et al., 1994). As discussed below more recent studies have provided further evidence for the existence of leptin-like molecules in fish.

1.6.1 *Detection of leptin-like immunoreactivity*

Later evidence for leptin's expression in fish was provided by immunolabelling of tissues from fish (Johnson et al., 2000; Pfundt et al., 2009; Gambardella et al., 2010; Russo et al., 2010). The presence of the hormone was detected using different polyclonal antibodies against mammalian leptin. Mammalian anti-leptin antibodies recognized bands of the appropriate size for leptin (16kDa) on Western blots of protein extracts of blood, brain, heart, stomach and liver of green sunfish (*Lepomis cyanellus*), bluegill sunfish (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), white crappie (*Pomonix annularis*), channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*) (Johnson et al., 2000). It was suggested that the presence of leptin in the brain was caused by binding of hormone by its receptor rather than leptin being synthesised in the brain itself (Johnson et al., 2000). In addition, immunohistochemical analyses have shown existence of leptin in the gastrointestinal tract of sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*) (Russo et al., 2010) and catshark (*Scyliorhinus canicula*) (Gambardella et al., 2010). This finding agrees with mammalian studies, where the stomach is a common site of leptin production (Bado et al., 1998).

1.6.2 *Identification of leptin-like genes*

The next piece of evidence to suggest that a leptin gene is present in fish was provided by the availability of whole-genome sequences in fish. Leptin-like genes were identified from investigations of the genomic synteny around mammalian leptin genes (Figure 10). For example, recently, leptin-like genes have been identified from pufferfish (*Takifugu rubripes*), the spotted green pufferfish (*Tetraodon nigrovirides*), medaka (*Oryzias latipes*) (Kurokawa et al., 2005), common carp (*Cyprinus carpio*) (Huising et al., 2006a), zebrafish *(Danio rerio)* (Gorrisen et al., 2009), Atlantic salmon *(Salmo salar)* (Ronnestad et al., 2010), arctic charr *(Salvelinus alpinus)* (Froiland et al., 2010) and grass (*Ctenopharyngodon idellus)* and silver carp *(Hypophthalmichthys molitrix)* (Li et al., 2010). The predicted fish leptin proteins show less than 25% amino acid sequence identity with mammalian leptins and the similarity between pufferfish and carp leptins is only slightly higher (Huising et al., 2006a). This considerable sequence dissimilarity between fish species is explained by the large evolutionary distance between cyprinids and pufferfishes, which diverged approximately 300 million years ago. Despite the low amino acid sequence identity, there is evidence that fish leptins are in fact orthologs of mammalian leptins. Firstly, they show conservation of gene structure, consisting of three exons, as in mammals, and the two coding exons are very similar in length to the corresponding exons of the human and mouse leptin genes (Kurokawa et al., 2005; Huising et al., 2006a) Secondly, in fish leptins, there is evidence for conservation of secondary and tertiary structure. Two conserved cysteine residues can be identified that together form a disulphide bridge, a conserved feature with mammalian leptins, and models of fish leptins, based on the crystal structure of human leptin confirm the characteristic four-helix bundle topology of class-I helical cytokines (Kurokawa et al., 2005; Huising et al., 2006a). Thirdly, the fish leptins cluster with mammalian leptins in phylogenetic analysis, supported by high bootstrap values (Gorrisen et al., 2009) (Fig.10). This suggests that they may possess specific leptin receptor binding affinity (Kurokawa et al., 2005).

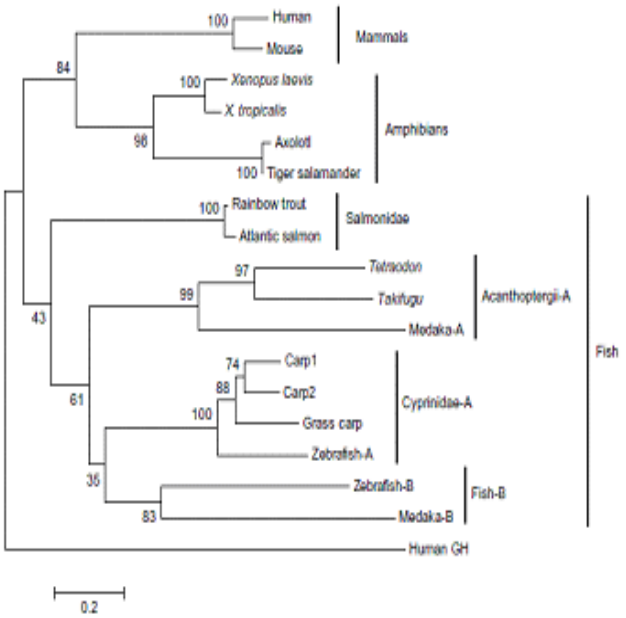


Figure 10 Peptide phylogenetic relationships of leptin and growth hormone (GH) using the neighbor-joining method in Clustal W and MEGA3. Numbers at nodes indicate the bootstrap value (%), obtained for 1000 replicates. GenBank Accession Nos. LEP: mouse, AAI25246; X. Laevis, NP_001089183; X. tropicalis, scafolld 11 (JGI ver. 4.1); axolotl, CO792338. GH: human, CAA23779 (Kurokawa et al., 2009).

In contrast to pufferfish and arctic charr, duplicate leptin genes have been identified in carp (Huising et al., 2006a; Li et al., 2010), zebrafish, medaka (Gorrisen et al., 2009) and Atlantic salmon (Ronnestad et al., 2010). The presence of duplicate leptin genes in these fishes suggests that duplicate leptin genes are a common feature of teleostean fishes. The duplicate zebrafish leptin genes, coding for leptin-a and leptin-b, share only 24% amino acid identity with each other and only 18% with human leptin. That both leptin-a and leptin-b are orthologs of human LEP and paralogs derived from whole-genome duplication early in the teleost lineage (Gorissen et al., 2009). The duplicate carp leptins have different coding regions, 5’ and 3’ untranslated regions, and introns. Moreover the sizes of the introns within the genomic sequences are different. These findings suggest that both carp leptin sequences are derived from separate genes rather than from the same gene through alternative splicing. Both carp leptin genes encode 171 amino acid leptin proteins that share 82 % amino acid identity (Huising et al., 2006a). Their high level of amino acid similarity suggests that both carp leptin genes are the likely result of a recent gene duplication event, possibly the recent tetraploidisation of the carp genome that occurred less than 16 million years ago (Huising et al., 2006a).

The duplicate salmon leptins show 71.6% similarity to each other with 22.4% (Lep-A1, 171aa) and 24.1% (Lep-A2, 175aa) identity to human leptin. The highest expression level of Lep-A1 was found in the brain, white muscle, liver, and ovaries. Lep-A2 had a lower expression than Lep-A1 in most tissues except for the stomach and mid-gut and kidney (Ronnestad et al., 2010). These results suggest that Lep-A1 is important in energy metabolism what agrees with mammalian model, whereas Lep-A2 may play a role in the digestive tract and liver (Ronnestad et al., 2010).

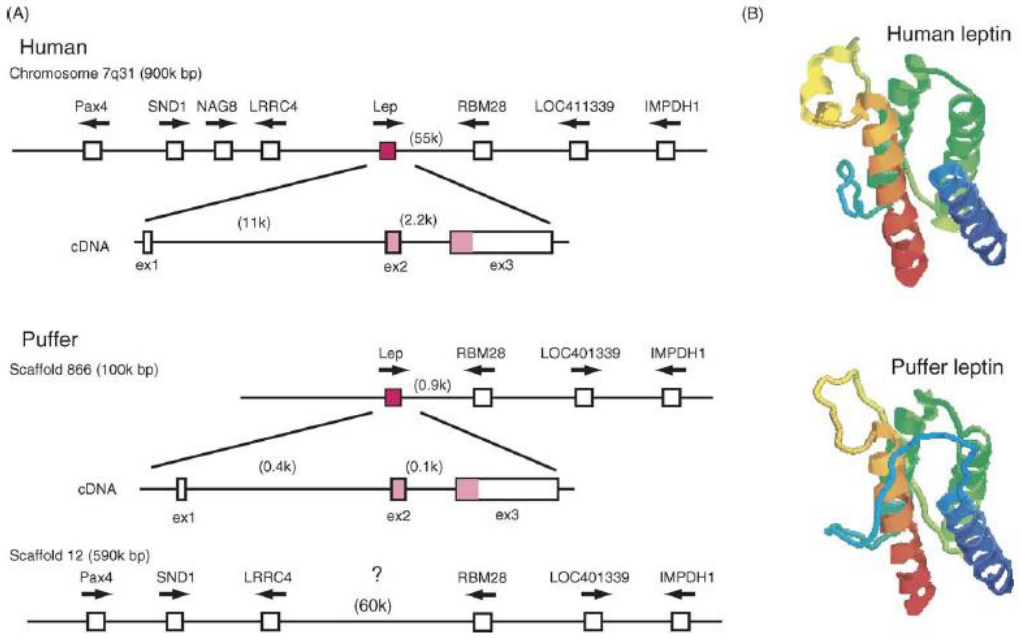


Figure 11 Comparison of leptin gene characterization between human and puffer (A) Gene arrangement and the cDNA structure of the leptin gene. Arrows indicate the direction of transcription of the genes. Shaded areas indicate open reading frame. GenBank accession nos.: Pax4, NM 006193; SND1, NM 014390; NAG8, NP 055226; LRRC4, NM 022143; RBM28, NP 060547; LOC401339, XP 379517; IMPDH1, NP 000874. (B) Ribbon diagram showing the tertiary structure of human and puffer leptin. Secondary and tertiary protein structures were modeled using the ProModII program at the SWISS-MODEL automated protein modeling server, based upon human leptin (1AX8.pdb) Protein Data Bank structure file (Kurokawa et al., 2005).

In addition, it has been demonstrated by dissection of embryo and larvae of Atlantic salmon, that leptin is supplied as a maternal transcript (Moen et al., 2010). Moreover, in larvae the highest expression of leptin has been found in the head and the large increase in leptin level has taken place one week after first-feeding (Moen et al., 2010).

1.6.3 *Leptin receptor*

A full-length LEPR gene has been identified from marine medaka (*Oryzias melastigma)* (Wong et al., 2007), pufferfish (*Takifugu rubripes*) (Kurokawa et al., 2008), Japanese medaka *(Oryzias latipes)* (Kurokawa and Murashita, 2009) and Atlantic salmon *(Salmo salar)* (Ronnestad et al., 2010). The predicted genes consist of 3348bp (21exons) (pufferfish; GenBank Accession No.AB385663), 3225bp (20 exons) (Japanese medaka; GenBank Accession No.AB457590) and 3441bp (Atlantic salmon; GenBank Accession No. AB489201), and encode 1116, 1074 and 1146 amino acids, respectively. The fish LEPR genes share less than 25% identity to mammalian leptin receptors (Figure 12). Sequences among the fish LEPR genes show 82-32.5% similarity at amino acid level. The amino acid sequences of Japanese medaka LEPR shares 81.8% and 47.4% identities with the LEPR of marine medaka and pufferfish, respectively. The identity between pufferfish and medaka is 46% (Kurokawa et al., 2008). The amino acid sequence of salmon LEPR has 32.5–42.6% identity with other fish LEPRs (Ronnestad et al., 2010). Despite the low amino acid sequence identity the fish LEPR genes contain all functionally important domains conserved among vertebrate LEPRs: three fibronectin type III (FN III) domains, the immunoglobulin (Ig) C2-like domain and a pair of repeated tryptophan/serine motifs (WSXWS) at an extracellular segment, and two JAK2-binding motif boxes and a STAT binding domain at an intracellular segment (Kurokawa and Murashita, 2009; Ronnestad et al., 2010). It is been demonstrated that there is five different LEPR isoforms presented in fish, which have different 3' ends of mRNA sequence (Ronnestad et al., 2010). Only one isoform is functional, containing all the important signalling domains (Ronnestad et al., 2010). In the crucian carp (Cao et al., 2011) and marine medaka (Wong et al., 2007), differential changes in expression of the long and secreted isoforms were reported in response to fasting and hypoxia, suggesting that short LEPR isoforms have a physiological role in teleost fish. In addition, hypoxia treatment induced expression of the short and long LEPR isoform in the gill, liver and heart (Cao et al., 2011; Wong et al., 2007) suggesting that leptin signaling pathway may play an important function in acclimation to hypoxia.

Expression of the LEPR in fishes was widespread, with the highest expression detected in the pituitary gland and ovary, moderate expression has been shown in brain, eye, heart, kidney, gill, skin, visceral adipose tissue, red muscle, liver and testis (Kurokawa et al., 2008; Ronnestad et al., 2010). These findings agree with pattern of mRNA

expression of the mammalian LEPR genes (Horev et al., 2000; Richards and Poch, 2003).

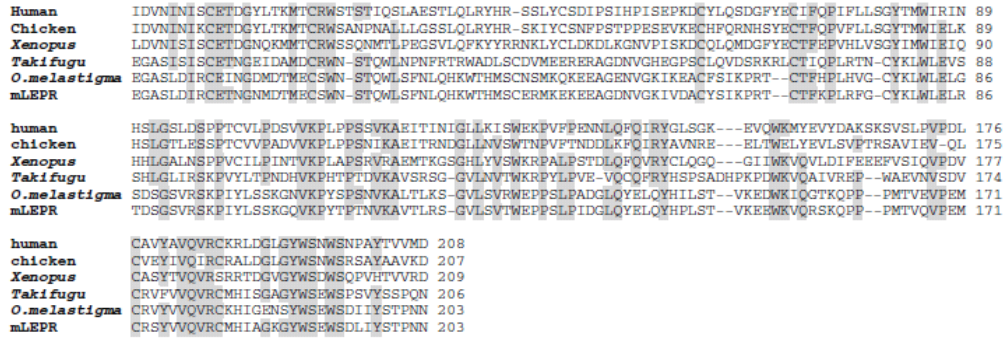


Figure 12 Comparison of the amino acid sequence of the putative leptin-binding region of LEPRs. Deletions are indicated by dashes and shaded areas indicate residues shared by >60% of all sequences. GenBank Accession Nos.: human, AAA93015; chicken, BAA94292; X. tropicalis, NP001037866; O. melastigma, ABC86922; mLEPR, AB457590 (Kurokawa and Murashita 2009).

1.6.4 *Effects of leptin administration*

To assess the role of leptin on food intake regulation in coho salmon (*Oncorhynchus kisutch*) (Baker et al., 2000), green sunfish (*Lepomis cyanellus*) (Londraville et al., 2002), goldfish (*Carassius auratus*) (Volkoff et al., 2003), and carp (*Cyprinus carpio*) (Huising et al., 2006), the injection of mammalian leptin has been performed. The results which have been obtained are unexpected compared with information about leptin in mammals. No clear effects have been found of human leptin on parameters such as growth, energy stores, gonad weight, and level of insulin, growth hormone or thyroxine (Baker et al., 2000, Londraville et al., 2002).

A number of reasons have been put forward to explain why injections of mammalian leptin in fish do not produce similar effects to those in mammals. Firstly, as discussed above, the sequence similarity between fish and mammal leptins appears to be low. Thus, mammalian leptins may not interact appropriately with the fish receptors. Secondly, it is possible that fish would be more responsive to mammalian leptin under different conditions, such as higher temperature, different seasons or developmental stages. Thirdly, it is possible that leptin has different functions in fish and mammals and

this may be related to the differences in energy metabolism between endotherms and ectotherms, where leptin's action may be slower when body temperature is lower (Londraville et al., 2002, Huising et al., 2006).

However, murine leptin injections increase intracellular fatty acid-binding protein (FABP) in green sunfish, which transports fatty acids across the cytoplasm to the site of their oxidation. The results provide evidence for a possible role of leptin in regulating lipid metabolism in fish, and that fish have receptors that recognize mammalian leptin (Londraville et al., 2002).

Unlike the situation with other fish species, leptin injection in goldfish (*Carassius auratus*) causes a reduction of food intake, in line with what has been reported in mammals showing that treatment with recombinant leptin increases energy expenditure and decreases food intake, body weight and body fat stores (Ashima et al., 2000). Moreover, fish co-injected with NPY or orexin A, orexigenic neuropeptides produced in the hypothalamus, and leptin had a food intake lower than that fish treated with NPY or orexin A alone (Volkoff et al., 2003). The result suggests an interaction between leptin, NPY and orexin A. This finding correlates with studies on mammals, where during fasting, reduced hypothalamic leptin signaling stimulates neuropeptide Y (NPY, which stimulates food intake) and inhibits corticotrophin releasing hormone (CRH, an inhibitor of food intake) production and release (Schwartz et al., 1996). Furthermore, results have been obtained, which suggest that the actions of leptin are mediated by cholecystokinin (CCK), which acts as a neurohormone to reduce food intake (Hsiao et al., 1983). These findings suggest that leptin plays an important role in the regulation of feeding and energy homeostasis in goldfish. It is not clear why the results of mammalian leptin injections in goldfish are different from those obtained from other species.

As might be expected the injection of recombinant fish leptins produces more bioactive effects in fish than mammalian leptins. For example, rainbow trout leptin suppressed food intake in rainbow trout *(Oncorhynchus mykiss)* (Murashita et al., 2008, Aguilar et al., 2010) and the same effect has been observed with recombinant carp leptin in grass carp (*Ctenopharyngodon idellus*), however food intake was reduced only on the first day after injection (Li et al., 2010). It is been demonstrated that leptin treatment decreased NPY mRNA levels in hypothalamus (Aguilar et al., 2010, Li et al., 2010).

Moreover, leptin administration increases levels in parameters involved in glucosensing (GK, PK and GSase activities; GK expression and glucose; glycogen and DHAP levels) (Aguilar et al., 2010). This anorexic effect is similar to that observed in mammals (Schwartz et al., 1996) and supports that the neuroendocrine pathways that control feeding by leptin are ancient and have been conserved through evolution.

Recombinant salmon leptin administration was performed on Atlantic salmon and showed significantly reduced growth rate and increased relative liver size (Murashita et al., 2011). This finding agrees with mammalian species, where leptin treatment reduces food intake and body weight (Mistry et al., 1997; Wetzler et al., 2004). This effect was linked to increased POMC expression (Murashita et al. 2010). These results differ from a study on coho salmon (*Oncorhynchus kisutch*), where human leptin did not cause any changes in body weight or any other physiological parameters (Baker et al., 2000).

1.6.5 *Changes in leptin expression with nutritional state*

The main site of gene expression of fish leptin is in the liver (Kurokawa et al., 2005; Huising et al., 2006a), which is different from the situation in mammals where leptin is primarily synthesised by adipose tissue (Zhang et al., 1994, Friedman et al., 1998). These different findings may be related to the fact that the liver is the major site of lipogenesis in non-mammals. This supports the observation that the liver, rather than adipose tissue, is one of the main sites of immunoreactive leptin in fish (Johnson et al., 2000). Leptin gene expression has been studied in carp liver in response to short and long-term fasting and refeeding, and in animals fed to satiation (Huising et al., 2007a) Leptin expression was not affected by fasting or refeeding, unlike the situation in mammals where fasting decreases leptin expression, and refeeding increases it (Saladin et al., 1995). NPY gene expression in the hypothalamus, which is increased by prolonged fasting in mammals (Clark et al., 1984, Levine et al., 1984) was not changed after a 6 week fast, but decreased hypothalamic gene expression of the inhibitory feeding peptide genes corticotrophin releasing factor (CRF), pro-opiomelanocortin (POMC) and thyrotrophin releasing hormone (TRH) was in line with what has been observed in mammals (Legradi et al., 1997). Similar to the effects of fasting, fish that were fed to satiation did not show increased leptin expression as might have been expected if leptin acts as a long-term satiety signal in fish. In contrast, increased leptin

expression was observed after ingestion of a single scheduled meal, providing evidence that leptin may act as a short term signal of satiety in carp. This result correlates to the postprandial increase in leptin mRNA that is observed in the hours following feeding in mice (Saladin et al., 1995) and is in agreement with the observation of an inhibitory effect on food intake following injection of recombinant mammalian leptin into the goldfish brain (Volkoff et al., 2003).

Long-term food restriction in Atlantic salmon lowered expression of the lep-A1 transcript in the fat-depositing tissues, visceral adipose tissues and white muscle but LEPR mRNA levels in brain, between fish fed reduced and full feeding regimes, did not show any difference (Ronnestad et al., 2010). Plasma concentrations of salmon leptin were also unaffected (Ronnestad et al., 2010), a result in line with a study on fasted rainbow trout in which no correlation was observed between plasma leptin and body condition (Kling et al., 2009).

Thus, leptin mRNA expression in fish is altered by changes in food intake, but there is limited evidence for involvement of leptin in the long-term regulation of food intake and energy metabolism as is the case in mammals (Huising et al., 2006a). These findings may be related to the differences in energy metabolism between endotherms and ectotherms. Ectotherms do not need to thermoregulate, thus their metabolic regulation is more flexible (Huising et al., 2006). For this reason the physiological role of leptin in ectotherms may differ quantitatively, if not qualitatively, from its role in mammals.

1.7 Leptin in non-mammalian vertebrates - amphibians

Evidence for leptin-like genes in amphibians was presented shortly after the discovery of leptin genes in fish. Leptin genes have now been described for four amphibian species: the tiger salamander (*Ambystoma tigrinum)* (Boswell et al., 2006), the axolotl (*Ambystoma mexicanum)* (Boswell et al., 2006), *Xenopus tropicalis* (Crespi and Denver, 2006), and *Xenopus laevis* (Crespi and Denver, 2006).

1.7.1 *Identification of leptin-like genes*

Leptin-like genes were identified in amphibians as a result of the availability of new genomic information in the form of genome sequence and EST libraries. First, a leptin-like gene found in tiger salamander (*Ambystoma tigrinum*) and axolotl (*Ambystoma mexicanum*) EST libraries was characterised (Boswell et al., 2006). Later, the coding region of the frog (*Xenopus laevis and Xenopus tropicalis)* leptin gene was reported (Crespi and Denver, 2006). Sequence identity between the salamander and *Xenopus* leptin-like genes is 66% at the nucleotide level over the coding sequence (Boswell et al., 2006).The amphibian leptin genes share less than 35% amino acid identity to mammalian leptins. However, several lines of evidence suggest that they represent the amphibian orthologues of mammalian leptin genes. Firstly, the mature peptide contains an identical number of amino acids to mammalian leptins (Boswell et al., 2006, Crespi and Denver, 2006). Secondly, the reported leptin genes have three exons and two introns, with the coding sequence in exons 2 and 3, similar to the genomic structure of mammalian leptin genes (Chmurzynska et al., 2003). Thirdly, analysis of the rate of non-synonymous substitutions in amphibian, mammal and fish leptin coding sequences was performed and followed the consensus pattern of vertebrate evolutionary divergence (Figure 13). Fourthly, comparison between the predicted tertiary structure of amphibian and human leptin has shown a conservation of the four-helix bundle structure, and the two cysteine residues required for formation of a disulfide bond have been conserved in all tetrapod species. Also, conserved leucines were found in salamander leptin, residues that are important for the formation of α-helices (Zhang et al., 1997; Boswell et al., 2006). Recombinant *Xenopus* leptin activated both *Xenopus* and mouse leptin receptors *in vitro* (Crespi and Denver, 2006).

Different expression patterns were identified in salamanders and Xenopus. In salamanders, the highest expression was observed in the skin, with lower expression also being seen in the brain, stomach, small intestine, colon, skeletal muscle, tongue, and fat body (Boswell et al., 2006). The strongest intensity of the PCR product in the frog was detected in the brain and heart, but also in liver, brain, pituitary gland, gastrointestinal tract, lungs, kidney, and gonads (Crespi and Denver, 2006). The tissue distribution of leptin mRNA is broader in amphibians compared with mammals, where expression was primarily observed in fat (Zhang et al., 1994). The wider range of expression in amphibians suggests that leptin may have a wider range of functions. In

Xenopus leptin is expressed throughout embryogenesis and tadpole development (Crespi and Denver, 2006). The expression of leptin mRNA was detected in frog oocytes and embryos before feeding stages and adipose tissue formation. The research suggests that this hormone may have adipocyte-independent roles in early development (Crespi and Denver, 2006). This finding agrees with the reports on mammals where widespread expression of leptin and its receptors has been identified in fetal tissues (Masuzaki *et al*. 1997, Hoggard *et al*. 1997).

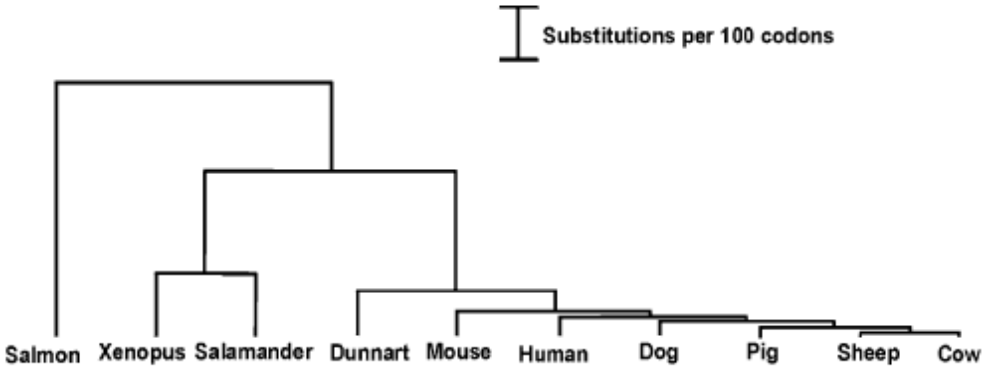


Figure 13 Phylogenetic tree constructed using non-synonymous substitutions for salamander and mammalian leptins. Trees were created by Growtree (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, Wisconsin, USA) with the unweighted pair group method using arithmetic averages. Sequences used were human (NM_000230), sheep (U84247), fat-tailed dunnart (AF067726), mouse (U18812), dog (AB020986), pig (AF052681), *Xenopus laevis* (AY884210), salmon (BI468126), and cow (U43943) (Boswell et al., 2006).

1.7.1 *Leptin receptor*

A full-length LEPR gene has been reported in *Xenopus tropicalis* (Crespi and Denver, 2006). The predicted gene consists of 26 exons and encodes 1,145 amino acids which corresponds to the exon structure and size of the receptor in mammals (Tartaglia et al., 1995). This frog LEPR protein shares only 37% amino acid sequence identity with human, however, sequence similarity is greatest in and around the ligand binding domain, transmembrane region, and the intracellular C-terminal region. Despite the low amino acid sequence identity phylogenetic analysis clearly placed the frog gene within a clade of the mammalian leptin receptor gene. A strong expression of LEPR mRNA was found in the brain, as is the case in mammals (Tartaglia et al., 1995). Expression of the

LEPR in *Xenopus* was widespread, with the highest expression detected in the pituitary gland, and strong levels of expression also observed in the skin, muscle, and testis. Lower but detectable levels of expression were found in different visceral organs and the ovary (Crespi and Denver, 2006). These findings correspond to the observations of widespread tissue distribution of leptin mRNA in *Xenopus*, indicating a broad range of leptin functions in amphibians.

1.7.2 *Effects of leptin administration*

To investigate the possible function of leptin in amphibians, injections of recombinant *Xenopus* leptin have been performed. Inhibitory effects on appetite were observed after central leptin injections in midprometamorphic tadpoles and juvenile frogs (Crespi and Denver, 2006). This result shows that leptin has anorectic activity in an ectotherm, which correlates with the results of leptin injection in mammals, where leptin rapidly lowers food intake (Mistry et al., 1997). Food-deprived frogs injected with leptin lost more weight than controls in the time periods examined, suggesting that leptin also increases energy expenditure as it does in mammals (Zhang et al., 1994). Thus, the function of leptin as a regulator of food intake and energy balance appears to have evolved before the emergence of amniote vertebrates. However no information is available on the effects of nutritional state on leptin gene expression in amphibians.

The LEPR is expressed in the hind limb of early prometamorphic tadpoles and lung. Injection of recombinant frog leptin induced hind limb growth (Crespi and Denver, 2006) and lung development (Torday et al., 2009). This finding suggests that leptin may have adipocyte-independent roles in early growth. The result agrees with evidence that leptin plays a role in mammalian limb (Hoggard et al., 2001) and lung (Torday and Rehan 2002; Torday et al. 2002) development.

1.8 The axolotl as a model amphibian

This thesis is focused on studying the biology of leptin in amphibians using the axolotl as a model species. Axolotls are well known neotonic amphibians, native only to Lake Xochimilco and Lake Chalco in central Mexico. The animal is a member of the Order Caudata which includes newts and salamanders. The closest relative of the axolotl is the tiger salamander. The scientific name of the axolotl, *Ambystoma mexicanum* was given by Shaw in 1798 (Shaw, 1798) and was described for the first time in 1828 by Cuvier, as the larva of an unknown salamander (Cuvier, 1828). In 1863, 34 axolotls were shipped to the Natural History Museum in Paris by General Forey of the French Expeditionary Forces in Mexico (Dumeril, 1870). Only 7 (6 black and 1 white axolotl) individuals were donated to Auguste Dumeril for scientific study, from which thousands of axolotls were bred and sent all over Europe (Dumeril, 1872). All the captive axolotls in the world were derived from those very animals imported to Paris (Smith, Armstrong, and Malacinski, 1989).

Axolotls are excellent experimental animals as they are easy to breed in captivity, inexpensive to feed, very hardy and moderately large. The animals are easily induced to reproduce compared to other salamanders in their family, which are almost never captive bred axolotls reach lengths up to 30 cm (12 inches), on average of 20 cm (9 inches) in length. The average mass of an adult animal is 85g (Brunst, 1955a). Axolotls play an important role in developmental studies (Bordzilovskaya, 1975) due to their amazing regenerative capabilities and large embryos, and because their large cells, are often used in histological studies (Brunst, 1955a; Smith, Armstrong, and Malacinski, 1989). The axolotl limb is used as a model of tissue and organ regeneration. Amputation anywhere between the shoulder and the hand induces the formation of a mass of dedifferentiated proliferating cells called the blastema that regenerates the missing limb, which becomes fully functional (Brockes, 1997).

Axolotls are also used in heart defect studies due to the presence of a mutant gene that causes heart failure in embryos. Since the embryos survive almost to hatching with no heart function, the defect is very observable (Trottier and Armstrong, 1977; Kulikowski and Manasek, 1977). Another attractive feature for research is the presence of several

colour morphs. Axolotls have four different colours, two naturally occurring colours (dark brownish-green - wildtype and black - melanoid) and two mutants (pale pink with black eyes - leucistic and golden, tan or pale pink with pink eyes - albino) (Dunson, 1974, Frost et al., 1984 and 1986) (Figure 14).

Figure 14 Axolotl colour morphs (www.caudata.org)

The axolotl is a neotonic amphibian, which means that it never undergoes metamorphosis, so the adult remains aquatic and gilled. The animal retains larval characteristics in the reproductive mature adult form. Neoteny appears to be maintained by a lack of thyroid stimulating hormone (TSH), which induces the thyroid to produce thyroxine (Prahlad and Delanney, 1965). In the axolotl, metamorphosis can be triggered under laboratory conditions via thyroid hormone injections like T3 (3, 3', 5'-triiodothyronine) (Swanberg and Norris, 1972, Norris and Platt, 1974), T4 (thyroxine) (Norris and Platt 1974), or TSH Norris et al., 1973, Taurog, 1974). The levels of those hormones are low in neotonic animals and drastically increase during metamorphosis (Platt, 1976). Tiger salamanders are able to undergo metamorphosis only in warm lakes, which suggests that some species can metamorphose naturally, depending on environmental and endocrinological conditions (Jenkin, 1970).

More recently, axolotls have been used in a variety of biological studies including cardiogenesis (Zhang et al., 2004, Denz et al., 2004, Cano-Martinez et al., 2010), embryogenesis (Bachvarova et al., 2004, Pelczar et al., 2010), genomics (Smith et al., 2009), thyroid hormone (TH) distribution (Page et al., 2007), TH induced metamorphosis (Page et al., 2008; Page et al., 2009), sex determination (Park et al., 2004; Smith and Voss, 2009) and organ and tissue regeneration (Schnapp and Tanaka, 2005, Monaghan et al., 2007, Menger et al., 2010, Satoh et al., 2010). In terms of

genomics resources, an integrated web portal ‘Sal-Site’ is available (http://www.ambystoma.org) to provide new tools for studying axolotls and tiger salamanders. It hosts the salamander genome project, the *Ambystoma* EST database and gene collection, and the *Ambystoma* map and marker collection (Smith et al., 2005).

1.9 Conclusions

Research arising from the discovery of leptin (Zhang et al., 1994) and experiments on leptin administration performed soon after that (Campfield et al., 1995; Pelleymounter et al., 1995; Halaas et al., 1995; Stephens et al., 1995) emphasised the role of leptin in mammals as an adiposity signal that circulates in the blood in proportion to energy stored as fat and acts in the brain to regulate energy balance. It had a major impact on obesity research as the neural circuits in the hypothalamus through which leptin acts to influence energy balance were identified (Sawchenko 1998). In particular, leptin's effects on body weight are mediated through effects on two separate populations of arcuate nucleus neurones that express the LEPR. One group expresses NPY and agouti related protein AgRP to increase food intake, and the other group expresses CART and POMC (from which alpha-MSH is derived), to reduce food intake (Lin et al., 2000). It became clear that the main role of these pathways is to coordinate responses to energy deprivation or restriction signalled by falling concentrations of plasma leptin (Baskin et al., 1999). Falling leptin concentrations therefore appear to be more important as a physiological signal than rising concentrations associated with obesity because they are linked to threats to survival such as starvation. In contrast, it became clear that high leptin concentrations in obese patients are linked to leptin resistance, reducing the possible effectiveness of leptin as an obesity treatment (Thorburn et al., 2000; Sinha and Caro 1998; Lee et al., 2002). Similarly, research on seasonal fattening cycles in mammals indicated that although leptin seems to coordinate short-term regulation of energy balance, seasonal obesity and leanness are not driven by changes in leptin production, and that seasonal fattening is associated with high leptin concentrations as a result of leptin resistance (Rousseau et al., 2003).

The discovery of leptin in mammals also stimulated research into its physiological functions other than the regulation of energy balance. It has been demonstrated that

leptin regulates other physiological systems like reproduction (Karlsson et al., 1997; Caprio et al., 1999; Kitawaki et al., 2000; Hoggard et al., 1997; Ludwig et al., 2000; Mounzih et al., 1998), immune system (Grunfeld et al., 1996; Sarraf et al., 1997; Bennet et al., 1996; Faggioni et al., 1998; Otero et al., 2006; Lord et al., 1998), cardiovascular system (Rahmouni et al., 2003; Bouloumié et al., 1998, Fukuda et al., 2003) or development (Schubring et al., 1996; Steppan et al., 2000; Felson et al., 1993) and that the effects on these systems that leptin exerts are largely consistent with an overall action of leptin as a signal of nutritional state. Examples of positive selection on the leptin gene in mammals associated with the development of novel functions are rare. Changes in normally conserved regions of leptin have been linked in seals to a role for leptin in the stretch-induced surfactant production pathway, and leptin production in the lungs (Hammond et al., 2005). Molecular cloning and expression of leptin in gray and harbor seal blubber, bone marrow, and lung and its potential role in marine mammal respiratory physiology (Hammond et al., 2005). However, leptin was also expressed in seal blubber, suggesting that its role as an adipose tissue signal has been retained.

For the decade following leptin's discovery, leptin genes had only been unequivocally identified in mammals, raising the possibility that the hormone was specific to this vertebrate group. The fact that leptin has an ancient evolutionary history was only revealed with the discovery of leptin-like genes in fish and amphibian genome databases (Kurokawa et al., 2005). Although cytokine signalling is present in invertebrates, leptin signalling appears to have arisen only after the divergence of urochordates and vertebrates (Liongue and Ward, 2007). While structural motifs have been conserved between fish, amphibian and mammalian leptins, the overall sequence similarity between fish and mammalian leptins can be less than 20%, raising the possibility that the functions of the hormone have changed during evolution.

To assess the idea that the role of leptin on food intake regulation has been conserved in different vertebrate groups, leptin administration experiments (often using mammalian leptin, but in some cases non-mammalian leptin) have been performed (Ashima et al., 2000; Murashita et al., 2008; Aguilar et al., 2010; Niewiarowski et al., 2000; Li et al., 2010). In most of the cases, treatment with recombinant leptin increases energy expenditure and decreases food intake, body weight and body fat stores (Ashima et al., 2000; Murashita et al., 2008; Aguilar et al., 2010), implying conserved function during evolutionary history. However in some studies, the effect of leptin administration was

only temporary (Li et al., 2010; Niewiarowski et al., 2000) suggesting that although a general regulatory effect on energy balance has been conserved, the specific effects of leptin may differ between vertebrate groups. It is possible that leptin has been deleted from the avian genome, perhaps related to alterations in metabolism linked to the extreme aerobic requirements of flight. In ectotherms such as fish, the regulation of leptin expression by long term food restriction or deprivation appears different from mammals, and may be related to metabolic regulation being more flexible because they do not have to thermoregulate (Huising et al., 2006).

Most of the research on leptin in non-mammalian vertebrates is being performed in fish. Research on amphibians is very limited by comparison (Boswell et al., 2006; Crespi and Denver, 2006). The studies have been performed on two species (*Ambystoma tigrinum; Xenopus tropicalis*) and the cDNAs identified share only 67% identity at the amino acid level (Crespi and Denver, 2006). This suggests that even within the same vertebrate group, leptin may have different functions due to genetic drift. More information is currently available for *Xenopus* than for the axolotl and it is the aim of this thesis to address this by extending investigation into the functions of leptin in salamanders. It is also the case that the effects of nutritional manipulation on leptin gene expression have not yet been investigated in any amphibian. This thesis will provide a new opportunity to investigate the functions of the leptin signalling system in these early tetrapod vertebrates, providing insight into the functional development of the system during vertebrate evolution.

1.9.1 *Aims and Objectives*

The aim of this thesis was to assess the structure and functions of leptin and LEPR in the axolotl. The project involved cloning the axolotl leptin and leptin receptor using information based on the sequence of the *Ambystoma mexicanum* leptin and the LEPR sequences from different species. The next step was to establish the pattern of tissue expression of leptin and its receptor in the adult and during development to extend investigation into the leptin signalling pathway in salamanders.

In order to understand the possible role(s) of leptin in the regulation of food intake and energy metabolism in amphibians, leptin and LEPR gene expression was studied in response to short-term restriced feeding and feeding to satiation.

Identification of amphibian leptin provides a scientific basis to clarify leptin's molecular and functional evolution.

Chapter 2. Materials and Generic Methods

2.1 Animals and tissues

All animals were obtained from a captive breeding colony at the University of Birmingham, United Kingdom. They were maintained in shallow dechlorinated tap water in plastic boxes and were fed daily with chopped liver. The animals were humanely killed before dissection by immersion in 0.1% tricaine methane-sulfonate (ethyl 3-aminobenzoate methane-sulfonate) anaesthetic (Sigma, Poole, Dorset, UK), in accordance with the United Kingdom Home Office Code of Practice. The three groups of animals used for tissue distribution studies were males and females of 6 months, 10 months and 3 years old. Body mass and length (snout to vent) was routinely recorded at the time of dissection (Table 2). Food was present in the gastrointestinal tract of all individuals at the time of dissection indicating that the animals were in a fed state. Following dissection, tissues were immediately incubated in RNAlater (Ambion, Applied Biosystems, Warrington, Cheshire, UK) at 4˚C overnight and were then frozen and stored at -80˚C.

Several axolotl embryonic and post-embryonic (hatched larvae, 44) stages were collected and stored in 1ml RNAlater (Ambion) at -80ºC until further processing. Axolotl embryonic development is divided into 44 stages based on external morphology and on features visible in dissected embryos (Harrison, 69; Bordzilovskaya, 89).

A

Adults	**1** male	**2** male	**3** male	**4** Male	**5** female	**6** female	**7** Male
Body mass (g)	92.6	113.3	101	116	108.2	103	93
Length (cm)	13	14	14	15	13.5	14	13

B

6-month old	**1**	**2**	**3**	**4**	**5**	**6**
Body mass (g)	13.2	15.5	16	16	24.9	9.5
Length (cm)	6.5	7	6.5	7	7.5	5.5

C

10-month old	**1** female	**2** male	**3** Female	**4** male	**5** Male
Body mass (g)	27.4	27.8	21.5	28	15.5
Length (cm)	9	9	7.5	8	8

Table 2 Animals used for tissue distribution studies including three age groups (6 months, 10 months and 3 years old). Body mass and length (snout to vent) was recorded at the time of dissection. (According to the guidelines for the format of theses, numbers and captions should appear at the bottom of the table/figure).

2.2 Nucleic acid extraction

Dissected tissues were stored in 1ml RNAlater (Ambion) at -80ºC until further processing. Total RNA was isolated from dissected tissues using TRI reagent according to the manufacturer's instruction (Helena, Gateshead, UK). Individual tissues were homogenised in TRI reagent in Lysing Matrix D using a FastPrep Instrument (Qbiogene, Bingham, Nottingham, UK) for 2x 30 sec at high intensity (5.5 m/s).

Isolated total RNA was treated with DNase I (1U) (Amplification Grade Deoxyribonuclease I; Invitrogen, Paisley, UK or Promega, Southampton, UK) for 30 min at 37ºC and inactivated by incubation at 75ºC for 10 min for digestion of the DNA. Visualization on a formaldehyde gel (2.9.1) showed that the RNA was not degraded.

Poly (A+) RNA was isolated using an Oligotex mRNA Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

Genomic DNA for PCR was isolated from dissected ovaries using TRI reagent according to the manufacturer's instructions (Helena, Gateshead, UK). The tissue was homogenised using a Fastprep Instument for 1x 40 sec at high intensity (4.5 m/s).

DNA and RNA concentration was determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Scientific, Loughborough, UK).

2.3 cDNA synthesis

First-strand cDNA was synthesized in 20 µl reactions with 2 µg of total RNA as template, using a SuperScript II synthesis kit (Invitrogen) and Oligo d(T)18 primer (Invitrogen). Reverse transcription was performed in 20 µl of solution containing 2 µg of total RNA, 1 µl of the oligo dT primer (500 µg/ml), 1 µl of dNTP mix (10 µM final concentration), 4 µl of 5x First-Strand Buffer, 2 µl of DTT (0.1 M) and 1 µl (50 U/µl) of SuperScript II Reverse Transcriptase and the mixture was incubated at 42 °C for 50 min. The reaction was stopped by raising the temperature to 70 °C for 15 min. First-strand cDNAs were diluted 5-fold with water and stored at -80ºC until further analysis by semiquantitative RT PCR or quantitative PCR.

2.4 PCR

PCR was performed using FastStart *Taq* polymerase (Roche Diagnostics, Lewes, East Sussex, UK) on a Gene Amp PCR system 9700 thermocycler block (Applied Biosystems). The reactions were carried out in 25 µl of solution containing 2.5 µl of 10x PCR reaction buffer, 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP (10 mM), 0.5 µl of

each primer (100 µM) and 0.2 µl of FastStart *Taq* polymerase (5 U/ µl).Primers and specific reaction conditions used for leptin, leptin receptor and cyclophilin are described below. Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/) and were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

2.5 Agarose gel electrophoresis

To prepare 100 ml of a 2% agarose gel, 2 g of agarose was added to 100 ml of 1xTAE buffer (50x TAE buffer: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA pH 8.0). The agarose was dissolved by boiling in a microwave oven. The solution was cooled to 60°C and 5 µl of ethidium bromide (10 mg/ml) was added. The gel was poured into the gel tray and left for 1h at room temperature to set. The samples, containing 6x loading buffer (Fermentas, York, UK), were then loaded, and the gel was run in 1xTAE buffer at 80 mV for 50 min. The DNA samples were visualized on a UV transiluminator and an image captured using Gel Doc Software Image Analysis (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

2.6 Molecular cloning

2.6.1 *Leptin*

To clone axolotl leptin, two sets of PCR primers were designed based on a Tiger salamander (*Ambystoma tigrinum*) leptin-like EST deposited in GenBank (Accession No. CN054256). The primers used were: forward primers 5’ATCCCAACCTTCCACTGTC, SalLep1 (positions 90–108 of Accession No.CN054256), 5’TCTTCTCCCGATGAACCTGAA, AxLep1 (positions 184-203 of Accession No. CO792338); and reverse primers 5’ACCTATCCAACGCAACTTTC, SalLep2 (positions 479–498 of Accession No.CN054256), 5’TTTGTGGGAGTTGGACACAA, AxLep2 (positions 594-613 of Accession No. CO792338) yielding reaction products: 409 bp, 425 bp, respectively.

PCR for leptin cDNA was performed (as described above), with an annealing temperature of 60°C and denaturing and extension steps of 94°C and 72°C, respectively. Times used were 15 sec denaturation, 30 sec annealing, and 30 sec extension, with an extension time for the final cycle of 7 min. PCR was carried out for 30 cycles. PCR amplification products were separated by electrophoresis on a 2% agarose gel, whereupon specific bands were excised and purified by the QIAquick gel extraction kit (QIAgen GmbH, Germany). Purified PCR products were cloned into pSC-A-amp/kan with subsequent transformation in StrataClone SoloPack competent cells according to the manufacturer's instructions (StrataClone PCR cloning kit, Stratagene, Agilent Technologies, Stockport, Cheshire, UK). Positive white colonies were cultured and plasmid DNA was extracted using a PureLink Quick Plasmid Miniprep kit (Invitrogen). The insert was sequenced from 6 clones in both directions using M13F and M13R, respectively.

Genomic DNA (300 ng) was also used as a template to amplify the axolotl leptin gene using FastStart *Taq* polymerase. The primers used were the same as for leptin cDNA amplification. The denaturation, annealing, and extension temperatures and times were 94°C, 15 sec; 60°C, 30 sec; and 72°C, 3:30 min; with the final extension step of 7min. PCR was carried out for 30 cycles. Visualization of PCR amplification product was shown on 1% agarose gel.

2.6.2 *Leptin receptor*

For analysis of tissue distribution of gene expression, PCR primers were designed from the cloned cDNA fragment of the axolotl leptin receptor (Accession No. GU562414). The primers used were: forward primer 5'-TCTCTTGTGAAACCGATGGA, AxLEPR1 (positions 6-25 of Accession No.GU562414); and reverse primer 5'-GCAACGAGCAGTCTTTTGATT, AxLEPR2 (positions 156-176) yielding a reaction product of 171bp. PCR was carried out as described above with an annealing temperature of 56°C and denaturing and extension steps of 94°C and 72°C, respectively. Times used were 15 sec denaturation, 30 sec annealing, and 30 sec extension, with an extension time for the final cycle of 7 min. PCR was carried out for 30 cycles. Amplification products were separated on a 2% agarose gel and visualized using ethidium bromide staining.

PCR was also used to amplify a fragment of axolotl leptin receptor gene using FastStart *Taq* polymerase and 300 ng genomic DNA as template. The primers used were the same as for LEPR cDNA amplification. The denaturation, annealing, and extension temperatures and times were 94°C, 15 sec, 54°C, 30 sec; and 72°C, 1:30 min; with the final extension step of 7 min. PCR was carried out for 30 cycles. Visualization of PCR amplification product was shown on 1.5% agarose gel.

2.6.3 *Cyclophilin*

To confirm the effectiveness of reverse transcription, PCR was also carried out to amplify a cDNA fragment of cyclophilin A. Primers were designed from an axolotl (*Ambystoma mexicanum*) EST (Accession No. BI818006). The forward primer was 5'-CTTCACAAACCACAATGGAAC, AxCycF1 (positions 212–232 of BI818006) and the reverse primer was 5'-ACAGATGAAAAACTGGGAGC, AxCycR1 (positions 340–359), yielding a reaction product of 148 bp.

PCR for cyclophilin A cDNAs was performed as described above with an annealing temperature of 60°C and denaturing and extension steps of 94°C and 72°C, respectively. Times used were 15 sec denaturation, 30 sec annealing, and 30 sec extension, with an extension time for the final cycle of 7 min. PCR was carried out for 30 cycles.

2.7 RTqPCR analysis

Quantitative real-time PCR assay was established for relative quantification of axolotl leptin and its receptor. Melting curve analysis, gel electrophoresis and sequencing assessed the identity of the products. RNA isolation using the Tri reagent resulted in high quality total RNA with A260/A280 ratios between 1.80 and 2.10. Total RNA isolated from 12 different tissues and was reverse-transcribed into cDNA for PCR analysis.

The assays for leptin, leptin receptor and cyclophilin were set up using the primers described above. All primers had Tms between 58ºC and 63.2ºC, and a GC content between 43 and 53% (Table 3).

Primers	Sequence	Tm (ºC)	GC (%)	Product size (bp)
Leptin F	5'ATCCCAACCTTCCACTGTC	58	53	409
Leptin R	5'ACCTATCCAACGCAACTTTC	58	45	
LEPR F	5'TCTCTTGTGAAACCGATGGA	63.2	45	171
LEPR R	5'GCAACGAGCAGTCTTTTGATT	63.2	43	
Cyclophilin F	5'CTTCACAAACCACAATGGAAC	49	43	148
Cyclophilin R	5'ACAGATGAAAAACTGGGAGC	58	45	

Table 3 Primers used for RTqPCR analysis

RTqPCR reactions were performed in a total volume of 25 µl, containing 2 µl of diluted cDNA template (1:5), 12.5 µl of SYBR greenER qPCR SuperMix universal (including Taq polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, and dNTP mix (with dUTP instead of dTTP); Invitrogen), 0.5 µl of forward and 0.5 µl of reverse primers (10 µM each). The PCR reaction was carried out on a PTC-200 Peltier Thermal Cycler Chromo 4 (MJ Research/Bio-Rad Laboratories, Hemel Hempstead, Herts, UK) with the following parameters: 1 cycle of 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for leptin and cyclophilin or 56°C for leptin receptor for 30 sec.

Specificity of RT-PCR products was confirmed with gel electrophoresis and resulted in a single product with the predicted length (leptin - 409 bp; LEPR - 171 bp; cyclophilin - 148 bp).

Fluorescent reading at high temperature at the end of the fourth PCR segment (just below the melting temperatures of our amplicons) melts unspecific PCR products (e.g., primer dimers) and assures quantification of specific product only (Pfaffl, 2001). Directly after the PCR, the machine performed a melting curve analysis by slowly (0.2 _C/s) increasing the temperature from 60 to 95ºC, with a continuous registration of changes in fluorescent emission intensity. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm). The

melting curve was read every 0.2 seconds and was held every 0.01 second. The absence of nonspecific products was indicated by observation of a single melting peak in melting curve (leptin 83°C; LEPR 78°C; cyclophilin 79°C) analysis by measuring the fluorescence intensity across the temperature interval from 60°C to 95 °C (Figure 15).

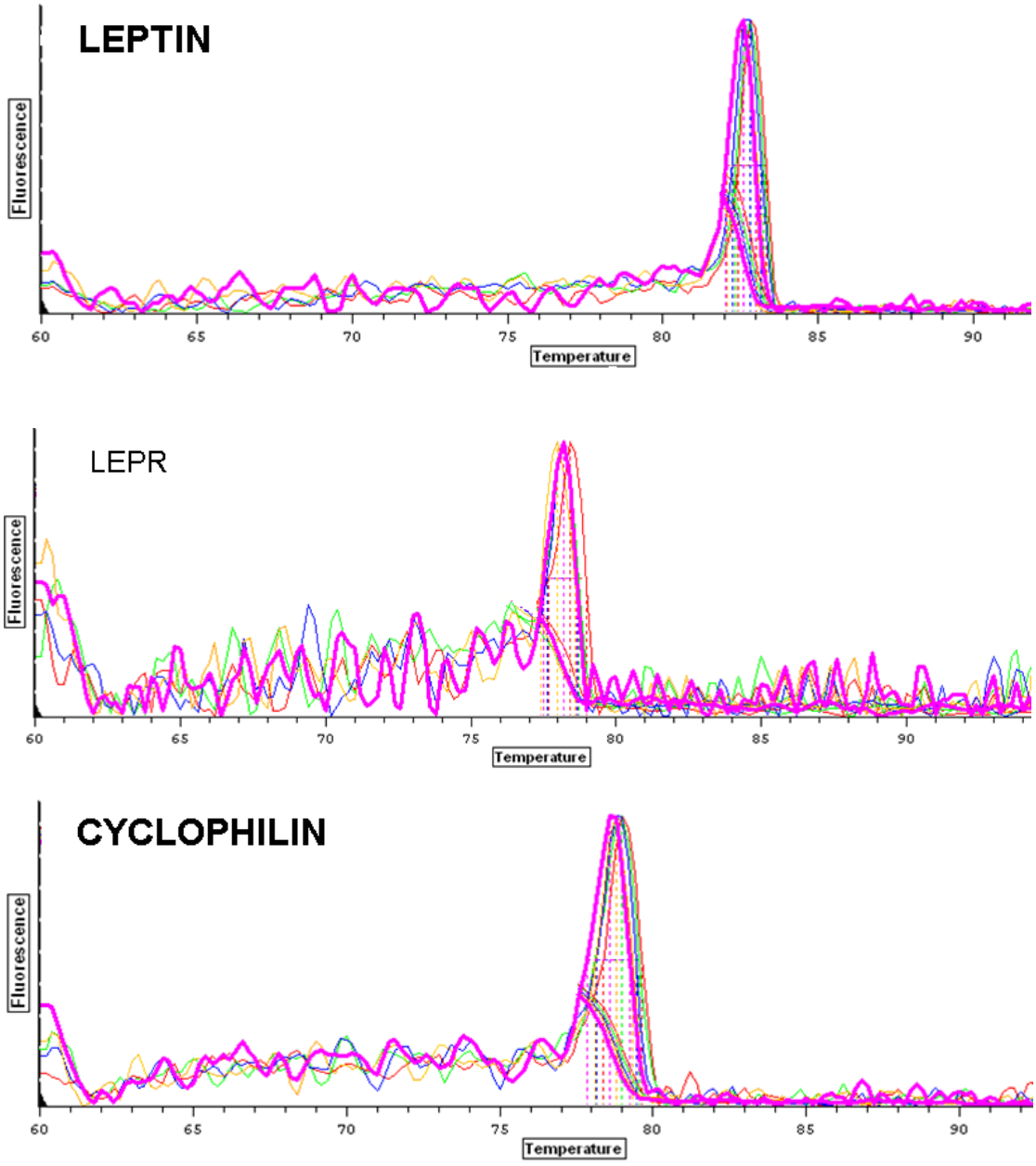


Figure 15 Melting curve analysis of the qrtRT-PCR amplification products using primers for leptin, leptin receptor and cyclophilin (Opticon Monitor software, Promega).

For each gene, a minimum of 5 independent replicates were carried out for statistical confidence, and the median of these values along with the standard error was calculated.

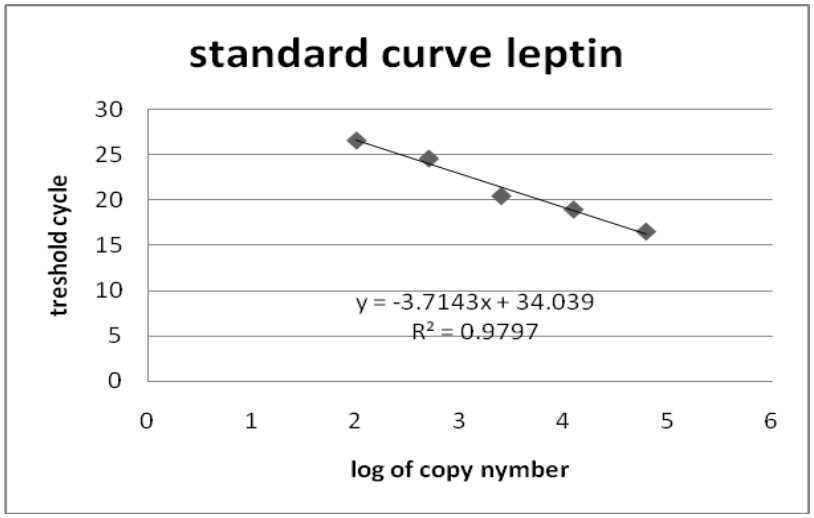
The samples were analysed and compared to axolotl cyclophilin as a reference gene, which gave similar results on the relative expression of leptin receptor, indicating that cyclophilin can be reliably used as a reference gene in the studies. A graph was plotted using the ratio values obtained and standard deviation of these values were calculated and shown as error bars in the graph.

Serial dilutions of plasmid DNA containing leptin and leptin receptor and cyclophilin inserts (target and reference genes), respectively, were made to assess PCR efficiency and which dilutions to use for the unknown samples. Calculation of PCR efficiency (E) was based on the slope of the relationship between log input cDNA vs the threshold cycle (Ct is defined as the point where the fluorescence increases above a background threshold level, which was determined as the second derivative maximum): $E=10^{[-\frac{1}{slope}]}$.

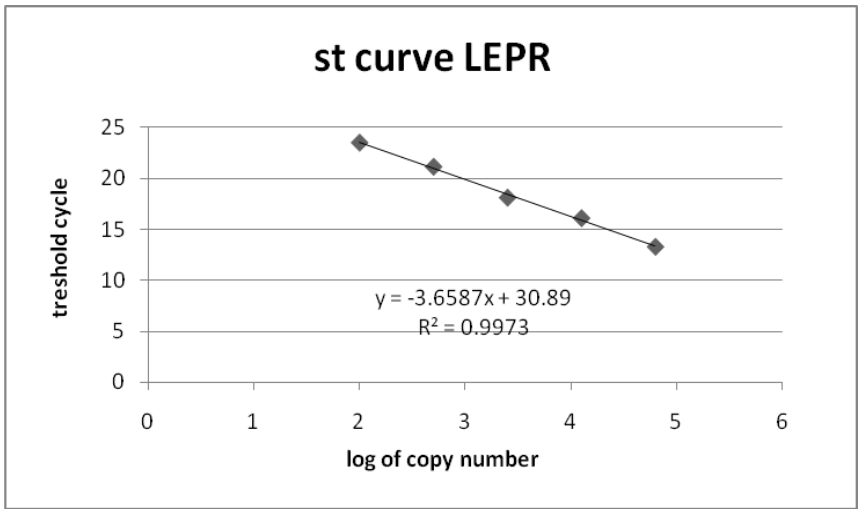
The Ct for leptin usually lay between 22 and 40, for LEPR 24 and 40, while those of cyclophilin were between 11 and 22. Using equal amounts of cDNA for the qRT-PCR, neither cyclophilin nor leptin and LEPR varied between samples taken from different animals.

To calculate PCR efficiency log [cDNA] serial dilutions vs Ct were plotted and gave $R^2 > 0.97$ (Figure 16). Investigated transcripts showed high real-time PCR efficiency rates: for leptin - 85.88%; LEPR - 91.64% and for cyclophilin - 98.27%.

A



B



C

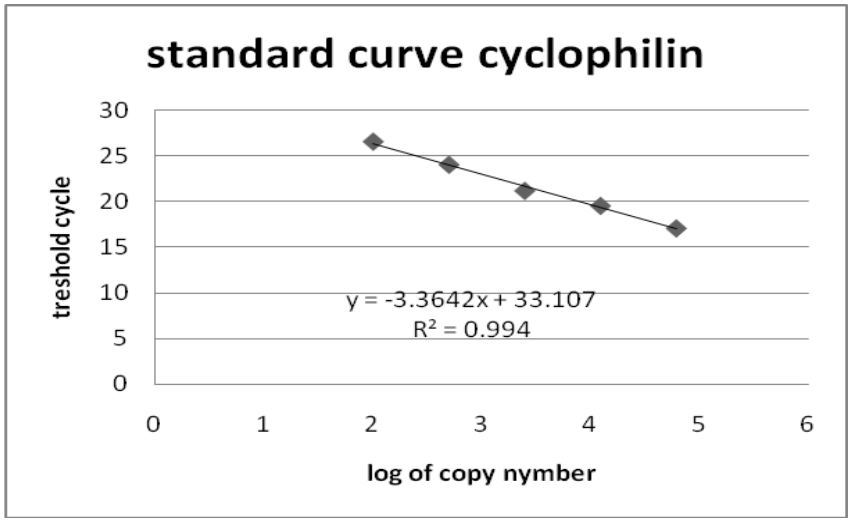


Figure 16 QrtRT-PCR dilution curves. Standard curve based on dilutions of leptin (A), LEPR (B) and cyclophilin (C) gene inserted into the pSC-A-amp/kan vector.

To quantify relative expression levels an efficiency-corrected relative expression method (Pfaffl, 2001; Roche Applied Science, 2001) was used:

$$Relative\ expression = \frac{E_{target}^{\Delta Ct(control-sample)}}{E_{reference}^{\Delta Ct(control-sample)}}$$

Each assay included no-template-controls (substituting cDNA with water or RNA) for each primer pair to confirm that reagents were not contaminated.

Data are expressed as means ± standard error of mean (SEM). The significance level was set at $p < 0.05$.

2.8 Statistics

The results obtained from real-time PCR for tissue expression were analysed by one-way and two-way Analysis of Variance (ANOVA) using SPSS software. Data were tested for normality and were log-transformed before analysis. Post hoc analysis was performed using Fisher's Least Protected Difference test. The level of statistical significance was set at $p<0.05$.

2.9 Northern blot

2.9.1 *Agarose gel containing 2.2 M formaldehyde*

To prepare 100 ml of a 1% agarose gel containing 2.2 M formaldehyde, 1 g of agarose was added to 72 ml of sterile water. The agarose was dissolved by boiling in a microwave oven. Subsequently, 10 ml of 10xMOPS electrophoresis buffer (10x MOPS: 0.4 M MOPS (pH 7.0) (3-[N-morpholino]propanesulfonic acid, 0.1 M sodium acetate, 0.01 M EDTA (pH 8.0)), 18 ml of formaldehyde (12.3 M) and 3 µl of ethidium bromide (10 mg/ml) were added to the agarose.

2.9.2 *RNA sample*

To each sample amount of poly(A^+) RNA, 2x RNA loading dye (Fermantas, R0641) was added and incubated for 10 min at 65^0C to denature RNA. The RNA samples and ready-to-use high range RNA ladder (Fermantas, SM0423) were loaded into the wells of the submerged gel, which was run in 1xMOPS electrophoresis buffer at 70 mV until the bromophenol blue had migrated ~5cm.

2.9.3 *Transfer to positive charged nylon membrane at alkaline pH*

The RNA was then transferred to a hybridisation membrane cut to the size of the gel. At this stage a corner of the membrane was cut to assist later in orientation. Transfer was carried out by vacuum blotting. The vacuum blotting apparatus was assembled during

the gel electrophoresis step as follows. A sheet of Whatman 3MM filter paper 5cm larger than the gel was cut and pre-wet in distilled water and placed on to the screen. The positively charged nylon membrane was cut to a slightly larger size than the gel, pre-wet in distilled water and positioned on the filter paper. RNA was transferred from the gel in 5x SSC with 10 mM NaOH (20x SSC: 175 g sodium chloride, 88 g trisodium citrate, pH 7.0 (HCl), H_2O to 1 litre). The pre-wet rubber mask was placed over the membrane. The rubber mask had a template cut such that the window is 5mm smaller than the gel. The gel was transferred in a position such that the opening in the rubber mask was in contact with the membrane. A suitable vacuum was applied. The transferred buffer (5x SSC with 10 mM NaOH.) was poured on to the surface of the gel. The transfer was carried out for 4 h after which fixing of the RNA was carried out by UV crosslinking (GS Gene Linker UV chamber,Bio-Rad, 150 mJ/cm2). Membranes not used immediately were stored between sheets of Whatman MM paper in sealed plastic bags at 4^0C.

2.9.4 *Hybridisation*

Up to 9 µg of extracted poly(A^+) RNA from axolotl testis was denatured at 65°C for 10 min and was then separated on a 1% agarose gel containing: 1x MOPS (10x MOPS = 0.4 M 3-(N-morpholino) propanesulfonic acid, 0.1 M sodium acetate, 0.01 M disodium EDTA, pH 7.0) and 2.2 M formaldehyde). Ethidium bromide (0.01 mg) was added to the gel and 1xMOPS running buffer. RNA transcript size was determined using a RNA size marker (Fermentas, York, UK) After electrophoresis, the RNA was transferred onto a positively charged nylon membrane (Roche, Burgess Hill, West Sussex, UK) by vacuum blotting with 5x SSC, 10 mM NaOH transfer buffer for 6 h and crosslinked (150 mJ/cm^2, GS Gene Linker UV chamber, Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Prehybridization was carried out by incubating the membrane in 10 ml of Ultrahyb-Oligo hybridization solution (Ambion) for 30 min at 42°C in a hybridization oven. For radioactive detection of the blotted RNA, three oligonucleotides designed from the cloned leptin and LEPR sequences (Sigma-Genosys, Haverhill, Suffolk, UK) were labelled with ^{32}P-ATP (6000 Ci/mmol, PerkinElmer, Beaconsfield, UK) using a Kinase Max kit (Ambion). The oligonucleotide probes used for the labelling and hybridization are as follows:

Primers	Sequence
Leptin1	5’GTCCATGTGTAGGCTGGAGAGGATGGCATGGAAGATCTCTAGGG
Leptin2	5’TCCGGGCATTGCAGCCAAGCAGAGAACTTAAGGCATGGAGAAG
LEPR1	5’GGTGCATTCATATACGTCATCATCCCGCAACGAGCAGTC
LEPR2	5’ACGTCATCATCCCGCAACGAGCAGTCTTTTGATTCCGAAG
LEPR3	5’TCCCGCAACGAGCAGTCTTTTGATTCCGAAGTGTTCTG

Table 4 Primers used for Northern Blot

All three probes for LEPR and two probes for leptin were added to the hybridization solution. Hybridization with agitation was performed overnight in a hybridization oven at 42°C. After hybridization, the membrane was washed twice for 30 min in 2x SSC, 0.5% SDS; once for 30 min in 1x SSC, 0.5% SDS; once for 30 min in 0.5x SSC, 0.5% SDS; and once in 0.1x SSC, 0.5% SDS, all at 42°C. The membrane was wrapped in clingfilm and exposed for 2 weeks to Kodak BioMax MS autoradiographic film. The film was processed by hand as follows: 30 sec in X-ray developer (Ilford Phenisol Developer, Herman technology Ltd., Town Lane, Cheshire, UK), 1 min in water bath, 1min in fixer (Polymax RT fixer, Kodak Professional, Hemel Hempstead, Hertfordshire, UK) and 5 min in water bath.

2.10 Molecular phylogeny

Phylogenetic trees were constructed by using the Maximum Likelihood method based on the Equal Input model (Tajima and Nei, 1984). The trees with the highest log likelihood were shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial trees for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total numbers of sites, the maximum parsimony method was obtained; otherwise BIONJ method with MCL distance matrix was used. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Chapter 3. Leptin Receptor

3.1 Introduction

To understand the physiological effects of leptin, it is necessary to study the structure and function of its receptor. Until recently, leptin receptor had only been identified in mammals, so information on the evolution of the leptin signalling pathway and its functions in vertebrates is very limited. Most of the research on leptin receptor in non-mammalian vertebrates is being performed in fish. A full-length LEPR gene has been identified from marine medaka (*Oryzias melastigma)* (Wong et al., 2007), pufferfish (*Takifugu rubripes*) (Kurokawa et al., 2008), Japanese medaka *(Oryzias latipes)* (Kurokawa, Murashita, 2009) and Atlantic salmon *(Salmo salar)* (Ronnestad et al., 2010). The fish LEPR genes share less than 25% identity to mammalian leptin receptors. Sequences among the fish LEPR genes show 82-32.5% similarity at amino acid level.

Although the chicken leptin gene is missing, there is evidence that a leptin-like signaling system is present in birds because receptor sequences have been cloned in the chicken and turkey that share greater than 90% sequence identity at both the nucleotide and amino acid level (Horev et al., 2000; Ohkubo et al., 2000; Richards et al., 2003). The identity between chicken and mouse LEPRs is 60% at amino acid level, indicating a relatively low similarity (Horev et al., 2000). Evidence also exists at the protein level for the LEPR in reptiles. LEPR-like immunoreactivity was detected in the pancreas (Paolucci et al., 2006), thyroid gland (Sciarrillo et al., 2005) and testis (Putti et al., 2009) of the lizard, *Podarcis sicula*.

Research on the amphibian LEPR is very limited. Only one LEPR gene has been reported in amphibians (*Xenopus tropicalis)* sharing 37% amino acid sequence identity with human (Crespi and Denver, 2006). Therefore, the aim of this chapter is to assess the structure and functions of leptin receptor in the axolotl via cloning the axolotl LEPR and establishing the pattern of its tissue expression in the adult and during development to extend investigation into the leptin signalling pathway in salamanders.

3.2 Methods

3.2.1 *Cloning of axolotl leptin receptor*

Amino acid sequences of *Xenopus tropicalis* (ABD63000), human (NP_002294), mouse (NP_666258) and Zebrafish leptin receptor (NP_001106847) were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2). Semi-degenerate primers were then designed using the CodeHop program (Rose et al., 1998). The primers used were: forward primer 5' GCCGAGATCTACGTGATCGAYGTNAAYAT (CHOPF2) and reverse primer 5' AGGAAGACGGGCTGGAAGRTRCAYTCRTA (CHOPR2), yielding a reaction product of 248bp. PCR was performed using cDNA from an axolotl hatched larva as template. A touchdown program was used with the annealing temperature decreasing from 63°C to 53°C, in increments of 0.5°C. Denaturing and extension steps were 94°C and 72°C, respectively. Times used were 15 sec denaturation, 30 sec annealing, and 30 sec extension, with an extension time for the final cycle of 10 min. PCR was carried out for 40 cycles. PCR products were visualised on a 2% agarose gel as described above. Amplification products were extracted from the agarose gel using a QIAquick gel extraction kit (Qiagen) ligated into pSC-A-amp/kan vector and transformed into Strataclone SoloPack competent cells using a StrataClone PCR cloning kit (Stratagene). Plasmid DNA was isolated using a PureLink Quick Plasmid Miniprep kit (Invitrogen) according to the manufacturer's instructions, and was then sequenced. The sequence was obtained in forward direction from seven clones amplified from testis. The partial LEPR sequence was submitted to GenBank and given the accession number GU562414.

Methods for PCR amplification of the LEPR fragment are described in Chapter 2 (General Methods).

3.2.2 *3' RACE*

To assess the 3' region of the axolotl LEPR gene, the 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) was used. Total RNA was extracted from the axolotl brain using the the TRIzol reagent following the manufacturer's instructions. The 3′ rapid amplification of cDNA ends (RACE) was used to attempt to generate

axolotl LEPR cDNAs. The 3′ ends of the cDNAs were amplified with a specific forward primer (5’-TCTCTTGTGAAACCGATGGA) and reverse random primers from the 3’ RACE cDNA amplification kit following the manufacturer's instructions. The denaturation, annealing, and extension temperatures and times were 94°C, 15 sec; 54°C, 30 sec; and 72°C, 3 min; with the final extension step of 7 min. PCR was carried out for 30 cycles. One hundred ng of control RNA was used to amplify 3’ RACE product as a positive control according to the manufacturer's instructions. PCR amplification products were separated by electrophoresis on a 1% agarose gel, and putative specific bands were excised and purified by the QIAquick gel extraction kit (QIAgen GmbH, Germany), cloned using a StrataClone PCR cloning kit (Stratagene) and sequenced.

3.2.3 *3’ and 5’ RACE*

To assess the 3’ and 5’ region of the axolotl LEPR gene, RACE reactions were performed using a SMART RACE cDNA amplification kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). First-strand cDNA was synthesized with 50 ng of polyA$^+$ RNA as template, isolated from the axolotl brain. The 5'-RACE and 3'-RACE reactions were performed with gene-specific primer (GSP) based on the partial axolotl LEPR gene (Accession No. GU562414, the nucleotide sequences obtained from the previous degenerate-primer PCR) and Universal Primer A Mix (UPM) according to the manufacturer's instructions. For the GSPs, GSP1 used for 5'-RACE was 5'-CGTCATCATCCCGCAACGAGCAGTCTTT; and GSP2 and GPS3 for 3'-RACE were 5'-TGACATGCAGATGGCATCGGGGCAACA; 5’-GTGGGGTGCGTGTGGACTGGTGCTTGC. Touchdown PCR was performed using the Advantage 2 PCR kit (Clontech). The reaction mix was prepared and the touchdown PCR was performed according to the manufacturer's instructions. The amplified products were purified from the gel, cloned and then sequenced. To ensure that the SMART RACE protocol was working efficiently, a positive control experiment was performed using the RACE- Ready cDNA generated from the Control Human Placenta Total RNA according to the manufacturer's instructions.

3.2.4 *DNA walking*

To obtain the 5′ region of the axolotl LEPR gene, cDNA and genomic walking were performed using the DNA Walking SpeedUp Premix Kit II (Biogene, Kimbolton, Cambs, UK). The 5' region was amplified using 50 ng of cDNA and 100 ng of whole genomic DNA, respectively. Primary PCRs were performed using adaptor primers (DW2-ACP 1-4) from the kit and an outer gene-specific primer TSP1 (LEPRR2 - 5'-GCAACGAGCAGTCTTTTGATT). PCR reactions were performed in 20 µl using Master Mix from the kit according to the to manufacturer's instructions. The cycling conditions were: 1x 94°C for 5 min, 42°C for 1 min, 72°C for 2 min; 30x 94°C for 30 sec, 60°C for 30°C for 30 sec, 72°C for 1:40 min and 1x 72°C for 7 min. Secondary PCRs were performed using primers DW-ACPN and TSP2 (LEPRR1 - 5'-TTCCGAAGTGTTCTGAATGGT). Three µl of the purified PCR products from the first PCR reactions were used with cycling conditions: 1x 94°C for 3 min; 35x 94°C for 30 sec, 60°C for 30 sec, 72°C for 1:20 min and 1x 72°C for 7 min.
The third PCR reactions used primers UP and TSP3 (LEPRR3 - 5'-GGTTGGTGGCAAAGAACAGT; LEPRR4 - 5'-ATGCCGCTGCTGTAGTACCT) containing 2µl of second PCR products as template. The cycling conditions were: 1x 94°C for 3min; 30x 94°C for 30sec, 65°C for 30sec, 72°C for 1:20min and 1x 72°C for 7min. PCR products of the third reaction were purified, cloned and sequenced.

3.2.5 *Use of LEPROT to design primers for the LEPR*

To obtain the 5' end of the axolotl LEPR gene, primers were designed to the 5' exons of the axolotl LEPROT sequence (Accession number CO789347) on the basis that the axolotl LEPROT and LEPR share common exons as they do in other vertebrates (Huang et al., 2002). The primers were: LeprOTF1 - 5'- GCCATACGAATCAGTGACGA; LeprOT2 - 5'-TCAGTGACGATACCGATGCT. PCRs were performed using the LEPROT primers as forward primers and either oligo dT primers, or LEPR gene specific primers (LEPR1 - 5' TTCCGAAGTGTTCTGAATGGT; LEPR2 - 5'-GCAACGAGCAGTCTTTTGATT; LEPRR3 - 5'-GGTTGGTGGCAAAGAACAGT; LEPRR4 - 5'-ATGCCGCTGCTGTAGTACCT) as reverse primers. PCR amplification was performed using the Expand High Fidelity PCR System (Roche). The PCR reactions were prepared according to the manufacturer's instructions and performed in a

total volume of 50 µl containing 200 µM of each dNTP, 300 nM of each primer, 1x Expand High Fidelity Buffer with 15 mM $MgCl_2$, 2.6 U of Expand High Fidelity enzyme mix and 50 ng of the axolotl brain cDNA as a template. The cycling conditions were: 1x 94°C for 2 min; 10x 94°C for 15 sec, 56°C for 30 sec, 72°C for 2 min; 20x 94°C for 15 sec, 56°C for 30 sec, 72°C for 2 min + 5 sec (cycle elongation for each successive cycle) and 1x 72°C for 7 min. The PCR products were cloned and sequenced as described in Chapter 2 (General Methods).

3.3 Results

3.3.1 *Cloning of axolotl leptin receptor*

A partial cDNA sequence, corresponding to a leptin receptor gene was isolated and sequenced from the axolotl (*Ambystoma mexicanum)* hatched larvae. The primer set was designed within the extracellular domain containing the ligand binding region, expected to be present in all forms of leptin receptor (Richards et al., 2003). The cDNA fragment consisted of 248 bp including the semi-degenerate primers (Figure 17) and the 190 bp axolotl-specific LEPR fragment was deposited in GenBank (Accession number GU562414). The predicted amino acid sequence shares 54-61% similarity with mammalian LEPRs, including 57% similarity with the human. Among non-mammalian LEPRs, sequence identity was 49% and 48% in the turkey and chicken, respectively, 46% in *Xenopus tropicalis*, and less than 30% with fish LEPRs (Figure 18). The cloned region is part of the extra-cellular domain and corresponds to the part of the C2 domain and the putative leptin-binding domain (Richards and Poch, 2003).

GCCGAGATCTACGTGATCGATGTAAACATTTCAATCTCTTGTGAAACCGATGG

AAAACTTACTAAAATGACATGCAGATGGCATCGGGGCAACATTATGTTGCTTGA

GAAAAGTACCTCTGAATTAAGGTACTACAGCAGCGGCATCTACTGTTCTTTGCCA

CCAACCATTCAGAACACTTCGGAATCAAAAGACTGCTCGTTGCGGGATGATGAC

GTA**TATGAATGCACCTTCCAGCCCGTCTTCCT**

S I S C E T D G K L T K M T C R W H R G N I M L L E K S T S E L R Y Y S S G I Y C S

L P P T I Q N T S E S K D C S L R D D D V

Figure 17 Nucleotide and amino acid sequence of LEPR from the axolotl. The positions of the semi-degenerate primers in the nucleotide sequence are in bold and underlined. The predicted amino acid sequence does not include the primers.

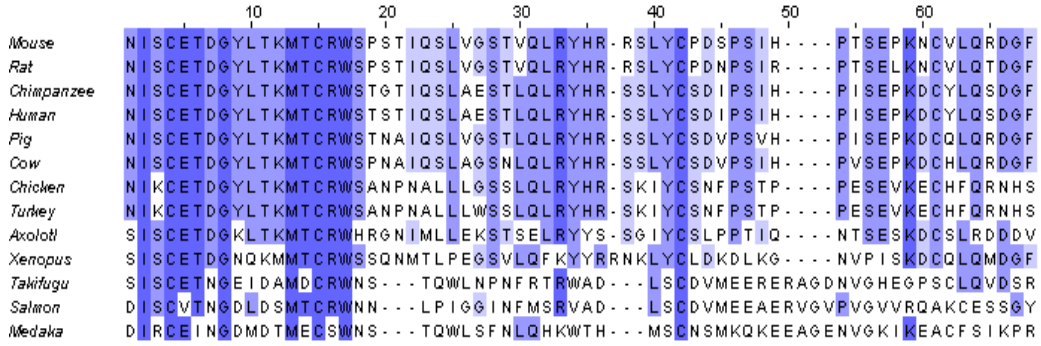


Figure 18 Amino acid alignment of vertebrate LEPRs on the basis of amino acid sequences of LEPR in chimpanzee (*Pan troglodytes*; XP_001161897), human (*Homo sapiens*; AAB09673), little brown bat (*Myotis lucifugus*; AAU47264), pig (*Sus scrofa*; ACT52816), cow (*Bos taurus*; DAA31276), sheep (*Ovis aries*; AAP33683), mouse (*Mus musculus*; CAM20702), rat (*Rattus norvegicus*; BAA12698), rhesus macaque (*Macaca mulatta*; AAF35388), chicken (*Gallus gallus*; AAF31355), turkey (*Meleagris gallopavo*; AAG40323), duck (*Anas platyrhynchos*; ACF17729), *Xenopus tropicalis* (ABD63000), Indian medaka (*Oryzias melastigma*; ABC86922), axolotl (*Ambystoma mexicanum*; GU562414, excluding primer sequences) and zebrafish (*Danio rerio*; NP_001106847) sequences.

In order to investigate the gene structure of LEPR, PCR amplification of axolotl genomic DNA was performed with the same primers used for cDNA amplification. This yielded a 171bp fragment, (Figure 19), the same product size as obtained using a cDNA template, indicating that the cloned LEPR fragment contained no introns. However, splice site prediction programs (www.fruitfly.org/seq_tools/splice.html;

http://www.cbs.dtu.dk/services/NetGene2/) predicted splice donor and acceptor sites with confidence scores of 0.90 and 0.37, respectively (at nucleotide positions 122-136; 116-133).

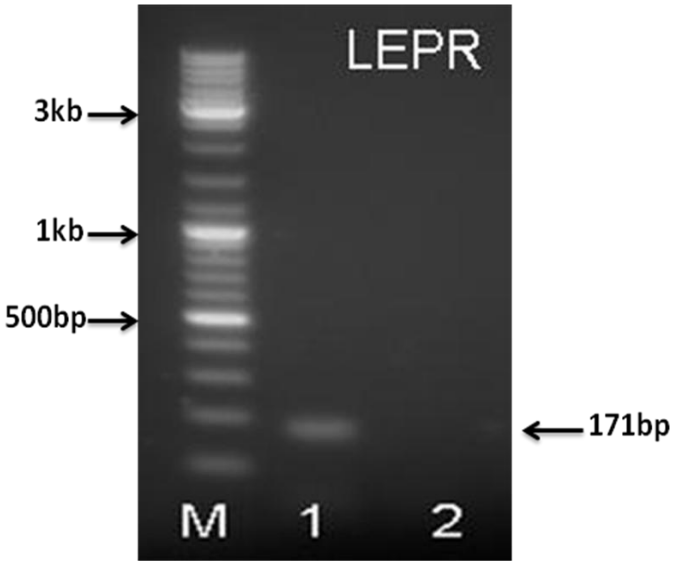


Figure 19 Amplification of axolotl genomic DNA using the cDNA primers. Lane 1 represents generated amplification product (171 bp) from axolotl genomic DNA template. M designates size marker and lane 2 represents no template control.

3.3.2 *Molecular phylogeny*

Phylogenetic analysis of the axolotl LEPR fragment in relation to other vertebrate LEPRS was performed using the deduced amino acid sequences and comparing it to the equivalent region of leptin receptor sequences available in GenBank (Figure 20). The phylogenetic tree shows that the evolutionary relationships between the axolotl LEPR with other vertebrate LEPRs are consistent with the consensus view of vertebrate evolution.

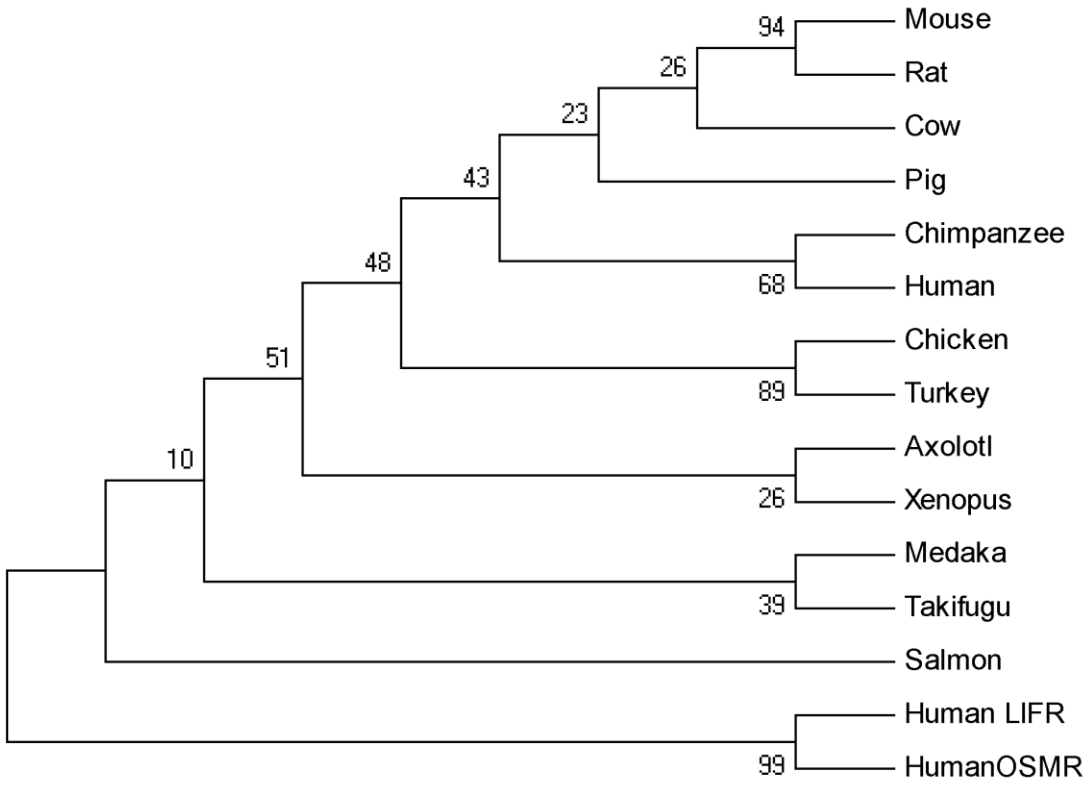


Figure 20 A phylogenetic tree (phylogram) constructed on the basis of amino acid sequences of LEPR in chimpanzee (*Pan troglodytes*; XP_001161897), human (*Homo sapiens*; AAB09673), pig (*Sus scrofa*; ACT52816), cow (*Bos taurus*; DAA31276), mouse (*Mus musculus*; CAM20702), rat (*Rattus norvegicus*; BAA12698), chicken (*Gallus gallus*; AAF31355), turkey (*Meleagris gallopavo*; AAG40323), *Xenopus tropicalis* (ABD63000), Indian medaka (*Oryzias melastigma*; ABC86922), axolotl (*Ambystoma mexicanum*; GU562414), *Takifugu rubripes* (BAG67079), Atlantic salmon (*Salmon salar;* BAI23197), human OSMR (AAI25210) and human LIFR (P42702). The tree was calculated using the Maximum Likelihood method based on the Equal Input model (Tajima and Nei, 1984) and drawn using the MEGA5 program (Tamura). The tree with the highest log likelihood (-1505.9443) is shown. The analysis involved 12 amino acid sequences. There were a total of 136 positions in the final dataset. Numbers at branch points represent the bootstrap value for 1000 replicates as percentages and indicate the statistical reliability of a node in the tree. Human oncostatin M receptor (OSMR) and leukemia inhibitory factor receptor (LIFR) were used as an outgroup.

3.3.3 *Attempts to access the full length of the axolotl LEPR cDNA*

In order to obtain the full length sequence of the LEPR cDNA, several techniques were performed including: PCR with oligo dA; 3' RACE; 5' RACE and DNA walking. However, none of the above methods succeeded in allowing the sequence beyond the cloned fragment to be identified.

To obtain the 3' region of the axolotl LEPR cDNA, PCR was performed using a gene-specific primer and oligo dA as a reverse primer. The experiment produced numerous products; however, following sequencing, none of them shared any similarities with the LEPR.

The 3′ end of the cDNAs were amplified with a specific forward primer and reverse random primer from the 3' RACE System for Rapid Amplification of cDNA Ends. The primers generated a 1.2 kb fragment from PCR of cDNA template extracted from axolotl brain (Figure 21). The sequence of this product did not show any similarities to LEPR. The positive control yielded an expected 720 bp fragment (Figure 22) indicating that the method was performed correctly.

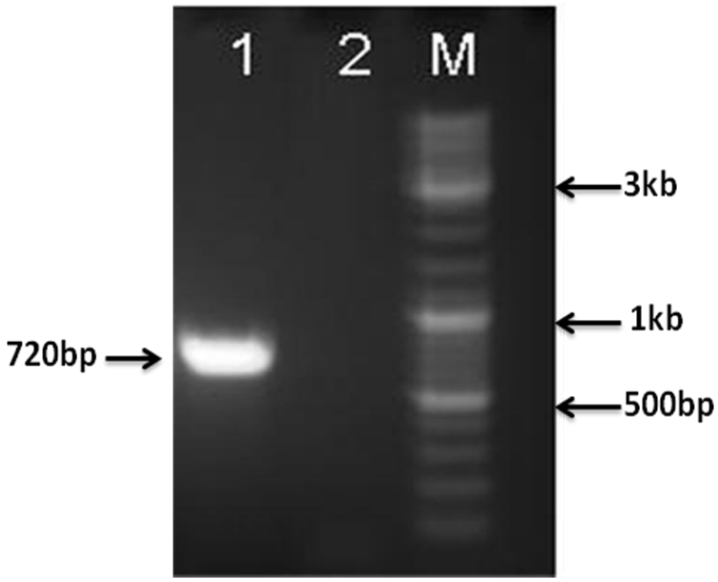


Figure 21 3' RACE product using control RNA. Lane 1 represents generated amplification product (720 bp); lane 2 - no template control and M designates size marker.

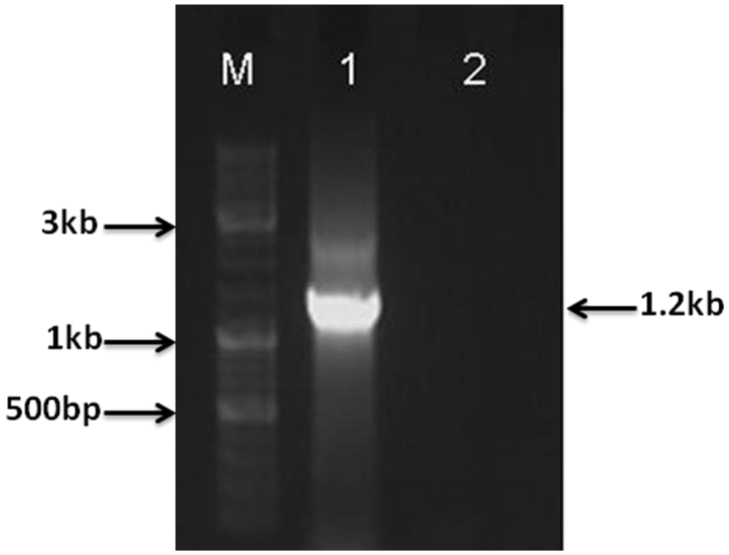


Figure 22 3’ RACE amplification of the axolotl LEPR gene. A 200-ng sample of RNA extracted from the axolotl brain was used for first strand cDNA synthesis. M represents size marker; lane 1 –yielded amplification product (1.2 kb) and lane 2 - no template control.

To assess the 3’ and 5’ region of the axolotl LEPR gene, RACE reactions were performed using a SMART RACE cDNA amplification kit. Axolotl LEPR specific primers were used in combination with a universal primer mix (UPM). The touchdown PCR was performed followed by nested PCR using LEPR-specific nested primer in combination with the nested universal primer (NUP). The PCR products were sequenced and analysed, however the sequences were not similar to LEPR. To ensure that the SMART RACE protocol works, a positive control experiment was performed using the RACE- Ready cDNA generated from the Control Human Placenta Total RNA. The reactions yielded the expected fragment sizes of 2.6 kb (3’ RACE) and 2.9 kb (5’RACE) (Figure 23).

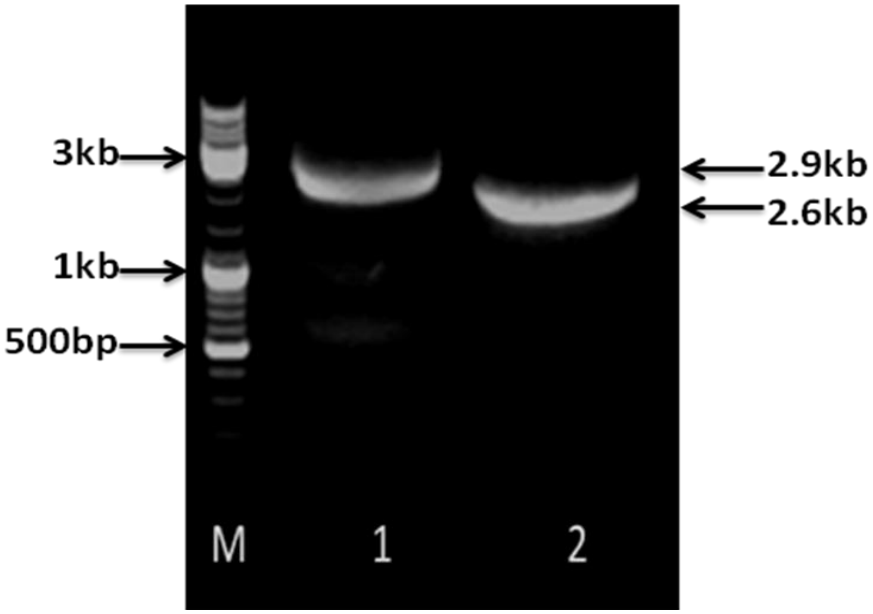


Figure 23 Positive control SMART RACE experiment. Lane 1 represents amplified 5' RACE fragment (2.9 kb); lane 2 – 3' RACE fragment (2.6 kb); M designates size marker.

To obtain the 5′ region of the axolotl LEPR gene, cDNA and genomic walking were performed using DNA walking SpeedUp Premix Kit II. The DNA walking experiment included three reactions: the first PCR reaction used the outer adaptor primers DW-ACP (1-4) from the kit and an outer gene-specific primer TSP1 (LEPRR2); the second reaction used primers DW-ACPN and TSP2 (LEPRR1); the third reaction used primers UP and TSP3 (LEPRR3; LEPRR4). PCR products of the third reaction were purified, cloned and sequenced (Figure 24). The sequences did not show any similarities to LEPR.

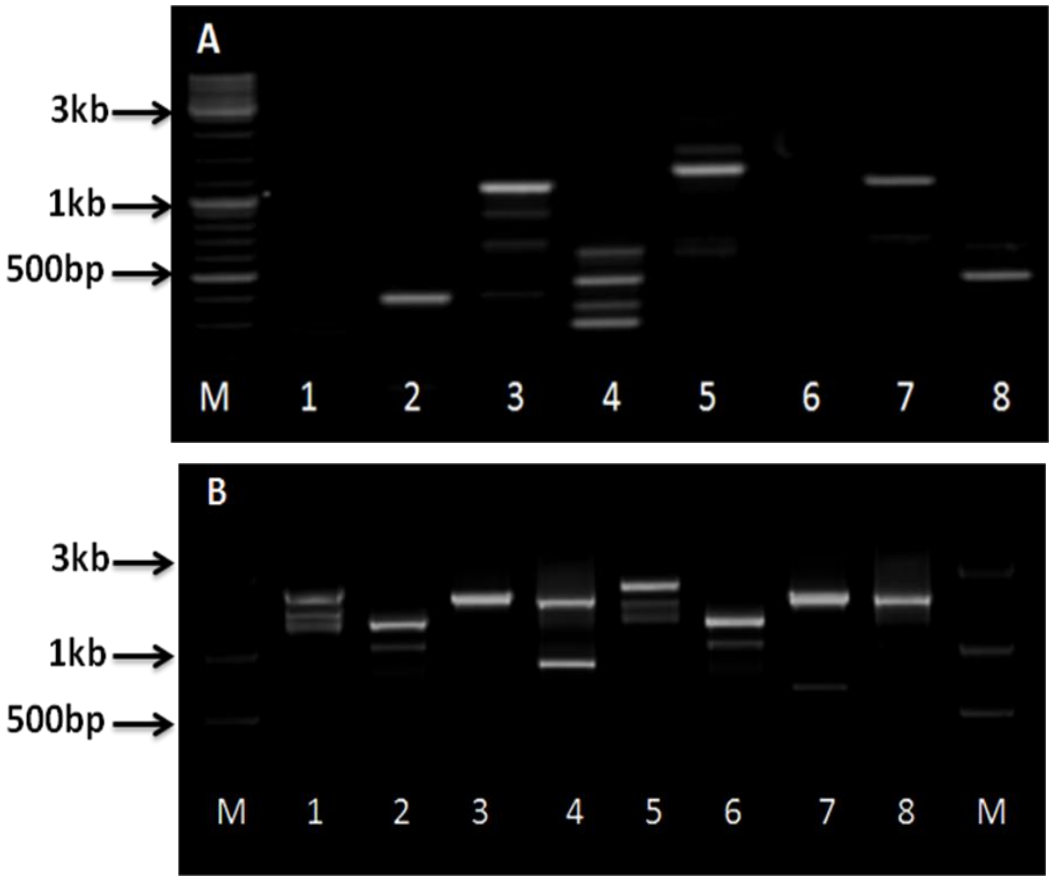


Figure 24 DNA walking; 50 ng of cDNA and 100 ng of whole genomic DNA were used for cDNA (A) and genomic walking (B) PCRs, respectively. DW-ACP1 (lane 1 and 5); DW-ACP2 (lane 2and 6); DW-ACP3 (lane 3 and 7) and DW-ACP4 (lane 4 and 8) were used for amplification of 5' region of the axolotl LEPR gene. As TSP3 primer LEPRR3 (lane1-4) and LEPRR4 (lane 5-8) were used. M represents size marker.

To obtain the 5' region of the axolotl LEPR gene, PCRs were performed using forward primers based on the axolotl LEPROT sequence. Either a range of LEPR gene specific primers (LEPR 1-4) or oligo dA were used as reverse primers. The experiment produced numerous products; however, following sequencing, none of them shared any similarities with the LEPR.

3.3.4 *Northern analysis*

Northern hybridization was carried out using poly(A)+ RNA extracted from axolotl testis with oligonucleotide LEPR probes designed from the cloned axolotl LEPR cDNA. Radioactive detection indicated a single band of RNA with a molecular size of approximately 3 kb (Figure 25).

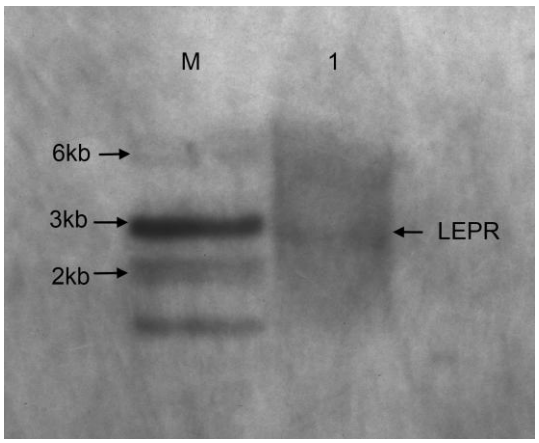


Figure 25 Northern hybridization of LEPR poly(A)+RNA isolated from axolotl testis. A 9 µg aliquot of poly(A)+ RNA was electrophoresed through a 1% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with 32P-labeled antisense axolotl leptin receptor oligonucleotides. The hybridization signal was captured digitally after overnight exposure to a film. The transcript detected is approximately 3 kb in size. M indicates RNA size ladder.

3.3.5 *Developmental expression of LEPR gene during axolotl embryogenesis*

The developmental expression of LEPR mRNA was analyzed by RT-PCR derived from 11 axolotl embryonic stages (9, 12, 15, 17, 20, 25, 29, 35, 37, 39, 42) and 1-post-embryonic stage (44- hatched larvae). LEPR expression peaked in early development at the late gastrula stage and became progressively weaker during neurulation so that it was barely detectable during the tailbud stages (stages 29-32), but increased markedly around the time of hatching (stages 41-43) (Figure 26).

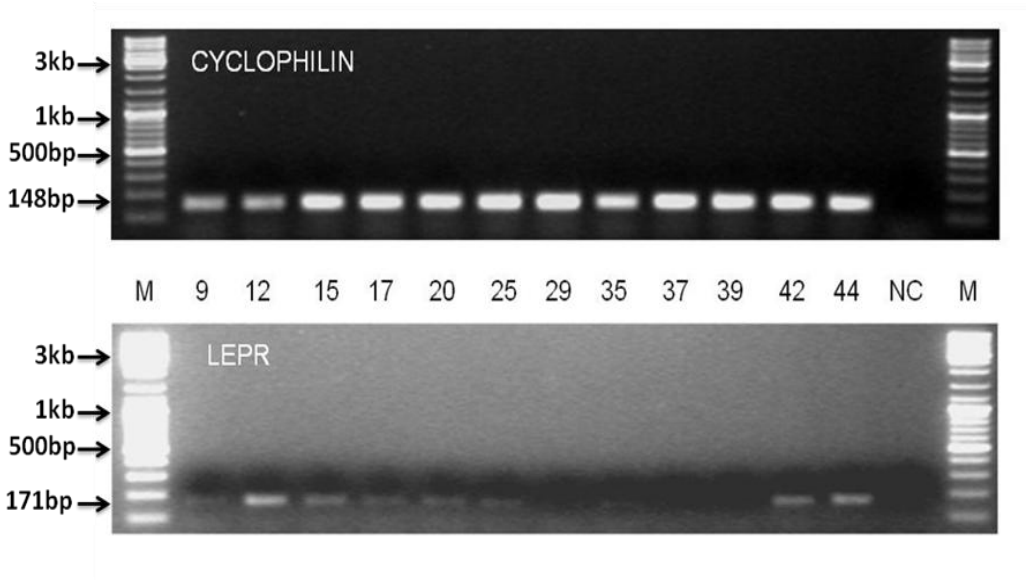


Figure 26 RT-PCR analysis of LEPR during embryogenesis and early larval development. Expression of cyclophilin in the same samples is shown for comparison.

3.3.6 *Tissue expression of axolotl LEPR gene using RT-PCR*

The distribution of axolotl LEPR expression was studied by RT-PCR on mRNA isolated from a variety of tissues in 5 individual animals (10-month old). Amplification of cyclophilin confirmed the effectiveness of reverse transcription. LEPR expression differed between the individuals studied. However, the strongest intensity of the PCR product was consistently observed in the brain with moderate expression also being seen in the skeletal muscles and stomach (Figure 27).

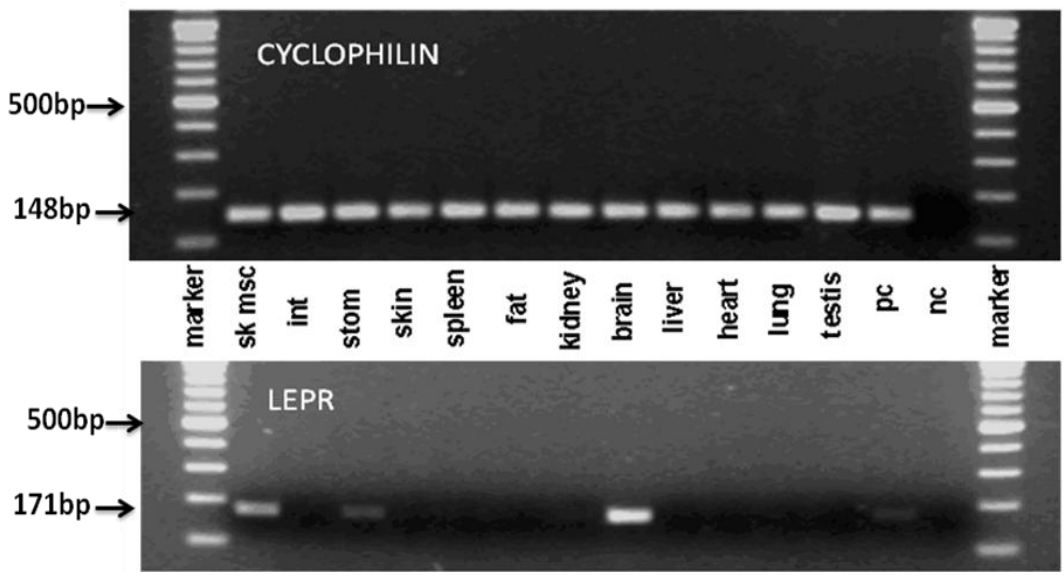


Figure 27 Tissue distribution of LEPR mRNA in the axolotl using RT-PCR. For each individual, the distribution for cyclophilin mRNA is shown in the upper panel, and for leptin mRNA in the lower panel. Identical lanes were used for leptin and cyclophilin in each individual. Tissue abbreviations: Sk Msc, skeletal muscle; Mid SI, mid small intestine; Stom, stomach; Fat, fat body; NC, negative control (no template); PC, positive control.

3.3.7 *Tissue expression of Tiger salamander LEPR gene using RT-PCR*

A partial cDNA sequence, corresponding to the cloned fragment of the axolotl LEPR cDNA was isolated and sequenced from Tiger salamander (*Ambystoma tigrinium)* skeletal muscle. The cDNA fragment consists of 171 bp and shares 100% similarity with the axolotl LEPR at the amino acid level. Tiger salamander LEPR tissue distribution was studied by RT-PCR on mRNA isolated from a variety of tissues in three different animals. To confirm the effectiveness of reverse transcription, PCR was also carried out to amplify a cDNA fragment of cyclophilin A. Leptin expression differed between the individuals studied. The strongest intensity of the PCR product was observed in the tongue, skeletal muscles, and stomach with moderate expression also being seen in the brain, testis and heart. Weak expression was observed in the skin and oesophagus (Figure 28).

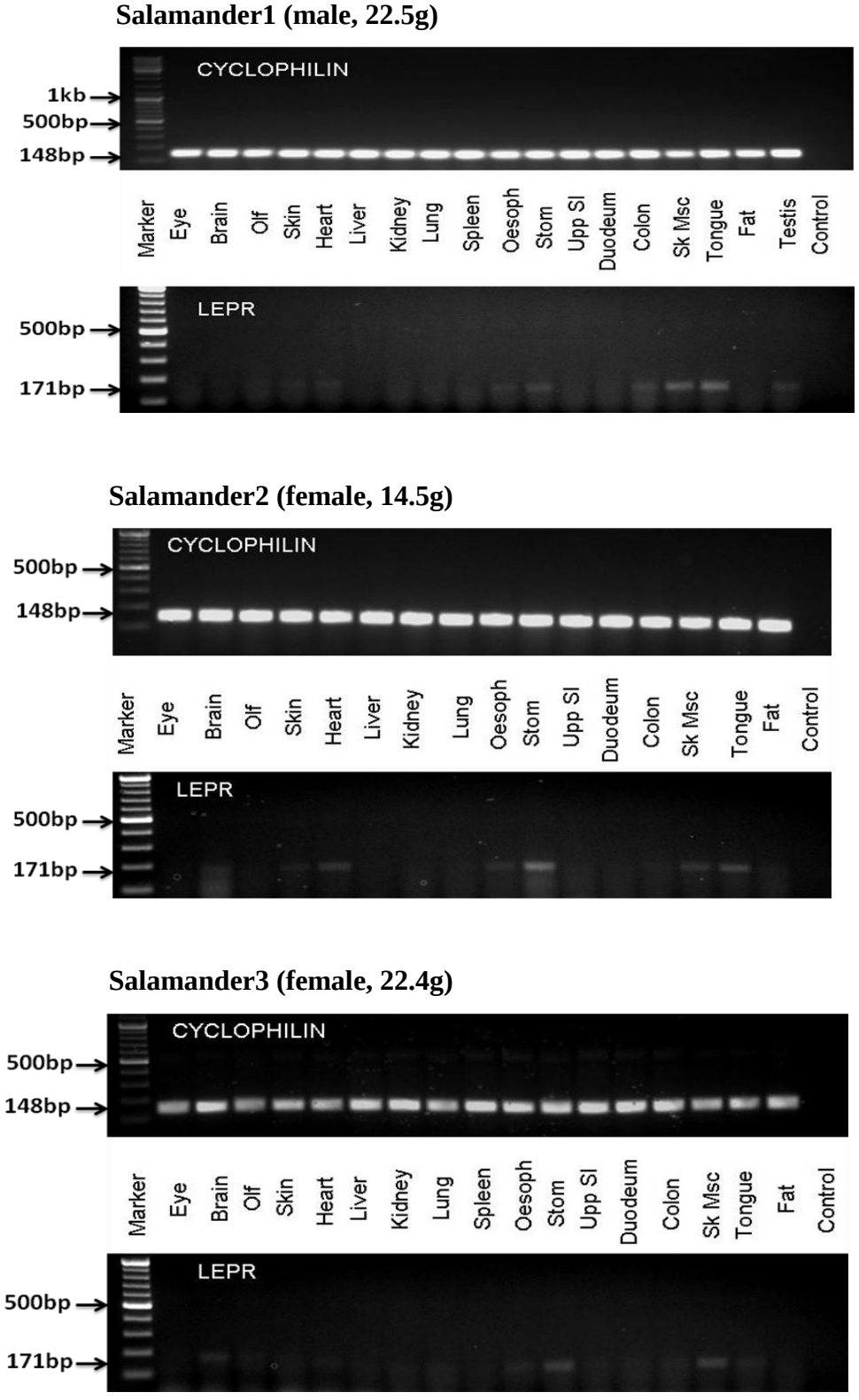
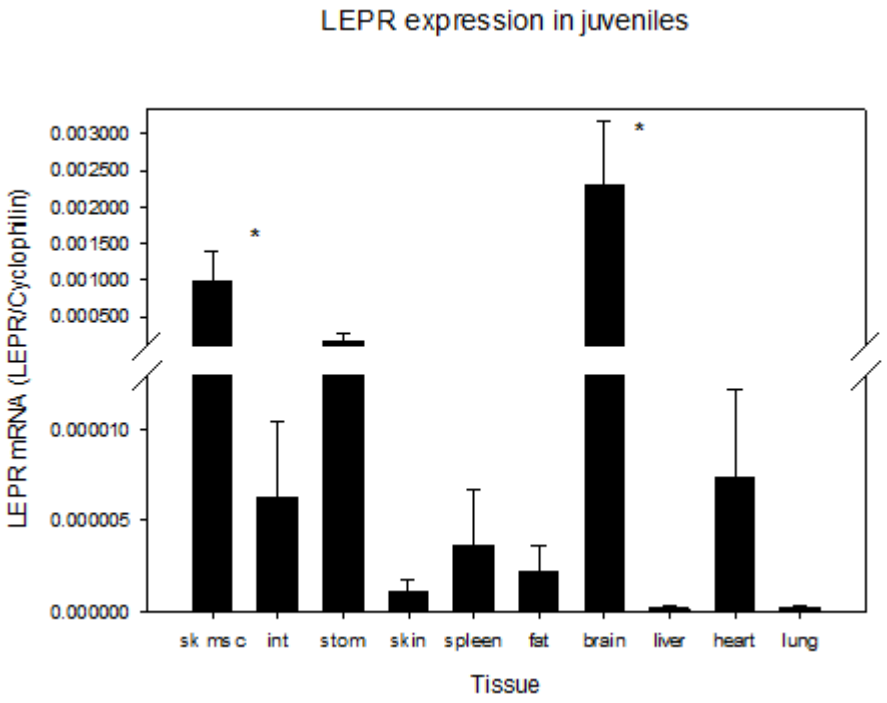


Figure 28 Tissue distribution of LEPR mRNA in the salamander using RT-PCR. For each individual, the distribution for cyclophilin mRNA is shown in the upper panel and for LEPR mRNA in the lower panel. Identical lanes were used for leptin and cyclophilin in each individual. Tissue abbreviations: Olf, olfactory bulb; Oesoph, oesophagus; Sk Msc, skeletal muscle; Upp SI, upper small intestine; Stom, stomach; Fat, fat body; Control (negative control - no template).

3.3.8 *Quantitative tissue distribution of axolotl LEPR using real-time RT-PCR*

The expression pattern of LEPR between tissues was established by quantitative real-time PCR in two different age groups of animals. The juveniles were 6-months old with no gonads developed; the adults were 3-years old. The tissue distribution of LEPR mRNA was broader in the juveniles compared with the adult animals (Figure 29A). In adults, expression differed significantly across tissues ($F(9,42)=2.48$, $p=0.015$) with the highest expression level detected in the brain and skeletal muscles. Expression in these two tissues was significantly higher than in the small intestine, spleen, fat, liver, heart and lung ($p<0.05$) (Figure 29B) which agrees with the qualitative RT-PCR results (Figure 27). In juveniles, LEPR expression also differed significantly across tissues ($F(10,45)=2.78$, $p<0.05$). The highest expression was in the skeletal muscle, brain and stomach, and did not differ significantly between these three tissues ($P>0.05$). Expression in the skeletal muscle and brain was significantly higher than in the small intestine, skin, spleen, fat, liver, heart and lung ($p<0.05$).

A



B

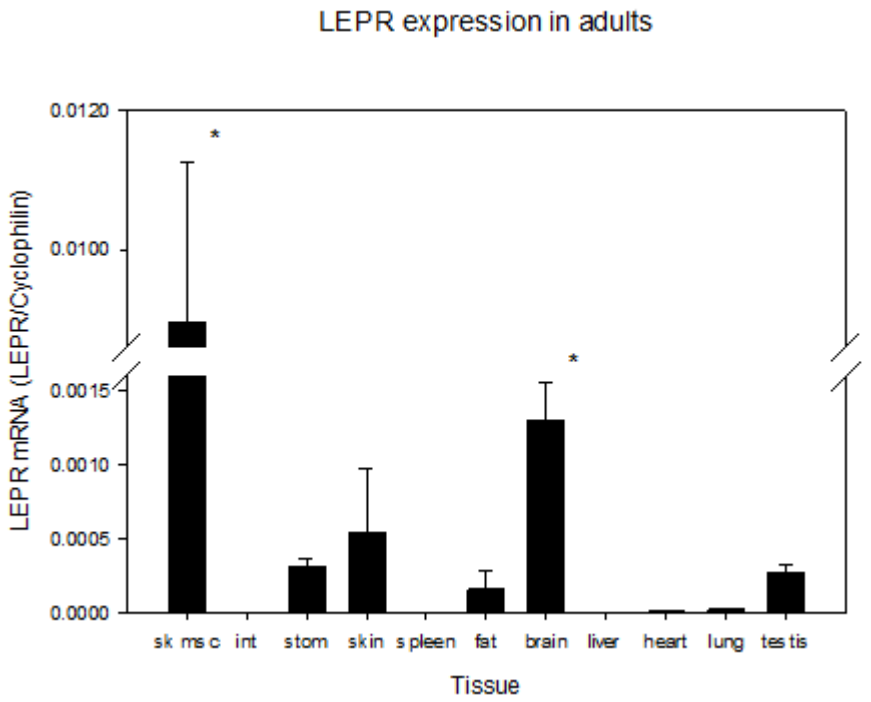


Figure 29 Expression of LEPR in the axolotl. The tissue distribution in two different age groups of animals (A, juveniles, 6 month old; B, adults, 3 year old) was analyzed by quantitative RT-PCR. Leptin level was normalized to the expression of the cyclophilin gene. Bars represent means (including SEM). Asterisks indicate significant differences for the skeletal muscle and brain compared with the small intestine, spleen, fat, liver, heart and lung (Fisher's least significant difference test; $p<0.05$).

In order to compare expression levels of LEPR between in the highest-expressing tissues between juveniles and the adults, another quantitative real-time PCR was performed. No significant differences were detected in tissue expression between the two age groups ($F(1,18)=2.45$, $p>0.05$) and there was no significant interaction between age and tissue expression (Figure 30).

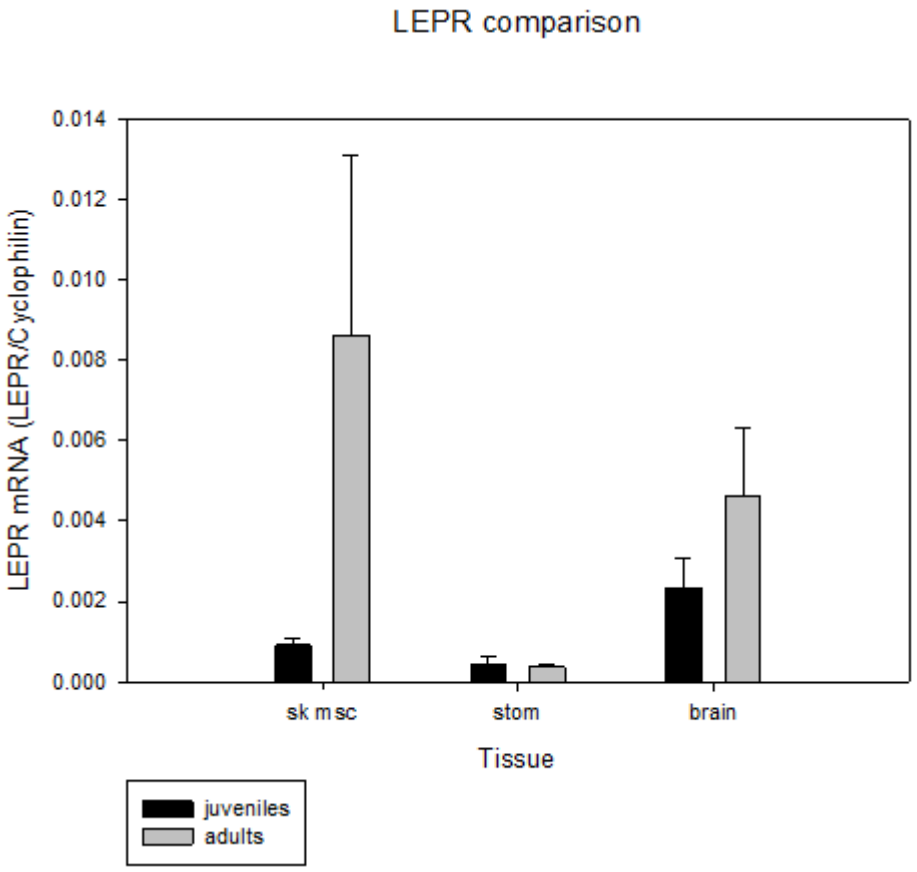


Figure 30 Comparison of expression levels of LEPR between juveniles and adults

3.4 Discussion

A partial sequence of an axolotl LEPR gene orthologue and its expression has been characterised. The predicted amino acid sequence shares 54-61% sequence similarity with mammalian LEPRs and 46% with the *Xenopus* LEPR. Northern hybridisation and RT-PCR analyses showed that the expression of the LEPR gene was widespread, including in embryonic tissues, with the highest expression being shown in the brain, stomach and skeletal muscle.

The sequence similarity of the cloned LEPR fragment is as would be expected. The results from the phylogenetic tree of fish, amphibian, avian and mammalian LEPR sequences demonstrate that the evolutionary relationship between leptin receptor molecules is consistent with the accepted consensus of vertebrate evolutionary divergence and that the sequence found is naturally present in the axolotl rather than being a cloning artifact. The amino acid sequence identity with the *Xenopus* LEPR at 46% is not close but is consistent with the 60% identify reported between the Tiger salamander and *Xenopus* leptin coding sequences (Boswell et al., 2006). The sequences of leptin and its receptor appear to be more conserved within salamanders because the leptin sequences of the axolotl and Tiger salamander are almost identical (Chapter 3) and the present study demonstrated that the cloned LEPR fragment is identical between the two species. In terms of the intron-exon structure of the axolotl LEPR, no evidence could be found in the present study, from PCR amplification of genomic DNA, for the presence of an intron in the cloned fragment. This was predicted by splice site prediction programs and also by sequence comparisons in the chicken and turkey and the pufferfish, where the fragment corresponds to the region between exons 11 and 12 (Richards and Poch, 2003, Kurokawa et al., 2008). This suggests that if the cloned axolotl LEPR sequence is correct, the intron/exon structure may differ from that in other vertebrates.

Further evidence that the cloned sequence does represent the axolotl LEPR is provided by the expression pattern observed in the present study. Northern hybridisation indicated that the gene is naturally expressed in the testis and that the axolotl LEPR transcript is approximately 3 kb in size. This lies close to transcript sizes reported in other animals, e.g. mouse, 4.5 kb; turkey, 4kb (Richards and Poch, 2003); *Xenopus*, 3.4kb (Crespi and Denver, 2006); pufferfish, 3.3kb (Kurokawa et al., 2008). A single transcript was detected, suggesting that one splice variant is predominantly expressed in the axolotl. Among other non-mammalian vertebrates, a LEPR splice variant has been detected in birds although its physiological significance is uncertain (Liu et al., 2007) and five splice variants were identified in Atlantic salmon, only two of which contained the transmembrane domain (Ronnestad et al., 2010).

The LEPR transcript is widely distributed among tissues, which suggests that leptin may have diverse physiological roles in amphibians as was also suggested for *Xenopus*

(Crespi and Denver, 2006). The strongest intensity of the PCR product was observed in the brain with moderate expression also being seen in the skeletal muscles and stomach. This finding agrees with the main sites of LEPR expression in mammals, and also in birds, *Xenopus*, and fish (Horev et al., 2000; Richards and Poch, 2003; Crespi and Denver, 2006; Kurokawa et al., 2008; Ronnestad et al., 2010) providing further evidence that the gene fragment cloned represents the axolotl LEPR. In all vertebrates, the LEPR is predominantly expressed in the hypothalamus, the part of the brain responsible for the control of food intake (Tartaglia et al., 1995). The expression pattern of the LEPR was similar to that of leptin, suggesting that leptin may have a paracrine function in these tissues. In mammals, gastric leptin is responsible for the short-term response to food intake mediated by satiety peptides such as cholecystokinin (Bado et al., 1998; Pico et al., 2003) and leptin expressed in skeletal muscle is involved in stimulation of glucose uptake (Cusin et al., 1998; Haque et al., 1999; Kamohara et al., 1997; Minokoshi et al., 1999). In contrast, a hemocrine action of leptin is predicted in tissues where only the receptor is expressed (Crespi and Denver, 2006).

In order to establish the expression pattern of LEPR between tissues more reliably, quantitative real-time PCR was performed in two different age groups of animals. The tissue distribution of LEPR mRNA was broader in juveniles compared with adult animals. The most widespread expression was observed in the smallest group of animals. In adults, the highest expression level was detected in the brain and skeletal muscles (Figure 29B) which agreed with the qualitative RT-PCR results (Figure 27). In juveniles, LEPR expression was high in the brain, skeletal muscles and stomach with moderate expression in the heart, skin, intestine, spleen and fat. Weak expression was observed in the liver and lung. However, when LEPR expression in skeletal muscle, stomach and brain was directly compared between the juvenile and adult animals by real-time PCR, no significant differences in expression with age were detected. In contrast, the analysis done for leptin in the fat, brain and heart shows that leptin expression is generally higher in the juveniles (Chapter 4).

The cloned Tiger salamander LEPR cDNA fragment was identical to the axolotl receptor sequence suggesting that the LEPR has been conserved in these closely-related species. Analysis of tissue expression was performed on the same cDNA samples used by Boswell et al. (2006) to characterise the leptin gene in the Tiger salamander. The LEPR tissue distribution was broader than that obtained for leptin expression in the

same animals (Boswell et al., 2006), however the expression pattern was similar with the highest levels in the brain, stomach, skeletal muscles and tongue (Figure 28). Faint LEPR expression was observed in the testis cDNA sample from the male salamander where strong leptin expression was reported in the previous study. However, although the skin showed strong leptin expression in one individual, it was not a site of LEPR expression. Overall, the tissue expression of the LEPR was very similar between the Tiger salamander and the axolotl, even though the Tiger salamanders sampled were metamorphosed animals.

The fact that expression of LEPR was found in axolotl embryos before feeding stages and before adipose tissue formation (late gastrula stage) suggests a potential role for the leptin system in amphibian embryonic growth and development. These finding agree with developmental expression of leptin and its receptor in *Xenopus tropicalis* (Crespi, Denver, 2006) and also in the Zebrafish (Liu et al., 2010). Although LEPR expression was not localised to specific tissues in the present study, the main site of LEPR expression in the embryonic zebrafish was in the notochord (Liu et al., 2010). In the present study a distinct peak in LEPR expression was observed around the time of hatching. Interestingly, a similar observation was made in the zebrafish when temporarily increased LEPR expression was associated with the switch in nutrition from yolk to external food and the transition to free swimming (Liu et al. 2010).

The general observation of LEPR expression during development in the axolotl is consistent with studies in mammals implicating leptin in the growth and development of the fetus, both through placental and fetal expression of the leptin and leptin receptor genes (Hoggard et al., 1997; Takahashi et al., 1999; Schubring et al., 1996; Heaney et al., 1996).

In conclusion, an axolotl cDNA fragment has been identified that shares similarities to characterised vertebrate LEPRs in its nucleotide and amino acid sequence and expression pattern. Conserved structure and function support the idea that the found sequence is orthologous to other vertebrate LEPRs. However, despite several attempts, using different methods, it was not possible to obtain a full-length cDNA sequence for the axolotl LEPR. The reasons for this are unclear. However, the similarity of the expression pattern to that observed in other vertebrates does provide compelling evidence that the fragment cloned in the present study does indeed represent the axolotl LEPR.

Chapter 4. Leptin

4.1 Introduction

To investigate the molecular evolution of leptin, it is crucial to identified leptin-like molecules and its function in non-mammalian vertebrates. The first evidence to suggest that the leptin gene has been conserved in non-mammalian vertebrates was based on Southern hybridization of a mouse leptin probe to genomic DNA from chicken and eels (Zhang et al., 1994). Afterwards, the cloning of a chicken leptin cDNA was reported (Taouis et al., 1998, Ashwell et al., 1999) and indicated that the identified cDNA shared 97% identity with mouse leptin at the amino acid level. The close similarity between mouse and chicken leptin induces doubt concerning the nature and origin of this sequence (Friedman-Einat et al., 1999, Dunn et al., 2001). Furthermore, analysis of the sequenced avian genomes suggests that leptin is absent (Pitel et al., 2010). However, there is evidence for a leptin-like gene in the genome of *Anolis carolinensis* (Boswell, 2011) together with leptin-like immunoreactivity in tissues from *Sceloporus undulates* and *Podarcis sicula* (Niewiarowski et al., 2000, Paolucci et al., 2001; Paolucci et al., 2006; Sciarrillo et al., 2005, Spanovich et al., 2005).

Therefore, information on the evolution of the leptin signalling pathway and its functions in non-mammalian vertebrates is very limited. More is known about leptin in fish than other non-mammals, beginning with the identification and characterisation of the leptin gene in the pufferfish (*Takifugu rubripes*) (Kurokawa et al., 2005). Full length leptin genes have since been identified in the common carp (*Cyprinus carpio*) (Huising et al., 2006a), zebrafish *(Danio rerio)* (Gorrisen et al., 2009), Atlantic salmon *(Salmo salar)* (Ronnestad et al., 2010), arctic charr *(Salvelinus alpinus)* (Froiland et al., 2010) and grass (*Ctenopharyngodon idellus)* and silver carp *(Hypophthalmichthys molitrix)* (Li et al., 2010). In contrast to pufferfish and arctic charr, duplicate leptin genes have been identified in carp (Huising et al., 2006a; Li et al., 2010), zebrafish, medaka (Gorrisen et al., 2009) and Atlantic salmon (Ronnestad et al., 2010), which derived from whole-genome duplication early in the teleost lineage (Gorissen et al., 2009). The predicted fish leptin proteins show less than 25% amino acid sequence identity with mammalian leptins and the identity between pufferfish and carp leptins is only slightly higher (Huising et al., 2006a). The discovery of leptin genes in fish is in agreement with

the detection of leptin-like immunoreactivity in the blood, brain, heart, stomach and liver of green sunfish (*Lepomis cyanellus*), bluegill sunfish (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), white crappie (*Pomonix annularis*), channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*) (Johnson et al., 2000), sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*) (Russo et al., 2010) and catshark (*Scyliorhinus canicula*) (Gambardella et al., 2010).

Evidence for leptin-like genes in amphibians was presented shortly after the discovery of leptin genes in fish. Leptin genes have now been described for four amphibian species: the tiger salamander (*Ambystoma tigrinum)* (Boswell et al., 2006), the axolotl (*Ambystoma mexicanum)* (Boswell et al., 2006), *Xenopus tropicalis* (Crespi and Denver, 2006), and *Xenopus laevis* (Crespi and Denver, 2006). The amphibian leptin genes share less than 35% amino acid identity to mammalian leptins. Although amphibian leptin was originally identified in the Tiger salamander, the axolotl is a more widely studied laboratory amphibian. It offers the opportunity to extend what is known about leptin in amphibians by allowing comparison with observations on the leptin gene expression in *Xenopus* (Crespi and Denver, 2006). The aim of this chapter is to use the axolotl leptin gene sequence to analyse the tissue distribution and developmental regulation of leptin gene expression.

4.2 Methods (see General Methods)

4.3 Results

4.3.1 *Cloning of axolotl leptin*

A partial cDNA sequence corresponding to a putative leptin gene was isolated from an axolotl hatched larvae. Primers were designed to amplify a fragment of axolotl leptin cDNA and the resulting sequence information was compared to an axolotl EST clone deposited in GenBank (Accession No. CO792338). The 425-bp fragment encodes 111 amino acid residues. The cDNA fragment isolated by PCR was 98.8% identical to the

EST sequence, differing by 5 gaps (at positions: 502, 520, 568, 580, 581 in axolotl EST sequence) and 5 substitutions (at positions: 425, 476, 551, 583, 584 in axolotl EST sequence). Furthermore, the axolotl cloned sequence shared 99% with salamander leptin (DQ64637) and differed by an A to G synonymous substitution at position 281 in the axolotl sequence (at position 476 in salamander sequence). The cDNA identified shares 67% with *Xenopus laevis*, 37% with human leptin, and 6-25% with fish leptins.

CTTCTCCCGATGAACCTGAAGGTGAGCGGCTTAGACTTCATTCCAGGAGAGCA
GTCCCTGGAGAGTTTGGACTCAGTAGACGAAACCCTAGAGATCTTCCATGCCATC
CTCTCCAGCCTACACATGGACAACATGGAACAGATCCTCAGCGACATTGAGAAC
CTCCGGCGCCTTCTCCATGCCTTAAGTTCTCTGCTTGGCTGCAATGCCC**G**GAAGA
GCGTGCATCCAGACACTCTGGGGAACCTGACAGAAGAGTATGCCAAGTCTCCAT
TCACAACGGAGAAAGTTGCGTTGGATAGGTTTCAGAAGAACCTTCACAGCATTG
TCAAACATTTGGAGCATACCTTGAGCTGCTGAAGGGAACAAAAACAGACTCTGG
GATATTGAACCCCACCATGAACATGG**TTGTGTCCAACTCCCACAAA**

V S G L D F I P G E Q S L E S L D S V D E T L E I F H A I L S S L H M D N M E Q I L S
D I E N L R R L L H A L S S L L G C N A R K S V H P D T L G N L T E E Y A K S P F T
T E K V A L D R F Q K N L H S I V K H L E H T L S C

Figure 31 Nucleatide and amino acid sequence of leptin gene from axolotl. The positions of the primers in the nucleotide sequence are in bold and underlined. An A to G substitution compared to the Tiger salamander and axolotl EST CO792338 is shown in red. The predicted amino acid sequence does not include the primers.

To investigate the leptin gene structure, PCR amplification of axolotl genomic DNA was performed with the same primers used for cDNA amplification. This yielded a 4.5 kb fragment (Figure 32). In contrast, a 409 bp product was obtained using a cDNA template, indicating that the cloned leptin fragment contained introns. Furthermore, splice site prediction programs (www.fruitfly.org/seq_tools/splice.html; http://www.cbs.dtu.dk/services/NetGene2/) predicted an exon-intron boundary with confidence scores of 0.55 and 0.77, respectively (at nucleotide positions 76-116; 86-106). The predicted splice donor and acceptor site corresponds to the position of the boundary in the mammalian leptin gene (between exon 2 and intron 2) (He et al., 1995).

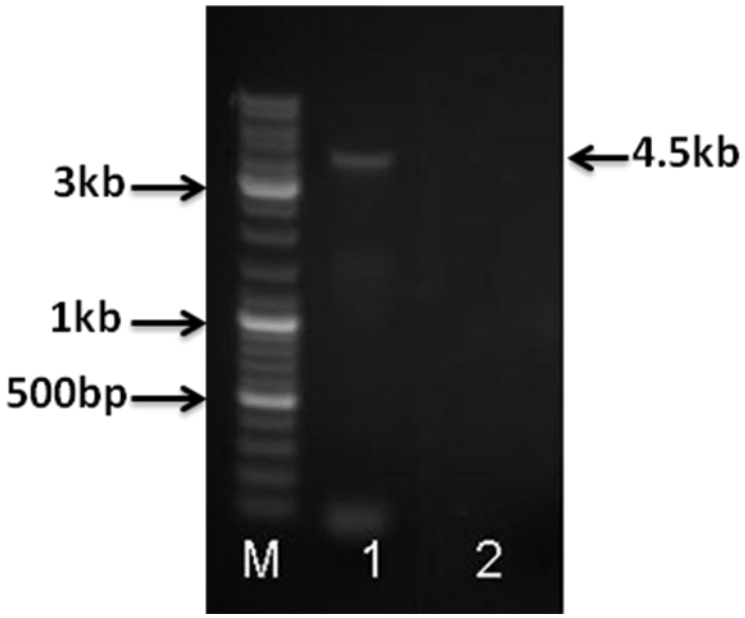


Figure 32 Amplification of axolotl genomic DNA. Lane 1 represents generated amplification product (4.5 kb) from 300 ng axolotl genomic DNA template. M designates size marker and lane 2 represents no template control.

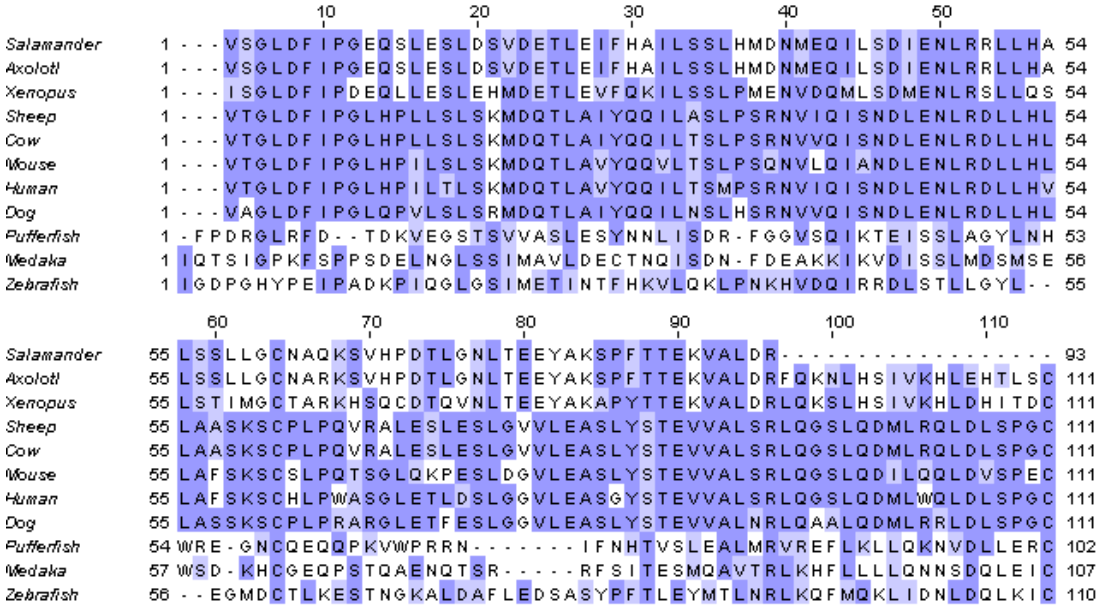


Figure 33 Amino acid alignment of vertebrate leptin on the basis of amino acid sequences of leptin in human (*Homo sapiens*; AAH69323), cow (*Bos taurus*; CAD54745), sheep (*Ovis aries*; Q28603), mouse (*Mus musculus*; ADM72802), dog *(Canis familiaris;* BAA35129), *Xenopus leavis* (NP_001089183), salamander *(Ambystoma tigrinum;*AAY68394) and zebrafish (*Danio rerio;*NP_001122048) sequences. The alignment was done in ClustalW (http://www.clustal.org/)

4.3.2 *Molecular phylogeny*

Phylogenetic analysis of leptin was performed in order to gain insight into the evolutionary history of this protein. Deduced amino acid sequences were compared to the equivalent region of leptin sequences available in GenBank. The alignments were used to construct a phylogenetic tree, which shows that the accepted evolutionary relationships between the species analyzed are consistent with the consensus view of vertebrate evolution (Figure 34) and group with *Xenopus* leptin.

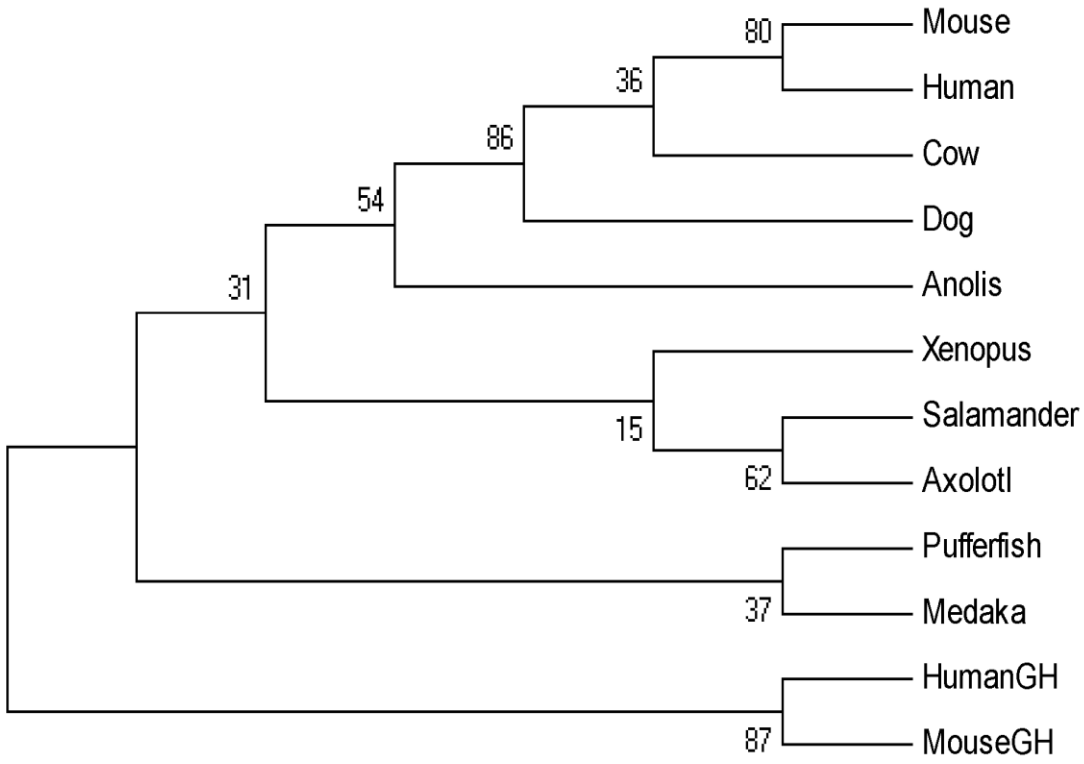


Figure 34 A phylogenetic tree (phylogram) was constructed on the basis of amino acid sequences of leptin in human (*Homo sapiens*; AAH69323), cow (*Bos taurus*; CAD54745), mouse (*Mus musculus*; ADM72802), dog *(Canis familiaris;*BAA35129), *Anolis carolinensis* (XP003229128), *Xenopus leavis* (NP_001089183), salamander *(Ambystoma tigrinum;*AAY68394), medaka *(Oryzias latipes;*BAD94448), pufferfish *(Takifugu rubripes;* BAD94444), axolotl (Ambystoma mexicanum), human growth hormone (GH; PO1241), and mouse growth hormone (GH; PO6880). The tree was calculated using the Maximum Likelihood method based on the Equal Input model (Tajima and Nei, 1984). The tree with the highest log likelihood (-3627.7182) is shown. The analysis involved 12 amino acid sequences. There were a total of 136 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Numbers at branch points represent the bootstrap value for 1000 replicates as percentages and indicate the statistical reliability of a node in the tree. Human and mouse growth hormone (human GH, mouseGH) were used as an outgroup.

4.3.3 *Northern analysis*

To investigate the presence of leptin in axolotl, Northern hybridization was carried out using poly(A)+ RNA extracted from axolotl testis. Radioactive detection was performed using RNA from the testis hybridized with axolotl leptin oligonucleotide probes yielding a band of RNA within an expected range of transcript sizes: 1.9 kb - 6 kb (Figure 35).

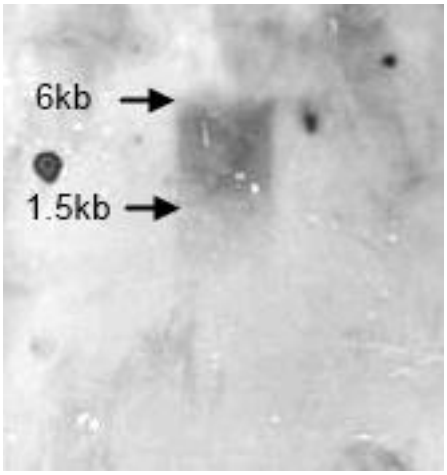


Figure 35 Northern hybridization of leptin poly(A)+RNA isolated from axolotl testis. A 9 µg aliquot of poly(A)+ RNA was electrophoresed through a 1% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with 32P-labeled antisense axolotl leptin receptor oligonucleotides. The hybridization signal was obtained after 2 week exposure to a film.

4.3.4 *Developmental expression of the leptin gene during axolotl embryogenesis*

The developmental expression of leptin mRNA in the axolotl was analyzed by qualitative RT-PCR. Leptin expression was not detectable during early development (neurulation) and tailbud stages (stages 29-32), however it increased markedly around the time of hatching (stages 41-43) so that it was detected only in the two last stages of embryonic development (stages 43 and 44) (Figure 36).

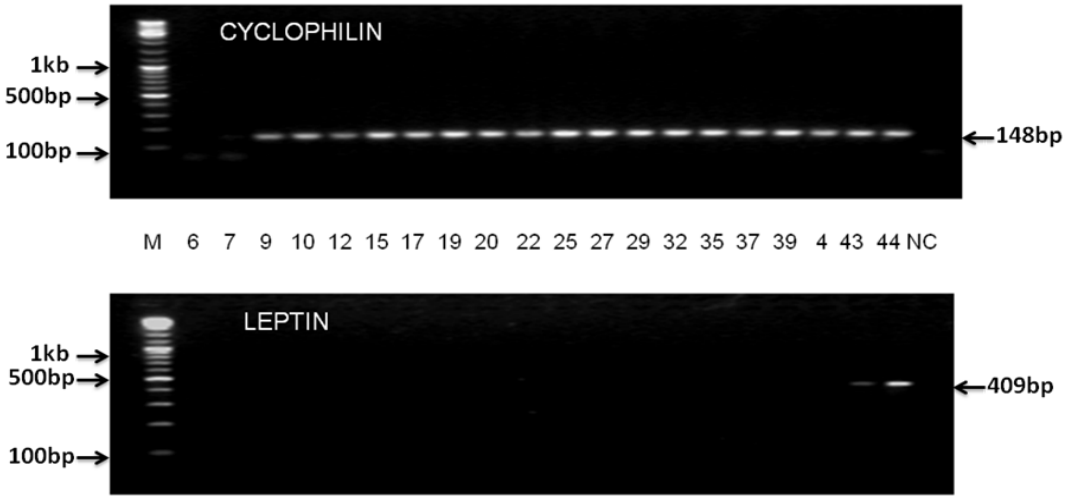


Figure 36 Developmental expression of leptin in the axolotl. Qualitative RT-PCR analysis of leptin during embryogenesis and early larval development. Amplification of cyclophilin indicates the presence of cDNA at the different developmental stages. M indicates DNA size ladder; NC – negative controls; the numbers – developmental stages.

4.3.5 *Tissue expression of axolotl leptin gene using RT-PCR*

The distribution of axolotl leptin expression was studied by RT-PCR on mRNA isolated from a variety of tissues in three different age groups of animals: 6 months old (no gonad development), 10 months old, and adults. Leptin expression differed between the individuals studied. The most widespread expression was observed in the youngest group of animals (Figure 37A). The strongest intensity of the PCR product was observed in the fat body and heart with moderate expression also being seen in the skin, skeletal muscles, and stomach. Weak expression was observed in the spleen, kidney and lungs. In the largest, adult, animals, leptin expression was detected mainly in the gonads and the heart (Figure 37C).

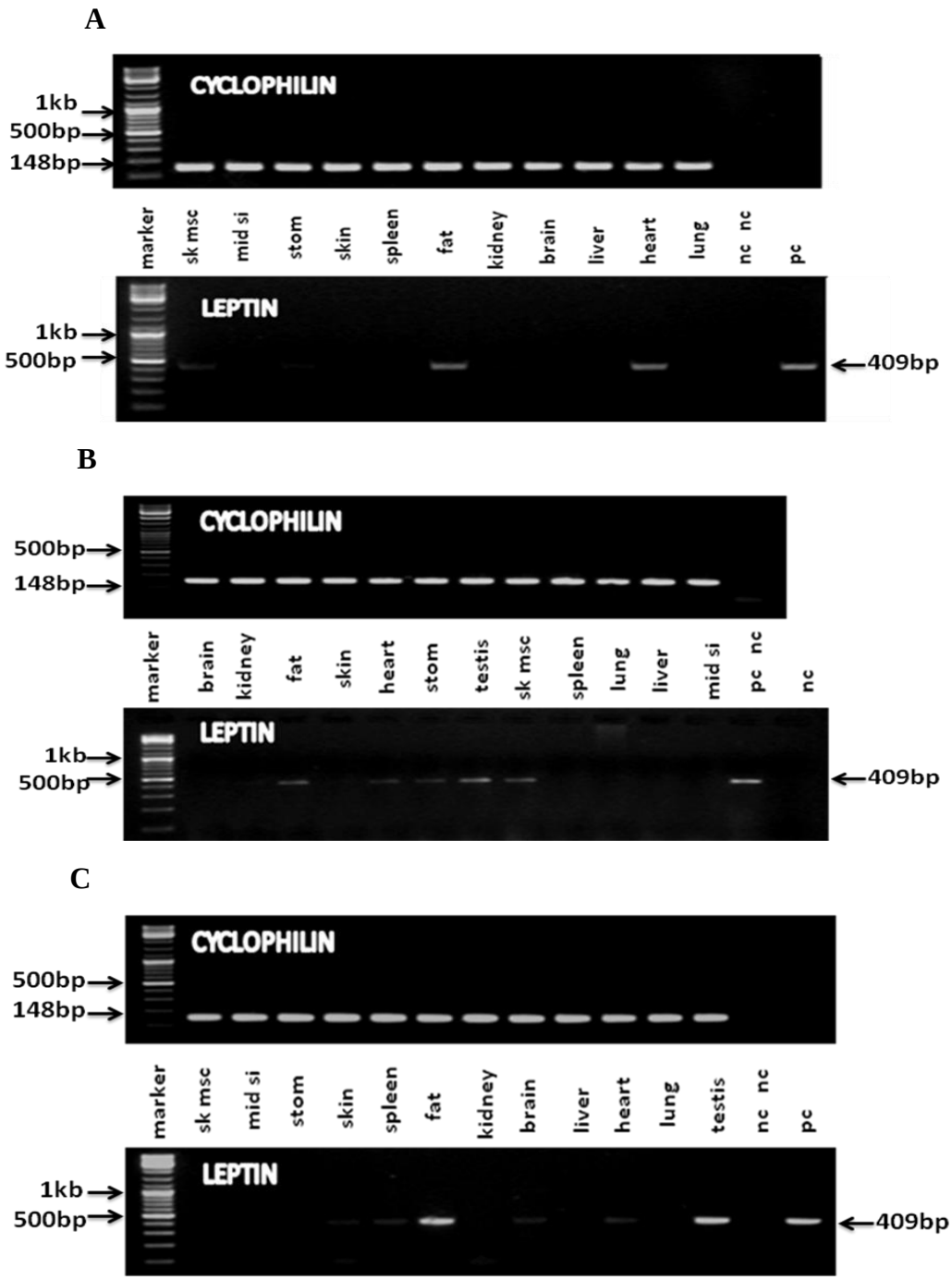
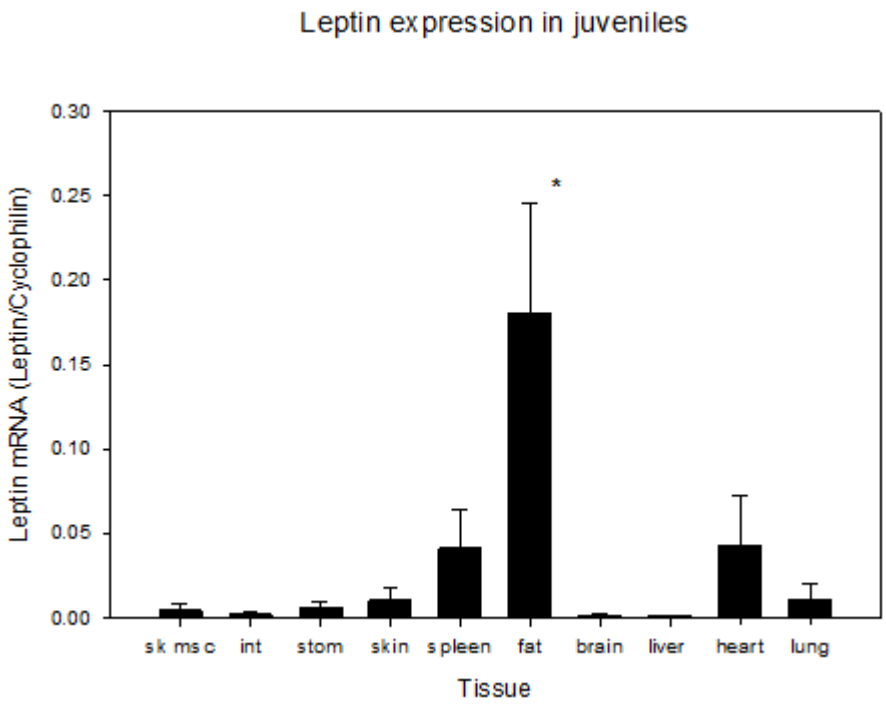


Figure 37 Tissue distribution of leptin mRNA in the axolotl using RT-PCR. Distribution is shown in representatives from three different age groups of animals (A, 6-month old animal; B, 10-month old male; C, 3-year old male). For each individual, the distribution for cyclophilin mRNA is shown in the upper panel, and for leptin mRNA in the lower panel. Identical lanes were used for leptin and cyclophilin in each individual. Note that the tissues sampled vary between individuals. Tissue abbreviations: Sk Msc, skeletal muscle; Mid SI, mid small intestine; Stom, stomach; Fat, fat body; NC, negative control (no template); PC, positive control.

4.3.6 *A quantitative real-time RT-PCR assay for axolotl leptin*

The expression pattern of leptin between tissues was established by quantitative real-time PCR in two different age groups of animals. The juveniles were 6-months old with no gonads developed; the adults were 3-years old. The tissue distribution of leptin mRNA was broader in the juvenile axolotls compared with the adult animals. In adults, the highest expression level was detected in the brain, fat, heart and testis (Figure 38B), which agrees with the qualitative RT-PCR results (Figure 37C), however expression did not differ significantly across tissues ($F(9,38)=2.136$, $p=0.059$). In contrast, in juveniles, leptin expression differed significantly across tissues ($F(9,46)=2.291$, $p=0.37$). The highest expression was in the fat body, which was significantly higher than in the skeletal muscles, small intestine, stomach, brain, spleen, liver and lung ($p<0.05$) (Figure 38A).

A



B

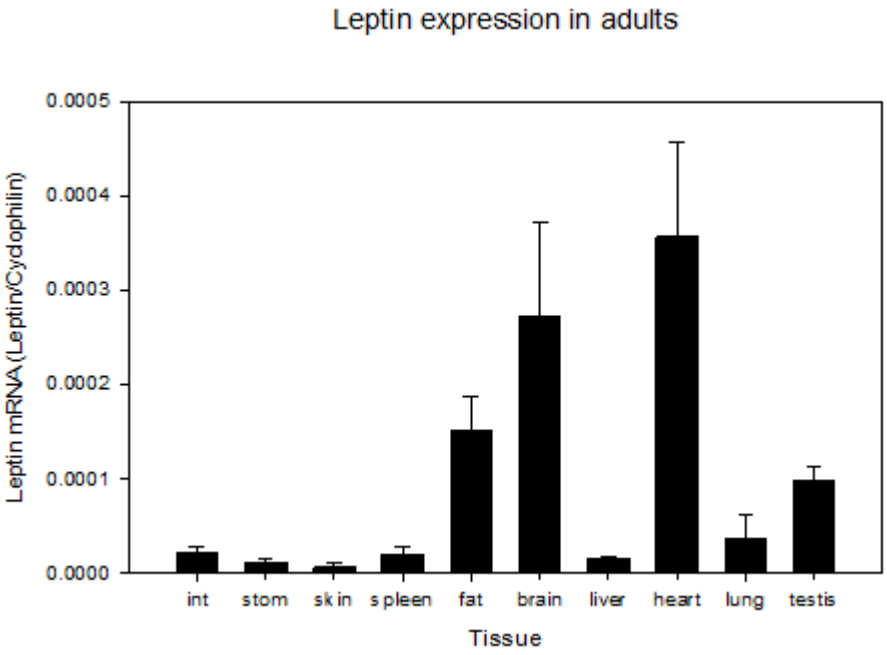


Figure 38 Expression of leptin in the axolotl. The tissue distribution in two different age groups of animals (A, juveniles, 6 month old; B, adults, 3 year old) was analyzed by quantitative RT-PCR. Leptin level was normalized to the expression of the cyclophilin gene. Bars represent means (including SEM). Asterisk indicates significant differences for the fat compared with the skeletal muscles, small intestine, stomach, brain, spleen, liver and lung (Fisher's least significant difference test; $p<0.05$).

In order to compare leptin expression levels between the juvenile animals and the adults, another quantitative real-time PCR was performed. Leptin expression across tissues was significantly ($F(1,14)=5.65$, $p<0.05$) higher in the juvenile animals, but no significant differences were detected by post hoc comparisons between individual tissues (Figure 39).

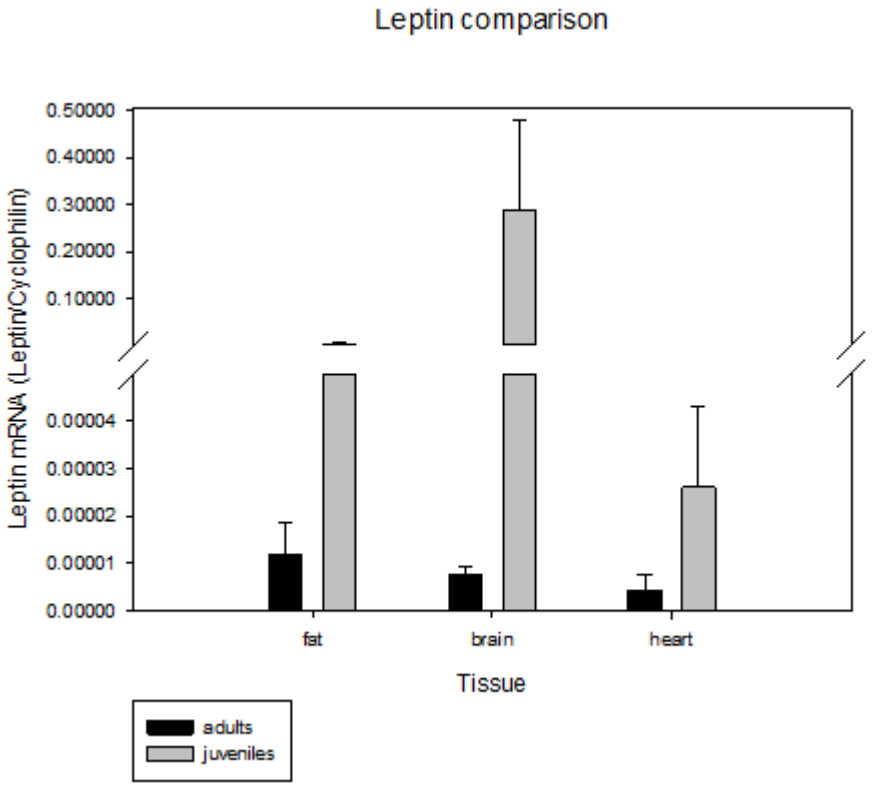


Figure 39 Comparison of expression levels of leptin between juveniles and adults

4.4 Discussion

An axolotl leptin-like molecule and its expression has been characterised. The identified sequence shares similarities on structural and expression pattern levels with mammalian leptin genes. The predicted amino acid sequence showed 37% identity with human leptin, 67% with Xenopus leptin and 99% with Tiger salamander leptin. The highest expression level was found in the fat, brain, heart and testis by analysing results from Northern blot, RT-PCR and real-time PCR.

The phylogenetic tree of mammalian, amphibian and fish leptin sequences shows that the identified sequence in naturally present in the axolotl and that the accepted evolutionary relationships between the species analysed are consistent with the consensus view of vertebrate evolution. A very high identity at the nucleotide level of

98% was found between the leptin fragment cloned and the equivalent sequence from the Tiger salamander and another axolotl EST sequence indicating that leptin has been highly conserved in these closely-related species. As previously shown in the Tiger salamander (Boswell et al., 2006) the axolotl leptin coding sequence studied contained an intron, as demonstrated by PCR amplification of genomic DNA. However, the evolutionary distance between the axolotl and the Tiger salamander corresponded to a difference in the size of the intron – the fragment amplified was 4.5 kb in the axolotl and 6 kb in the Tiger salamander. The general finding that the leptin coding sequences contains two exons is consistent with mammalian (He et al., 1995) and fish (Kurokawa et al., 2005) leptin gene structure.

Northern hybridisation analysis demonstrated that the axolotl leptin transcript lies within the range of transcript sizes reported in other animals (human - 3.4 kb; mouse - 4.5 kb; bat – 2.3 kb; Tiger salamander – 1.9 kb) (He et al., 1995; Zhao et al., 2003; Boswell et al., 2006). This result shows that the cloned gene is naturally expressed in the testis.

The expression pattern of leptin between tissues was established by quantitative real-time PCR in two different age groups of animals. The tissue distribution of leptin mRNA was broader in the juveniles compared with the adult animals. In juveniles, the strongest intensity of the PCR product was observed in the fat body and heart with moderate expression also being seen in the skin, skeletal muscles, and stomach. Weak expression was observed in the spleen, kidney and lungs. In adult axolotls leptin expression was detected mainly in the fat, testis and heart. In the closely-related tiger salamander (investigated in three individuals by qualitative PCR), the highest leptin mRNA was observed in the skin and testis and moderate expression in the brain, stomach, small intestine, colon, skeletal muscle, tongue, and fat body (Boswell et al., 2006). Thus the general pattern of expression was comparable between the two species, although stronger expression was observed in the skin in the tiger salamander, and no leptin expression was reported in the heart. When leptin expression in the fat, brain and heart was directly compared, between juvenile and adult axolotls the overall level of leptin expression across tissues was significantly higher in the juveniles. This may be linked to the general observation of more widespread leptin expression across tissues in both axolotls and tiger salamanders, and suggests that leptin gene expression may be developmentally regulated in salamanders (Boswell et al., 2006). In contrast, however,

the analysis done for LEPR in the brain, skeletal muscles and stomach demonstrated no significant difference in expression with age (Chapter 3).

The general pattern of leptin expression in salamanders is generally comparable with observations in *Xenopus laevis*, even though the amino acid sequence similarity between the two species is only 60%. In Xenopus, the strongest intensity of leptin expression was detected in the brain and heart but also in liver, brain, pituitary gland, heart, gastrointestinal tract, lungs, kidney, and gonads (Crespi and Denver, 2006). Amphibans and teleost fish show more widespread tissue distribution of leptin expression compared to mammals, suggesting a wider range of leptin functions. In teleost fish, the liver is consistently a strong site of leptin expression and adipose tissue weak (Copeland et al. 2011). Conversely, in amphibians, expression is more mammal-like in that expression is greater in adipose tissue compared to liver. This may be related to metabolic differences between these ectothermic taxa.

In addition to the fat body, the most prominent sites of leptin expression in the axolotl were the heart, brain and testis. The expression of leptin in the heart may be related to reports in mammals of a hypertrophic effect of the hormone on cardiac muscle cells (Karmazyn et al., 2007) while leptin acts in the mammalian brain to regulate neuronal plasticity and excitability (Harvey, 2007). Moderate leptin expression was observed in the testis in the sexually mature axolotls and strong expression in the tiger salamander. Reports of leptin expression in the testis are rare in mammals, although it has been linked to testicular development in the mouse (Herrid et al., 2008). As leptin expression was also prominent in the gonads of *Xenopus laevis*, the local production of leptin may be more significant in controlling gonadal function in amphibians compared to mammals. It was observed that in the axolotl, the fat body is situated next to the testis and is reduced in size as the testes develop suggesting interplay between these organs during reproductive development where leptin may act as a link between adipose tissue and the reproductive system.

During axolotl embryonic development, leptin expression was found before feeding stages and before adipose tissue formation (late gastrula) and it increased markedly around the time of hatching. This finding suggests a potential role for the leptin system in amphibian embryonic growth and development, which agrees with developmental expression of leptin in *Xenopus tropicalis* (Crespi and Denver, 2006) and also in the

Zebrafish (Liu et al., 2010). It is likely that the peak in leptin expression at hatching, associated with the transition to free-feeding is linked to the establishment of brain circuits controlling energy metabolism. For example, in Atlantic salmon, a peak in leptin expression was observed in the head after the larvae starting feeding which was associated with peaks in expression of the POMC and CART genes known to be regulated by leptin in mammals (Moen et al., 2010).

In conclusion, an axolotl cDNA fragment has been identified that shares similarities to other characterised vertebrate leptins in its nucleotide and amino acid sequence and expression pattern. Identification of leptin-like genes in amphibians and fishes demonstrates that leptin is of ancient origin in vertebrates rather than having evolved recently in mammals. The more widespread expression of leptin in amphibians and fish, including prominent expression in tissues other than fat, suggest a wider range of leptin functions in non-mammalian vertebrates compared to mammals.

Chapter 5. Changes in leptin expression with nutritional state

5.1 Introduction

In order to understand the possible role(s) of leptin in the regulation of food intake and energy metabolism in amphibians, it is essential to study changes in leptin expression due to nutritional state. The effects of feeding status in non-mammalian vertebrates have been mostly investigated in fish. Leptin gene expression has been studied in carp (Huising et al., 2007a), rainbow trout (Kling et al., 2009) and Atlantic salmon (Rønnestad et al., 2010) in response to short and long-term fasting, refeeding and feeding to satiation. Leptin expression was unaffected by fasting, feeding to satiation or refeeding in carp (Huising et al., 2007a), unlike the situation in mammals where fasting decreases leptin expression, and refeeding increases it (Saladin et al., 1995). In contrast, long-term food restriction in Atlantic salmon lowered expression of the lep-A1 transcript in the fat and white muscle, however plasma concentrations of salmon leptin did not show any difference. The same results were obtained from rainbow trout (Kling et al., 2009). Moreover, increased leptin expression was observed after ingestion of a single scheduled meal, providing evidence that leptin may act as a short term signal of satiety in carp (Huising et al., 2007a). This result correlates to the postprandial increase in leptin mRNA that is observed in the hours following feeding in mice (Saladin et al., 1995) and is in agreement with the observation of an inhibitory effect on food intake following injection of recombinant mammalian leptin into the goldfish brain (Volkoff et al., 2003).

Thus, leptin mRNA expression in fish is altered by changes in food intake, but there is limited evidence for involvement of leptin in the long-term regulation of food intake and energy metabolism as is the case in mammals (Huising et al., 2006a). These findings may be related to the differences in energy metabolism between endotherms and ectotherms. Ectotherms do not need to thermoregulate, thus their metabolic regulation is more flexible (Huising et al., 2006). For this reason the physiological role of leptin in ectotherms may differ quantitatively, if not qualitatively, from its role in mammals. However, this has not been tested in ectothermic vertebrates outside fish. Although the effects of leptin on feeding behaviour have been studied in *Xenopus laevis*

(Crespi and Denver, 2006), the effects on nutritional state on leptin gene expression have not yet been studied in amphibians. To address this, the effects of feeding status and mRNA levels in selected tissues were examined in two groups of axolotls which had been either fed to satiation (every day), or fed every other day for 14 days. If leptin expression is regulated in a similar way to mammals, a significantly higher level of leptin expression would be expected in the fed, compared to the restricted-fed group.

5.2 Materials and methods

Axolotls (6 months old) were purchased from The Ambystoma Genetic Stock Centre, University of Kentucky, USA and were maintained at the Comparative Biology Centre, Newcastle University. Five days after arrival in Newcastle, during which they were allowed to recover and acclimatise, the axolotls were divided into two groups of twelve animals. They were housed individually in tanks at 20˚C in dechlorinated tap water, and were fed commercial amphibian pellets. The first group was fed to satiation (every day) and the second group was fed every other day over a 14-day period. At the end of the experiment, the animals were humanely killed at 24 h after the last feeding time by immersion in 0.1% tricaine methane-sulfonate (ethyl 3-aminobenzoate methane-sulfonate) anaesthetic (Sigma, Poole, Dorset, UK), in accordance with the United Kingdom Home Office Code of Practice. Following dissection, tissues were immediately incubated in RNAlater (Ambion, Applied Biosystems, Warrington, Cheshire, UK), stored at 4˚C overnight, and were then frozen and stored at -80˚C.

Nucleic acid extraction, cDNA synthesis, RTqPCR analysis for leptin and LEPR and statistics were performed according to the General Methods Chapter.

PCR was also carried out to amplify a cDNA fragment of axolotl NPY. Primers were designed based on an axolotl NPY EST deposited in GenBank (Accession No. AY660754). The primers used were: forward primer 5'-TGCTAACCTTTGCCCTGTCT, AxNPYF1 (positions 168-187 of Accession No. AY660754), and reverse primer 5'-GATCACCAAACCGGGATCTA, AxNPYR1 (positions 407-416 of Accession No. AY660754) yielding reaction product: 249 bp.

PCR for cyclophilin A cDNAs was performed as described in the General Methods Chapter with an annealing temperature of 60°C and denaturing and extension steps of 94°C and 72°C, respectively. Times used were 15 sec denaturation, 30 sec annealing, and 30 sec extension, with an extension time for the final cycle of 7 min. PCR was carried out for 30 cycles.

5.3 Results

5.3.1 *Body mass*

The effect of the nutritional manipulation was demonstrated by the differences in body mass between control and restricted-fed axolotls. Restricted feeding for 14 days (fed every other day) resulted in significantly lower body mass and length ($p<0.001$) compared to normally fed axolotls (fed every day) (Figure 40). Fed axolotls increased from 5.79 ± 0.19 to 12.48 ± 0.5 g during the14-day experimental period, while restricted-fed axolotls exhibited a gain in body mass from 5.94 ± 0.24 to 7.96 ± 0.29 g. Thus there was a 47.6% increase in body mass in the fed group, compared to a 25.9% increase in the restricted-fed group.

5.3.2 *Leptin and LEPR expression*

Expression of leptin and LEPR were investigated in fat, brain and heart for leptin, and brain, stomach and skeletal muscles for LEPR because gene expression had previously been demonstrated in those tissues (Chapters 3 and 4).

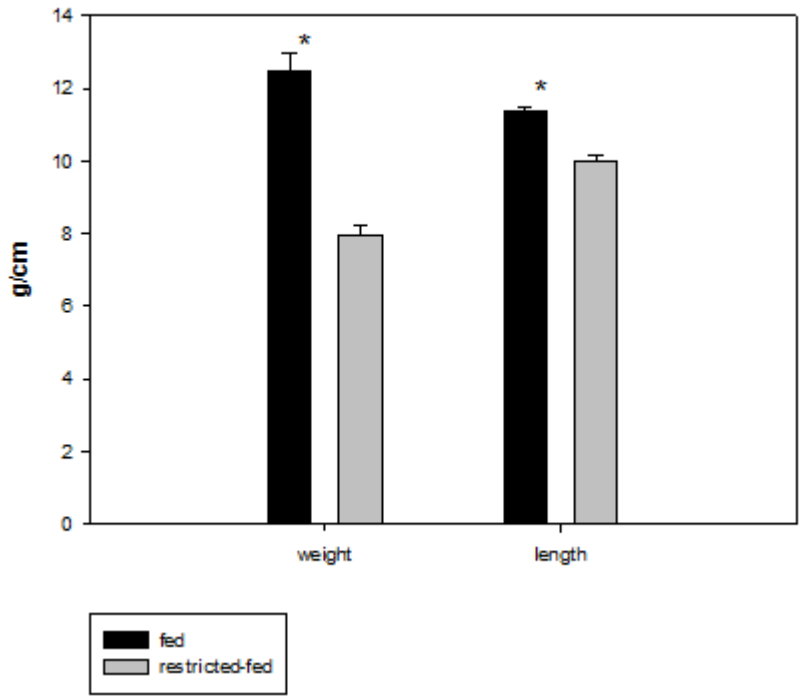
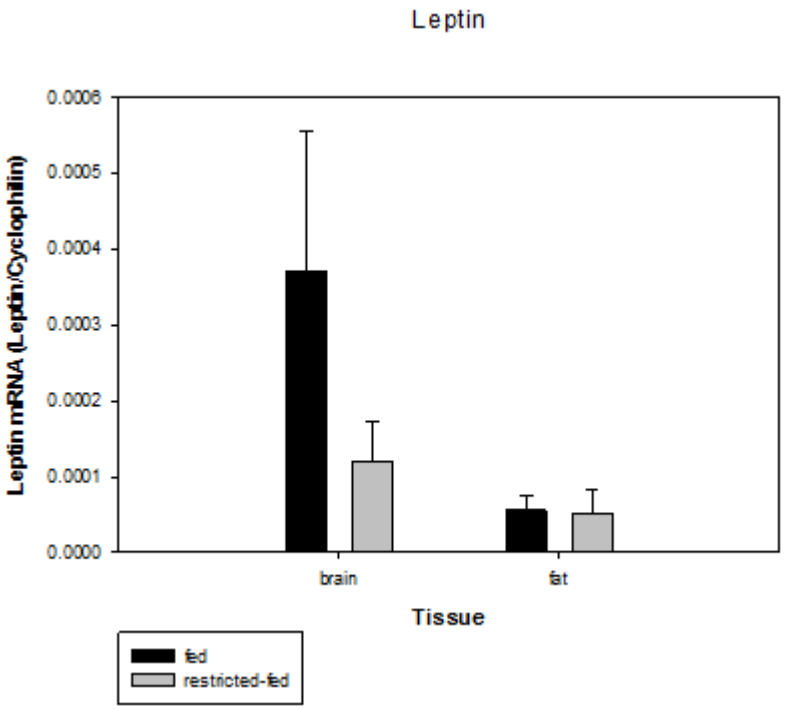


Figure 40 Effect of restricted feeding for 14 days on axolotl size. Bars represent means (including SEM). Asterisks indicate significant differences in body mass and length between fed and restricted-fed groups of animals (Fisher's least significant difference test; $p<0.001$).

In all analysed tissues there was a tendency towards a lower leptin and LEPR expression of restricted-fed axolotls, although no significant differences were detected in any tissue between the groups. Analysis for leptin expression in fat and brain demonstrated a trend for higher leptin expression in the fed group (Fat: $F(1,18)=2.068$; $P=0.169$; Brain: $F(1,18)=1.559$; $P=0.229$) (Figure 41A). The expression of leptin in heart was not detectable. Expression of LEPR in brain and skeletal muscles was also not affected by feeding regime, however showed a tendency towards a higher expression in the fed group of animals (Skeletal muscle: $F(1,20)=0.000$, $P=0.992$; Brain: $F(1,17)=0.091$ $P=0.767$) (Figure 41B). The expression of LEPR in stomach was not detectable.

A



B

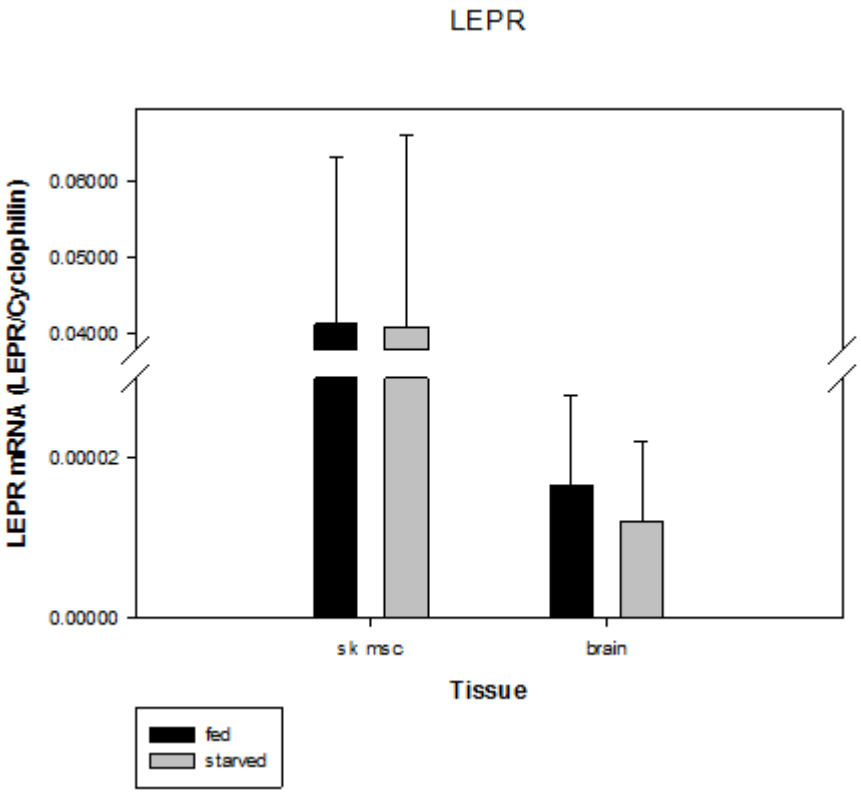


Figure 41 Effect of restricted feeding of axolots for 14 days on leptin (A) and LEPR (B) expression in selected tissues. Leptin and LEPR levels were normalized to the expression of the cyclophilin gene. Bars represent means (including SEM).

5.3.3 *NPY expression*

NPY gene expression is regulated by leptin in mammals and was investigated here as an additional marker of nutritional status, Higher NPY expression was found in the restricted-fed group of animals, however there was no significant difference between the groups ($F(1,15)=2.211$; $P=0.159$).

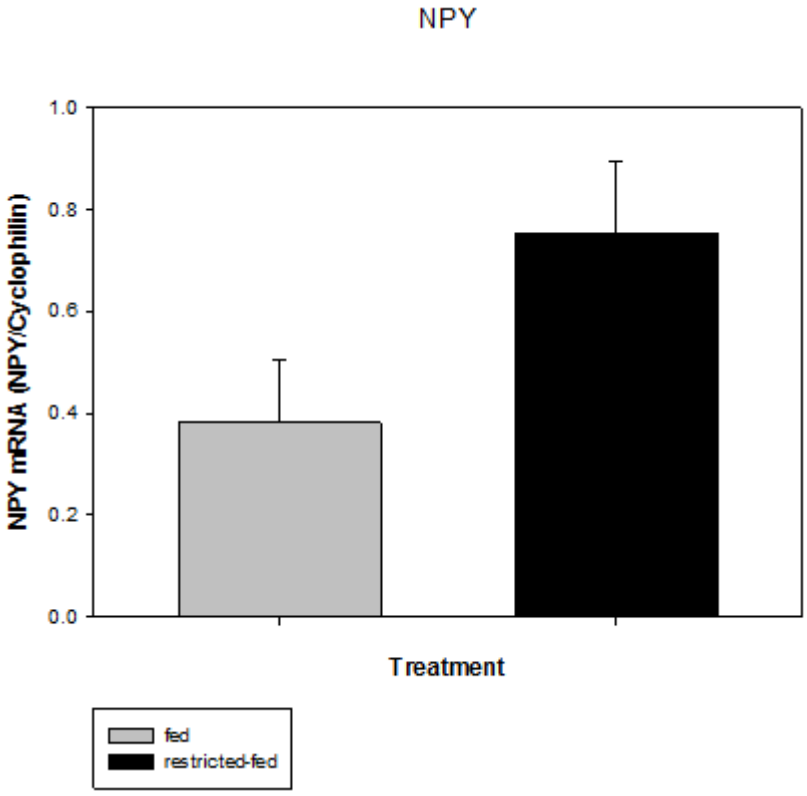


Figure 42 Effect of restricted feeding of axolotls for 14 days on NPY expression in the brain. NPY mRNA levels were normalized to the expression of the cyclophilin gene. Bars represent means (including SEM).

5.4 Discussion

From mammalian studies (Saladin et al., 1995), it would be predicted that leptin expression would be higher in the fully fed axolotls compared to the restricted-ffed animals. However, no significant differences were found in leptin and LEPR expression between the experimental groups, suggesting that the nutritional regulation of these genes may be more similar to the pattern in fish than mammals.

The impact of the nutritional manipulation was demonstrated by a significant difference in body mass between the fed and restricted-fed animals. Axolotls that were fed to satiation grew twice as fast as restricted animals. However the clear difference in nutritional state was not reflected in increased leptin expression in the fed group at the termination of the experiment. Moreover, after 14 days of fasting, the brain expression of orexigenic NPY was not significantly changed, however it showed a tendency towards higher NPY expression level in fasted animals. This results show similarity with mammalian model, where leptin decreases NPY expression and it the same time reduces food intake (Lewis et al., 1993; Mizuno and Mobbs, 1999; Stephens et al., 1995). However, because the difference was not significant, it is unclear whether NPY gene expression is regulated by leptin in amphibians as it is in mammals.

Thus, the effects of manipulating the feeding level in axolotls more closely resembles the situation in some teleost fish studies where leptin gene expression was unaffected by fasting, feeding to satiation or refeeding (Huising et al., 2006a; Rønnestad et al., 2010). This constrasts to the situation in mammals, where fasting decreases leptin expression, and refeeding increases it (Saladin et al., 1995). Thus, leptin mRNA expression in fish is altered by changes in food intake, but there is limited evidence for involvement of leptin in the long-term regulation of food intake and energy metabolism as is the case in mammals (Huising et al., 2006a).

Collectively these findings may be related to the differences in energy metabolism between endotherms and ectotherms. Ectotherms do not need to thermoregulate, thus their metabolic regulation is more flexible (Huising et al., 2006a). For this reason the physiological role of leptin in ectotherms may differ quantitatively, if not qualitatively, from its role in mammals.

For further study, the design of the experiment should be changed. The fasting period may not have been long enough to significantly decrease leptin expression level of the restricted-fed group. Both groups at the termination of the experiment had food present in the stomach, which indicates that there is scope for a greater degree of food restriction. The timing of killing the animals may also have influenced the results because both groups had been fasted for 24 h. If the fed group had received food shortly before they were killed, it is possible that increased leptin expression may have been detected in the stomach as suggested by mammalian studies (Bado et al., 1998).

In conclusion, short-term fasting did not result in any significant changes in leptin expression in the restricted-fed animals, however it showed a tendency towards lower leptin and LEPR expression in the restricted fed axolotls. These are the first data on the regulation of leptin expression in amphibians. The results indicate that the regulation of leptin expression by nutritional state more closely resemble the situation in other ectotherms such as teleost fish. However, there were trends for leptin and NPY gene expression to change in a similar pattern to mammalian studies, so further experiments need to be performed in amphibians to confirm whether there is indeed a distinct pattern between ectotherms and endotherms.

Chapter 6. Concluding Summary

Until 2005, leptin-like molecules had only been identified in mammals, so that little is known about the molecular evolution of the hormone and its functions. However, in the last five years, more information has become available as leptin and leptin receptor genes have been sequenced in fish, amphibians and reptiles. Most of the research on leptin in non-mammalian vertebrates is being performed in fish, though research on amphibians is very limited by comparison.

Partial cDNAs corresponding to putative leptin and leptin receptor genes from axolotl hatched larvae were isolated and sequenced. The partial leptin (425bp) and leptin receptor (248bp) cDNA sequences share 37% and 61% identities, respectively, with human leptin at the amino acid level. Data from phylogenetic analysis, Northern blot, RT-PCR and RTqPCR analysis have shown that the cloned fragments are not artifacts and are naturally expressed in the axolotl.

Cloning the axolotl LEPR was a key component of the work because no sequence information was previously available. A year was devoted to attempting to obtain full-length sequence for the LEPR using PCR, 3'- and 5'-RACE, and DNA walking methods but these were not successful for reasons that remain unclear.

Despite the unsuccessful attempts to obtain the full-length axolotl LEPR sequence, the evidence presented in this thesis strongly suggests that the cloned fragment is indeed a natural ortholog of the mammalian LEPR. The phylogenetic analysis indicates that the axolotl LEPR groups with the *Xenopus* LEPR, consistent with the consensus view of vertebrate evolution. Furthermore, Northern hybridisation detected a transcript size of approximately 3kb, which lies within the range of transcripts reported in *Xenopus* and teleost fish and shows that the LEPR gene is naturally expressed in the testis. Only a single transcript was detected in axolotl testis polyA+ RNA, suggesting the presence of a single splice variant in that tissue at least. The existence of alternative splicing of the LEPR, observed in mammals, birds, and teleost fish, has not been reported in amphibians, so its significance for this Class remains to be determined.

Importantly, the LEPR tissue distribution, with the highest expression in the brain and skeletal muscles, agrees with the main sites of LEPR expression in mammals, and also

in birds, *Xenopus*, and fish (Horev et al., 2000; Richards and Poch, 2003; Crespi and Denver, 2006; Kurokawa et al., 2008; Ronnestad et al., 2010), where leptin receptor is predominantly expressed in the hypothalamus, the part of brain responsible for the control of food intake, implicating conserved function of LEPR. This finding suggested conserved function of LEPR and provides further evidence that the gene fragment cloned represents the axolotl LEPR. In addition, LEPR expression in axolotl embryos before feeding stages and before adipose tissue formation indicates that leptin signalling pathway may be involved in amphibian embryonic growth and development, showing that leptin may have diverse physiological roles in amphibians. These finding agree with developmental expression of leptin and its receptor in *Xenopus tropicalis* (Crespi, Denver, 2006) and also in the Zebrafish (Liu et al., 2010). The increased expression of both leptin and the LEPR around the time of hatching in all three species may be particularly significant because this is associated with the time that larvae are making the transition from internal to external food sources and are beginning free-swimming. Thus, leptin signaling may play an important role in this developmental transition.

To investigate the cell types in which leptin and the LEPR were expressed in the axolotl, several months were spent working on in-situ hybridisation (using both isotopic and non-isotopic approaches). The aim of this experiment was to localize leptin and LEPR mRNA within the axolotl tissues showing the highest expression of leptin and its receptor, as was reported recently for the zebrafish LEPR (Liu et al., 2010). However, it was not possible to detect hybridisation reliably, even when control probes such as oligo(dT) were used to detect all mRNAs, and different methods of tissue preservation were tested. The approach of using specific antibodies was not achievable due to a lack of antibodies against amphibian leptin and LEPR. It would have been preferable to have performed this work in a laboratory optimised for in situ hybridisation but unfortunately this was not possible.

The work presented in this thesis provides compelling evidence that the cloned fragments in the present study represent the axolotl leptin and its receptor. This has laid a platform for future investigations centred on the function of axolotl leptin and the LEPR.

Furthermore, in order to understand the possible role(s) of leptin in the regulation of food intake and energy metabolism in amphibians, changes in leptin and LEPR expression were investigated due to nutritional state. The effects of feeding status on

leptin and the LEPR in non-mammalian vertebrates have been confined to teleost fish, where leptin expression increased (Kling et al., 2009) or was unaffected by fasting, feeding to satiation or refeeding (Huising et al., 2007a; Rønnestad et al., 2010), unlike the situation in mammals where fasting decreases leptin expression, and refeeding increases it (Saladin et al., 1995). The impact of the nutritional manipulation was demonstrated by a significant difference in body mass between the fed and restricted-fed animals. Axolotls that were fed to satiation grew twice as fast as restricted animals. However, short-term fasting did not result in any significant changes in leptin expression in the restricted-fed animals, nevertheless it showed a tendency towards lower leptin and LEPR expression of fasted axolotls. Moreover, the brain expression of orexigenic NPY was not significantly changed, however it showed a tendency towards higher NPY expression level in fasted animals. This results show similarity with mammalian model, where leptin decreases NPY expression and it the same time reduces food intake (Lewis et al., 1993; Mizuno and Mobbs, 1999; Stephens et al., 1995). These findings indicate that the regulation of leptin expression by nutritional state more closely resemble the situation in other ectotherms such as teleost fish. Ectotherms do not need to thermoregulate, thus their metabolic regulation is more flexible (Huising et al., 2006). For this reason the physiological role of leptin in ectotherms may differ quantitatively, if not qualitatively, from its role in mammals.

Comparative studies on leptin are needed to understand its function in all vertebrates and invertebrates. The aim of the present study is to increase an understanding of the physiological effects, diversity of functions and evolutionary history of leptin. Identification of leptin-like genes in amphibians, conserved structure and function demonstrate that leptin is of ancient origin, rather than this hormone has evolved recently in mammals. This thesis provides opportunity to investigate the functions of the leptin signalling system in these early tetrapod vertebrates, providing insight into the functional development of the system during vertebrate evolution.

6.1 Future work

The cloned region of LEPR is part of the extracellular domain. The full length LEPR isoform containing the extracellular and transmembrane domains together with

intracellular motifs is considered to be the fully functional receptor. In additional, shorter, isoforms the intracellular domain is truncated or absent (Richards and Poch, 2003). Several isoforms of LEPR genes were identified in mammals (Cioffi et al., 1996), chicken (Horev et al., 2000) and teleosts (Cao et al., 2011; Wong et al., 2007). Therefore, full-length sequence of LEPR should be cloned, and intracellular region should be used for the gene expression assay. Obtaining the full sequence awaits the sequencing of the axolotl genome, although this is not straightforward owing to its large size compared to other vertebrates (Voss et al., 2001). It is possible that a traditional cDNA library screening approach may be more successful.

Moreover, to investigate the cell types in which leptin and the LEPR were expressed in the axolotl and to localize leptin and LEPR mRNA within the axolotl tissues, in-situ hybridisation should be performed.

Furthermore, to investigate evolutionary conversation of leptin action on food intake regulation and metabolism, the effect of leptin administration should be studied. The expected results of this study would be reduction of food intake and body weight loss (Schwartz et al., 1996), however administration of leptin in fish showed unexpected effects (Baker et al., 2000, Londraville et al., 2002) compared with information about leptin in mammals. In contrast, intracerebroventricular injections of recombinant *Xenopus* leptin strongly decreased food intake in juvenile frogs, and tadpoles were only sensitive to the inhibitory effects of exogenous leptin from the midprometamorphic developmental stage (Crespi and Denver, 2006). These results support the conservation of leptin's role as an anorectic signal in non-mammalian vertebrates.

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