SEARCH FOR LEPTIN OR A LEPTIN-LIKE MOLECULE IN THE
SERUM AND THE FAT-STORING TISSUES OF SEA LAMPREY,
*PETROMYZON MARINUS*

by

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A Thesis submitted in conformity with the requirements
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The objective of this research was to search for a leptin or a leptin-like protein, which may play a role in lipid metabolism during the life cycle of *P. marinus*. Probing Western blots of *P. marinus* sera from different intervals of lamprey life cycle with mammalian leptin antibodies detected a 65 kD protein present in higher concentrations in the larvae (growth and metamorphic stages) compared to the upstream migrants. The absence of this protein from the sera of *Lampetra appendix* (larvae and adults) suggests that this protein is species specific. A 16-17 kD protein was detected in the nephric fold of transformers stage 2 and 4 and 100 kD protein in the fat column and muscle and 50 kD protein in the nephric fold, fat column and muscle of all intervals tested. Mammalian leptin antibodies also detected a 116 kD protein in eel serum. The identity of these immunoreactive proteins is not known but competition studies show that they most likely share antigenic determinant(s) with leptin protein.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES AND TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Lamprey metamorphosis</td>
<td>2</td>
</tr>
<tr>
<td>Ob protein: Leptin</td>
<td>10</td>
</tr>
<tr>
<td>OBJECTIVE</td>
<td>20</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>21</td>
</tr>
<tr>
<td>Collection of blood and tissue samples</td>
<td>22</td>
</tr>
<tr>
<td>Tissue preparation for SDS-gel electrophoresis</td>
<td>23</td>
</tr>
<tr>
<td>Protein determination assay</td>
<td>23</td>
</tr>
<tr>
<td>SDS-polyacrylamide gel electrophoresis (PAGE)</td>
<td>24</td>
</tr>
<tr>
<td>Electroelution of proteins from SDS-PAGE</td>
<td>24</td>
</tr>
<tr>
<td>Antibody production</td>
<td>25</td>
</tr>
<tr>
<td>Serum IgG purification</td>
<td>26</td>
</tr>
<tr>
<td>Electrophoretic blotting of SDS-polyacrylamide gels</td>
<td>27</td>
</tr>
<tr>
<td>Immunological detection of leptin on nitrocellulose membrane</td>
<td>28</td>
</tr>
<tr>
<td>Competition studies</td>
<td>29</td>
</tr>
</tbody>
</table>
Two-dimensional (Crossed) immunoelectrophoresis (CIE) | 30
Immunoprecipitation of mammalian and lower vertebrate sera | 32
Serum from thyroid hormones and goitrogen treated animals | 32

**RESULTS**
Western blots | 34
Crossed Immunoelectrophoresis | 57
Immunoprecipitation of serum from mammals or lower vertebrates | 60

**DISCUSSION**
Ob Sc immunoreactive serum proteins | 65
Identity of 65 kD protein | 68
Ob Sc immunoreactive tissue extract proteins | 70
Ob A2 immunoreactive tissue extract proteins | 71
Ob A1 and Ob A2 immunoreactivity to serum proteins | 72

**CONCLUSION**

**LITERATURE CITED**
LIST OF FIGURES AND TABLES

Figure | Facing Page
--- | ---
1. Schematic representation of leptin secretion and action during fed and starved states. | 12
2. Lamprey (P. marinus) and eel serum immunoblots. | 37
3. Immunoblots of P. marinus, L. appendicis and eel serum. | 40
4. SDS-polyacrylamide gel and immunoblot of P. marinus serum from different periods of lamprey life cycle. | 43
5. SDS-polyacrylamide gels and immunoblots of P. marinus nephric fold, fat column and muscle tissue extracts. | 46
6. Immunoblot of P. marinus nephric fold and fat column tissue extracts probed with Ob A2 antiserum. | 49
7. Immunoblots of P. marinus sera treated with thyroid hormones and KCIO₄. | 52
8. Immunoblots of P. marinus ammocoete and adult sera probed with A65 antiserum. | 55
9. Crossed immunoelectrophoresis of P. marinus ammocoete and adult sera. | 58

Table | Page
--- | ---
1. Pattern of immunoreactivity of leptin antibodies (Ob Sc, Ob A2 and Ob A1) with lamprey and eel sera and lamprey adipose tissue extracts. | 64
INTRODUCTION

Lamprey life cycle

Lampreys and myxinoids are the only remaining representatives of the most primitive vertebrates (Hardisty, 1982). They likely possessed a common agnathan (jawless vertebrate) ancestor, with the myxinoid (hagfish) being the more ancient offshoot, from which lamprey diverged about 500 million years ago (Goodman, 1981). Fossil records dating back to 280 million years ago show that the living lampreys, Petromyzoniformes have experienced very few morphological changes (Bardack and Zangerl, 1971). Lampreys can be distinguished by two different adult life history types resulting in the classification of parasitic and the non-parasitic species (Hardisty and Potter, 1971b). Parasitic species are divided into two groups, those that feed in the sea and those that are restricted to fresh water systems (Hubbs and Potter, 1971). The fresh water parasitic lampreys, such as the Petromyzon marinus in the Great Lakes (Smith, 1971) and the Lampeeta fluviatilis in Lake Ladoga of Gulf of Finland (Berg, 1948 in Hubbs and Potter, 1971), have had a marine origin (Hubbs and Potter, 1971). Most members of the non-parasitic species (brook lampreys) are closely related and morphologically similar to a specific parasitic species. It is hypothesized that the non-parasitic species and their corresponding parasitic species have been derived from a common parasitic ancestor such that together they are called “paired or satellite species” (Hardisty and Potter, 1971c).

Lampreys exhibit a complex life cycle. The larvae (ammocoetes) mainly reside within burrows in silt deposits of rivers and streams and feed on algae and detritus for 2-7 years (Hardisty and Potter, 1971a, Potter, 1980). Given suitable environmental and
physiological conditions, the larval growth phase ends and metamorphosis commences. In *P. marinus*, metamorphosis begins in mid-July and takes about 3-4 months (Youson, 1997) and there are seven stages (1-7) of development (Youson and Potter, 1979). This is a phase of developmental changes in which the animal goes through major morphological and physiological alterations in preparation for adult life (Youson, 1980). The juveniles of the parasitic species, migrate downstream to the sea (anadromous species), to a larger body of fresh water (landlocked species) or within their natal stream where they feed on blood and body fluids of teleost fishes; in the first two cases feeding results in rapid growth (Hardisty and Potter, 1971b). The feeding period in parasitic species such as *P. marinus* lasts from 12 to 21 months (Applegate, 1950; Smith 1971). At the end of this period, feeding stops and the upstream migration begins concomitant with sexual maturation. Once sexually mature, the animal spawns and dies (Larsen, 1980). In the non-parasitic lampreys the downstream migration of the young adult is minimal and, since parasitic feeding is absent, they commence sexual maturation shortly after metamorphosis; spawning and death follows shortly thereafter (Malmqvist, 1980).

The ancestral history of lampreys and the complex morphological and biochemical changes during metamorphosis render them valuable as a research model in the field of endocrinology and developmental biology (Youson, 1999).

**Lamprey metamorphosis**

*The role of thyroid hormone*

There are several factors involved in regulation of lamprey metamorphosis and it is thought that the interaction of several hormones orchestrate this developmental interval
(Youson, 1994). So far, only thyroid hormones (TH) have been implicated with the metamorphic phenomenon (Youson, 1997). The larval lamprey has an endostyle (subpharyngeal gland) instead of a typical vertebrate thyroid gland with follicles (Wright and Youson, 1976). One of the functions of the endostyle is to synthesize two thyroid hormones, 3,5-triiodothyronine (T₃) and thyroxine (T₄), along with their precursors 3-moniodothyrosine and 3,5-diiodothyrosine (Barrington and Sage, 1972). Another function of the endostyle is to produce mucus. The secreted mucus contributes to the mucus of the central food cord which extends the length of the pharynx and moves towards the esophagus (Mallatt, 1981). As water passes over these mucous strands, the gills extract oxygen from water and food particles such as diatoms, desmids, protozoans and detritus (Creaser and Hann, 1929; Schroll, 1959 in Barrington and Sage 1972) are trapped and moved into the intestine. Morphological changes during metamorphosis transform the endostyle into the adult thyroid gland with follicles (Barrington and Sage, 1972; Wright and Youson; 1976). The mucus-secreting cells make no contribution to the adult thyroid since they completely degenerate during metamorphosis (Hardisty and Baker, 1982).

Serum thyroid hormone levels increase gradually during the larval growth phase reaching a peak prior to metamorphosis (Youson et al., 1994), decrease during metamorphosis and stay constant throughout the remainder of the life cycle (Wright and Youson, 1977; Lintlop and Youson, 1983a; Youson et al., 1994). The decline of serum thyroid hormone levels (T₄ and T₃) in metamorphosing lamprey (Wright and Youson, 1977; Lintlop and Youson, 1983; Youson et al., 1994) is contrary to the metamorphic scenario in amphibians (Galton, 1988) and flounder (Tagawa et al., 1990). This view that
thyroid hormone is involved in metamorphosis is supported by experiments with thyroid hormone administration, which in anuran tadpoles (Etkin, 1968) and flounder larvae (Inui and Miwa, 1985) initiates metamorphosis and inhibits (to a certain degree) lamprey metamorphosis by favoring the larval phase, that is, fewer animals metamorphose (Youson et al., 1997). Thyroid hormone inhibitors, goitrogens like propylthiouracil (PTU) and potassium perchlorate (KClO₄), have been used in similar studies to further prove the role of thyroid hormone in metamorphosis in vertebrates. Administration of goitrogens delays metamorphosis in anuran tadpoles and flounder larvae (Miwa and Inui, 1987; Galton, 1988) and induces metamorphosis in lamprey larvae (Holmes and Youson, 1993; Youson et al., 1995; Manzon and Youson, 1997). In these animals, measurement of thyroid hormone (T₄ and T₃) levels are depressed compared to the control (untreated larvae) (Youson et al., 1995; Manzon and Youson, 1997). This lends support to the hypothesis that reduction in the systemic levels of thyroid hormone does play a role in induction of lamprey metamorphosis.

An important relationship exists between lipid metabolism and serum thyroid hormone levels; this relationship is important to the primary focus of this thesis. Elevated thyroid hormone levels coincide with increased food assimilation and liopogenesis and a decline of serum thyroid hormone levels coincides with a non-trophic period and lipid depletion (Youson et al., 1994; Kao et al., 1998). This subject will be treated in more detail below.

The involvement of other hormones by directly or indirectly affecting thyroid hormone levels, has been implicated in the regulation of metamorphosis in lamprey but
Further research is required to show any direct evidence (Youson and Sower, 1991; Youson, 1997).

Environmental cues: Temperature

The regular nature of the timing of metamorphosis suggests that seasonal changes in the environment such as photoperiod and temperature may play a role in the induction of metamorphosis (Youson, 1994). Eddy (1969) showed that indeed environmental cues such as light and temperature might be involved in initiation of metamorphosis. She demonstrated that pinealectomy inhibits metamorphosis in Lampetra planeri ammocoetes and this view was supported by subsequent studies on P. marinus (Cole and Youson 1981). Landlocked species of P. marinus in the tributaries of the Great Lakes metamorphose at mean summer temperatures of 21°C (Holmes and Youson, 1994). Since metamorphosis takes place at the same time every year, coinciding with rise in water temperature, it has been suggested that the seasonal rise in water temperature is essential for commencement of metamorphosis, given that other criteria are met (Youson et al., 1993; Holmes and Youson, 1994). The importance of seasonal rise in temperature to the onset of metamorphosis has been the subject of many investigations. Incubation of animals at the time of metamorphosis at temperatures lower than normal (13 °C) results in lower incidence of metamorphosis and retardation of the metamorphic process (Youson et al., 1993). Furthermore, when premetamorphic ammocoetes (see later) of sea lamprey, Petromyzon marinus, were incubated at 21°C for nine months, only one ammocoete metamorphosed and the experimental animals suffered substantial loss in weight and mass (Holmes and Youson, 1994). This led the investigators of this study to suggest that presumptive metamorphic larvae require a winter-chill period prior to
metamorphosis during which low temperatures induce anabolism whereby the animal might increase its lipid reserves. This interval of anabolism is followed by the normal increase in environmental temperature, the cessation of feeding in certain sized animals and the induction of the catabolism of lipids.

**Physiological preparation**

The discussion of temperature requirements for metamorphosis leads to another factor involved in initiation of metamorphosis, which is physiological preparation. Parasitic and non-parasitic species of lamprey go through a period of starvation during metamorphosis and during the last period of their life, which is characterized by sexual maturation, spawning and death. Energy is required for the processes of metamorphosis and reproduction and in the case of parasitic species, for upstream migration as well. Lipid reserves are the source of energy during metamorphosis (Lowe et al., 1973; Moore and Potter 1976; O’Boyle and Beamish, 1977). Body composition data for *P. marinus* is supported by histological examinations, which show that there is a greater amount of lipid found in ammocoetes prior to metamorphosis than in the postmetamorphic adult (Youson et al., 1979). Furthermore, measurements of total body lipid content show that at early larval stages, *P. marinus* body lipid content constitutes 4% of total wet body weight; at the beginning of metamorphosis the lipid content reaches a peak of 13.5% and during metamorphosis the lipid levels fall to 7.9% (Lowe et al., 1973; Beamish et al., 1979; Youson et al., 1979). The hypothesis is that accumulation of a sufficient amount of lipid which results in increase in mass is necessary for the larvae of *P. marinus* to proceed into metamorphosis (Potter et al., 1978b).
Measurement of mass and length of many larvae of *P. marinus* have shown that they have to be of specific length and mass (size) to enter the metamorphic phase of the life cycle (Potter et al., 1978b; Youson et al., 1993; Holmes and Youson, 1994). Once the animal is of the critical length, it goes through a period of arrested growth such that there is a switch in metabolism from protein anabolism to lipogenesis and accumulation of lipid in several fat depot sites (O’Boyle and Beamish 1977; Potter 1980). The fat is deposited in the liver, kidney, fat column, muscle (myosepta), subcutaneous tissue and gonads (O’Boyle and Beamish, 1977; Youson et al., 1979). The increase in mass during this period brings the animal to the threshold value for the commencement of metamorphosis. This threshold value is called condition factor (CF) and is expressed as:

\[ \text{CF} = \text{mass (g)} / \text{length (mm)}^3 \times 10^6 \]

The critical length and mass when used with CF can predict animals which are about to enter metamorphosis (Holmes and Youson, 1994) and these animals are called premetamorphic ammocoetes. For the landlocked *P. marinus* the critical length and mass (size) of the premetamorphic ammocoetes have to be at least 120 mm and 3.0 g, respectively (Youson et al., 1993) and a minimum CF value 1.50 (Youson, 1994). As the animal goes through metamorphosis the CF is relatively constant up to stage 1-2 due to the retention of the fat constituting a large (13.5 %) component of the body mass (Potter et al., 1978b). Starting from stage 3 the CF decreases with the depletion of fat reserves (Potter et al., 1978b; Youson et al., 1979; Kao et al., 1997b); weight declines while the length stays relatively constant (Potter, 1980; Youson, 1994).
Lipid metabolism and importance of fat reserves

Supporting evidence for accumulation of lipid and mobilization and catabolism of stored material (lipid) during metamorphosis (O'Boyle and Beamish, 1977; Youson et al., 1979; Bird and Potter, 1981) comes from studies done on lipid metabolism during this non-trophic period. The changes in lipid metabolism are studied by monitoring the activity of enzymes involved in lipogenesis and lipolysis of triglycerides, which are the major form of lipid found in the lamprey (Hardisty and Rovainen, 1982). The fluctuations of lipid levels are more closely studied in the liver, kidney and intestine of landlocked sea lamprey, *P. marinus*. Lipogenesis, the process of fatty acid and triacylglycerol synthesis, is assessed by the activity of acetyl-CoA carboxylase enzyme (ACC), the rate-limiting enzyme of de novo fatty acid synthesis, and diacylglycerol acyltransferase (DGAT), the terminal enzyme in triacylglycerol synthesis (Kao et al., 1997a,b). Lipolysis is monitored by the activity of triacylglycerol lipase (TGL) since it is responsible for catabolism of triacylglycerol (Kao et al., 1997a,b) which in other poikilotherms results in mobilization of fatty acids for energy use (Sheridan, 1994). According to recent studies, patterns of lipid metabolism in kidney, liver and intestine divide lamprey metamorphosis into two intervals. The first interval spans from the larval phase to stage 3 of metamorphosis. During this time, lipogenesis increases and lipolysis is reduced in the liver and kidney; this is indicated by the decrease in plasma fatty acids in conjunction with the increase of lipogenic enzyme activity (Kao et al., 1997b). Also, there is enhanced lipolysis in the intestine. The higher ACC activity of the kidney compared to the liver and the intestine confirms the view that the kidney is one of the major lipid storage organs (Youson et al., 1979; Kao et al., 1997b).
Reversal of the above processes is observed in the second interval of lipid metabolism that starts from stage 3 and lasts up to stage 6 of metamorphosis. During this time, lipolysis increases and lipogenesis decreases in the kidney and the liver and the opposite occurs in the intestine. The higher TGL activity in the liver compared with the kidney and the intestine suggests that the liver plays a major role in lipid mobilization during metamorphosis (Kao et al., 1997b). This finding is consistent with an earlier report which showed that during metamorphosis there is a decrease in the activity of malic dehydrogenase enzyme, NADP-specific isocitrate dehydrogenase and hexose monophosphate shunt dehydrogenase, enzymes that are responsible for the supply of substrates (e.g. Acetyl-CoA, glycerol-3-phosphate, NADPH) used in synthesis of lipids (Sheridan and Kao, 1998). The changes in lipid metabolism of liver, kidney and intestine are concurrent with morphological changes occurring in these organs during metamorphosis. This suggests that the energy requirement of these and other metamorphic processes is derived from the lipid accumulated during the larval growth phase and early metamorphic stages (Youson et al., 1979; Kao et al., 1997b).

Although Kao et al. (1997) examined the lipid metabolism in specific organs, there is evidence which shows that lipid depletion in lamprey during metamorphosis and other non-trophic stages (upstream migration) is a whole body event (Lowe et al., 1973; Moore and Potter, 1976; Bird and Potter, 1981). For example, most of the fat in the fat column is depleted during metamorphosis and this tissue is transformed into the major hematopoietic site (Potter et al., 1978a). Lipid mobilization also occurs in the musculature, subcutaneous fat, and myosepta of the trunk region (Youson et al., 1979). The pattern of lipid accumulation and depletion during the larval life (growth phase and
metamorphic phase) could mean that these and other metamorphic changes can not be initiated until sufficient fat is accumulated to provide the necessary energy which is required for the non-trophic period of metamorphosis. In fact, in the ammocoetes of the P. marinus, accumulation of sufficient amounts of lipid is essential to fulfill the CF requirement of a premetamorphic animal, such that ammocoetes which satisfy the specific length requirement but lack the necessary fat reserves will not undergo metamorphosis (Youson et al., 1993; Holmes and Youson, 1994). On this assumption it could be expected that the organism has a mechanism for measuring the accumulation of fat and triggering metamorphosis when the appropriate level is attained.

**Ob protein: leptin**

Recently, a hormone was identified in mammals that signals the brain about the status of fat reserves in the body (Zhang et al., 1994) resulting in a reduction of food intake. Coleman (1973), who was working in the Jackson laboratories, found that obese (ob) mice and diabetic (db) mice exhibit the same diabetic symptoms associated with obesity, namely non-insulin dependent diabetes mellitus (NIDDM) syndrome. These mice are characterized by obesity, hyperglycemia, hyperinsulinemia, hyperphagia, insulin resistance and sterility (Coleman, 1973). The similarity of the phenotype caused by two different genes led to the belief that obese and diabetic mice suffer from the same defect (Coleman, 1973). From parabiosis studies it was concluded that the ob and normal mice have an intact food integration center which responds to a satiety signal present in the normal mouse and the db mouse has the satiety signal but the food integration center, the hypothalamus, seems to be defective (Coleman, 1973). This satiety signal was identified
by positional cloning of the ob gene which is expressed in the adipose tissue (Zhang et al., 1994). Friedman et al. (1994) identified the ob gene product, as a 167 amino acid protein hormone that regulates food intake and energy expenditure. They named this ob protein, leptin. The ob/ob mice are leptin deficient or produce a truncated form of leptin (Zhang et al., 1994) and db/db mice have a genetic defect in the long isoform of the leptin receptor, ob-R (Lee et al., 1996) which results in a truncated form of the leptin receptor. The evidence for the existing defects in the ob/ob and db/db mice comes from administration of leptin to these obese mice. It was shown that recombinant leptin injection results in weight loss, decrease in plasma insulin and glucose levels, increase in thermogenesis and decrease in food intake in ob/ob mice (Figure 1 A; Halaas et al., 1995; Maffei et al., 1995; Pelleymounter, 1995). However, diabetic (db/db) mice were not affected by this treatment since they have a defective leptin receptor (Tartaglia et al., 1995). Leptin receptors have been found in the hypothalamus (Tartaglia et al., 1995) as well as in many other tissues. The distribution of receptors suggests that in addition to serving as a lipostat, leptin may also have other physiological roles (Figure 1 A). For example, leptin suppresses insulin secretion in (perfused pancreas and isolated islet cells) of normal and ob/ob mice but not in Zucker fa/fa rats (diabetic rats with defective ob receptor; Emilsson et al., 1997). In the kidney, administration of leptin to normally hydrated mice has a diuretic effect (Serradeil-Le Gal, 1997). In the intestine, leptin has a rapid inhibitory effect on sugar absorption (Lostao et al., 1998). Leptin stimulates proliferation and differentiation of hematopoietic stem cells (Bennett et al., 1996; Cioffi et al., 1996; Gainsford et al., 1996).
Figure 1. (A) Schematic representation of leptin secretion and action. Leptin is secreted from white adipose tissue (WAT) and is transported by blood into the brain. Leptin binds to leptin receptor (Ob-R) in the hypothalamus where it acts to inhibit secretion of neuropeptide Y (NPY), which results in decrease in thermogenesis in the brown adipose tissue (BAT) and decrease in food intake. This function of leptin is responsible for decrease in mass and ultimately results in inhibition of leptin secretion. Leptin can also have direct effects on peripheral tissues. Leptin stimulates the reproductive axis, stimulates diuresis, increases sugar absorption in the intestine, stimulates proliferation of hematopoietic stem cells, inhibits triglyceride synthesis in adipocyte and non-adipocyte cells. In the pancreas, leptin inhibits insulin secretion and it has been suggested that insulin decreases leptin secretion by a negative feedback loop. (+), secretion; (-) inhibition; †, increase; ‡, decrease. Modified from Trios and Mantzoros, 1997 and Houseknecht et al., 1998.

(B) Schematic representation of neuroendocrine response to starvation. Upon starvation, insulin and leptin hormone secretion decreases, the hypothalamic-pituitary-adrenal axis is stimulated and the thyroid axis and the reproductive axes are suppressed. Diminished leptin hormone levels result in increase in NPY expression in the hypothalamus which is responsible for suppression of the reproductive axis and thermogenesis in BAT. Studies suggest that leptin hormone repletion in starving mice is responsible for reduction of suppression of thyroid hormone levels and decrease of HPA axis activity (Flier, 1997). Modified from Trios and Mantzoros, 1997.
Leptin also plays a role in the stimulation of the reproductive axis. Leptin administration to infertile ob/ob mice restores reproductive function lending support to findings which suggest a link between fertility and adiposity (Mounzih et al., 1996; Chehab et al., 1997). In adipocyte and non-adipocyte cells, leptin inhibits lipogenesis by inhibiting ACC enzyme gene expression and enhancing lipolysis, which suggests an autocrine/paracrine mode of action (Bai et al., 1996). A more recent study has implicated a role for leptin in regulation of fatty acids and triglyceride homeostasis in non-adipocytes (Unger et al., 1999).

**Presence of leptin or leptin-like molecule in lamprey, *P. marinus***

Since lipid accumulation and metabolism are critical to metamorphosis in *P. marinus*, the question arises whether a leptin-like factor is involved in this developmental process. As the body fat content of larval lampreys increases to a specific level, which satisfies the minimum CF value, leptin could reach a threshold level at which it can trigger metamorphosis. However, since lipogenesis in lamprey is an ongoing process until stage 1-2 of metamorphosis, leptin levels might continue to increase and reach a new threshold level at which it would evoke other physiological processes specific to leptin. One such action would be fat depletion by the direct effect of leptin on both adipocytes and non-adipocytes, such as that seen in mammals (Shimabukuro et al., 1997; Zhou et al., 1997).

Considering the effect of leptin on thyroid hormone secretion in mammals, this proposed scheme of action of lamprey leptin or a leptin-like molecule fits in with what is known about the role of thyroid hormone in lamprey metamorphosis.
Evidence of an existing relationship between leptin and thyroid hormone in mammals comes from studies performed on starved mice (Figure 1B). Upon starvation, serum thyroid hormone levels, as well as expression of leptin mRNA, drop and secretion of glucocorticoid increases (Ahima et al., 1996). The drop in the thyroid hormone levels is responsible for decrease in metabolic rate which is essential to conserve energy in the starving organism (Flier, 1998). Leptin repletion reverses the physiological effects of starvation. That is, administration of recombinant leptin to starved mice results in depression of hypothalamic-pituitary-adrenal axis activation and reduction of suppressed thyroid hormone (Hamann and Matthaei, 1996). More specifically, leptin administration reverses the suppression of thyrotropin releasing hormone (TRH) expression in the paraventricular nucleus (PVN) of the hypothalamus (Legradi et al., 1997).

The above results show the effect of leptin on thyroid hormone, however, there is also evidence of a thyroid hormone effect on leptin. Administration of thyroid hormone to hypothyroid mice inhibits leptin mRNA expression (Fain et al., 1997). Since leptin stimulates thyroid hormone secretion and thyroid hormone in turn inhibits leptin mRNA expression and secretion, then a negative feedback loop might be operating between these two hormones (Fain et al., 1997). If a leptin-like molecule exists in lamprey and this feedback loop between leptin and thyroid hormone is operational, then prior to metamorphosis when there is a surge in thyroid hormone levels, leptin secretion might be suppressed but its level could be sufficient to signal the brain about the fat content of the body. However, as thyroid hormone levels drop by stage 1-2 of metamorphosis then suppression of leptin could be relieved and the increased level could induce several responses such as weight loss.
Insulin is another agent that is involved in the complex interplay of endocrine and neuroendocrine responses elicited by leptin; insulin also plays a role in lamprey lipogenesis which is essential for metamorphosis (See below). There is evidence that in mammals leptin seems to be involved in regulation of insulin secretion. Emilsson et al. (1997) provided proof for a direct effect of leptin on pancreatic islet B-cells. They showed that in the studies of perfused pancreas of ob/ob mice, leptin inhibited basal insulin secretion. Furthermore, in isolated islets of ob/ob and wild type mice, both of which have leptin receptors, leptin inhibited glucose stimulated insulin secretion. However, the same treatments when applied to fa/fa rats with defective leptin receptors failed to produce any changes. Furthermore, it has been shown that the molecular basis for insulin inhibition by leptin seems to be at the transcriptional level. Leptin injection to ob/ob mice results in inhibition of preproinsulin mRNA levels (Seufert et al., 1999). In addition to the effect of leptin on insulin secretion, insulin administration to normal mice and ob/ob mice causes an increase in leptin mRNA expression (Saladin et al., 1995). These results show that there could be a negative interaction operating between leptin and insulin in mammals (Remesar et al., 1997).

In lampreys, serum insulin levels are higher in ammocoetes than in stage 4 metamorphosing animals but there is a gradual increase around stage 6 (Youson et al., 1994). These changes in the insulin levels are concomitant with morphological changes in the islets where small islets of B-cells in larvae are replaced by large aggregations of B-cells and D-cells (Elliott and Youson, 1986). If a leptin-like molecule exists in lampreys and shares functional similarities with mammalian leptin then as insulin levels increase leptin synthesis might increase. Increased levels of leptin in turn might be
responsible for depression of insulin levels during metamorphosis. However, the importance of insulin to lamprey metamorphosis appears to be due to its involvement in lipid metabolism (Kao et al., 1999b). Therefore, if leptin is present in lamprey then, indirectly (by affecting insulin levels) leptin may regulate lipid metabolism. Anti-lamprey insulin (Plisetskaya, 1980) injected into larvae (Youson et al., 1992) results in an increase in plasma fatty acid concentration and increase in triacylglycerol lipase activity in the liver of the larvae. In a more recent study (Kao et al., 1999b), it was shown that insulin administration to larvae and stage 6 metamorphosing animals induces reduction in lipolysis (decrease in triacylglycerol lipase activity) in kidney and liver (and muscle in the larvae) and increase in lipogenesis. Lower levels of plasma fatty acids, greater lipid concentration in kidney and muscle of the larvae, increase in de novo fatty acid synthesis (higher activity of ACC) and increase in triacylglycerol synthesis (higher activity of DGAT) indicate the enhanced level of lipogenesis. The changes in lipid metabolism induced by insulin are analogous to lipid metabolism in spontaneous metamorphosis as it has been shown by measurement of the activities of enzymes involved in lipid metabolism (Kao et al., 1997a,b).

Studies show that indirectly, insulin could affect lipid metabolism in lampreys by interaction with other hormones like thyroxine/triiodothyronine (Kao et al., 1999a,b). It has been suggested that insulin and thyroid hormone act synergistically to enhance lipid accumulation in the larval stage and as thyroid hormone levels decrease during metamorphosis this synergism is no longer in effect and lipolysis of lipids begin (Kao et al., 1999b). This effect of thyroid hormone is in agreement with the proposed mode of action of leptin or a leptin-like molecule in lamprey. That is, high levels of thyroid
hormone in lamprey larvae coincide with lipogenesis when leptin levels are proposed to be suppressed and low thyroid hormone levels with lipolysis when leptin is proposed to promote lipid depletion. On this assumption a leptin-like molecule in lamprey not only would it be responsible for the fluctuations of thyroid hormone and insulin levels, but by doing so, it might also regulate lipid metabolism in lamprey.

In this brief account of the dynamics of lamprey metamorphosis and leptin regulation of the fat depot and energy homeostasis in mammals, the emergence of a few common factors can be noted. In the mouse, leptin secretion results in decrease in food uptake and loss of fat reserves (Zhang et al., 1994; Pelleymounter et al., 1995; Halaas et al., 1996) which are analogous to the physiologic state of metamorphosing lamprey (Youson et al., 1979; Kao et al., 1997a,b; Youson, 1997), although the status of food intake in lamprey might be independent of leptin effect. Also, there are key hormones that are involved in lamprey lipid metabolism during metamorphosis, which are regulated by leptin expression and secretion in mammals. These hormones are insulin (Emilsson et al., 1997) and thyroid hormone (Ahima et al., 1996; Fain et al., 1997; Flier 1998) and these hormones are important in lamprey metamorphosis. A lipostat like leptin could play an important role in the initiation of metamorphosis in lamprey and depletion of fat reserves for energy use during metamorphosis. An accumulation of lipid during the arrested growth phase (length remains constant but the mass increases) of the lamprey larval life is essential to fulfill the size requirement of a premetamorphic animal (Holmes and Youson, 1994) and, once sufficient amount of lipid is stored leptin could relay messages to the central nervous system about the fat content of the body and, subsequently, metamorphosis will be triggered. However, a second and a more
important function of leptin in lamprey would be depletion of fat reserves possibly by regulating hormone secretion and by promoting depletion of triglycerides and inhibiting their synthesis in adipocytes and non-adipocytes.
OBJECTIVE

The objective of this research is to determine whether there exists a leptin or a leptin-like protein, which may play a role in utilization of lipid reserves during lamprey metamorphosis. Preliminary studies done by Friedman et al. (1994) showed hybridization of a mouse leptin probe to eel genomic DNA. This observation along with the cloning of chicken leptin which shares 96% homology with murine leptin (Taouis et al., 1998) led to the belief that leptin might be conserved in lower vertebrates. These results when considered in conjunction with the role of lipid in lamprey metamorphosis gave some hope for the presence of leptin in the organism. My approach to finding leptin or a leptin-like molecule in lamprey was by using antibodies made against mouse and human leptin to probe Western blots containing serum and tissue homogenates of lamprey from various periods of the life cycle. This approach was adopted since adipocytes and serum of obese mammalian subjects have been used in Western blots to identify, and in some cases, to quantifying leptin (MacDougald et al., 1995; Halaas et al., 1995; Maffei et al., 1995; Pelleymounter et al., 1995).
MATERIALS AND METHODS

The larvae (ammocoetes) of *Petromyzon marinus* (at least 105 mm and 2 g) were collected from several streams of the Great Lakes watershed (Oshawa Creek, Fish Creek, Rifle River, Harris River and Bronte Creek). Larvae of *Lampetra appendix* were collected from Bowmanville Creek which is a Lake Ontario tributary. The larvae were captured using an electroshocking device and the upstream-migrant (pre-spawning) adults of *P. marinus* and were captured by netting in Duffin’s Creek. The larvae of both species were transported to the aquatic facilities at University of Toronto at Scarborough and they were maintained in fiberglass tanks filled with a 5-6 cm of clean industrial sand for burrowing and supplied with aerated, dechlorinated tap water at ambient temperatures (12-21°C). They were maintained in artificial light-dark photoperiod, which mirrored the ambient light cycle throughout the year. The larvae were fed baker’s yeast once a week (1 g/animal per week). Large ammocoetes (premetamorphic larvae) of *P. marinus*, also called condition factor ammocoetes (CF), at least 120 mm and 3.0 g, were captured in the spring (Fish Creek, Bronte Creek and Harris River) and they were periodically checked for external signs of metamorphosis. The metamorphosing animals (transformers) were staged (1-7) according to criteria of Youson and Potter (1979). Some animals that metamorphosed to juveniles were eventually provided with rainbow trout (*Oncorhynchus mykiss*) upon which they fed. Completely metamorphosed, *L. appendix* adults, which were reared in the laboratory, were also used for the study. For purposes of positive control for leptin, male Wistar rat and male Balb-C mouse were obtained from the Animal Care facilities at University of Toronto at Scarborough. Fresh water North
American eel, *Anguilla rostrata* was obtained from the laboratory of Dr. D.G. Butler at the Ramsay Wright Building, University of Toronto.

**Collection of blood and tissue samples**

Lampreys and eels were anaesthetized by immersion in 0.05% (w/v) tricaine methanesulfonate (MS-222, Syndel Laboratories Ltd., Vancouver, British Columbia, Canada) and length and weight measurements were recorded. Blood samples from ammocoetes and metamorphosing individuals were collected into heparinized capillary tubes by severing the caudal vasculature. The animals were sacrificed by decapitation. The blood was allowed to clot overnight at 4°C and then centrifuged at 7000 g for five minutes. The serum was collected and stored at −20 °C. Blood samples from feeding juveniles, adults and eels were collected by heart puncture from anaesthetized animals. Mouse and Wistar rat blood was collected from the tail vein. These animals were anaesthetized with Somnotol injection (50 mg/kg) and along with feeding juveniles, adults and eels were sacrificed by decapitation. The blood from two mammals was processed in the same manner as above. Blood was also obtained from one human female (53 kg age 25) and one human male (70 kg age 62) with assistance of the Health Center at the University of Toronto at Scarborough. Lamprey tissues such as liver, nephric fold (which included the whole nephrogenic tissue with the gonads (testes or ovaries), intestine, muscle and fat column were obtained and liver and epididymal fat were removed from Wistar rats. The tissues were frozen in liquid nitrogen and stored at −70 °C.
Tissue preparation for SDS-gel electrophoresis

Extracts of tissues were prepared by homogenizing the tissues in 2 × lysis buffer [30 % (w/v)]. The buffer used was modified from protocols provided for Western blotting and immunoprecipitation by Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Transduction Laboratories (Lexington, KY, USA). The buffer contained 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 % NP-40 (Nonidet P-40), 0.1 % SDS (sodium-dodecyl sulphate), 0.5 % DOC (sodium deoxycholate), 0.025 % sodium azide and 1 % PMSF (phenylmethylsulphonyl fluoride).

The extracts were prepared by hand, using a glass homogenizer with a teflon pestle, and centrifuged for 30 minutes at 16000g in the cold (4°C). The infranatant was collected and stored at -20°C.

Protein determination assay

A microprotein assay was utilized for protein quantitation of serum and tissue extract samples (Bradford, 1976). Bradford reagent was prepared by dissolving 0.1 g of Coomassie brilliant blue G-250 (Bio-Rad; Mississauga, Ont, Canada) in 50 ml of 95 % ethanol and 100 ml of 85 % phosphoric acid and made up to 1 liter with distilled water. Bovine serum albumin V (Fisher; Unionville, Ontario, Canada) was used to generate the standard curve. The serum samples were directly used in the assays. To avoid interference from the lysis buffer the protein of the tissue extract samples was precipitated with acetone (6 v acetone/v of extract). The samples were centrifuged for five minutes and the protein precipitate was washed twice with acetone and dissolved in 0.05 M NaOH for use in microprotein assay.
SDS-polyacrylamide gel electrophoresis (PAGE)

The method used for SDS-polyacrylamide gel electrophoresis was that of Laemmli (1970).

Sample preparation for SDS-PAGE

Samples of sera and tissue extracts were mixed with 2 × SDS sample buffer at 1:1 (v/v). The buffer contained 4 × Tris.Cl/SDS, pH 6.8, 20 % (w/v) glycerol, 4 % (w/v) SDS, 3.1 % DTT and 0.001 % (w/v) of bromophenol blue. After mixing with 2 × sample buffer the samples were boiled for 5 minutes and centrifuged at 12000 g for five minutes. The supernatant (4 μl for serum and 30-80 μg of protein for tissue extract) was loaded on to the gel.

The gels were cast in 10 × 10 cm and 24 × 15 cm glass plates. Sera were fractionated using 10 % separating gel and 5 % stacking gel. 12.5-15 % separating gels were used for the separation of proteins in tissue extracts to improve resolution of the bands. A constant voltage of 100 volts was applied to the gels until the tracking dye, 0.002 % bromophenol blue, reached the bottom of the gel (2-2.5 hours). The gels were removed from the glass plates and stained with 0.25 % Coomassie blue R250 (Sigma; Oakville, Ont, Canada) made in methanol: acetic acid: distilled water (5:1:5 v/v/v) to visualize the proteins. The gels were incubated in the stain for 1 hour on a platform shaker then destained in methanol: acetic acid: distilled water (4:0.5:5 v/v/v) for two hours with gentle shaking. The gels were photographed after destaining.

Electroelution of proteins from SDS-PAGE

Larval lamprey serum was electrophoresed on 7 % SDS-polyacrylamide slab gel
(24 cm × 15 cm). The band with the immunoreactive serum protein (65 kD) as identified in the immunoblots (see later section) was cut out from the gel and placed in the elutrap (Schleicher & Schuell; Dassel, Germany). The elutrap was placed in an electrophoresis chamber and both the elutrap and the electrophoresis chamber were filled with electrophoresis buffer (25 mM Tris-HCl pH 8.3 and 192 mM glycine). The electroelution was carried out at constant voltage of 85 volts (25 mA) for 20 hours at 4°C. The protein from each gel representing 90 µl of serum (10 gels in total representing 900 µl of serum) was eluted into 0.8-1 ml electrophoresis buffer. The volume of the buffer was reduced by pressure dialysis to 0.25-0.5 ml. The eluted sample usually contained 25 µg-40 µg of protein as quantified by Bradford microprotein assay. The eluted sample was used in immunoblots to verify the elution of 65 kD protein.

Antibody production

Immunization procedure for production of leptin antibody (Ob A2)

Prior to immunization, pre-immune serum was obtained from a 2 Kg, white New Zealand female rabbit (Charles River; St. Constant, Quebec, Canada). This rabbit was injected subcutaneously at two locations in the shoulder regions using 100 µg of mouse recombinant leptin (Sigma) in 400 µl of PBS (phosphate buffer saline, pH 7.4) emulsified in 400 µl of Freund’s complete adjuvant (Sigma). Twenty-one days later a booster injection was administrated with the antigen being emulsified in Freund’s incomplete adjuvant (Sigma). Blood was collected from an ear vein 32 days after the first injection and the effectiveness of the antiserum was tested by a Western blot, with recombinant leptin, lamprey and eel sera. The rabbit was exsanguinated 40 days after the first
injection at the Animal Care facilities at University of Toronto, Medical Sciences Building.

*Immunization procedure for production of antibody against the 65 kD lamprey protein (A65)*

Pre-immune serum was obtained from another female 2 Kg New Zealand white rabbit. This rabbit was immunized using 100 µg of eluted 65 kD lamprey protein (described in Results) in 500 µl of Tris-Glycine buffer (electrophoresis buffer) emulsified in 500 µl of Freund’s complete adjuvant. Booster injections were administrated 17 days and 43 days after the first injection. The effectiveness of the antiserum was tested in a similar manner as the leptin antiserum.

**Serum IgG purification (A65 antiserum)**

IgG was collected from the serum using the protocol supplied with the Econo-Pac serum IgG purification columns (Bio-Rad). The serum salt content and pH was adjusted by dialyzing serum against rabbit serum application buffer (20 mM Tris-HCl pH 8 containing 28 mM NaCl) overnight at 4°C. The column was pre-washed with 1.5-M sodium thiocyanate (40 ml) and then, the column was equilibrated with 70 ml of application buffer. The dialyzed serum sample (4 ml) was applied to the column and IgG was eluted with 20 ml of application buffer in 1 ml fractions. The presence of IgG was determined by checking the absorbency of the fractions using the Spectrophotometer set to 280 nm. The fractions with absorbency higher than 0.1 were combined and used as probes in an immunoblotting procedure which contained recombinant leptin and lamprey serum.
Electrophoretic blotting of SDS-polyacrylamide gels

The procedure outlined by Towbin et al. (1979) and Burnette (1981) for electrophoretic blotting of SDS-polyacrylamide gels was followed with minor modifications. Serum (4 μl) or tissue extracts (60 μg-100 μg of protein) and mouse recombinant leptin (300 ng) were first subjected to electrophoresis as described above. At the end of the electrophoresis the gels were equilibrated in transfer buffer, 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol for 45 minutes. The proteins were transferred from gel to nitrocellulose as follows using a BioRad Mini transblot electrophoretic transfer cell. Briefly, the gel cassette was placed in a large container filled with transfer buffer. One fiber pad was wetted and placed on the cassette followed by 2 pieces of Whatman #1 filter paper cut to the size of the fiber pads. One strip of Whatman 3MM filter paper cut to the size of the gel was wetted and placed on top of the thin filter papers followed by the gel. A sheet of nitrocellulose (0.45-μm pore size; Schleicher & Schuell) which was cut to the size of the gel was wetted and placed on top of the gel. Whatman 3MM filter paper of the same size was placed on top of the nitrocellulose membrane followed by two Whatman #1 filter papers and the fiber pad. The gel holder cassette and an ice pack (Bio-ice unit) was placed into the transfer chamber and it was filled with cold transfer buffer. The blotting was carried out by applying constant amperage (250 mA) for 3 hours to transfer protein of tissue extracts (12.5-15 % separating gel) and 1.5 hours for serum proteins (10 % separating gel).

To determine the successful transfer of proteins from the gel, the nitrocellulose membrane was stained for 5 minutes in 0.5 % Ponceau S (Sigma) made in 1 % glacial
acetic acid and 99 % distilled water. The blot was destained for 2 minutes in distilled water.

**Immunological detection of leptin on nitrocellulose membrane**

The protocol followed was that provided with the Enhanced Chemiluminescence kit (Amersham Pharmacia Biotech; New Jersey, New York, USA).

**Immunoblots**

The blots were soaked overnight (16 hours) at 4°C in 5 % (w/v) low fat milk powder in Tween Tris-buffered saline (10 mM Tris-HCl, 0.25 M NaCl, 0.25 % Tween-20 pH 7.5), to block non-specific binding sites. The blocking buffer was removed by washing the membranes in Tween-Tris buffered saline (TTBS) at room temperature for 15 minutes and twice for 5 minutes with fresh changes of TTBS.

**Incubation with Primary Antibody**

Three leptin antibody preparations were used to probe the blots. The first one was purchased from Alpha Diagnostic International (San Antonio, TX, USA). It was a polyclonal antibody (neat serum) made against the whole leptin molecule (Ob A1). The second antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). It was an affinity purified polyclonal antibody raised against a peptide corresponding to 137-156 amino acids mapping at the carboxy terminus of human Ob-gene product (Ob Sc). The third antibody (Ob A2) was produced in our laboratory (see above) using 146 amino-acid, mouse-recombinant leptin (Sigma). Ob Sc antibody was used at a dilution of 1:1000 and the Ob A1 and Ob A2 antisera at 1:500. The blots were incubated with the primary antibody for 2 hours with gentle agitation at room temperature. Another wash
was performed as before and the blots were incubated with the secondary antibody (horseradish peroxidase [HRP]-linked, donkey anti-rabbit IgG; Amersham Pharmacia Biotech) for 1.5 hours at 1:1000 dilution, as recommended by the manufacturer. The secondary antibody was removed by washing with TTBS for 15 minutes followed by washing four times for 5 minutes each with fresh changes of TTBS. The bound immunoglobulins were visualized by incubating the blots with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) for 1 minute then exposing to autoradiography film (Sterling diagnostic imaging; NewWark, DE, USA) for variable time intervals (15 seconds, 1.5 minutes, 2.5 minutes and 10 minutes).

*Dot blots*

The optimum dilutions of the primary antibodies (titer) were determined by dot blots. In this procedure, equal amounts of antigen (mouse recombinant leptin) were placed on a strip of nitrocellulose. Then, the membranes were incubated in blocking buffer for 1 hour at room temperature with gentle shaking. After the blocking step the membranes were treated as in the procedure outlined for the immunobLOTS.

*Competition studies (Antibody-antigen specificity test)*

The Ob Sc antibody was preabsorbed with Ob Sc blocking peptide (Santa Cruz Biotechnology), which was the antigen used to produce the Ob Sc antibody. The protocol used for competition studies was that supplied by Alpha Diagnostics. The primary antibody at a dilution of 1:1000 (10 µl) was incubated with 20 µl (4 µg) or 40 µl (8 µg) of blocking peptide for 1 hour at 4°C. This preparation of absorbed antibody was incubated with immunoblots (instead of the primary antibody) to observe possible
blocking of the immunoreactive signal in lamprey serum and the mouse recombinant leptin. In some tests the primary antibody was pre-absorbed with lamprey (120 μl) or eel serum (120 μl) and this preparation was applied to Western blots of lamprey serum, eel serum and recombinant leptin samples.

Two-dimensional (Crossed) Immunoelectrophoresis (CIE) using A65 antiserum

This method is useful for quantitation of mixtures of proteins and the analysis of the composition of protein mixtures (Walker, 1988). Although, Western blotting is a far more sensitive method, CIE was used to determine the presence or absence of the 65 kD ammocoete protein in sera of other periods of the life cycle. The following procedure was used. Laurell’s buffer stock solution (Laurell, 1965) was made which contained 13.1 g sodium barbital, 2.1 g barbital and 0.4 g calcium lactate in 1 L. Clean glass plates (7.0 x 7.0 cm) were placed on a level platform and 7.5 ml of 2 % agarose (w/v) made in Laurell’s buffer at 1:5 dilution, was heated to 55 °C and was rapidly poured onto the plates. Once the agarose solidified, a hole was cut at one end of the agarose by a 2 mm diameter punch and the plate was placed on the platform of a horizontal electrophoresis apparatus such that the hole was at the negative electrode. About 1500 ml of the Laurell’s buffer, diluted in water (1:5), was added to both wells of the apparatus and then 7 μl of serum (ammocoete and adult in different holes) was added to the holes and electrode wicks were applied to each side of the agarose gel. Electrophoresis was run at a constant voltage of 200 volts (7 ma per plate) for 1.5 hours. At the end of the first dimension, the agarose plates were removed from the apparatus and strips of agarose in
line with the holes were cut out and placed on separate plates. Agarose of different formulations were applied to each plate. First, 300 µl A65 antibody was added to 4.7 ml of 2% agarose (55°C) and poured onto the plate with an agarose strip. To test for the absence of the 65 kD protein in the adult serum, the 300 µl of A65 antiserum was absorbed with 600 µl of adult serum then the mixture was centrifuged. The supernatant was added to 4.1 ml of 2% agarose (55°C) and poured onto the plate with agarose strip on it (one plate with ammocoete serum agarose strip and another with adult serum agarose strip). After the agarose solidified, the plates were placed onto the platform of the apparatus and the electrode wicks were placed on each side of the plate taking care that the hole was at the negative electrode. A constant voltage of 50 volts (1.4 ma per plate) was applied to the apparatus for 16 hours at room temperature. At the end of the 16 hours the plates were removed, the surface of the gels were covered with wet Kimwipe and a piece of blotting paper was cut to the size of the gel and placed on top of the Kimwipe. A stack of paper towel 2 cm high was placed on top of the blotting paper and a small weight was placed on top. When the paper towels were wet to the top, the plates were removed and the agarose was dried using a hair-dryer. The gels were stained for 5 minutes by placing the plates in 0.25 % Coomassie Blue G made in 95 % ethanol: glacial acetic acid: distilled water (4.5: 1: 4.5 v/v/v). Then they were destained using the destain of SDS-polyacrylamide gels (see above).
**Immunoprecipitation of mammalian and lower vertebrate sera**

Sera from mouse, human, lamprey and eel were used in this procedure. The protocol followed was that supplied by the manufacturer of Protein A-agarose (Santa Cruz Biotechnology). The amount of serum used was adopted from immunoprecipitation procedures applied in studies of mammalian leptin (Halaas et al., 1995; Campfield et al., 1995). 100-500 µl of serum was incubated with 10 µl (optimum dilution of antibody) of Ob Sc or Ob A plus 0.5 ml of immunoprecipitation buffer for 1 hour on a rotating platform at 4°C. Agarose beads with bound Protein A (20 µl per reaction, as recommended by the supplier) was added to the antigen-antibody complex and incubated for another 1 hour under the same conditions. The samples were centrifuged at 12000 g for 4 minutes and the supernatant was discarded. The beads were washed with 1 ml of immunoprecipitation buffer and centrifuged for 4 minutes. The washes were repeated six times. The antigen-antibody complex was removed from the beads by boiling for 5 minutes in 30 µl of 2 x SDS sample buffer. After centrifuging the immunoprecipitates for 5 minutes at 12000 g, 30 µl supernatant was loaded onto SDS-polyacrylamide gels and immunoprobed with both antibodies on Western blots.

**Serum from thyroid hormone (T₃ and T₄) and goitrogen (KClO₄) treated animals**

Sera were obtained from animals which were part of an experiment designed to block KClO₄-induced metamorphosis in CF larvae of *P. marinus* by exogenous thyroid hormones (TH) and the effects of KClO₄ and TH on serum TH concentrations (Manzon and Youson, 1998). Briefly, *P. marinus* larvae (≥ 120 mm) were randomly assigned to glass aquaria for either 4, 8 or 16 weeks of treatment or as untreated controls. The
experimental groups received 0.05 % KClO₄, T₄ at a concentration of 1 mg/L and T₃ at a concentration of 1 mg/L. The procedure for blood collection and storage of serum, was as mentioned above. Two samples from each experimental treatment (KClO₄, T₃ and T₄) after 16 weeks of treatment were used in Western blotting.
RESULTS

Mammalian leptin has been detected in the serum of rodents and humans using Western blotting (Halaas et al., 1995; Maffei et al., 1995; Frederich et al., 1995). Since antibodies vs mammalian hormones have been successfully used in our laboratory to detect certain lamprey hormones in lamprey serum and tissues (Elliot and Youson, 1986; Cheung et al., 1991), the first step in search of leptin or a leptin-like molecule began with probing Western blots of lamprey serum and tissue extracts with antibody against mammalian leptin.

Western blots

Lamprey, eel and mammalian sera

Western blots were probed with antibody raised against the whole mouse recombinant leptin molecule (Ob A1; Alpha Diagnostic International). The Ob A1 antiserum was not immunoreactive with sera from lamprey larvae and adults (4 μl). Since leptin is secreted from white adipose tissue in mammals (Zhang et al., 1994) and Western blots of adipocytes (3T3-L1; MacDougald et al., 1995) have successfully detected leptin, the Ob A1 antiserum was used to probe Western blots of adipose tissue extracts of lamprey larvae and adult (nephric fold and fat column; Youson et al., 1979). Only the positive control (recombinant mouse leptin) was immunoreactive (16 kD) with the Ob A1 antibody. Wistar rat serum (4 μl) and epididymal fat and liver tissue extracts (40 μg of protein) were also probed on Western blots for leptin as another source for positive control but immunoreactivity of the antibody to mammalian leptin in serum and tissue extracts was not detected (Data not shown). Since leptin is present in low
concentrations in serum (Halaas et al., 1995; Pelleymounter et al., 1995) this approach may have been ineffective in detecting leptin in a small amount of serum of normal rat (vs diabetic rat). Therefore, an immunoprecipitation technique was then considered essential, which allows precipitation of proteins using large volumes of serum. However, this approach required more antibody and resulted in the incentive to produce antibodies (Ob A2) using mouse recombinant leptin purchased from Sigma (see below for immunoprecipitation results).

At the same time an attempt was made to try leptin antibody with a different specificity, i.e. antibody against an amino acid sequence mapping at the carboxy terminus of the Ob protein (Ob Sc antibody). Since eel genomic DNA was shown to hybridize to a mouse leptin probe (Zhang et al., 1994) and eel is a teleost phylogenetically closer to lamprey than is a mammal, it was decided to use eel serum in the immunobloting procedures as well. Immunoblots of lamprey and eel sera (4 μl) from 4 individual animals and the positive control (recombinant mouse leptin) when probed with the Ob Sc antibody showed the presence of the positive control (16 kD), immunoreactivity at 65 kD in two larval intervals and two metamorphosing stages of lamprey, and no immunoreactivity in the serum of adult P. marinus. The Ob Sc antibody was also immunoreactive with 116 kD and ~40 kD proteins in eel serum (Figure 2A). The detection of the ~40 kD eel signal in all replicate experiments was not consistent like the 116 kD protein. Therefore, it was decided that the 40 kD signal might be due to non-specific interaction. A negative control in which the blots were incubated with only the secondary antibody showed no immunostaining for both sera and recombinant mouse
**Figure 2.** (A) Lamprey (*P. marinus*) and eel serum immunoblot. Lamprey serum (4 μl; individual animal from each interval) and 2, 4, and 8 μl of eel serum (one animal) were electrophoresed on a 10 % SDS-polyacrylamide gel and then blotted onto nitrocellulose and probed with Ob Sc antibody at 1:1000 dilution. The blot shows the 65 kD protein present in the larval growth phase (ammócoete and CF) and two stages of metamorphosis (Stage 2 and 4) but it is absent in the adult serum (upstream-migrant). Note the 116 kD immunoreactive protein in eel serum and another signal of ~40 kD. The second immunoreactive signal in the eel serum sample was not consistently detected like the 116 kD protein in replicate experiments (E.g. See figure 2 B). This immunoreactivity might be attributed to non-specific interaction of antibody with eel serum proteins.

A: Ammócoete serum  
CF: Condition factor ammócoete serum  
T1: Stage 1 transformer serum  
T4: Stage 4 transformer serum  
Sp: Adult serum  
Lp: Recombinant mouse leptin (300 ng)

(B) Lamprey and eel serum immunoblot. Lamprey and eel sera (4 μl; individual animal from each stage) were electrophoresed on a 10 % polyacrylamide gel. The proteins were blotted onto nitrocellulose and immunoprobed with Ob Sc antibody (1:1000). The blot shows the Ob Sc immunoreactive with 65 kD and 116 kD lamprey and eel serum proteins, respectively.

A: Ammócoete serum  
T2: Stage 2 transformer serum  
T4: Stage 4 transformer serum  
Sp: Adult serum  
Lp: Recombinant mouse leptin (300 ng)
(C) Competition study. An immunoblot with the same lamprey and eel sera samples (individual animal from each stage) used as in figure (A) but the immunoblot was probed with Ob Sc antibody (1:1000) preabsorbed with 4 μg of Ob Sc blocking peptide which is the antigen used to make the Ob Sc antibody. The 65 kD and 116 kD signals are blocked to the same extent as the mouse recombinant leptin. Arrowheads indicate the position of the blocked proteins (116 kD in eel and 65 kD in lamprey).
leptin. The same samples were immunoprobed with Ob A2 antiserum made against recombinant leptin (produced in laboratory) and, although recombinant leptin was detected, positive signals in the lamprey and eel samples were not detected. The pattern of immunoreactivity of Ob A2 was very similar to that of Ob A1 and like the Ob A1 antiserum, in other experiments, Ob A2 failed to detect mammalian leptin in rat and human sera.

Antigen-antibody specificity

The immunostaining of a 116 kD protein in eel serum and a stage-specific 65 kD protein in lamprey serum with the Ob Sc antibody appeared to be significant. However, the authenticity of this immunoreactivity had to be determined. Competition studies were carried out to determine the specificity of the antibody to the lamprey and eel proteins. Using Ob Sc blocking peptide, which is the peptide used to generate the Ob Sc antibody, these competition studies showed that recombinant leptin, lamprey and eel proteins from six individual animals were blocked to the same extent when blots were immunoprobed with primary antibody (Ob Sc) preabsorbed with Ob Sc blocking peptide (Compare Figures 2 B and C). To further test the specificity of the lamprey and eel signals to the Ob Sc antibody, the primary antibody was preabsorbed with either lamprey or eel sera (10 µl of antibody preabsorbed with 120 µl of serum). Although, it was expected that some blocking of the recombinant mouse leptin and the signals present in lamprey and eel sera would occur, no blocking of these signals was observed (Figure 3 B, data for blot blocked with eel serum is not shown). Perhaps these proteins (116 kD and 65 kD) are present in amounts in serum insufficient for the complete absorption of the primary antibody.
Figure 3. (A) An immunoblot of sera from the lampreys, *P. marinus* and *L. appendix*, and the eel. The sera (4 μl; individual animal from each interval) were electrophoresed on a 10% SDS-polyacrylamide gel then blotted onto nitrocellulose and immunoprobed with Ob Sc antibody (1:1000). The Ob Sc antibody was immunoreactive with 65 kD and 116 kD proteins present in the sera of *P. marinus* and eel, respectively, but no immunoreactivity was present in *L. appendix* serum. Note that the 65 kD signal is present in larval (A) and metamorphosing stages (T1 and T4) of *P. marinus* but is absent in the serum of upstream-migrant adults (Sp). The Ob Sc antibody is immunoreactive with recombinant leptin (Lp).

A: Ammocoete serum  
T1: Stage 1 transformer serum  
T4: Stage 4 transformer serum  
Sp: Upstream-migrant adult serum  
LA: *L. appendix* ammocoete serum  
LT: *L. appendix* adult serum  
Lp: Mouse recombinant leptin (300 ng).

(B) Competition study. Same samples as in Figure (A) but the immunoblot was treated with Ob Sc antibody (1:1000) preabsorbed with 120 μl of lamprey *P. marinus* ammocoete serum. The Ob Sc immunoreactive signals including the mouse recombinant leptin were not blocked. Similar results were obtained when Ob Sc antibody was preabsorbed with eel serum, i.e. no blocking of the Ob Sc immunoreactivity was observed.
(A)

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To determine whether the 65 kD protein was present at other periods in the life cycle of *P. marinus*, Western blots were made using sera of all periods from larva to adult. Figure 4 A shows the serum proteins from seven individual animals as seen in Coomassie Blue stained gel. Using the Ob Sc antibody the Western blots showed that the 65 kD protein was present in sera of animals from all periods except the feeding juvenile and the upstream-migrant adult (Figure 4 B). Competition experiments were performed using Ob Sc antibody preincubated with the Ob Sc blocking peptide. The 65 kD signal was blocked to the same extent as the recombinant mouse leptin in all the same serum samples examined (Figure 4 C).

It was important to establish whether this 65 kD protein was characteristic of the larval growth and metamorphic phases of lampreys in general. Therefore, serum samples of another species of Petromyzoniformes was obtained for Western blot analysis. The sera of larvae, stage 4 metamorphosing animals (not shown in figure 3 A) and adults (pre-spawning) of the fresh water (brook) lamprey, *Lampetra appendix* showed no immunoreactivity to Ob Sc antibody, that is, the 65 kD protein was absent in the serum of this species (Figure 3 A).

*Lamprey tissue extracts*

In an attempt to find the tissue which is responsible for the synthesis of the lamprey 65 kD protein, lamprey tissue homogenates were prepared and immunoprobed with Ob Sc antibody in Western blots. Since in mammals, leptin is synthesized in adipose tissue, extracts of lamprey tissues known to contain fat (nephric fold, fat column, muscle, liver and intestine) were used for Western blotting analysis (Figure 5 A and B) and probed with Ob Sc and Ob A2 antibodies. Three animals were used for every
Figure 4. (A) *P. marinus* sera (4 μl) from different periods of the life cycle (individual animal from each interval) were electrophoresed on a 10 % SDS-polyacrylamide gel and stained with Coomassie Blue.

A: Ammocoete serum

CF: Condition factor ammocoete serum

T2: Stage 2 transformer serum

T4: Stage 4 transformer serum

T6: Stage 6 transformer serum

P: Feeding juvenile serum

Sp: Upstream-migrant adult serum

MM: Wide range molecular markers (Sigma)

(B) Immunoblot of *P. marinus* sera from different periods of the life cycle. Lamprey serum (4 μl) from the same periods of lamprey life cycle as in Figure 4 A were electrophoresed on 10 % SDS-polyacrylamide. The proteins were transferred onto nitrocellulose and immunoprobbed with Ob Sc antibody (1: 1000). The Ob Sc antibody detected the 65 kD protein in only the larval (ammocoete) period (growth and metamorphic phases) of the life cycle but not in later intervals (P and Sp). Immunoreactivity was noted with the 16 kD leptin (Lp) representing recombinant mouse leptin (300 ng).

(C) Competition study. Lamprey serum (4 μl) of the same intervals of the lamprey life cycle as in Figure 4 A and B but the immunoblot was probed with Ob Sc antibody (1:1000) preabsorbed with 8 μg of Ob Sc blocking peptide. The 65 kD protein is blocked in all samples to the same extent as the mouse recombinant leptin. Arrowheads indicate the position of the blocked signals in lamprey sera and leptin.

43
**Figure 5.** (A) Lamprey *P. marinus* fat column tissue extracts (80 μg protein, representing three animals from each interval) electrophoresed on a 12.5 % SDS-polyacrylamide gel and stained with Coomassie Blue.

A: Ammocoete

T2: Stage 2 transformer

T4: Stage 4 transformer

T6: Stage 6 transformer

MM: Wide range molecular markers (Sigma)

(B) Lamprey nephric fold tissue extract (80 μg protein, representing three animals from each interval) electrophoresed on a 12.5 % SDS-polyacrylamide gel. Samples represent the same periods of lamprey life cycle as in Figure (A).

(C) An immunoblot of lamprey nephric fold, fat column and muscle tissue extracts from two metamorphosing stages (T2 and T4). Tissue extracts containing 80 μg of protein (representing three animals from each stage) were electrophoresed on a 12.5 % SDS-polyacrylamide gel then blotted onto nitrocellulose and immunoprobed with Ob Sc antibody (1:1000). The Ob Sc antibody was immunoreactive with: a 100 kD protein in the muscle and fat column; a 50 kD and 40 kD protein common to muscle, fat column, and nephric fold; and a ~16-17 kD protein present in the nephric fold.

NF: Nephric fold tissue extract

FC: Fat column tissue extract

MS: Muscle tissue extract

T2: Stage 2 transformer

T4: Stage 4 transformer

Lp: Mouse recombinant leptin (300 ng).
(D) Competition study. Same tissue extracts as in Figure 5 C but the immunoblot was treated with Ob Sc (1:1000) antibody preabsorbed with 8 μg of Ob Sc blocking peptide. The 100 kD and 50 kD signals are blocked effectively, ~16-17 kD signal in nephric fold is blocked to the same extent as the mouse recombinant leptin, and the 40 kD is similar to that in Figure C.
tissue from each interval of the life cycle. Tissue extracts of intestine were also probed with Ob Sc antibody as a negative control since intestine is not a fat-storing tissue. The results showed no immunoreactivity (Data not shown). However, the blots of fat-storing tissues immunoprobed with Ob Sc antibody revealed immunoreactivity at 50 kD which was common to nephric fold, fat column and muscle tissue extracts of all periods of the lamprey life cycle which were tested [Larvae, transformer stage 2, 4, feeding juvenile and upstream-migrant adult (data not shown)]. In addition, a weak 40 kD and a strong 100 kD signals were present in muscle and fat column of the same samples. A low molecular weight protein, approximately 16-17 kD was also detected in the nephric fold homogenates of stage 2 and stage 4 transformers only (Figure 5 C). Competition experiments were performed as before using the Ob Sc blocking peptide. The 100 kD and the 50 kD signals were blocked completely and the 16-17 kD protein was blocked to the same extent as the recombinant leptin signal (Figure 5 D). It was concluded that the immunoreactivity observed in the lamprey tissues was specific. The Western blots were performed with the proper negative control using incubation with secondary antibody only; the secondary antibody showed weak immunoreactivity to the 40 kD protein present in the tissue extracts (Figure 6 A). It was concluded that the 40 kD signal was due to non-specific interaction of rabbit IgG with lamprey protein.

The Ob A2 antiserum was used in Western blots of adipose tissue extracts (same as above) from P. marinus. The Ob A2 antiserum at dilutions of 1:500 and 1:2500 showed immunoreactivity with many proteins in the tissue extracts of larvae and adults (Figure 6 B). In fact, the pattern of immunoreactivity was the same as that seen when
Figure 6. (A) Ammocoete and adult nephric fold, fat column and muscle tissue extracts (80 µg protein, representing three animals from each interval) were electrophoresed on a 12.5 % gel then blotted onto nitrocellulose and immunoprobed with the secondary antibody (horseradish peroxidase (HRP)-linked, donkey anti-rabbit IgG; Amersham Pharmacia Biotech) at a dilution of 1:1000. This negative control shows that the secondary antibody was immunoreactive with the 40 kD protein detected in previous figures; the 40 kD signal is due to non-specific interaction.

(B) An immunoblot of lamprey (P. marinus) nephric fold and fat column tissue extracts using Ob A2 antiserum. Tissue extracts containing 80 µg (representing three animals from each interval) of protein were electrophoresed on a 12.5 % SDS-polyacrylamide gel. The proteins were blotted onto nitrocellulose and immunoprobed with Ob A2 antiserum (antibody made vs. the whole recombinant mouse leptin) at a dilution of 1:500. The Ob A2 antiserum was immunoreactive with several lamprey proteins in each tissue extract and also with mouse leptin (Lp).

NF: Nephric fold tissue extract
FC: Fat column tissue extract
MS: Muscle tissue extract
Lp: Mouse recombinant leptin (300 ng).
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40 kD

(B)

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100 kD

50 kD

40 kD

16 kD
SDS-polyacrylamide gels of these tissues were stained with Coomassie Blue. The same samples were immunoprobed with pre-immune rabbit serum to identify possible immunoreactivity of endogenous rabbit immunoglobulins with lamprey proteins but no immunoreactivity resulted. This suggested that there was extensive non-specific binding of the antibody with lamprey proteins and at this point, the results could not be used to draw any conclusions.

*Serum from KClO₄ and thyroid hormone treated larvae*

In mammals, thyroid hormone has been shown to inhibit leptin secretion by a negative feedback loop (Fain et al., 1997). In order to determine if the 65 kD protein present in the serum of *P. marinus* in the larval period was a leptin like molecule, sera from individual larvae treated with either the goitrogen KClO₄, T₃ or T₄ hormones were probed on Western blots with Ob Sc antibody. A difference in the immunoreactivity of the sera from animals treated with KClO₄ was not expected since this treatment induces metamorphosis and sera from metamorphic animals had been already tested and shown to have positive immunoreactivity. The results showed that the 65 kD protein was present in all sera tested with no variation between the larvae treated with thyroid hormone (Figure 7 A) and KClO₄ (Figure 7 B) compared with the control (untreated) larvae.

*A65 antiserum*

The molecular weight of the lamprey 65 kD serum protein differed from that of mammalian leptin. Nonetheless, the presence of this protein in a phase specific and a species specific pattern in *P. marinus* was significant. An attempt was made to isolate this protein to produce antibodies and try the antibody in blots with recombinant mouse leptin, sera and tissue extracts of lamprey (See Materials and Methods). The objective
Figure 7. (A) An immunoblot of lamprey sera from KClO₄-treated animals. Serum samples (4 μl) from two control animals (untreated larvae) and two KClO₄-treated animals were electrophoresed on 10 % SDS-polyacrylamide gel then blotted onto nitrocellulose and probed with Ob Sc antibody at 1:1000 dilution. The blot shows the 65 kD Ob Sc immunoreactive protein in all samples, the antibody also is immunoreactive with leptin (Lp).

C: Serum from control (untreated larva)

K1: Serum sample from KClO₄ treated animal which did not undergo metamorphosis

K2: Serum sample from KClO₄ treated animal at stage 3 of metamorphosis

Lp: Recombinant mouse leptin (400 ng)

(B) An immunoblot of lamprey sera from thyroid hormone treated animals. Serum samples (4 μl) from one control animal (untreated larva), two thyroxine (T₄) treated larvae and two triiodothyronine (T₃) treated larvae were electrophoresed on 10 % SDS-polyacrylamide gel then blotted onto nitrocellulose. The blot was probed with Ob Sc antibody at 1:1000 dilution. The results show the 65 kD Ob Sc immunoreactive protein in all samples.

C: Serum sample from control (untreated larva)

T₄: Serum sample from T₄ treated larva

T₃: Serum sample from T₃ treated larva

Lp: Recombinant mouse leptin (400 ng)
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16 kD

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16 kD
was to see if this antibody would be immunoreactive to mouse recombinant leptin and to proteins present in serum and the tissue extracts.

The 65 kD protein was isolated from 10% polyacrylamide gels by elution (See Materials and Methods). The purity of the eluted material was checked by probing Western blots of eluted material with Ob Sc antibody. Once the A65 antiserum (produced against the isolated 65 kD lamprey larval protein in the laboratory) was prepared, Western blots of lamprey larva and adult sera from individual animals, mouse recombinant leptin and eluted 65 kD protein were probed with the A65 antiserum. The immunoreactivity observed in the serum samples was intense and the immunoreactivity at the position of 65 kD was not discernable (Figure 8 A). When the blot was compared with a Coomassie Blue stained gel of the same samples, the antiserum appeared to have been immunoreactive with all the proteins found in the lamprey serum. The same results were obtained even when the rabbit immunoglobulins were isolated from the antiserum using an IgG affinity column (See Materials and Methods). Greater dilutions of the antiserum (1: 300000) did not eliminate these signals. The A65 antiserum was not immunoreactive with mouse recombinant leptin but it was immunoreactive with the isolated 65 kD protein which suggested that the antibody was produced against the larva 65 kD protein. Since the 65 kD protein was detected in larval serum and absent in the adult serum, it was thought that absorbing the antibody with adult serum might eliminate some of the signals which are common to larva and adult sera from the larva sample, except the 65 kD protein. The results showed that the intensity of the immunoreactivity was reduced, and there were some differences between the pattern of immunoreactivity of absorbed A65 antiserum and the untreated antibody (Compare Figure 8 A and B). In
Figure 8. (A) An immunoblot of lamprey ammocoete and adult sera (individual animals from each interval) and the 65 kD protein eluted from ammocoete serum. The sera were electrophoresed on a 10 % SDS-polyacrylamide gel then blotted onto nitrocellulose and immunoprobed with the A65 antiserum (antibody made vs the 65 kD ammocoete serum protein) at a dilution of 1:150000. The A65 antiserum was immunoreactive with all lamprey serum proteins including the isolated 65 kD protein. No immunoreactivity was present with mouse recombinant leptin (Lp; see arrowhead).

A 1: Ammocoete serum 1 µl
A 2: Ammocoete serum 0.5 µl
Sp 1: Upstream-migrant adult serum 1 µl
Sp 2: Upstream-migrant adult serum 4 µl
Lp: Mouse recombinant leptin (300 ng)
AE: Isolated 65 kD ammocoete serum protein

Arrowheads indicate the position of the 65 kD protein in the ammocoete and adult sera.

(B) Same samples as in Figure 8 A but the immunoblot was treated with A65 antibody (1:150000) preabsorbed with adult serum to eliminate proteins common to both larval and upstream-migrant adult periods. The intensity of the immunoreactivity was reduced but several lamprey immunoreactive signals were still present and the 65 kD protein seemed to be absent.

Arrowheads indicate the position of the 65 kD protein and recombinant mouse leptin (16 kD).
addition there was no evidence for immunoreactivity of a 65 kD protein in the larval and adult sera with the absorbed antiserum.

Since the A65 antiserum was immunoreactive with many proteins in the serum, it was decided to employ the crossed immunoelectrophoresis technique to determine the specificity of the A65 antiserum.

**Crossed Immunoelectrophoresis**

CIE showed that the A65 antiserum was immunoreactive with proteins present in the larval and adult sera (Figure 9 A). When the plates were compared by superimposing one plate on the other, it was found that the major antigen-antibody complex peak in the larval plate (Figure 9 A, 65 kD) corresponded to the small peak in the adult plate. This led to the conclusion that the peaks represented the same protein. Judging from the intensity and the size of the peak present in the larval plate, this peak is assumed to be the 65 kD protein. The antigen-antibody immunoprecipitate peak in the adult plate was considerably smaller than the peak corresponding in the larval plate. Since the area under the antigen-antibody complex peak is proportional to the amount of antigen present (Walker, 1988), it was concluded that the 65 kD protein is present in the adult serum at lower concentrations than in the larva. Pre-incubation of the antiserum with the adult serum showed no immunoreactivity in both adult and larva sera (Figure 9 B). This result confirmed the view that the 65 kD protein is present in both sera. Perhaps the absence of the 65 kD protein in the Western blots of both life cycle intervals when they were probed with A65 antiserum preabsorbed with adult serum (Figure 8 B) was due to the blocking of this signal.
Figure 9. (A) Crossed immunoelectrophoresis of sera from an ammocoete and an upstream-migrant adult of *P. marinus*. In the first dimension 7 μl of ammocoete (individual animal) and adult sera (individual animal) were electrophoresed on plates containing 2 % agarose gel. In the second dimension, 300 μl of A65 antiserum was in the agarose. The intensity and the size of the peak of the antigen-antibody complex indicate the presence of the 65 kD protein in the ammocoete serum. The smaller peak (C) suggests the presence of a contaminant in the A65 antiserum. The antigen-antibody complex peak in the adult serum corresponds to the 65 kD protein present in the ammocoete serum; the size of the peak indicates that the protein is present at lower concentration in the adult compared to the ammocoete.

(B) The plates contain the same samples as in figure (A) but in the second dimension the agarose contained 300 μl of A65 antiserum that was preabsorbed with 600 μl of adult serum. The absence of antigen-antibody complex supports the view that the 65 kD protein is common to both ammocoete and adult sera.
There was an additional peak in the larva serum (Label C in Figure 9 A), which suggested that there was at least one contaminant present in the immunogen used for immunization.

**Immunoprecipitation of serum from mammals or lower vertebrates**

Leptin is found at a very low concentration in the serum of lean mammals (12.5 ng/ml in mice and 5-15 ng/ml in humans) (Maffei et al., 1995; Haynes et al., 1997). Therefore, in order to detect circulating leptin, studies use immunoprecipitation to isolate leptin in relatively large amounts of serum. To take into account the possibility that leptin in the lamprey is present at concentrations too low to detect directly on a blot using 4 μl of serum, immunoprecipitation was employed using lamprey, eel, rat, mouse and human sera. The immunoprecipitates were then subjected to electrophoresis and Western blotting. A 16 kD protein was not detected in the mammalian and fish sera using Ob A2 and Ob Sc antibodies. Although, sensitivity of ECL blotting technique is less than 1 pg of antigen according to the manufacturer’s specification, Ob A2 antiserum and the Ob Sc antibody did not detect authentic recombinant leptin when the immunoprecipitation procedure was used with amounts lower than 100 ng and 50 ng, respectively. Furthermore, the Ob Sc antibody did not immunoprecipitate the lamprey 65 kD nor the eel 116 kD lamprey and eel serum proteins respectively, detected in previous immunoblots. In the light of the fact that immunoprecipitate of leptin was not obtained from mouse or human sera, it would seem that the immunoprecipitation conditions used should be modified.
DISCUSSION

Metamorphosis in the larvae of lamprey *P. marinus* is characterized by transformation and regression of larval structures and development of adult tissues and organs (Youson, 1980). This process involves complex interaction of hormonal, environmental, metabolic and nutritional factors (Youson, 1994). An important physiological condition for spontaneous metamorphosis to proceed is the accumulation of lipid at specific fat depot sites (Youson et al., 1997). Once the larva is of specific length and accumulation of lipid brings it to a specific size (length and weight), the minimum condition factor criterion is satisfied and metamorphosis is triggered (Holmes and Youson, 1994) given that other conditions, environmental and hormonal, are met (Youson, 1997). During this non-trophic period of metamorphosis the accumulated fat reserves are depleted for energy use (Sheridan and Kao, 1998). The importance of these fat reserves to metamorphosis suggests that a signal from the adipose tissue might relay messages about the status of the fat depot that would subsequently result in initiation of metamorphosis. The following is a brief summary of literature, which provided the basis for the research of this thesis.

Leptin, a 167 amino acid protein hormone in mammals, is synthesized in the adipose tissue and transported to the brain in the blood where it exhibits its effects by release and inhibition of factors which ultimately cause decrease in food intake, increase in energy expenditure, and increase in physical activity (Houseknecht et al., 1998). Centrally, leptin acts by inhibiting the expression of neuropeptide Y (NPY) (Stephans et al., 1995) which is a potent stimulator of food intake (Clark et al., 1985), an inhibitor of brown fat thermogenesis and causes an increase in insulin and corticosteroid levels.
(reviewed in Rohner-Jeanrenaud et al., 1996). Also, leptin stimulates thermogenesis by sympathetic activation of brown adipose tissue (BAT) via the hypothalamus.

Peripherally, leptin acts by attenuating insulin action, in part by inhibiting insulin secretion from pancreatic B-cells (Kieffer et al., 1996; Emillson et al., 1997). Shimabukuro (1997) and Zhou (1997) have found other peripheral pathways of leptin action such as the depletion of triglycerides from nonadipocytes and adipocytes. Furthermore, leptin inhibits esterification of free fatty acids to triglycerides and induces oxidation of free fatty acids, which generates energy.

A hormone like leptin could play a significant role in lamprey fat homeostasis. Studies have shown that leptin mRNA expression and plasma levels are highly correlated with size of adipose tissue and body mass index (BMI) in lean and obese mice and humans (Frederich et al., 1995; Maffei et al., 1995). That is, leptin mRNA expression increases as the size of the adipose tissue increases and serum leptin levels drop as body mass decreases. As was mentioned above, the amount of adipose tissue of lamprey P. marinus fluctuates during its life cycle. In the larval period, lipid content is 4% of total body wet weight but it reaches a peak of 13.5% prior to metamorphosis and during metamorphosis lipid levels drop to 7.9% (Lowe et al., 1973; Beamish et al., 1979; Youson et al., 1979). Studies of lipid metabolism in lampreys by monitoring plasma fatty acid levels and activity of lipogenic and lipolytic enzymes, have supported this pattern of lipid accumulation and lipid depletion (Kao et al., 1997b; Sheridan and Kao, 1998). These studies suggest that lipogenesis is the predominant metabolic pathway from the late larval phase to stage 3 of metamorphosis and lipolysis takes over in stage 3 to stage 7 of metamorphosis. Since leptin levels are highly correlated with the amount of fat
reserves in mammals (Frederich et al., 1995; Maffei et al., 1995), if a leptin-like molecule is present in lamprey, then as large amounts of lipid accumulate prior to metamorphosis the resulting rise in leptin levels might signal the brain about the status of fat reserves and result in initiation of metamorphosis. As lipogenesis continues to stage 1-2 of metamorphosis, leptin levels might continue to increase and trigger other physiological effects specific to leptin such as depletion of triglycerides and inhibition of triglyceride synthesis by direct action of leptin on adipocytes and nonadipocytes. Another effect of leptin in lamprey could be regulation of thyroid hormone and insulin. Leptin is involved in secretion of thyroid hormone, as elucidated in experiments with starving rodents (Ahima et al., 1996; Fain et al., 1997), and in the secretion of pancreatic hormone insulin (Kieffer et al., 1996; Emilsson et al., 1997). If a leptin-like molecule is present in lamprey then perhaps it is responsible for fluctuations of these hormone levels and consequently it is responsible for change in lipid metabolism during the larval period (growth and metamorphic phase).

The approach for a search of a leptin or a leptin-like molecule in lamprey was by probing immunoblots of lamprey serum and lipid-containing tissue extracts with antibodies made against mammalian leptin. The antibodies used, Ob A1, Ob A2 and Ob Sc, had different patterns of immunoreactivity with lamprey and eel sera and tissue extract proteins (See table 1). Except for the 16–17 kD protein detected in the nephric fold of metamorphosing animals (stage 2 and 4), an immunoreactive 16 kD protein was absent in sera of lamprey and the positive controls (rat and human sera). The SDS-polyacrylamide gels used in the Western blots did not allow good separation of serum
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Table 1. Pattern of immunoreactivity of leptin antibodies (Ob Sc, Ob A2 and Ob A1) with lamprey and eel sera and lamprey adipose tissue extracts. React, immunoreactivity observed; block, blocking observed with Ob Sc antibody preabsorbed with Ob Sc blocking peptide, (+), positive; (-), negative; NS, non-specific reaction; N/A, experiment was not carried out.
protein for volumes of serum greater than 10 μl. If a lamprey leptin, like mammalian leptin, circulates at very low concentration, then larger volumes of serum should be used to detect this molecule. Immunoprecipitation allows precipitation of proteins from large volumes of serum but the immunoprecipitation of as much as 500 μl of lamprey serum with the leptin antibodies (Ob A2 and Ob Sc) failed to show evidence of a 16 kD molecule immunoreactive to leptin antibodies. However, for several reasons it could not be concluded that leptin was absent from lamprey serum. First, an attempt to detect leptin using the immunoprecipitation procedure in positive controls (rat and human serum) was also unsuccessful suggesting that perhaps a modified procedure needed to be used. Furthermore, when the sensitivity of the immunoprecipitation method and the ECL system was tested using different amounts of recombinant leptin, the lowest amount of leptin detected was 50 ng. Leptin is present at very low concentration in sera of mice (12.5 ng/ml) and humans (5-15 ng/ml) (Maffei et al., 1995; Haynes et al., 1997). If leptin is found at similar concentrations in the sera of lampreys, then larger volumes (> 500 μl) of serum must be used to detect leptin. In addition, the amino acid sequence of the presumed lamprey leptin may not have been conserved during the course of evolution, that is the mammalian leptin is not very similar to lamprey leptin consequently the mammalian leptin antibody may not precipitate lamprey leptin.

*Ob Sc immunoreactive serum proteins*

The Ob Sc antibody did, however, show a strong signal on Western blots with a 65 kD protein in lamprey larval serum and 116 kD protein in eel serum. Proof for the antigen-antibody specificity came from antigen competition experiments. The 65 kD and 116 kD signals along with the recombinant leptin signal were blocked to the same extent
after the antibody was absorbed with the Ob Sc blocking peptide, which is the peptide used to produce the Ob Sc antibody. Thus, the immunoreactivity was not due to non-specific antigen-antibody interaction. The Ob Sc antibody is an affinity-purified polyclonal antibody, that can react with lamprey and eel proteins. In cases of cross-reactivity, the cross-reacting proteins most likely share an epitope related or identical to that present in the immunogen (Drenckhahn et al., 1993). Therefore, the 65 kD and the 116 kD proteins might have one or more of the antigenic determinants of the carboxy terminal end of leptin. The immunoreactivity observed in the eel serum was of some interest since preliminary results had demonstrated that a clone of mouse leptin hybridizes with eel genomic DNA (Zhang et al., 1994).

The immunoreactivity in the lamprey serum raises the following question: Could this 65 kD protein represent some form of leptin? One possibility for the higher molecular weight would be that the 65 kD protein is made up of 4 repeated domains (which would account for the high molecular weight), each one with antigenic similarity to mouse leptin. The lamprey leptin and mammalian leptin relationship might be similar to the relationship that has been shown with lamprey and mammalian albumin. SDS-1 serum protein of adult lamprey P. marinus is homologous to the mammalian albumin but has a molecular weight of 160 kD and consists of seven domains instead of three domains like the mammalian albumin (Gray and Doolittle, 1992). Another possibility is that this protein has a number of functional domains, one of which could be leptin like. The 65 kD protein could be a large molecule from which the mammalian leptin has evolved.

No studies were carried out to determine the function of the 65 kD protein but its presence in a phase-related fashion meets an expectation of a putative lamprey leptin. It
was expected that if there was a lamprey leptin that levels would increase as lipid accumulated in the larva and would remain at high levels to induce lipid depletion during metamorphosis. Therefore, the presence of the 65 kD protein in the larval, immediately premetamorphic, and metamorphosing intervals agrees with the proposed scheme of lamprey leptin secretion during lamprey life cycle.

When an antibody (A65 antiserum) to 65 kD protein was used in Western blots, it was not immunoreactive with the recombinant mouse leptin. This suggested that mouse leptin did not have an antigenic determinant which was recognizable by the A65 antiserum (see later section). The A65 antiserum was immunoreactive with the 65 kD protein eluted from larva serum but it was difficult to determine the specificity of A65 antiserum to lamprey serum proteins by Western blot since it was also immunoreactive with all lamprey serum proteins (larva and adult). Perhaps pre-clearing the lamprey serum with rabbit IgG might discount some of these signals as non-specific interaction.

An attempt to analyze the immunoreactivity of A65 antiserum with lamprey serum using crossed immunoelectrophoresis (CIE) resulted in a conclusion that the 65 kD protein is present in lamprey serum at larval and adult periods of the life cycle with the latter possessing a lower concentration of the protein. This protein was absent in the Western blots of upstream-migrant adult serum probed with Ob Sc antibody. The detection of the 65 kD protein in the serum of the adult by using CIE with A65 antiserum suggests that there might be some specific difference in the amino acid sequences of the putative leptin-like molecule in larvae and adults. The adult form may not contain the epitope(s) immunoreactive with the Ob Sc antibody such that the protein in adults could only be detected by the antibody made against the whole molecule (A65).
The CIE results also showed the presence of a contaminant in the A65 antiserum. It is possible that the 65 kD immunogen was contaminated one or more proteins or the 65 kD band may in fact be made up of a few proteins with very similar molecular mass. Thus antibodies were produced against more than one protein (Drenckhahn et al., 1993).

Various procedures can be used to analyze the purity of the 65 kD protein. First, the use of two-dimensional polyacrylamide gel electrophoresis might resolve this 65 kD band into more than one protein provided that they have different isoelectric points. Second, high performance liquid chromatography (HPLC) can be used to fractionate proteins or peptides present in eluted sample (65 kD band) and determine the fraction which contains the immunoreactive 65 kD protein in a Western blot.

Identity of 65 kD protein

The functional and structural identity of the 65 kD protein is not yet known, but the temporal appearance of this protein could satisfy the criterion that was proposed for a leptin-like molecule in *P. marinus*. At the moment, this 65 kD protein appears to be species specific since it is absent in serum of *L. appendicis*. The absence of the 65 kD signal from the larval and adult sera of this species has a few implications. It could be that this protein is present in *L. appendicis* with some variation in the amino acid sequence such that it is not immunoreactive with the Ob Sc antibody. On the other hand, the absence of this protein might reflect a major difference between larvae of parasitic and non-parasitic species.

Although, there is no evidence that fat reserves are an essential requirement for initiation of metamorphosis in *L. appendicis*, this species has been shown to have a minimum size criterion (at least 155 mm and 5.4 g) for commencement of metamorphosis.
(Holmes et al., 1999). However, the average condition factor is only 1.39 in early metamorphosing stages compared to minimum of 1.50 in *P. marinus* (Holmes et al., 1999; Youson, 1994). These data suggest that physiological preparation (a significant weight-length relationship) might not be as important to *L. appendix* metamorphosis as is in the case of *P. marinus*. As a result, a lipostat like leptin might not be required in *L. appendix*.

Alternatively, it could be that the 65 kD protein is not a leptin like molecule. One could argue that the results of the Western blots of thyroid hormone treated larvae support this view. If indeed this protein is a leptin-like molecule, like mammalian leptin its production should be inhibited by thyroid hormone (Fain et al., 1997). Therefore, there should have been a distinct quantitative difference between the immunoreactivity of 65 kD protein in the serum of thyroid hormone treated larvae and the untreated larvae. The results of this experiment suggest that the secretion of this protein is not affected by thyroid hormone. At least there are no obvious differences in the Western blot. Subtle differences might be found if densitometry of the blots is performed. However, it should be kept in mind that, it is difficult to make correlations between the role of thyroid hormone in mammals and lamprey since thyroid hormone might have different functions in each organism. Another observation which supports the view that 65 kD protein is not a leptin-like molecule is the fact that leptin decreases food uptake in mammals in part by inhibiting secretion of neuropeptide Y in the hypothalamus. In lamprey larvae, feeding and breathing are linked processes (Randall, 1972). In mammals food intake is a neurohormonal behavior; the animal (mammal) can see and smell the food but does not feed due to the effect of high levels of circulating leptin on the hypothalamus. However, the
animal can still get oxygen since feeding and breathing are two separate processes. The conclusion that lampreys stop feeding at the onset of metamorphosis is based on the modifications of the physical structure of the respiratory apparatus and the digestive system (Youson, 1980). Therefore, a hormone like leptin is not needed to modify the feeding behavior.

These observations alone are not sufficient to conclude that the 65 kD protein is not a leptin-like molecule. This protein may lack some of the mammalian characteristics of leptin (decrease in food intake and inhibition by thyroid hormone) due to differences in the demands of the organisms and their environment, but it may still act as a lipostat and facilitate depletion of fat reserves in *P. marinus*.

**Ob Sc immunoreactive tissue extract proteins**

The results from the immunoblots of lamprey tissue extracts showed a strongly immunoreactive 100 kD protein in the muscle and the fat column and a weakly immunoreactive 50 kD protein common to nephric fold fat column and muscle. These signals were detected in all stages tested: larva, metamorphosing stage 2, 4, feeding juvenile and upstream-migrant adult. Antigen competition studies effectively blocked the 100 kD and 50 kD signal along with the positive control recombinant mouse leptin. After establishing the specificity of the antigen-antibody immunoreactivity no further tests were carried out to determine the identity of these signals. The immunoreactivity of the 100 kD in the muscle and the fat column and 50 kD protein in the nephric fold, muscle and fat column, suggests that they could contain amino acid sequences similar to a leptin determinant(s) and they do not seem to be associated with metamorphosis or a specific phase of the lamprey life cycle. In future studies, these bands can be eluted like the 65
kD protein and purified. The purified proteins can be subjected to trypsin degradation and sequenced. However, to determine if the tissue-extract proteins are in fact leptin or leptin-like molecule, which have mammalian features, a bioassay can be performed. For example, the purified immunoreactive peptides can be injected into ob/ob mice or premetamorphic larvae and check for weight loss, increase in energy expenditure and normalization of insulin levels.

A signal of ~16-17 kD was detected in the nephric fold of metamorphosing stage 2 and 4 animals but not the larvae. Competition studies showed that the antigen-antibody interaction was specific. The presence of this signal in the nephric fold extracts is of some interest since the nephric fold is one of the major fat storage sites in lamprey (Youson et al., 1979; Kao et al., 1997a,b). Furthermore, since I hypothesized that a leptin-like molecule would induce lipolysis, the rate of lipolysis in the kidney (nephric fold) during metamorphosis is greatest (Kao et al., 1997b). However, Western blotting alone is not sufficient to draw conclusions about the identity of this signal.

Ob A2 immunoreactive tissue extract proteins

Proteins from various tissue extracts, 100 kD, 50 kD and the 16 kD were detected with the Ob A2 antiserum, but many of the other tissue proteins were immunoreactive as well. Incubation of the tissue extract blots with only the preimmune serum revealed no immunoreactivity and the secondary antibody eliminated only one band as non-specific interaction. However, a more effective approach might be the pre-clearing of the samples with rabbit IgG. Western blotting might then show that some of these signals are due to binding of rabbit IgG by lamprey proteins.
Ob AI and Ob A2 immunoreactivity to serum proteins

Immunoblots probed with Ob AI and Ob A2 antisera, which were made against the entire recombinant mouse leptin molecule, showed strong immunoreactivity to recombinant mouse leptin but did not show immunoreactivity for the 65 kD protein in lamprey. The Ob AI and Ob A2 antisera apparently do not contain paratopes immunoreactive to the carboxy terminus, which is presumably, the immunogenic determinant being detected in the 65 kD protein of lamprey serum. Perhaps, the carboxy terminal end of the leptin molecule may not be as immunogenic when it is part of the whole molecule as it is when separated from the rest of the leptin molecule. Similarly, the A65 antiserum may not recognize recombinant mouse leptin on Western blots if the antigenic determinant is not available.

A curious property of the Ob Sc antibody was revealed when it was used to probe the blocking peptide on a Western blot. This Ob Sc blocking peptide is supplied by the manufacturer of the Ob Sc antibody for use in competition studies, and is, according to the manufacturer, the antigen used to produce the Ob Sc antibodies. The results showed a signal of 66-70 kD for the blocking peptide. In a series of conversations with the manufacturer, they insisted that the Ob Sc blocking peptide is 1 kD peptide consisting of only 19 carboxy terminal amino acids (137-156) of mouse leptin and since it will run off a 12.5-15 % SDS-polyacrylamide gel, it is not expected to show up in the Western blots. They cannot explain why the blocking peptide gives an intense 66-70 kD band on a Western blot with the Ob Sc antibody purchased from them. Although, it is difficult to determine the reason for the difference between the results obtained by the Ob AI and Ob A2 antisera and the Ob Sc antibody, the pattern of immunoreactivity of the antibodies
made against the whole leptin molecule (Ob A1 and Ob A2) is the same. They both failed to detect the 65 kD protein in lamprey larval serum and 116 kD eel serum protein. This further suggests that the leptin molecule and the leptin peptide might possess different epitopes such that when used as immunogens, the antibodies produced have different specificities. It may also suggest that the lamprey and eel serum immunoreactive proteins have the same antigenic determinant recognizable by the Ob Sc antibody.
CONCLUSION

The search for leptin or a leptin-like protein in the lamprey, *P. marinus*, resulted in finding mammalian leptin antibody immunoreactive proteins in serum (65 kD) and tissue extracts (100 kD, 50 kD and 16-17 kD). The antibody directed towards the carboxy terminal of mammalian leptin also immunoreacted with a 116 kD protein in eel serum. The significance of the 65 kD protein lies in the fact that the protein has a temporal appearance, i.e. it is present at a higher concentration in the larval period (both growth and metamorphic phases) compared to the adult. Furthermore, the 65 kD protein is absent in serum of larvae and adults of brook lamprey, *L. appendix*. These results suggest that this protein is specific to the larval period of life cycle of *P. marinus* species of lamprey when adipose tissue is abundant. The temporal appearance of this protein satisfies what was proposed at the commencement of the research for a putative lamprey leptin. However, the data collected during this research are not sufficient to ascribe a role for this protein in fat accumulation and mobilization during larval period of the life cycle.

From the lamprey tissue immunoreactive proteins the 16-17 kD protein is of interest since it was detected in the nephric fold of stage 2 and 4 metamorphic animals. The importance of this finding lies in the fact that this tissue is a major fat storage site in the larva and during metamorphosis (stage 3 - stage 7) and the greatest amount of lipid depletion occurs in this tissue. The immunoreactivity of eel serum protein (116 kD) is also of some interest since eel was one of the lower vertebrates whose genomic DNA was shown to hybridize to mouse leptin probe (Zhang et al., 1994).

A subsequent, effective approach to finding leptin in lamprey would be to search for the leptin gene using mammalian probes in adipose tissue of lamprey (fat column and
nephric fold). The positive results of cross-species hybridization of mouse leptin probe to genomic DNA, specifically to eel, gives some expectation that the gene is present in fish and potentially in the lamprey.

The identity of a leptin-like protein in lamprey is important for it could shed some light on the regulation of lipid metabolism and subsequently, metamorphosis in lamprey. Furthermore, such a discovery might also provide some explanation for the versatile functions of leptin, which may have evolved early on in the evolution of vertebrates.


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