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DISTRIBUTION OF PROOPIOCORTIN (POC) AND PROOPIOMELANOTROPIN (POM) mRNA DURING DEVELOPMENT OF THE SEA LAMPREY: A QUANTITATIVE SPATIAL AND TEMPORAL *IN SITU* HYBRIDIZATION STUDY.

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy and Physiology

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ABSTRACT

The sea lamprey, *Petromyzon marinus*, undergoes a complex life cycle beginning with a filter feeding larval stage followed by a seven stage non-trophic metamorphosis, an actively feeding parasitic adult stage and spawning adult stages. The lamprey has ancient origins and conservative evolution but the full extent and the role of hormones the lamprey pituitary secretes is unknown. Recently, two POMC-like pituitary prohormones proopiocortin (POC) and proopiomelanotropin (POM) have been characterized from adult sea lamprey. POC and POM are multi-domain prohormones. POC encodes a nasohypophysial factor (NHF), ACTH, a MSH, and β-END and POM encodes MSH-A, MSH-B and β-END. In this study, radiolabelled riboprobes were generated specifically to the NHF domain of POC mRNA and MSH-B domain of POM mRNA in order to examine the expression of POC and POM during development of the sea lamprey by *in situ* hybridization. POC expression appears evenly distributed throughout most cells of the rostral pars distalis (RPD) throughout the entire life cycle. POC expression also occurs in scattered cells of the caudal pars distalis (CPD) at stage 5 of metamorphosis and by the spawning stage POC expression is mainly distributed in the dorsal aspect of this region. POM expression was completely confined to most cells of the pars intermedia (PI) at all stages examined. Computer assisted image analysis was utilized to quantitatively determine the relative amounts of POM and POC expression throughout development. The signal densities (area fraction) of expression signal and an estimate of the total volume of POM expressing cells in the PI and POC expressing cells in the RPD and CPD was calculated. Analysis of POM expression revealed very high signal densities in larval animals which decreased by early metamorphosis, steadily increased and reached high levels by late metamorphosis (stages 6 and 7) and even higher levels in spawners. Volumetric analysis revealed that the net volume of POM expressing cells is at its lowest in larval animals and increases during development. Therefore the decrease in POM signal density early in metamorphosis is compensated by an increase in the total volume of the PI and POM expressing cells, such that the total amount of POM expressing cells is greater in the early metamorphic animals than that of the larval animals despite the lower signal densities. Analysis of signal density and volumetric measurements of POC expression revealed that POC expression in the RPD is low in larval animals and steadily increases during development reaching very high levels by the spawning stages. POC expression in the CPD, first visible at stage 5, increases steadily throughout the remainder of metamorphosis and reaches the highest levels of expression in spawning animals. These results would implicate the role of POM and POC perhaps not in the initiation of metamorphosis but in some processes necessary for development of the lamprey. The very high levels of POM and POC expression in spawning animals suggest a role in the sexual maturation of lampreys.

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To my parents

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ACTH	-	Adrenocorticotropic hormone
AF	-	Area fraction
AH	-	Adenohypophysis
AN	-	Anterior neurohypophysis
APES	-	3-aminopropyltriethoxysilane
bp	-	Base pair
CLIP	-	Corticotrophin-like intermediate lobe peptide
CF	-	Condition factor
CPD	-	Caudal pars distalis
cpm	-	Counts per minute
DEPC	-	Diethylpyrocarbonate
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
END	-	Endorphin
FSH	-	Follicle stimulating hormone
GH	-	Growth hormone
Hy	-	Hypothalamus
Inf	-	Infundibulum
ISH	-	*In situ* hybridization
JP	-	Joining peptide
LH	-	Luteinizing hormone
LHRH	-	Luteinizing hormone releasing hormone
LPH	-	Lipotrophin
Me	-	Median eminence
MS-222	-	tricaine methanesulfonate
MSH	-	Melanocyte stimulating hormone
NCF	-	Noncondition factor
NHF	-	Nasohypophysial factor
NP	-	N-terminal peptide of β-LPH
NTB	-	Nitroblue tetrazolium
NTP	-	N terminal peptide
P	-	Parasitic adult
PAS	-	Periodic acid-Schiff
PBS	-	Phosphate buffered saline
PD	-	Pars distalis
PFA	-	Paraformaldehyde
pGEM-4Z	-	Promega ™Gemini plasmid vector
PI	-	Pars intermedia
PN	-	Pars nervosa
POC	-	Proopiocortin
POM	-	Proopiomelanotropin

POMC	-	Proopiomelanocortin
PRL	-	Prolactin
PS	-	Prespawner
PT	-	Pars tuberalis
RIA	-	Radioimmunoassay
RPD	-	Rostral pars distalis
S	-	Spawner
^{35}S-CTP	-	Cytidine 5'-[α-thio]triphosphate (radioactive)
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	-	Signal peptide
SSC	-	Saline sodium citrate
S-UTP	-	Uridine 5'-[α-thio]triphosphate (non-radioactive)
TEA	-	Triethanolamine
TSH	-	Thyroid stimulating hormone
III	-	Third ventricle

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1. INTRODUCTION

1.1 Cyclostomes

Lampreys and hagfishes represent the only two remaining jawless fishes surviving from the Paleozoic era. Lampreys (Petromyzontiformes) and hagfishes (Myxiniformes) are taxonomically placed in their own vertebrate superclass Agnatha (without jaws) in the class of Cyclostomata separated from gnathostome (jaw mouthed) vertebrates (Holmes and Bull, 1974; Forey and Janvier, 1993). At one time, common features between the two cyclostomes suggested a close relation between them. Both fishes lack jaws, internal ossification, scales and paired fins. Other shared characteristics include pouch-like gills and complex tongues (Forey and Janvier, 1994). However, these shared anatomical features are insignificant when differences in biochemistry, physiology and karyotypes are considered. As of yet no molecular evidence has provided conclusive answers to the relationship between the two species (Stock and Witt, 1992; Forey and Janvier, 1994). Interestingly, lampreys and higher vertebrates share numerous physiological and morphological features not shared by hagfishes. Paleontological evidence suggests lampreys and hagfishes have long separate histories, and that lampreys are more closely related to gnathostomes than either is to hagfishes (Forey and Janvier, 1993). However, more conclusive molecular analyses are needed to better define the evolutionary taxa of these species.

1.2 Lamprey life cycle

Lampreys have a complex lifestyle beginning with the development of eggs to the larval, or ammocoete form, which live burrowed in silt banks of rivers, followed by a seven

stage metamorphosis or transformation and adult life (Potter *et al.*, 1978; Hardisty, 1979; Youson and Potter, 1979). The length of the larval stage is variable (2 to 7 years), and depends on the richness of food (microorganisms upon which they filter-feed), population densities and seasonal water temperatures. The timing and induction of metamorphosis, which occurs over a 2-3 month period, is synchronized with environmental and hormonal cues in all lamprey species (Potter *et al.*, 1978). Metamorphosis varies directly with water temperature under experimental conditions and the role of hormones in metamorphosis is not clearly defined (Holmes and Youson, 1993). A condition factor (CF=weight (g)/length^3 (mm) X 10^6) of 1.5 or greater, in conjunction with a minimum size (120 mm), and minimum weight (3.0 g) requirement, is a predictor of pending metamorphosis of sea lamprey under controlled laboratory conditions (Youson *et al.*, 1993; Holmes and Youson, 1993). At the end of the metamorphic phase, the resulting young adult, or juvenile lamprey, may follow two different species dependent patterns of development.

Of the 31 recognized lamprey species, fourteen are parasitic and begin to feed on the blood, body fluids and tissues of teleost fish after metamorphosis. Parasitic lamprey migrate downstream toward lakes to begin their feeding stage which may vary between one and three years. Nine of the parasitic lamprey species are anadromous and will migrate towards marine waters to continue feeding. Three species of anadromous lampreys have given rise to landlocked forms, one example being the sea lamprey, *Petromyzon marinus* of the Great Lakes system (Hardisty and Potter, 1971). The end of the parasitic adult stage is marked by migration upstream to spawn, then the lampreys die. The non-parasitic or brook lampreys do not feed in their adult stage, are nonmigratory and are thus restricted to fresh waters, have

a reduced duration of adult life of six to nine months and do not grow any larger than the ammocoete (Hardisty and Potter, 1971).

1.3 Impact of lampreys in the Great Lakes

Parasitic landlocked sea lamprey prey on large commercial and sporting fishes causing major damage to fish stocks of the Great Lakes fishing industry which as of 1994 was valued at $2-4 billion (Great Lakes Fisheries Commission, 1994 written communication). Lamprey control methods now in use include the use of lampricides, in streams and tributaries, such as 3-trifluoromethyl-4-nitrophenol, toxic to larval and adult lampreys (Meyer and Schnick, 1983). However, lampricides are not proving to be a favourable means of control as they are costly, not effective at all locations in large bodies of water and have uncertain long term ecological effects (Meyer and Schnick, 1983). Alternative control methods require increased understanding of lamprey biology and these are actively being pursued. In this aspect, lamprey metamorphosis has been identified as the period of vulnerability and possible point of control and intervention of the parasitic lamprey. As of yet, little is known and understood of the developmental, environmental and hormonal factors involved in the initiation and completion of metamorphosis. The role of pituitary hormones is likely to provide answers to lamprey development since they play a major role in the developmental processes and sexual maturation in all other vertebrates. Information gained of the factors initiating and controlling metamorphosis may be of value in developing biological methods of controls as this period in the lampreys' life cycle may be a period when the animal is most vulnerable to factors causing mortality (Hardisty and Potter, 1971).

1.4 The pituitary

In general, very little variation exists between the pituitary, or hypophysis of vertebrates (Gorbman *et al.*, 1983). The pituitary is comprised of neural and epithelial structures represented by the neurohypophysis and adenohypophysis, respectively. The neurohypophysis is derived from a ventral outgrowth of the floor of the diencephalon and remains attached to the brain by a neural stalk (infundibulum). The adenohypophysis is derived from an epithelial diverticulum of the embryonic oral cavity named Rathke's pouch (Gorbman *et al.*, 1983). The lamprey pituitary is anatomically similar to that of other vertebrates but does diverge from this plan in that the adenohypophysial tissue has a nasohypophysial origin (Hardisty, 1979). The nasohypophysis is derived from the somatic ectodermal thickening of the olfactory placode of the early embryo which sinks down in a common depression, the nasohypophysial pit (Larsen and Rothwell, 1972). The nasohypophysial stalk, developing from the pit, extends posteriorly below the forebrain, and it is from the nasohypophysial stalk that the adenohypophysial tissue proliferates (Larsen and Rothwell, 1972).

1.4.1 Gnathostome pituitary

The adenohypophysis can be subdivided into three regions: the pars tuberalis (PT) which surrounds the infundibulum, the pars distalis (PD) and the pars intermedia (PI). (Figure 1.1.).

The PD is composed of several types of hormone secreting parenchymal cells. Other types of cells include numerous capillary endothelial cells and fibroblasts that produce

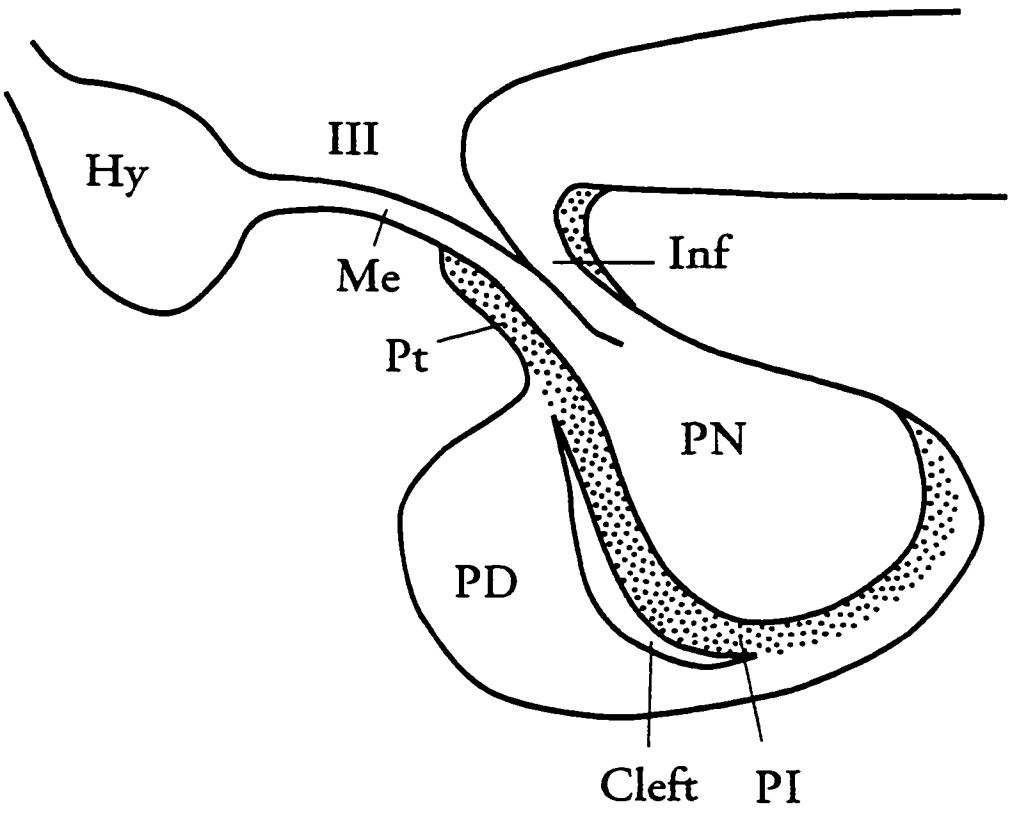


Figure 1.1. Diagram of a sagittal section through the pituitary of a typical mammal. Hypothalamus, Hy; Infundibulum, Inf: Pars distalis, PD; Pars tuberalis, PT; Pars intermedia, PI; Pars Nervosa, PN; Median eminence, Me; Third ventricle of the brain, III; Cleft, remnants of Rathke's pouch. Modified from Gorbman *et al.* (1983).

networks of fibers which support the cords of hormone secreting cells. Two classes of parenchymal cells have been described in the pars distalis: chromophobes, and chromophils. Stains such as haematoxylin, eosin, periodic acid-Schiff (PAS), and orange G are commonly used to study pituitary cytology (Bancroft and Cook, 1984). The staining properties of the chromophilic cells are dependent on the contents of the hormone-containing secretory granules in the cells and can be differentiated into acidophils, which bind acid dyes, and basophils which are stained by basic dyes. Acidophils contain simple proteins, ie., growth hormone or prolactin, and stain with eosin or orange G. Basophils contain glycoproteins, ie., gonadotrophic, corticotrophic, thyrotrophic hormone or melanocyte-stimulating hormone, which stain with PAS or haematoxylin (Bancroft and Cook, 1984). Most chromophobic cells form a supporting network for the parenchymal cells of the adenohypophysis. Some may represent degranulated chromophilic cell types.

The pars intermedia, absent in some vertebrate species (birds and some mammals) and rudimentary in man, consists of chromophobic and basophilic cells (Krause and Cutts, 1986). The pars intermedia is usually separated from the remainder of the adenohypophysis by a cleft or split which is the residual lumen of Rathke's pouch.

The pars tuberalis, when present (absent in agnathans, elasmobranchs and teleosts), is a small funnel-shaped region which surrounds the infundibulum (Gorbman *et al.*, 1983). This region consists of acidophils, basophils and chromophobes (Krause and Cutts, 1986).

The neurohypophysis is most constant in structure. Variations in the neurohypophysis among vertebrates include differences in shape, a greater degree of development of the pars nervosa in terrestrial vertebrates and the lack of a median eminence

in primitive vertebrates (lampreys and hagfishes) (Gorbman *et al.*, 1983).

In the pars nervosa, neurosecretion takes place into capillaries that lead directly to veins and then into the systemic circulation (Junqueira *et al.*, 1986). The second type of neurosecretion occurs at the median eminence which has a common blood supply with the pars distalis. Hormones originating within neurons in the hypothalamus are secreted from axons in contact with the capillaries of the median eminence, and rather than joining the general venous drainage of the brain, gather into short portal vessels that extend to the pars distalis and break up again into capillaries affecting the release of pituitary hormones within the adenohypophysis (Junqueira *et al.*, 1986).

1.4.2 Lamprey pituitary

Morphologically, the lamprey adenohypophysis is divided into three distinct regions or lobes: rostral (RPD) and caudal (CPD) pars distalis, and the posterior pars intermedia (PI). Each region of the lamprey adenohypophysis consists of solid convoluted cell cords, separated by connective tissue septa. (Figure 1.2.).

The lamprey adenohypophysis is much more differentiated than the neurohypophysis and a distinct pars intermedia is situated ventral to and in close contact with the posterior neurohypophysis separated by a thin vascularized septum, the plexus intermedia (Holmes and Bull, 1974). The pars distalis is situated ventral to the anterior neurohypophysis, separated by a layer of connective tissue. The lamprey neurohypophysis consists of a thin anterior section that is really the floor of the diencephalon of the brain. The posterior part is slightly thickened and is the terminating neurohaemal structure for neuron terminals whose cell

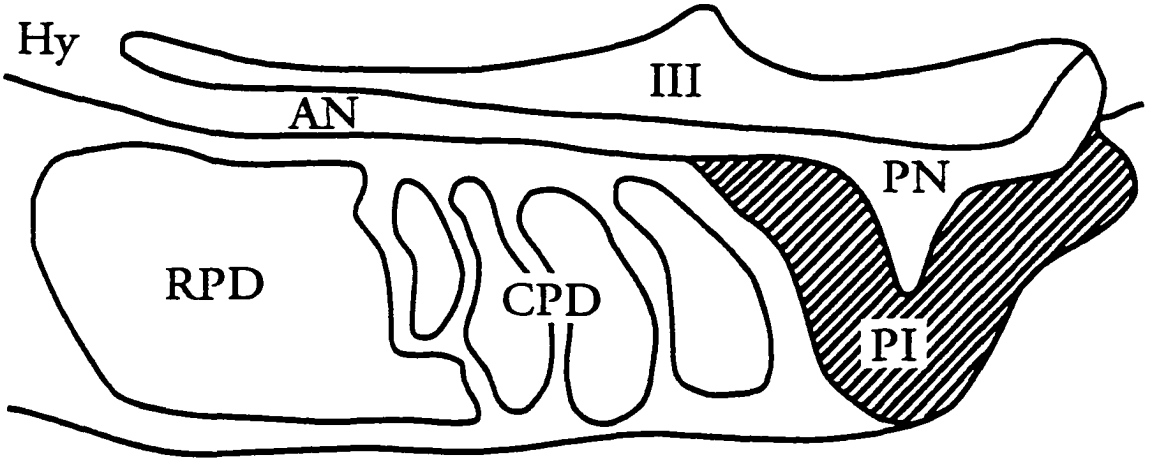


Figure 1.2. Diagram of a sagittal section through the pituitary of a lamprey. Hypothalamus, Hy; Anterior neurohypophysis, AN; Posterior neurohypophysis, PN; Rostral pars distalis, RPD; Caudal pars distalis, CPD; Pars intermedia, PI; Third ventricle of the brain, III. Modified from Dores *et al.* (1984).

bodies are in the preoptic region of the hypothalamus (Sterba, 1972). Blood drainage of the RPD and CPD is separate from that of the PI and the neurohypophysis. There is no portal system nor is there direct innervation between the hypothalamus and the PD as lampreys lack a median eminence (Bage and Fernholm 1975; Gorbman *et al.*, 1983). The only possible way for the hypothalamus to regulate secretion by the pars distalis would seem to be by diffusion of the neurosecretions (at the level of the anterior neurohypophysis) across the connective tissue barrier that separates them (Gorbman *et al.*, 1983).

1.5 Morphological changes in lamprey adenohypophysis during development

An intact adenohypophysis (AH), has been implicated for the initiation and completion of metamorphosis of the lamprey (Joss, 1985) and changes in activity of cell types in the AH may be directly related to events in the lamprey life cycle (Wright, 1989). Using partial hypophysectomy techniques, Joss (1985) found that an intact pars distalis is necessary for the complete transformation of the Southern Hemisphere lamprey *Geotria australis* (anadromous and parasitic) from larva to adult. The RPD is required for initiation of metamorphosis but without the CPD, transformation can only proceed to metamorphic stage 3 suggesting that more than one pituitary hormone is involved in the metamorphosis of lampreys. The assumption that more than one hormone is involved in the process of metamorphosis is supported by cytological and immunocytochemical studies of the larval, pretransforming, metamorphic and adult stages of the lamprey pituitary (for review see Hardisty and Baker, 1982). Cytogenesis in the lobes of the pars distalis progresses throughout development. Temporal cytodifferentiation of the rostral pars distalis differs

from that of the caudal pars distalis. These changes have been described by cell activity, and staining properties of the lobes which correlate just prior to and at the onset of metamorphosis (Joss, 1985). These morphological changes will be discussed below.

1.5.1 Larval

The nasohypophysial stalk in the sea lamprey may be a source of adenohypophysial cells throughout most of the larval stage as well as during metamorphosis (Wright, 1983). In young ammocoetes (2.5 cm - 9.0 cm in length) the adenohypophysis is enveloped by connective tissue but not separated into distinct regions and lacks fibrovascular septa that divide the gland into lobes as seen in post-metamorphic animals (Percy *et al.*, 1975). In *P. marinus*, subdivision of the adenohypophysis by connective tissue into its three lobes occurs when the ammocoete reaches an approximate length of 9.0 cm (Percy *et al.*, 1975; Hardisty, 1979) representing a larval animal 3 to 5 years old.

Cytogenesis of the pars distalis proceeds slowly throughout larval life and is thought not to be completed until metamorphosis (Joss, 1985). The adenohypophysis of the larval anadromous sea lamprey contains three granulated, secretory cell types while that of the adult contains eight or nine, reflective of differences in the function and developmental state of the gland in the ammocoete compared to the adult (Wright, 1983). Each region of the larval adenohypophysis has been characterized on the basis of ultrastructural morphology by a single granulated cell type. Eighty to ninety percent of the cells in the larval RPD are granulated cells (Wright, 1983) and are basophilic (Hardisty, 1979). Because the RPD is necessary to initiate metamorphosis, the basophilic cells, the only secretory cells, may be

involved in the initiation of lamprey metamorphosis (Wright, 1989). The granulated cells of the CPD only represent 10-20% of the cells and are morphologically (and presumably functionally) different from those of the RPD (Wright, 1983).

The PI of larval sea lamprey consists primarily of granulated cells (Wright, 1983). The presence of at least two types of secretory vesicles in addition to the electron dense granules in cells of the PI suggests that these cells may be involved in the synthesis of a number of substances (Wright, 1989).

1.5.2 Metamorphosis

The fine structure of the RPD during most of metamorphosis remains similar to that of larval sea lamprey (Wright, 1989). During metamorphosis the number of chromophobes decrease while the basophils increase in number as well as the amount of stainable material within the cell (Percy *et al.*, 1975). Through stages 1-5 of metamorphosis, the RPD is similar to that of the larval AH in that there is only one granulated and one non-granulated cell type but by stages 6 and 7 a second type of granulated cell appears (Wright, 1989). The CPD displays the most changes throughout metamorphosis. At the onset of metamorphosis the cells of the CPD appear inactive and non-granulated cells predominate. During stage 3 and by stage 4 almost all cells of the CPD appear more synthetically active and granulated (Wright, 1989).

By stage 5 most cells of the CPD have become granulated. Four granulated, secretory cell types are identified in the CPD of the newly metamorphosed animal. The ultrastructural morphology of the PI appears similar to that of the larva throughout metamorphosis (Wright,

1989).

1.5.3 Adult

Comparisons of cell types identified (on the basis of morphology) in the AH during metamorphosis with those in the AH of upstream migrant adult lamprey must be made with caution as many changes are occurring during metamorphosis that allow the lamprey to accommodate to its adult lifestyle. During the lampreys' upstream migration they become sexually mature (Wright, 1989). Changes in activity and morphology of all cells may be directly related to these events. The RPD of the adult stage is composed of two (Percy *et al.*, 1975) or three (Larsen and Rothwell, 1972) granulated cell types; the CPD consists of three granulated cell types (Larsen and Rothwell, 1972; Percy *et al.*, 1975) and the pars intermedia consists of one granulated cell type.

In sexually maturing animals (upstream migrant) and spawning animals, the dominant cell type in the RPD is the basophil whose granulation becomes more intense as the animal approaches spawning (Hardisty, 1979). In the spawning stage, the basophilic cells of the CPD become inactive and their cytoplasmic granules are depleted. Carminophils demonstrating presence of glycogen, or glycoproteins (Junqueira *et al.*, 1986) in the CPD also increase in number and in staining (Hardisty, 1979).

In the PI of the adult lamprey, a single cell type exists at different stages of secretory activity (Hardisty, 1979). The upstream migrant PI contains a range of various undifferentiated to active cell types (Larsen and Rothwell, 1972). At spawning, the cells are depleted of their secretory granules, presumably discharged into the capillary space between

the neurohypophysis and the PI (Hardisty, 1979).

1.6 Pituitary hormones of gnathostomes

Six principal hormones are produced and secreted by the cells in the adenohypophysis. Those of the pars distalis include: luteinizing hormone (LH); follicle stimulating hormone (FSH); prolactin (PRL); growth hormone (GH); thyroid-stimulating hormone (TSH); and adrenocorticotrophic hormone (ACTH) (Steger and Peluso, 1982). In species with a pars intermedia, melanocyte-stimulating hormone (MSH) is secreted (Steger and Peluso, 1982).

Growth hormone stimulates somatic and bone growth as well as carbohydrate and fat metabolism. Growth hormone increases the release of insulin and therefore amino acid uptake and protein synthesis (Gorbman *et al.*, 1983). Prolactin stimulates mammary function in mammals, has a freshwater osmoregulatory function in fishes, and modulates thyroxine action in amphibian metamorphosis; TSH stimulates thyroid cells to produce and release thyroid hormones; FSH stimulates gametogenesis; and LH stimulates steroidogenesis.

A number of peptides produced and secreted by cells of the adenohypophysis are a result of post-translational processing of a precursor hormone proopiomelanocortin (POMC). POMC is the common precursor of ACTH, lipotrophin (LPH), β-endorphin (END), and the melanotrophins (α-,β-, and γ-MSH). ACTH stimulates growth and multiplication of adrenal cortical cells as well as corticosteroid hormone synthesis and secretion; γ, α, and β-MSH all have been shown to affect adrenal (cortisol and aldosterone) secretions (Bertagna, 1994); α-MSH and β-MSH are involved in the dispersion of melanin pigment in melanocytes and

cause darkening of the skin in lower vertebrates (Gorbman *et al.*, 1983; Bertagna, 1994); LPH mobilizes fat, disperses melanin pigment, and stimulates the adrenal cortex; and END has an analgesic or opiate-like action on the central nervous system (Gorbman *et al.*, 1983).

The function of the pars tuberalis has not yet been established. Due to its anatomic association with the pituitary stalk and median eminence, it cannot be ablated without destruction of surrounding tissue to examine its possible role(s) (Steger and Peluso, 1982). Immunohistological and other histological investigations have described the presence of luteinizing hormone-containing cells (Steger and Peluso, 1982).

Neurohypophysial hormones include the vasotocins and oxytocin. Arginine vasotocin, found in all vertebrate classes and replaced by vasopressins in adult mammals, regulates water balance by acting as an antidiuretic and regulates blood pressure by direct vasopressor activity (Gorbman *et al.*, 1983). Only mammals produce oxytocin, which is involved in uterine muscle contraction and ejection of milk from the mammary glands (Gorbman *et al.*, 1983).

1.7 Pituitary hormones of lampreys

Assays using extracts from *P. marinus* pituitary on various species including rats, and bullfrogs, were found to exhibit neurohypophysial arginine vasotocin activity (Sawyer *et al.*, 1961; Sawyer, 1965). Following the administration of arginine vasotocin, free fatty acid concentrations in *P. marinus* are raised, suggesting stimulation of lipid mobilization, and the notion that this hormone may serve to protect the tissues from depletion of carbohydrate reserves (Hardisty and Baker, 1982). Arginine vasotocin was subsequently, and until

recently the only pituitary hormone isolated and characterized from adult sea lampreys (Lane *et al.*, 1988). The difficulty in isolating and identifying lamprey pituitary hormones probably stems from the difficult methods used to obtain large quantities of hormones from the small pituitaries (0.7 mg wet weight in adult *P. marinus*) (Lane *et al.*, 1988). Suzuki *et al.* (1995) have sequenced the vasotocin cDNA from adult lamprey, *Lampetra japonica*, and reported it to be a 1179 base pair sequence encoding the vasotocin precursor of 157 amino acid residues, consisting of a 22 amino acid signal peptide and a 135 amino acid vasotocin domain, and a long 3' non-coding region of 655 bases.

A homodimeric glycoprotein, nasohypophysial factor (NHF), has been isolated from the pituitaries of adult *P. marinus* by SDS-PAGE and characterized by deglycosylation and reduction properties (Sower *et al.*, 1995). The protein consisted of two similar subunits suggestive of a gonadotrophin. However, NHF has no immunoreactive or sequence similarity to any other pituitary hormone. Antisera generated against NHF have demonstrated immunoreactivity localized in the RPD, PI and olfactory system of developing larval lampreys and in the majority of cells of the RPD, a few cells of the CPD, and blood of upstream migrant adult lampreys (Sower *et al.*, 1995). The monomer of NHF is a 121 amino acid residue sequence with no resemblance to any other sequenced pituitary hormone and although the function of this glycoprotein is not known, its presence in the developing olfactory and adenohypophysial tissues, as well as the blood of adult lampreys suggests an important function (Sower *et al.*, 1995).

Recently, two cDNAs encoding POMC-like hormones have been sequenced from the pituitary cDNA libraries of upstream, pre-spawning adult sea lamprey *P. marinus* (Heinig

et al., 1995, Takahashi *et al.*, 1995a).

1.8 Molecular structure of POMC

1.8.1 Gnathostome

Proopiomelanocortin (POMC) mRNA was first isolated from bovine pars intermedia and characterized as approximately 1200 bases long, encoding a peptide with a molecular weight of 31 kDa (Nakanishi *et al.*, 1979). Only one POMC gene exists in gnathostomes. The gene consists of three exons and two introns. The large 3' (and third) exon contains the nucleotides coding for all the biologically active peptides and the majority of the N-terminal precursor (Figure 1.3.). POMC is a member of the opioid family and a precursor molecule of corticotrophin (ACTH), met-enkephalin derived from lipotrophin (LPH), melanotrophin (α-MSH, β-MSH, and γ-MSH) and endorphin (END) (Naito *et al.*, 1984; Ma *et al.*, 1994) (Figure 1.3.).

The POMC sequence is highly conserved among all examined species expressing this prohormone. The 'spacer' sequences separating the coding region domains are poorly conserved. The sequence of POMC mRNA has been determined in several species of mammals (Douglass *et al.*, 1984), amphibians (Martens *et al.*, 1985; Hilario *et al.*, 1990) and teleosts (Soma *et al.*, 1984). A signal sequence of 26 amino acids necessary for translocation of the precursor through the endoplasmic reticulum is present at the N-terminus. In all jawed vertebrates, the POMC molecule is similar in regard to the organization of the different domains of the precursor and the amino acid sequence; the ACTH sequence in the middle, and β-MSH and β-endorphin are at the C-terminus (Douglass *et al.*, 1984; Soma *et al.*,

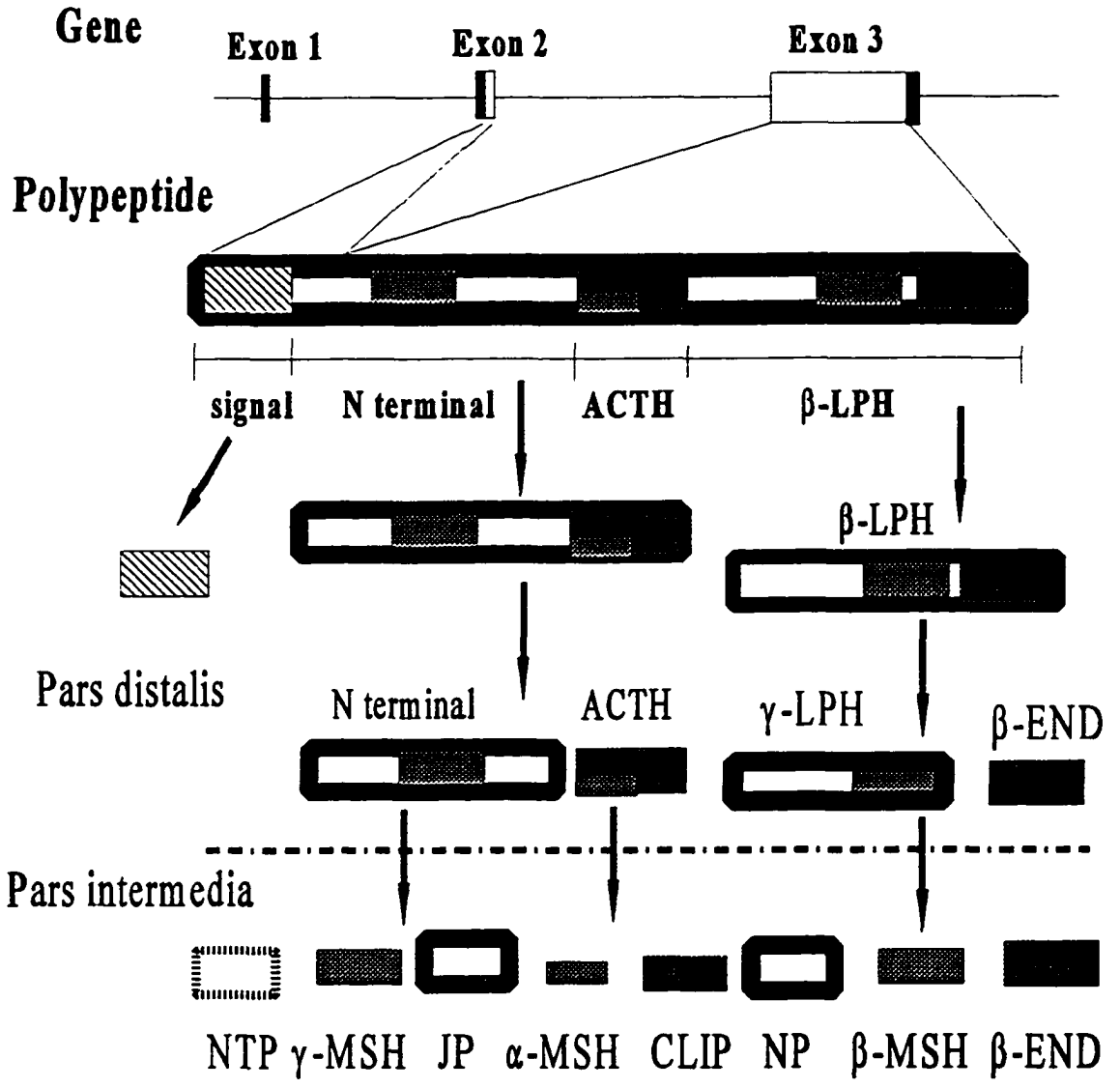


Figure 1.3. Structure of the human gene coding for POMC and its polypeptide structure. The boxed regions contain the coding regions for the precursor polypeptide. Tissue-specific processing in the two lobes of the pituitary, the pars distalis and the pars intermedia is illustrated. Adrenocorticotrophin, ACTH; Beta-lipotrophin, β-LPH; Gamma-lipotrophin, γ-LPH; Beta-endorphin, β-END; N terminal peptide, NTP; Corticotrophin-like intermediate lobe peptide, CLIP; Gamma-melanocyte stimulating hormone, γ-MSH; Joining peptide, JP; Alpha-melanocyte stimulating hormone, α-MSH; N-terminal peptide of β-LPH, NP; Beta-melanocyte stimulating hormone, β-MSH. Modified from Hanneman *et al.* (1989).

1984; Martens *et al.*, 1985; Hilario *et al.*, 1990).

1.8.2 Lamprey

In lamprey a POMC isoform consisting of a 986 bp cDNA sequence, encodes a 278 amino acid open reading frame of NHF, an MSH sequence, ACTH and β-END but not to a functional β or γ-MSH domain and is designated as proopiocortin, POC (Figure 1.4.). The MSH domain of POC at the N-terminal part of ACTH is not chemically identical and therefore not assigned as α-MSH (Takahashi *et al.*, 1995b).

A second POMC isoform consisting of a 2045 bp cDNA with a 245 amino acid open reading frame encoding MSH-A, MSH-B and a different β-END but no ACTH has been designated proopiomelanotropin, POM (Figure 1.5.). The MSH-A and MSH-B are not similar to α-MSH or any other vertebrate β or γ-MSH and are therefore not designated as such (Takahashi *et al.*, 1995a).

These results implicate the molecular evolution and functional divergence of the POMC gene suggesting that lamprey hormones ACTH and MSH are produced by proteolytic cleavage of tissue specific prohormones from 2 genes. This is significantly different from other vertebrates where the hormones are produced by tissue specific proteolytic cleavage of a single prohormone expressed from a single gene (Takahashi *et al.*, 1995a).

1.9 Distribution of POMC

It is widely accepted that a variety of mammalian pituitaries express POMC in the corticotrophs of the pars distalis and in melanotrophs of the pars intermedia (Nozaki and

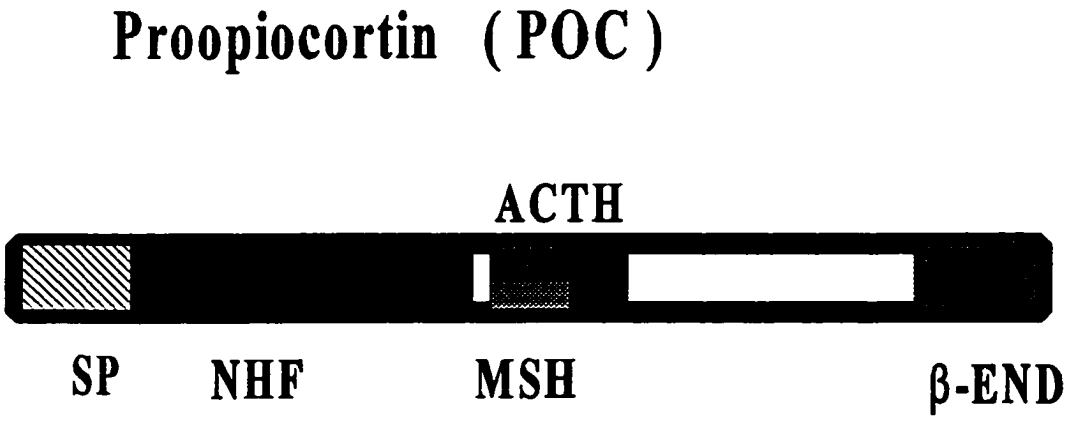


Figure 1.4. Proopiocortin structure deduced from cDNA sequence (Heinig *et al.*, 1995). Boxed regions represent polypeptide domains of the precursor molecule. Signal peptide, SP; Nasohypophysial factor, NHF; Adrenocorticotrophin hormone, ACTH; Melanocyte stimulating hormone, MSH; Beta-endorphin; β-END. Modified from Takahashi *et al.*, (1995a).

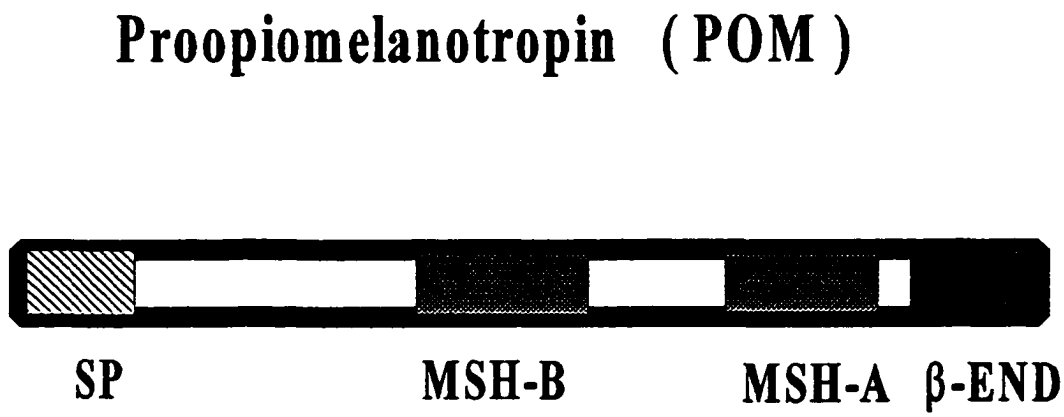


Figure 1.5. Proopiomelanotropin structure deduced from cDNA sequence (Takahashi *et al.*, 1995a). Boxed regions depict polypeptide domains. Signal peptide, SP; Melanocyte stimulating hormone A and B, MSH-A, MSH-B; Beta-endorphin, β-END. Modified from Takahashi *et al.* (1995a).

Gorbman, 1984; Naito *et al.*, 1984; Dores and McDonald, 1992). POMC expression occurs early in the developing fetal pig and its pattern of expression differs in the lobes of the pituitary and regions of the brain during development (Ma *et al.*, 1994). Expression of fetal pig POMC mRNA was localized by *in situ* hybridization (ISH) first in the corticotrophs of the pars distalis, then in the melanotrophs of the pars intermedia and last in the extra-pituitary brain tissues, including the anterior hypothalamic area, the arcuate nucleus, the nucleus medialis thalami, fasciulus tegmenti and entorhinal cortex (Ma *et al.*, 1994).

There is also evidence that POMC is not only produced by pituitary and brain cells but is also expressed in a number of other tissues and is specifically cleaved depending on the cell and tissue type (Bhardwaj and Luger, 1994). Northern blot analysis has revealed that POMC mRNA is present in human epidermal keratinocytes as well as in melanocytes (Bhardwaj and Luger, 1994). The full POMC fragment is expressed in the bovine hypothalamus and pituitary; a smaller fragment is found in the hypothalamus, adrenal medulla, thyroid, thymus, duodenum and lung. The smaller mRNA of the adrenal however lacks the 5' end and therefore may not be translated in this tissue (Hanneman *et al.*, 1989). POMC mRNA is also found in the ovaries of frogs (Nabissi *et al.*, 1995).

1.10 Distribution of POMC end products in gnathostomes

1.10.1 Gnathostomes

Different peptides are derived from POMC in different tissues via processes which occur in a well defined sequence as the peptides move through the cellular organelles. The precursor is transported across the endoplasmic reticulum via its hydrophobic N-terminal

signal sequence which is removed prior to its complete translation. POMC is then processed through the Golgi apparatus and ultimately its end products are stored in secretory granules before being secreted by exocytosis (Bertagna, 1994). Peptides derived from POMC undergo processing including cleavage from the precursor hormone at specific sites. Modifications include: glycosylation, phosphorylation, acetylation, amidation, sulphation and methylation (Hanneman *et al.*, 1989; Bertagna, 1994).

The domains of all polypeptide precursors are flanked by pairs of basic amino acid residues (pairs of lysine and/or arginine). POMC contains eight sequences of two, and one sequence of four basic amino acids which are potential cleavage sites for processing enzymes. The nature of the POMC end-products depends on the cleavage specificity of the tissue (Bertagna, 1994). Trypsin-like and carboxypeptidase-like enzymes are involved in the cleavage reactions to produce the bioactive peptides (Hanneman *et al.*, 1989). These proteolytic enzymes have recently been identified as a superfamily of enzymes called prohormone convertases (PCs), PC1 and PC2 (Bertagna, 1994).

In corticotrophs, only PC1 is present, and its proteolytic action is limited such that only four cleavage sites (Lys-Arg sites only) are used for processing and ACTH is a major end product (Figure 1.3.). Other peptides generated are N-terminal fragment (NT), joining peptide (JP), β-LPH, small amounts of γ-LPH and β-END (Bertagna, 1994). Melanotrophs contain both PC1 and PC2, and their synergistic action leads to a more extensive proteolysis generating the smaller fragments: γ-MSH, ACTH is cleaved to α-MSH and corticotrophin-like intermediate lobe peptide (CLIP), and β-LPH is cleaved to β-MSH, and β-END (Bertagna, 1994).

The largest peptides derived from POMC include the N-terminal peptide, ACTH, and β-lipotrophin (LPH). The N-terminal peptide is processed to yield γ-MSH and ACTH is processed further in some tissues to yield α-MSH and CLIP (Hanneman *et al.*, 1989) (Figure 1.3.). α-MSH corresponding to the N-terminal 13 residues of ACTH is highly conserved among many species (Hadley, 1988). β-LPH gives rise to γ-LPH and β-MSH and β-endorphin. β-endorphin contains the sequence for met-enkephalin but is not known to be cleaved from β-endorphin *in vivo* (Eipper and Mains, 1980).

Again, major POMC end products in corticotrophs of the PD are ACTH, β-lipotrophin and β-endorphin, while α-MSH and β-endorphin are the major end products in melanotrophs in the pars intermedia (Douglass *et al.*, 1984; Naito *et al.*, 1984; Soma *et al.*, 1984; Martens *et al.*, 1985; Hilario *et al.*, 1990). The major site of synthesis of POMC-derived peptides are in the adenohypophysis, however they have also been found in the human pancreas, gastrointestinal tract, placenta, thyroid, mast cells and the reproductive tract of male rat. POMC-derived peptides are also found in human peripheral leukocytes (Hanneman *et al.*, 1989).

While it is clear that ACTH and MSH are expressed or located in the pars distalis and pars intermedia respectively in higher vertebrates, very little is known of the presence, pattern of expression and role of proopiomelanocortin-related peptides in the pituitary and brain of lower vertebrates. Frog brain extracts have shown α-MSH-like, β-endorphin-like, and ACTH-like reactivity (Vaudry *et al.*, 1975; Jégou *et al.*, 1983). Proopiomelanocortin related peptides, such as α-MSH, corticotrophin-like intermediate lobe peptide (CLIP), β-MSH, and endorphin have also been isolated from the pituitary corticotrophs and

melanotrophs of the chum salmon, *Oncorhynchus keta* (Dubois *et al.*, 1979; Naito *et al.*, 1984).

1.10.2 Lamprey

Proopiomelanocortin-like related peptides have been detected in the adult lamprey pituitary (Dores *et al.*, 1984; Dores and McDonald, 1992; Nozaki *et al.*, 1995). Using specific antisera generated against synthetic mammalian POMC-related peptides, ACTH-like immunoreactivity has been identified in the RPD of sexually mature brook lamprey, *Lampetra lamotteni* (Dores *et al.*, 1984). α-MSH-like immunoreactivity was localized in the pars intermedia, and met-enkephalin-like immunoreactivity in the anterior neurohypophysis, RPD and PI (Dores *et al.*, 1984). Neither β-LPH nor β-END immunoreactivity was observed countering the notion that ACTH and β-LPH are derived from the same precursor (Dores *et al.*, 1984). Screening *P. marinus* PI by RIA with synthetic leu-enkephalin antisera revealed the presence of met- but not leu-enkephalin suggesting that this opioid may be derived from another precursor (Dores and MacDonald, 1992) as in gnathostomes both of these peptides are derived from the β-LPH region of POMC.

More recent work on POMC-derived peptide distribution involved the use of antisera generated against synthetic lamprey ACTH, MSH-A and MSH-B (Nozaki *et al.*, 1995) from the recently identified and deduced sequences of POC and POM. Using these antisera, ACTH and MSH-A immunoreactivity was found in most cells of the RPD, and a few scattered cells of the CPD of upstream migrant *P. marinus* (Nozaki *et al.*, 1995). It must be considered that preabsorption of anti-MSH-A with MSH-B abolished MSH-A

immunoreactivity in the PD, indicating that MSH positive material in the PD has a lower antigenic specificity than that in the PI. ACTH, MSH-A and MSH-B share amino acid core sequence similarities and antisera cross reactivity may be due to shared protein structures (Nozaki *et al.*, 1995). MSH-A and MSH-B immunoreactivy was also found in all cells of the PI (Nozaki *et al.*, 1995).

1.11.1 Function of POMC-derived peptides in lampreys

1.11.2.1 ACTH

Other than arginine vasotocin, no other lamprey pituitary hormone has been isolated and therefore no direct bioassays to elucidate their role(s) have been possible. However, lamprey pituitary extracts containing fractions of ACTH (based on *in vitro* adrenal ascorbic acid depletion assay), have been reported to cause moulting in toads (Larsen and Rothwell. 1972). *L. planeri* treated with mammalian ACTH exhibit interrenal hyperplasia and cellular changes similar to those observed in other vertebrate adrenocortical cells after ACTH treatment (Hardisty and Baker, 1982). However, administration of ACTH to *P. marinus* failed to show any consistent pattern of plasma concentrations of various corticosteroids (Hardisty and Baker, 1982). Both cortisol and mammalian ACTH have been found to raise blood sugar levels in the lamprey *L. fluviatilis* (Hardisty and Baker, 1982). Eastman and Portanova (1982) bioassayed steroid production using rat adrenal cells incubated with larval and adult lamprey *Lampetra aepyptera* brain and pituitary extracts (synthetic ACTH as standard) and reported ACTH bioactivity measured as steroid production by the adrenal cells. Also, bioassays of *L. fluviatilis* pars distalis extracts create cytochemical changes (alteration

of the reducing potential) in the zona reticularis of guinea pig adrenal tissue similar to that of standard ACTH (Baker and Buckingham, 1983) thus suggesting corticotrophin related activity in pars distalis extracts. More recently, a bioassay of the steroidogenic activity of lamprey ACTH was demonstrated by its ability to stimulate the conversion of radiolabelled 11-deoxycortisol to 11-deoxycorticosterone and deoxycorticosterone when incubated with spawning lamprey interrenal tissue (Takahashi *et al.*, 1995b).

1.11.2.2 MSH

The role of MSH in dispersion of melanin granules in the melanophores of amphibia is well established (Smith-Gill and Carver, 1981). Pituitary extracts from the lamprey *L. fluviatilis* cause melanin dispersion when tested on frog and demonstrated MSH activity in a lizard (*Anolis spp.*) skin bioassay (Baker and Buckingham, 1983). Also, pallor and concentration of melanin granules caused by partial and total hypophysectomy of the lamprey suggests a MSH is secreted by the pituitary (Larsen and Rothwell, 1972). However, attempts to demonstrate definitive similarities between gnathostome α-MSH and lamprey melanotrophic factor have revealed dissimilarities in their immunoreactivity and molecular charge (electrophoretic R_f values), suggesting that lamprey melanotrophin is not homologous to gnathostome α-MSH (Baker and Buckingham, 1983). The melanin-dispersing activities (assessed visually) of synthetic peptides MSH-A and MSH-B by *in vitro* frog skin assay revealed that MSH-B was 10 and 100 times more potent than salmon α-MSH and MSH-A respectively (Takahashi *et al.*, 1995b).

1.12 Research objectives

Pituitary hormones are centrally involved in the control of developmental processes and sexual maturation in vertebrates. However there has been very little information on the role played by pituitary hormones in larval lamprey development, initiation and completion of metamorphosis and sexual maturation. Now that two new pituitary prohormones have been identified in the lamprey, it will be important to understand their role in development. Preliminary Northern blot analysis of total RNA from larval, metamorphic and adult lamprey pituitaries suggests that the expression of both POC and POM are developmentally regulated. *In situ* hybridization studies of POM and POC mRNA expression during development will provide precise and more informative localization of POM and POC in the pituitary and brain of the developing lamprey particularly during metamorphosis. Ultimately correlation of temporal and quantitative variations in the expression of lamprey pituitary hormones with entry into and progression through metamorphosis will indicate those hormones likely involved.

The objectives of this research are:

1) To determine the temporal and spatial distribution of POM and POC mRNA in the pituitary and brain of *P. marinus* in larval, metamorphic and adult stages.

2) To quantitate the relative amounts of POM and POC mRNA in the pituitary and brain of *P. marinus* during development.

2. MATERIALS AND METHODS

2.1 Collection and maintenance of specimens

Sea lampreys, *Petromyzon marinus*, at different stages of their life cycle, were collected from various sources and maintained in the Fish Health Unit, Atlantic Veterinary College. Non-condition factor (NCF) <1.5, and condition factor (CF) ≥1.5, ammocoetes were collected in June from streams in the Canadian Lake Ontario watershed and tributaries of the Saint John River, New Brunswick and Putnam Creek, New York. Young (juvenile) parasitic adults were collected in late November from a fish farm off the River Philip, Nova Scotia, and prespawning adults were collected in late May - early June at a fish ladder operated by the Department of Fisheries and Oceans Canada on the LaHave River, in New Germany, Nova Scotia. Juvenile parasitic adults (130 to 179 mm in length and 5.2 to 8.4 g in weight) were sampled while feeding on Atlantic salmon, *Salmo salar*. Young larvae, raised and maintained in the laboratory, were collected at sixty and seventy days post fertilization. All animals were maintained on a 12:12 light-dark photoperiod.

Larval and metamorphic animals were maintained at ambient water temperatures at 17-18°C. Juvenile parasitic adults were maintained at water temperatures between 10 and 11 °C. Prespawners were acclimated to and kept at 18°C. Young larvae were raised and maintained in water kept at 18°C. Rainbow trout, *Oncorhynchus mykiss*, approximately 20 centimetres in length, supplied by the Cardigan Salmonid Enhancement Centre in Cardigan, PE, and Atlantic hagfish, *Myxine glutinosa*, supplied by the Huntsman Marine Science Centre, St. Andrews, NB, were used for negative control tissues.

Larvae with CF≥1.5 were considered potential candidates to undergo metamorphosis

and were checked regularly for external signs of metamorphosis from late June through November. The sizes of CF larvae sampled ranged between 145 and 165mm and weights ranged between 4.7 to 7.5 g. Identification of the metamorphic stages was determined using external morphological characterizations described by Youson and Potter (1979). The sizes of NCF larvae sampled ranged between 114 and 153mm with weights ranging between 1.7 and 3.9 g. The metamorphic animals ranged between 125 and 145 mm in length and weights ranging between 3.0 and 4.9 g. Sexually mature lamprey were identified using external morphological criteria described by Piavis (1961). The length of upstream migrant and sexually mature lamprey ranged between 620 and 900 mm. Animals of this size weigh between 260 to 500g.

The brain and pituitary from young larvae (60 and 70 days post fertilization), ammocoetes, (NCF and CF), metamorphic (stages 1, 3 through 7), young parasitic adults, prespawning (upstream migrant), sexually mature lampreys, rainbow trout and hagfishes were processed for *in situ* hybridization. Three to five animals were sampled for each stage in the life cycle. Stage 2 metamorphic animals were not available for this study.

2.2 Tissue fixation and processing

Animals were anaesthetized in 0.05% tricaine methanesulfonate (MS-222) and decapitated. The entire cranial region of the larval, metamorphic and young adults and only the brain and pituitary of the large prespawning and spawning lampreys, Atlantic hagfishes and rainbow trout were fixed in 4% paraformaldehyde (PFA) in 1 X phosphate buffered saline (PBS), pH 7.2, for 45 minutes at room temperature then dehydrated in graded ethanol

series, cleared in xylene and embedded in paraffin. Young larvae were fixed *in toto*, in formalin and processed as above. All solutions used were prepared or treated with 0.1% diethylpyrocarbonate (DEPC) water which is a strong RNase inhibitor. Serial longitudinal or transverse sections, six micrometers thick, were mounted on precleaned glass slides subbed for 2 minutes in 2% 3-aminopropyltriethoxysilane (APES) (Sigma Chemical Company, St. Louis, Missouri, United States) in acetone, dried overnight at 42 °C and stored at -20 °C with desiccant until use. Some longitudinal sections of ammocoetes, parasitic, and spawners pituitaries were processed for routine staining with haematoxylin and eosin.

2.3 Preparation of RNA probes.

2.3.1 Proopiocortin (POC)

The Pst I fragment of the POC cDNA sequence (Heinig *et al.*, 1995) (Fig. 2.1), 115 base pairs (bp) long, was subcloned into the Pst I site of pGEM-4Z vector (Promega, Madison, Wisconsin, United States). The 115 bp fragment which was used to synthesize sense and antisense radiolabelled riboprobes encodes a portion of the NHF domain unique to POC mRNA. For transcription of the antisense or sense probes the pGEM-4Z vector was restricted and linearized by overnight digestion with HincII and HindIII restriction enzymes (New England Biolabs, Mississauga, Ontario), respectively.

BASE COUNT 234 a 267 c 334 g 190 t

1 ctgcaacgca aagcaacact [gaaaaagggg aaaagtgctg ctggaatgat gggaaactgc
---**Signal peptide**----------
61 tctcgactgc tgcttctgct ggagatgctg tcaatcatct ccccgtctgc cagtgccatg
----------**NHF**---
121 tgctgggcac ggctggacca ggggtgcttc accgactgca agaaatac * tg cagcaatggg
----
181 acacgggcag gcacgccggc ggcggtgctg gagaatctgc tggcatgcgt gcagctcaaa
----
241 tgcagcgacg acggtgatga caacgacgac gacgctcccc tgc * tgcagtg gatcgcaagc
----
301 agagccgaat cccgcagcga tttcgacatc gccaacaaca agtggtggct cgtccgctgg
----
361 ggtggacaga gtggcctgag tggcgagggt ggcgagagtg gtggaagtcc gagggtggag
----
421 caggtggatt tggcggggca ggtggagtcc tccccggcga gtagttccag ccaggccaag
----------**ACTH-**
481 cgttccgtgt cctcccccaa gtacgccatg gggcatttcc gctggggcag ccccgataag
----
541 gccaccatcc gcaagcgcag accggtgcga cccaacacgt ccgacagccc cgagatccca
----
601 gactacgcct tcaatggggt ggaaggcccg gcagacgacg cgggcgactc cgtgttcatg
----
661 agccgcaggg agacgccgga cgcggccggg caccgtggag tggacgaggc ggcggcgacg
----
721 ggggaagatg ccgaggttgg aaataaagac ggggtcttcc gcgtgcctcc gccattcaaa

781 cgctacggtg gcttcatgaa agtgatgcaa gagattgacc attggccact ggtgccagta
---**β-END**----------
841 atccgcaagg tcatgcacaa ggagagcaca aagtcgctct gagtgcctcg cgtgttgagg
----
901 caacgcagtg ctttgtttaa gtcggttaat gaaacgtcga tttgatttgc atgcattgat

961 gtcaggatgc atttgtaggt gtcgtttagt taaattcaaa aata] aaaaaa tctattaatc

1021 gcttc

FIGURE 2.1 Sea lamprey cDNA sequence of **proopiocortin** mRNA. Square brackets "[ ]" depict POC cDNA sequence (Heinig *et al.*, 1995). ACCESSION number D55628. Asterisks "*" depict the two Pst I sites for generation of the 115 base pair insert.

2.3.2 Proopiomelanotropin (POM)

A 361 bp cDNA fragment was subcloned into pGEM-4Z vector using the AvaI and SphI restriction sites in the POM sequence (2045 bp) (Takahashi *et al.*, 1995a), and the respective restriction enzymes (New England Biolabs) (Fig. 2.2). The 361 bp fragment encodes the entire MSH-B domain unique to the POM cDNA sequence as well as some noncoding spacer sequence.

The POM insert was digested overnight with EcoRI or HindIII (New England Biolabs) to linearize the template for transcription of the antisense and sense riboprobes, respectively. There is a 58.2% identity in a 134 nucleotide overlap in the C terminal region of POC and POM cDNA sequences and no identity at all between the POC (115bp) and POM (361 bp) sequences being used as probes for expression of POC and POM.

Run-off transcripts from the appropriate templates were made using the Riboprobe Gemini System II (Promega, Madison, Wisconsin). The *in vitro* RNA transcripts for sense and antisense probes were synthesized in a reaction containing 40mM Tris-HCl, pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl, 20 units RNasin® ribonuclease inhibitor (Promega), 100 mM dithiothreitol (DTT), 2.5 mM each of ATP, GTP, UTP, 1.0 μg linearized template, 200 μCi at 40mCi/ml [^{35}S]CTP (Amersham International, Arlington Heights, IL, USA) and 20 units of T7 RNA polymerase (Promega) (POM antisense, POC sense) or 20 units of SP6 RNA polymerase (Promega) (POM sense, POC antisense). The reaction mixture was incubated for 30 minutes at 37 °C. Following this process another 20 units of polymerase were added followed by another 30 minute incubation at 37°C.

BASE COUNT 504 a 575 c 515 g 451 t

```
   1 acccgccttt gctcacaaga cgacgacgcc tcaccgctcc tcacctcaag aggctcacaa

  61 tggccactac gagcagtgct ccaacccgga gctcctcatt gccgtgccgt gtcgctctcc
     ------Signal peptide----------
 121 tactgtcggg cctcatcgcc ctcctgggtc ccgcggcatc caggagtgct cctctcgtct
     ------------------------------------------------------------
 181 gcctacaggc gtgcgagagc tgtctggaac ccgcccagcc tgagccgctc tgctggatgc

 241 agtgcc * tcgg ggagtgcagt cgccttgcgg caccatctgc tgacggctcc gagattgtgc

 301 tccttggagg aggaggagga gatgaggcgc cagagggagg agaggtctcc gccgacaagc

 361 gggtccagga gagcgccgac ggctaccgga tgcaacactt ccgctgggga cagcctctgc
         MSH-B----------
 421 ccggcaagaa gcggcagccg gagcagagcc agggggtccc tcttggcatg gggagcgacg
     ----
 481 aaaacgcacg ggtggtgaac ggagggcagg cttgggacga agggtggacc ctcgacgacc

 541 aggcaaacga ggtgaacgcg cggcagtggt cggcggcacc gtccaagaag gactcgacgc

 601 ctctga * gcat gcagaaggag aacccggagc tctaccagat gaaccacttt cggtgggggc
                                MSH-A----------
 661 agccgccgac gcacttcaag cagaagcgct acggcggctt catgcgcaag tcgccgggct
     ------------------------  β-END----------
 721 acgcgcacct gaagccgctg gtcacgttct tccgggacgt catgaagaac gacagcccga
     ------------------------------------------------------------------------
 781 caatgctcaa taattaggcg gcagaccccc cccccaccga gcgacggagc gaaggggatc
     --------------------
 841 catgcgcggg cgggaggttg ttgccacagt tgccgtggct gtttttgttt ttggggaggt
 901 aatgctgtaa cagctccggt gccttatagt ttctggagaa aaagtggccc gtgaaatttc
 961 acggtgagag tgccccccca cccttcccct gcaaatccca ccccaaaacc tcccgtttct
1021 cggcacttat gactgggatt gctcatcatc atacaacact attccccgcc gtcaatttct
1081 ataactccat aagaatataa tggtgaaaat tctagattta aagctttcgt gactgctagg
1141 atccatactc tttggttcta ttgggtaaag tcacgtaagt gaaataacgt gaccaaagag
1201 tataatggtg aaagttgtat ggtttatttt atttttcgcc aaggtcaaaa aaggccatag
1261 ggtgtgcaaa gctcatcggt agtacaacag ggccctggtt ctttcccgtc agatcgagcc
1321 ccatatttga ctgcacaatg aaaaccagtc caagtcagcg ctgtgactga gactgactca
1381 cagtttcgaa ggaatcgtct aaataactga aatggattaa aaaaacacac ctacagtgtg
1441 gttggtggct aggtttttta cagatacaac caaagagtgg acagaatggc ctcgtgttta
1501 ccaccaccgt tattaccaaa cactgcctta cacgactgaa aactattgga acactctgag
1561 caatcgccct cattcacgaa cggtcatagt gtttttaata ggttcattat agctattcac
1621 aaaaaacgaa gtcttcaacc ccaccacctt aaataaaagt cacagaaagc acggcatgat
1681 ttgcgtgtca agctgaaaca ttcgtgtgca atggtgtgct ggaatttgaa tttggtaaac
1741 gcccgtgatt tttcttcacc agcaccaatg cggacaagac agcattgcct gcgcaataat
1801 gaccaccacc tctctgtcaa ctgatcctct ctctttaaca tcacaccctc acccgtatgt
1861 atcaacgttg tactcccaaa cacgctctgg catcacgcat aatgaccaat aacaaactcc
1921 cgatgcgcat ttaactcctc cgcgtgcgtt tgtccttcag tccgctagtt atgacactct
1981 ctcgttgtcg acacgccacg tgcctattta tgtgctcact tgaaataaac acatgtaacc
2041 cactt
```

FIGURE 2.2 Sea lamprey cDNA sequence of **proopiomelanotropin** mRNA. ACCESSION number D55629. From Takahashi *et al.*, (1995a).
Asterisks "*" depict Ava I and Sph I restriction sites used to generate the 361 base pair insert.

2.4 Probe purification

The DNA template was removed following a 15 minute DNase digestion reaction with 15 units of RNase-free DNase 1 at 37 °C. Removal of unincorporated nucleotides and precipitation of radiolabelled RNA probe were performed by phenol-chloroform-isoamyl extraction and precipitation with ammonium acetate (7.5M) and cold absolute ethanol. The RNA pellet was briefly washed with 70% ethanol and resuspended in 5µl of 0.2M DTT. The resuspended probe was either used immediately or stored at -70° C for up to 3 days before use.

Probe was applied to tissue in the presence of cold competitor which was made by transcribing pGEM-4Z vector without an insert, using either the SP6 or T7 polymerase in the presence of nonradioactive nucleotides, one being S-UTP (Mandel Scientific, Lachine, Quebec, Canada). Amounts between 50-100 ng of cold competitor were added to 100-200 ng of radioactive probe, boiled for 3 minutes and immediately suspended in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 1mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.05% yeast tRNA, 0.05% polyadenylic acid, 50 mM DTT, 10% dextran sulfate) to a final specific activity of 3-4 X 10^4 cpm/µl.

2.5 Pre-hybridization treatments

Tissue sections were brought to room temperature and warmed overnight at 42 °C before use to firmly attach the sections to the subbed slides. The sections were deparaffinized in three 3-minute changes of xylene and rehydrated through graded ethanols

and equilibrated in 1 X phosphate buffered saline (PBS) for 5 minutes. Polar and charged groups, and non-specific binding sites were blocked by incubating in freshly prepared 0.1M triethanolamine-Cl (TEA), pH 8.0 for two minutes, followed by two 5 minute washes in 0.25% acetic anhydride in TEA buffer.

2.6 Hybridization

Ten microliters of radiolabelled probe, diluted to 3-4 X 10^4 cpm/µl with hybridization buffer in the presence of cold competitor, were added to each pretreated tissue section. Slides were allowed to incubate for 17-18 hours at 45 °C in a moist chamber containing a solution of 50% formamide, 0.3M NaCl, 10 mM Tris-HCl, and 1 mM EDTA.

2.7 Post-hybridization washes

Non-specific and mismatched probes were removed by a series of stringent washes. Three wash solutions were used in this process: solution A (50% formamide, 2X SSC, 20 mM ß-mercaptoethanol), solution B (50% formamide, 2X SSC, 20 mM ß-mercaptoethanol, 0.5% Triton X-100), solution C (2X SSC, 20 mM ß-mercaptoethanol). Two sets of 15 minute washes in each of solution A and B are done at 50 °C , (90% and 94% stringency (see Appendix A for formula) for POM and POC respectively (Leitch *et al.* 1994)) followed by two, two minute washes in solution C at room temperature. Tissue sections were then rinsed in RNase buffer (10 mM Tris-Cl, pH 7.5, 5mM EDTA, 0.3M NaCl) for 30 seconds at 37 °C. Following this process RNase I A, from bovine pancreas, (10 mg/ml) (Pharmacia Biotech, Dorval, Quebec, Canada) was added to the buffer to a final concentration of 20 µg/ml and

incubated for 30 minutes at 37 °C followed by a final rinse in only RNase buffer for 30 minutes at 37 °C. Sections were washed in a second series of stringent washes in two 30 minute washes in solution C and A at 45 °C, (86% and 89% stringency (see Appendix A for formula) for POM and POC respectively (Leitch *et al.*, 1994)) followed by two 5 minute washes in 2X SSC at room temperature. Sections were dehydrated and air dried for approximately 12 hours before autoradiography.

2.8 Autoradiography

Slides were dipped in full strength Kodak NTB-2 emulsion (Eastman Kodak Company, Rochester, NY) at 42 °C under safelight conditions and air dried in the dark. Slides were sealed in light tight boxes, and stored at 4 °C for an exposure time of 12 hours before being developed. Slides were developed at room temperature in Kodak D-19 (diluted 1:1 in distilled water) for 4 minutes, washed in distilled water for 10 seconds and fixed in Kodak Rapid Fix for 5 minutes and washed in two 5 minute distilled water washes. Air dried slides were examined under the light microscope. Some slides were counterstained with Harris's haematoxylin. Photographs of hybridized, and counterstained sections were generated using a Zeiss, D-7082, transmitted-light photomicroscope (Zeiss, West Germany).

2.9 Negative Controls

Sense, vector sequence, and non-radioactive riboprobes were transcribed from the same templates used to make the anti-sense probes and hybridized to lamprey brain and pituitary sections. Atlantic hagfish, and rainbow trout pituitary and brain sections were also

hybridized with both POM and POC radiolabelled riboprobes.

2.10 Quantification of expression

The number of slides prepared, each with four to six sections, consisting of the entire pituitary of each animal varied with the stage of development, that is the size of the pituitary. In general, the number of slides generated consisting of the entire pituitary were: 8 (+/-2) for ammocoetes (CF and NCF); 9 (+/-3) for metamorphic animals stages 1, 3 and 4; 14(+/-2) for metamorphic animals stages 5, 6 and 7 and juvenile parasitic adult animals; and 42 (+/-10) for prespawning and spawning animals. Each *in situ* hybridization trial consisted of 15 to 18 systematically sampled slides. At least three slides per animal per stage, one from each lateral and one from the medial aspect of the pituitary (with four to six sections each), were selected for each trial. Therefore, a minimum of three and maximum of five stages were used in each trial to compare relative amounts of expression throughout the given stages of development. One trial deviated from this in that it consisted of two slides (one medial, one lateral) per animal and compared nine different stages of development. Alternate sections on each slide were incubated (probed) with either the POM or POC probe.

To measure the amount of hybridization of probe to pituitary sections, all sections were examined using identical illumination parameters on an Olympus BH-2 light microscope. Projected images were captured using a Panasonic WV-BP500 camera and digitized into a 512 X 512 matrix using the Scanning Electron Microscope Image Collection and Processing System (SEMICAPS) computerized microscopic image analysis system. The digitized pituitary images were then exported to ImagePC © (Alpha 9 release, Scion

Corporation) (NIH-Image software). The appropriate pituitary region of interest was selected from the image and a standardized procedure was used to distinguish background staining from radiolabelled cells in all sections. The density of hybridization in the selected sections of pituitary was encoded as one of 256 shades of gray ranging from black to white. The ImagePC [c] software enabled the operator to determine threshold value by visual inspection. Pixels with grey values above the threshold were labelled as black and those below the threshold labelled as white by the computer. The number of black labelled pixels were designated as hybridized clusters and automatically measured by the computer as an area in millimetres squared, and calibration was done by the operator. The original total tissue area selected was then measured. All sections hybridized for each animal in each trial (4-6 sections) were measured in this way. Sections where the background levels were too high or tissue morphology was poor were omitted from analysis.

Measurements were exported to a statistical software package and an area fraction (area occupied by signal (black) divided by total area of selection) was calculated. A oneway analysis of variance, with multiple comparisons was calculated for each trial measured. Mean values of area fractions in individual trials were plotted and results were examined for repeating patterns of expression between trials. Data were analyzed using Minitab release 10.1 software.

Volumetric measurements were also done to consider any changes in the total volume of cells expressing either POM or POC as the pituitary developed and increased in size. Cavalieri's method (mean-value theorem) was used to calculate an estimated volume of the PI, RPD and CPD throughout development (Gundersen *et al.*, 1988; Cruz-Orive, 1990).

Area fraction values, obtained by computer assisted quantification, were then used to calculated an estimate of the volume of the lobes that contained signal, that is the volume of cells expressing either POM or POC. Analysis of covariance and regression analysis was performed on the volumetric data for every trial, to determine any temporal patterns of expression throughout development (Minitab release 10.1).

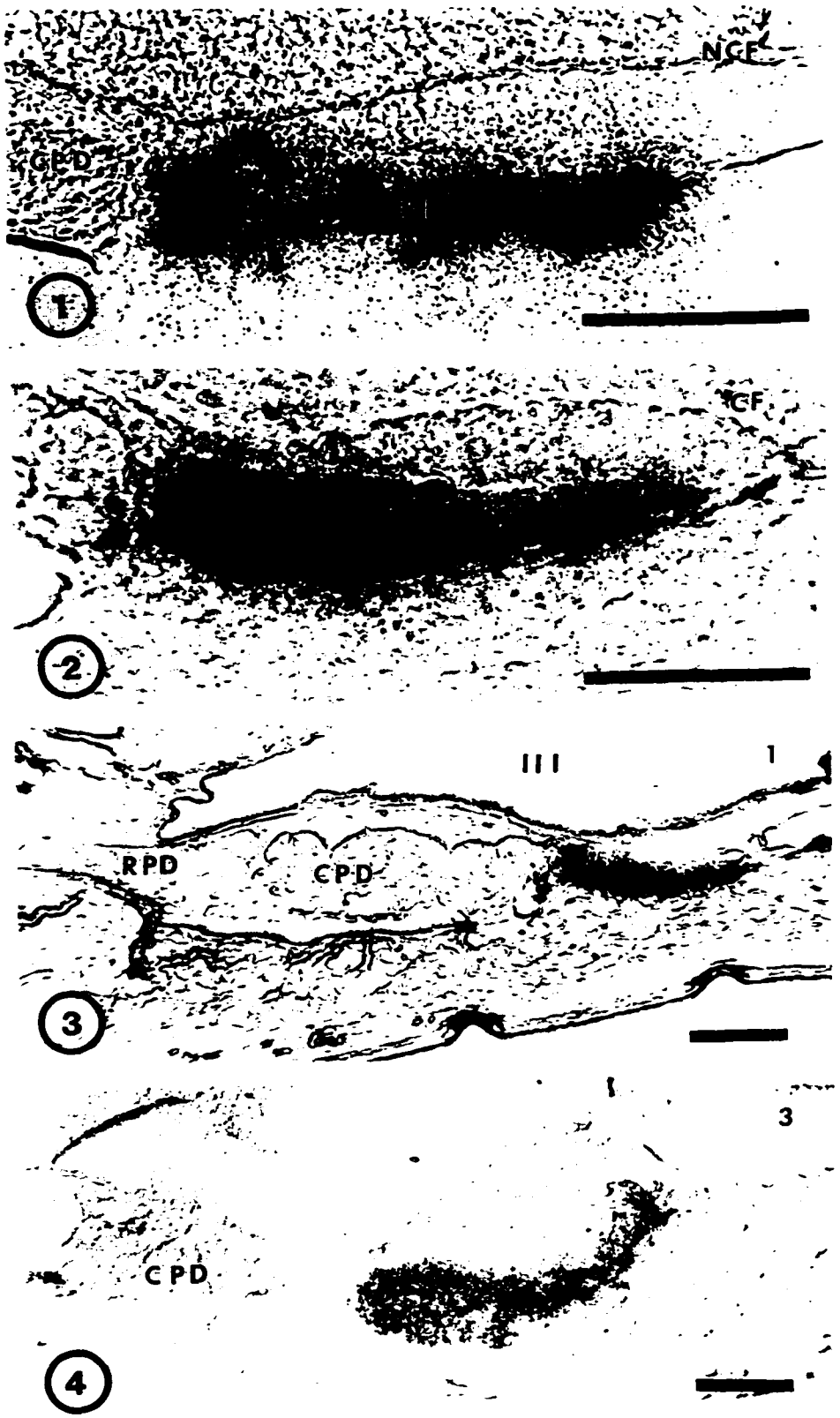
3. RESULTS

3.1 Proopiomelanotropin (POM)

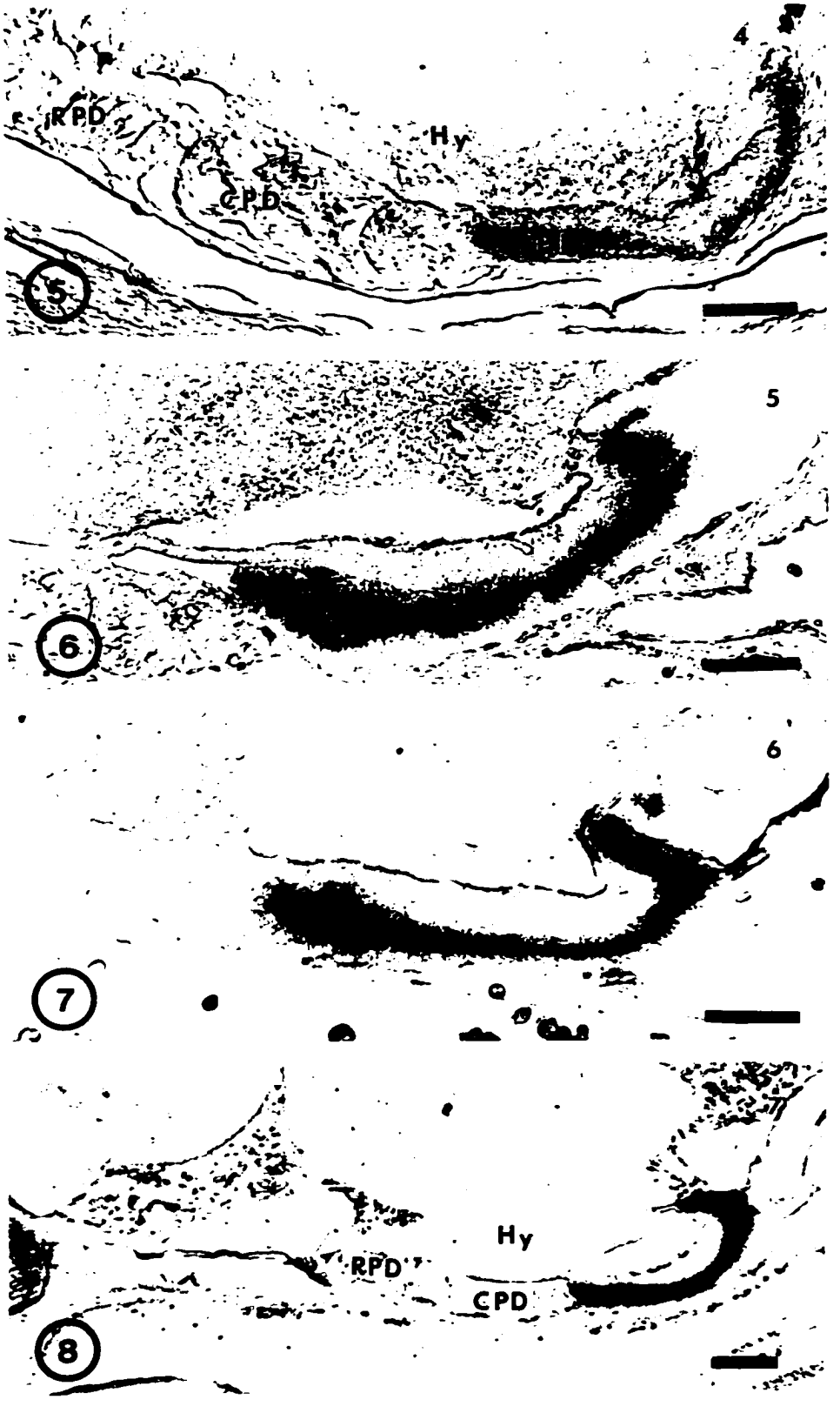
3.1.1 Qualitative observations

The expression of proopiomelanotropin (POM) was examined in brain and pituitary sections of young larvae 60 day post-fertilization (n=2) and 70 day post-fertilization (n=2), NCF larvae (n=7), CF larvae (n=4), metamorphic stages: 1 (n=2), 3, 4, 5, 6, 7 (n=4 per stage), juvenile parasitic adult (n=6), prespawners (n=4, 2 male and 2 female) and spawners (n=3, 1 male and 2 female). Proopiomelanotropin is expressed in most cells of the pars intermedia (PI) during all stages of the lamprey life cycle (Figs. 3.1 to 3.11) except 60 and 70 day post-fertilization young larvae (not shown). Expression of POM could be visualized in NCF larvae and the signal appeared in all cells of the PI (Fig. 3.1.). There was no specific hybridization of the POM probe to any other cells within the brain or pituitary. Analysis of POM expression patterns were made on sagittal sections. In addition, transverse sections of spawning lamprey PI were also examined to exclude possible spatial differences throughout the lobe of the pars intermedia (Fig. 3.11). The POM hybridization signal was distributed in most cells throughout the medial-lateral aspect of the PI. Although it appeared that all cells were hybridized, it was not possible to conclude that every cell expresses POM. Some signal may be due to scatter of radioactivity emitted on emulsion film creating signal not directly overlying bound probe. For example, in Figs. 3.1 and 3.2, scattered signal can be seen immediately (approximately 15 μm) about the perimeter of the PI. Specific POM hybridization could not be visualized beyond that of background levels in young larvae (n=2,

Figures 3.1 to 3.4. Proopiomelanotropin (POM) expression in sagittal sections of the pars intermedia (PI) during development. Caudal pars distalis, CPD; Rostral pars distalis, RPD; Pars intermedia, PI; Third ventricle of the brain, III.
Bar = 100 μm.
Figure 3.1. Noncondition factor (NCF) larva; **Figure 3.2.** Condition factor (CF) larva; **Figure 3.3.** Metamorphic stage 1 (1); **Figure 3.4.** Metamorphic stage 3 (3).

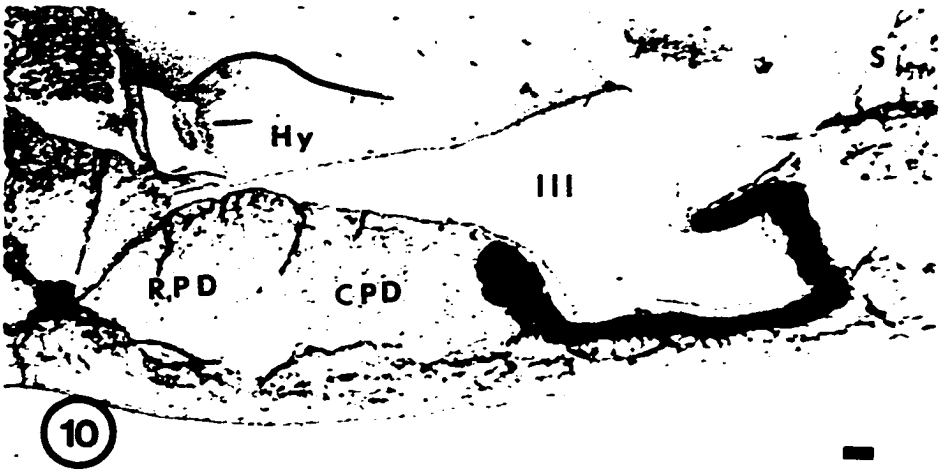

NCF
CPD
1
CF
2
III
1
RPD
CPD
3
3
CPD
4

Figures 3.5 to 3.8. Proopiomelanotropin (POM) expression in sagittal sections of the pars intermedia (PI) during development. Caudal pars distalis, CPD; Rostral pars distalis, RPD; Pars intermedia, PI; Hypopthalamus, Hy. ' * ' denotes non-specific hybridization. Bar = 100 μm.
Figure 3.5. Metamorphic stage 4 (4); **Figure 3.6.** Metamorphic stage 5 (5);
Figure 3.7. Metamorphic stage 6 (6); **Figure 3.8.** Metamorphic stage 7 (7).


4
RPD
Hy
CPD
5
5
6
6
7
Hy
RPD
CPD
8

Figures 3.9 to 3.10. Proopiomelanotropin (POM) expression in sagittal sections of the pars intermedia (PI) during development. **Figure 3.11.** POM expression in a transverse section of the PI. Caudal pars distalis, CPD; Rostral pars distalis, RPD; Pars intermedia, PI; Hypopthalamus, Hy; Third ventricle of the brain, III.
Bar = 100 μm.
Figure 3.9. Parasitic stage (P); **Figure 3.10.** Spawning animal (S).
Figure 3.11. Spawning animal (S).


P
III
9


S
Hy
III
RPD
CPD
10


III
S

60 and 70 day old) (results not shown).

By observation it was evident that the levels of expression between stages varied. In general, a consistent pattern of expression was observed in every trial. POM appears to be expressed at very high levels in both NCF and CF larvae as illustrated by the strong signal intensity during these stages (Figs. 3.1 and 3.2). Expression is also high in stage 1, but signal intensity does not appear as strong as that of the larval stages (Fig. 3.3). By metamorphic stages 3 and 4, expression appears substantially decreased (Figs. 3.4, 3.5). Expression appears to be up regulated throughout the remainder of metamorphosis and is apparent by the greater signal intensities seen in these stages. It becomes difficult to visualize any differences in signal intensities in late metamorphosis (stages 5 through 7) (Figs. 3.6, 3.7, 3.8). It was more difficult to visualize differences, either higher or lower, of POM expression between adjacent metamorphic stages. For example, stage 3 and 4 (Figs. 3.4, 3.5), and stage 6 and 7 (Figs. 3.7, 3.8) appeared to be quite similar. Expression during the juvenile parasitic adult stage (Fig. 3.9) appears greater than that in most of the metamorphic animals, at least the early and mid stages, 1 through 4. The signal intensities of larval, late metamorphic and adult animals appear similarly high but the signal intensity of POM expression seems to be greatest during the spawning stages (Figs. 3.10, 3.11).

3.1.2 Quantification of POM expression

To corroborate the qualitative observations, computer assisted image analysis was performed. The density of signal was calculated as an area fraction (AF) of area occupied by hybridization signal (expression) over a defined total region of pituitary lobe (entire pars

intermedia). Nine different trials were examined by one way analysis of variance with Fisher's pairwise comparisons (individual error rate 0.05), each comparing multiple (at least 3) stages, with multiple (at least 4) hybridized sections per stage. Mean AF values generated by comparing the levels of POM expression between different stages revealed statistical significance ($p < 0.05$) for each trial (Table 3.1).

Although the RNA transcription and the *in situ* hybridization protocols were optimized, not all variables could be precisely controlled in every trial. Therefore, the levels of expression between trials could not be grouped together. Probe concentrations were not uniform between all trials due to experimental limitations and deviations, including the exact amount of DNA template, the amount of *in vitro* RNA transcription and radioisotope incorporation, and precise probe dilution. Another experimental variable was the limited availability of animals (due to seasonal dependent metamorphosis and time of stage collection), and limited amount of probe per trial. Due to these limiting factors, it was not possible to examine every stage of development in any individual trial. Therefore, the mean values of relative expression (AF) for each trial were plotted as histograms and used to interpret a general pattern of expression throughout development (Fig. 3.12 A-I). A histogram of all the data from all nine trials is illustrated in figure 3.13.

Statistical analysis of the area fraction data supports the qualitative patterns of expression described above. Trial 6 best illustrates the signal intensities (levels of expression) of 9 of the 11 stages examined in this study (Fig. 3.12.F). Although this histogram represents only one trial, it demonstrates the general pattern of POM expression which is repeatedly illustrated in all other trials and represented in figure 3.13 for all data.

Table 3.1. Mean area fraction values of POM expression in the PI.

	Stage of development											
Trial	NCF	CF	1	3	4	5	6	7	P	PS	S	p-value
1	0.6561				0.6107	0.7804			0.0872			0.0370
2	0.5432	0.7967			0.4663				0.6846			0.0030
3		0.7952			0.4825				0.6520	0.8910		0.0000
4	0.6291				0.3230	0.3981				0.8310		0.0000
5	0.5490		0.3347		0.3282	0.4423				0.7597	0.7653	0.0000
6	0.5430	0.7771	0.5373	0.4462	0.4908	0.6232		0.6394	0.5701	0.8090		0.0010
7				0.3516	0.3951	0.4939		0.6771		0.7897		0.0000
8				0.4639		0.4597	0.6322	0.6257	0.7559			0.0010
9					0.6876	0.7073	0.7618	0.8091	0.8319		0.8623	0.0000

Figure 3.12. Histograms of each of the nine trials representing mean area fraction values of the signal density of POM expression in the PI throughout development. Values were generated by computer assisted quantification and one-way ANOVA. Noncondition factor (NCF) and condition factor larvae (CF); metamorphic stages 1, 3 to 7; juvenile parasitic adult (P); prespawners (PS) and spawners (S).

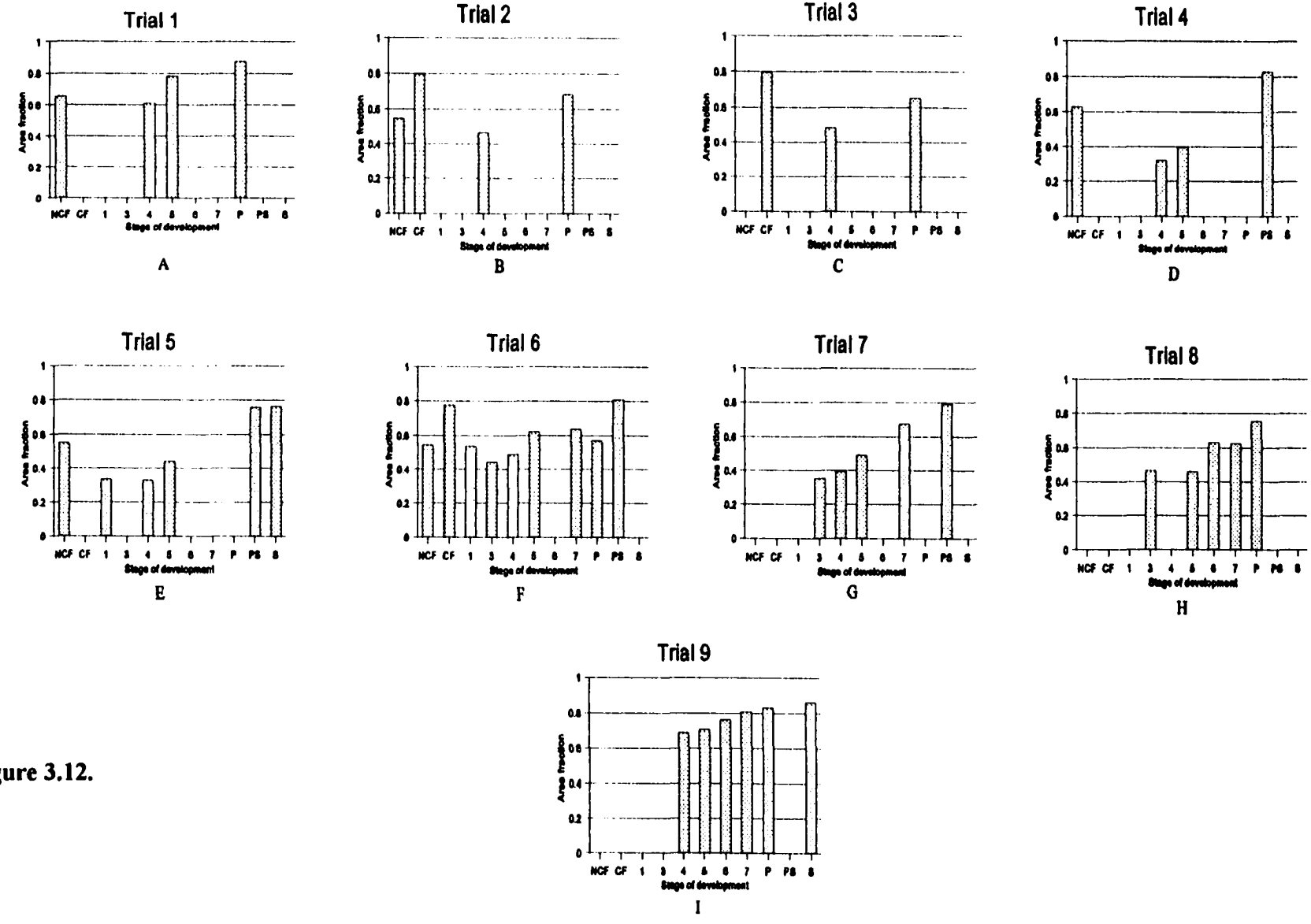

Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Trial 7
Trial 8
Trial 9
Area fraction
Stage of development
NCF CF 1 3 4 5 6 7 P PS S
A
B
C
D
E
F
G
H
I

Figure 3.12.

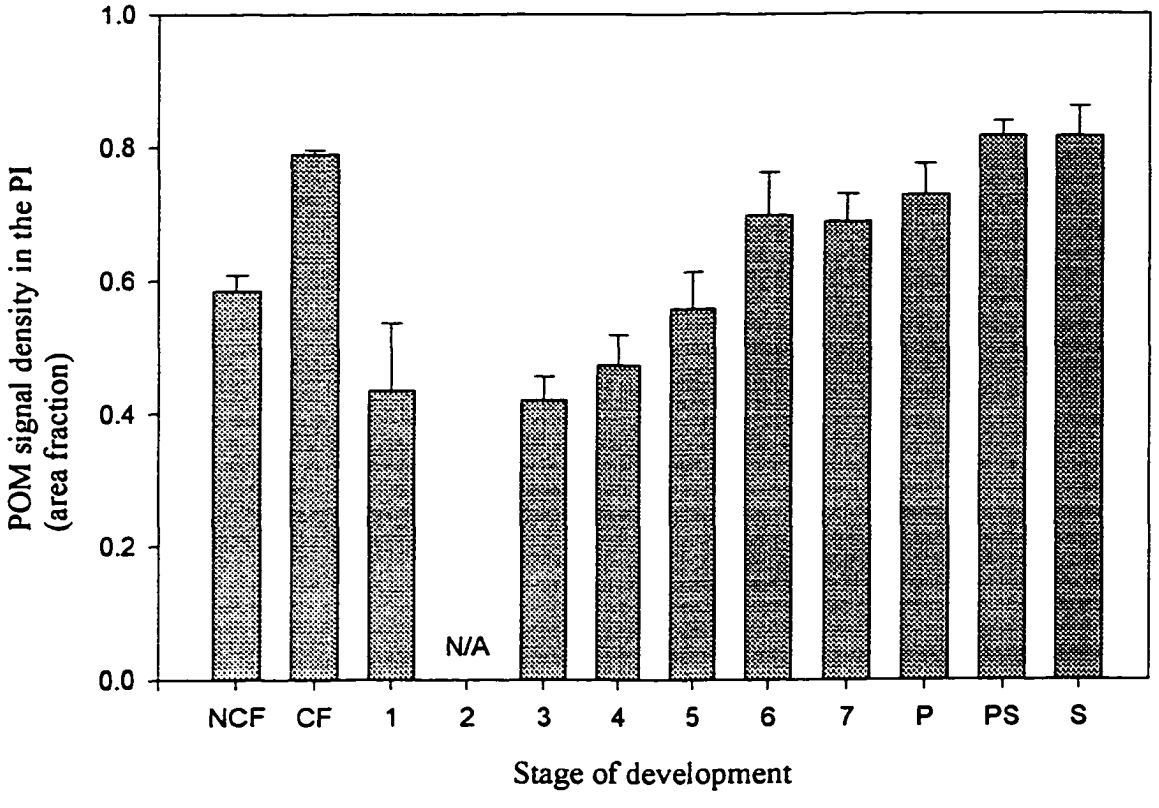


Figure 3.13. Histogram of the mean values from all nine trials of the signal density of POM expression in the PI during development. Bars represent S.E. of mean values. Noncondtion factor (NCF) n=7 and condition factor larvae (CF) n=4; metamorphic stages1 n=2, 3 to 7 n=4; juvenile parasitic adult (P) n=6; prespawner (PS) n=4 and spawners (S) n=3. Stage 2 metamorphic animals were not available for this study.

The signal density of POM expression is high in both NCF and CF larvae. The density decreases somewhat by stage 1, and more so by stage 3. Although stage 2 could not be examined (unavailability of animals), the pattern would predict that the density of signal would be lower than that at stage 1 but equal to or higher than stage 3. Signal increases gradually throughout the remainder of metamorphosis, and the highest density is seen during the spawning stages. It is interesting to note that the levels of signal density during the CF stage is as comparably high as that of parasitic and spawning stages (Fig. 3.13).

Fisher's pairwise comparisons of area fraction data was employed to reveal which, if any, of the stages expressed at significantly different levels, as determined by density of signal, relative to each other. Expression of POM in CF animals is significantly greater than all other stages of development except that of spawning animals, where the levels of expression are comparable. Although the levels of expression appeared to be similar in NCF and CF animals, quantitative analysis revealed that expression in CF animals is actually significantly greater. Therefore an increase in signal density occurs between NCF and CF animals. Analysis of stages 1 and 3 revealed that the signal density of POM expression is at its lowest levels (not significantly greater than any other stage) during these stages, as illustrated by the histograms. All stages but stage 1, were significantly greater than stage 3, again describing the similarity of expression between these two stages and that stage 3 is possibly the stage of lowest levels of POM expression. The density of POM expression during the larval stages (NCF and CF) and stage 6 and older were significantly greater than stages 4 and 5. There was no significant difference in density of expression between stages 6 and 7 as well as between stage 7 and juvenile parasitic adults. The density of expression

of spawning animals was significantly greater than all stages except CF larvae, indicating that density of signal between these two stages is similar.

Area fraction data revealed that signal densities varied throughout development. That is, in general, the density of signal was very high in larval animals, decreased in early metamorphic animals, gradually increased throughout development and reached very high levels in the adult stages. Area fraction data considers the density of signal in a given area and not the changes in volume of the pituitary as it develops.

Volumetric data was generated to reveal the relative net volume of cells expressing POM in an estimated total volume of the pars intermedia in a given animal and stage. The total number of serial sections incorporating the PI was calculated for every animal used in this study. Area measurements required to calculate area fraction values and the volume estimation mean-value theorem (Cruz-Orive, 1990) were used to calculate the total volumes of the PI and the volume of cells expressing POM in the PI during development. For volumetric analyses, up stream migrant pre-spawner and sexually mature spawning data were grouped together and classified as 'spawners'. Negative volume values generated for larval animals from the regression analysis of volume data of all stages (larval through to spawners) are a result of the large volumes of spawning animal pituitaries which are much greater than those of all other stages in this study. The spawning stage represents the oldest and most mature stage which can be sampled. No animals could be sampled between the period when the lamprey completes metamorphosis (juvenile parasitic adult) and the period of the spawning migration. After metamorphosis, lampreys migrate to the sea and are difficult to sample and assign approximate age. The amount of time between these two stages may be

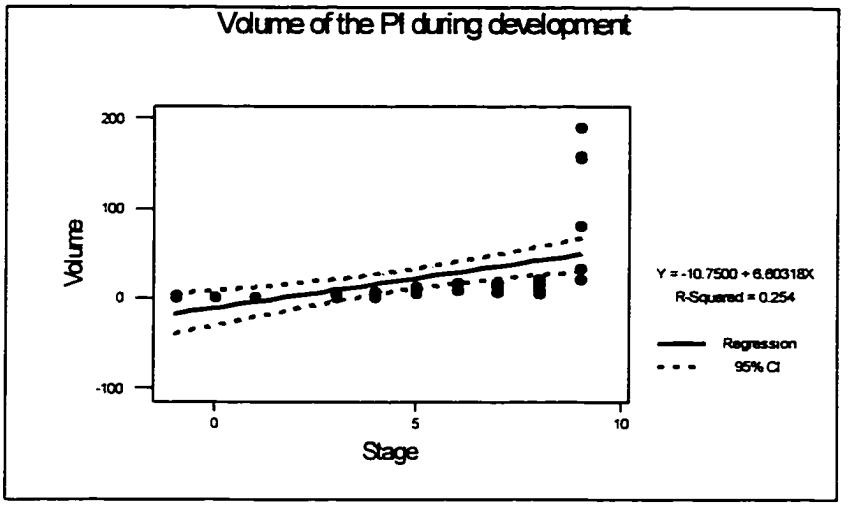
a long one, two or more years, allowing for greater development and increase in size as the lamprey feeds. The large disparity between juvenile parasitic adults and spawners stems from the tremendous amount of growth which occurs over an extended period of time between these two stages, as opposed to the relatively similar sizes between all other stages in the life cycle which may only be a few months apart. Therefore, the volume data for spawning animals is an outlier in the data set.

The outlier data creates a left shift in the linear regression plots assigning negative volume data to NCF, CF and stage 1 animals. The linear regression plots of total PI volume and volume of POM expression (Fig. 3.14.C) also illustrate that the volume of expression in NCF and CF is greater than the PI itself which is not real. To determine the effect of the outlier spawning data that data was removed and the remainder re-analysed. Therefore two separated data sets were analysed, one including the volume data for spawning animals (Fig. 3.14) and one without (Fig. 3.15).

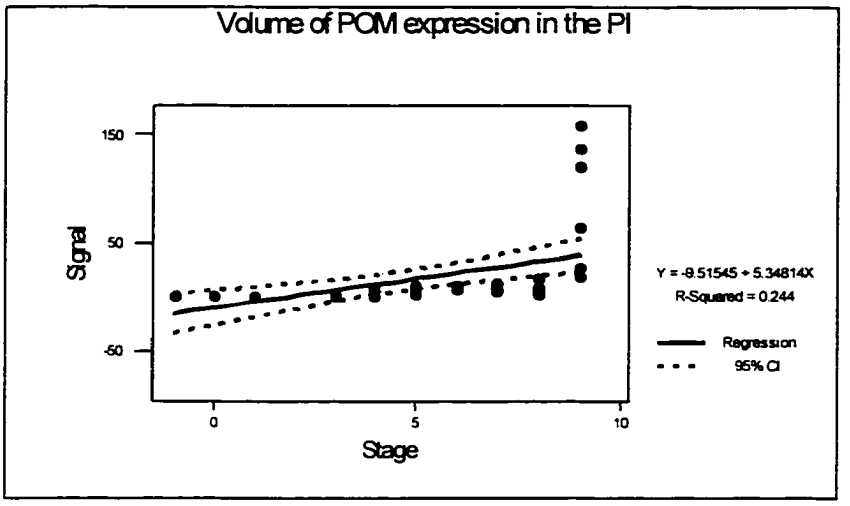
The linear regression of volume of POM expression in the PI not including the spawning data, revealed greater correlation coefficients than the data set including the outlier data (compare $r=0.713$ and $r=0.494$) ($p<0.001$) (Fig. 3.15). The slopes of the lines were also much less in the data set not including spawner data (compare Fig. 3.15 B, where $m=0.98$ to Fig. 3.14 B where $m=5.35$) indicating a more subtle change in volumes of expression during development (larval to parasitic stage). The greater slope in Fig. 3.14 B was biased by the large outlier spawner data.

The fitted regression lines not including spawner data (Fig. 3.15), indicate that as the pituitary develops and increases in size, the volume of cells expressing POM also increases.

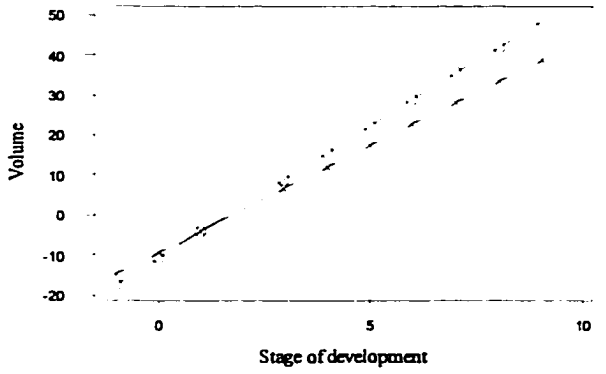
A



B



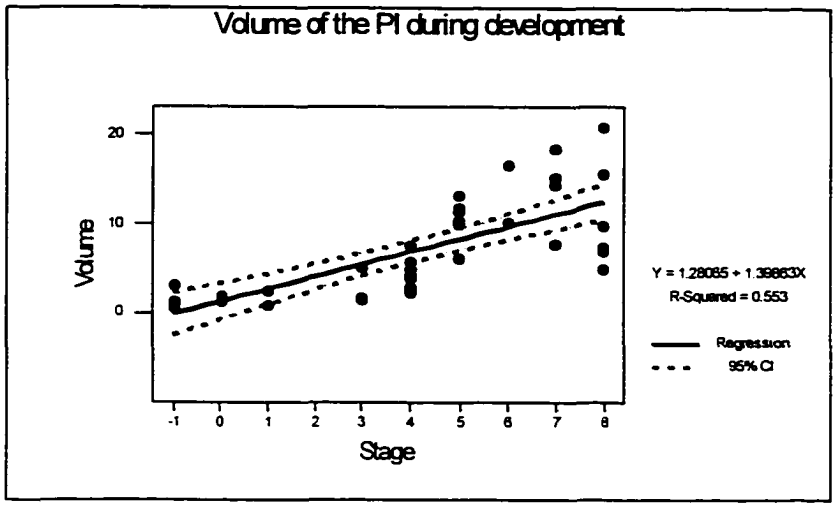
Volume of POM expression in the PI during development
Fitted regression plots.



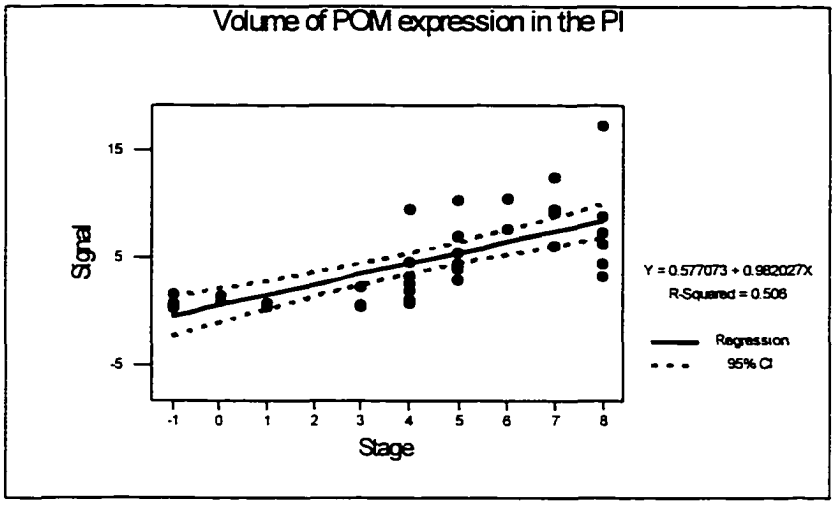
C

Figure 3.14. **A**. Scatter plot of total volume (μm^3) of the PI. **B.** Scatter plot of volume of signal (cells expressing POM) during development. Dashed line represents the 95% confidence interval of the linear regression (solid line) of volume data. **C**. Fitted regression plots of figure A (line with "o") and B (line with "x"). Stages -1, 0: NCF and CF; 1 to 7: metamorphic stages; 8 and 9: parasitic and spawning stages.

A



B



C

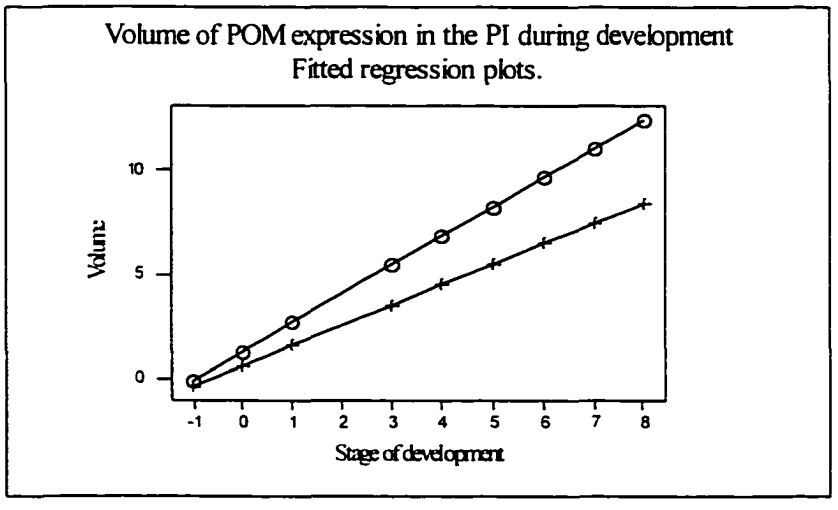


Figure 3.15. **A.** Scatter plot of total volume (μm^3) of the PI. **B.** Scatter plot of volume of signal (cells expressing POM) during development not including the spawning stage. Dashed line represents the 95% confidence interval of the linear regression (solid line) of volume data. **C.** Fitted regression plots of figure A (line with "o") and B (line with "+"). Stages -1, 0: NCF and CF; 1 to 7: metamorphic stages; 8: parasitic stage.

The difference between the total volume of the PI and the volume of cells expressing POM diverge throughout development. This is indicative of the presence of other cells not hybridized.

The only deviation between area fraction data (Fig. 3.13) and volume data (Fig. 3.15) is the great signal density seen in the larval stages (NCF and the peak at CF animals) followed by the decrease in expression in the early metamorphic animals. It appears that although signal densities vary, the net volume of cells and therefore total expression of POM generally increases throughout development.

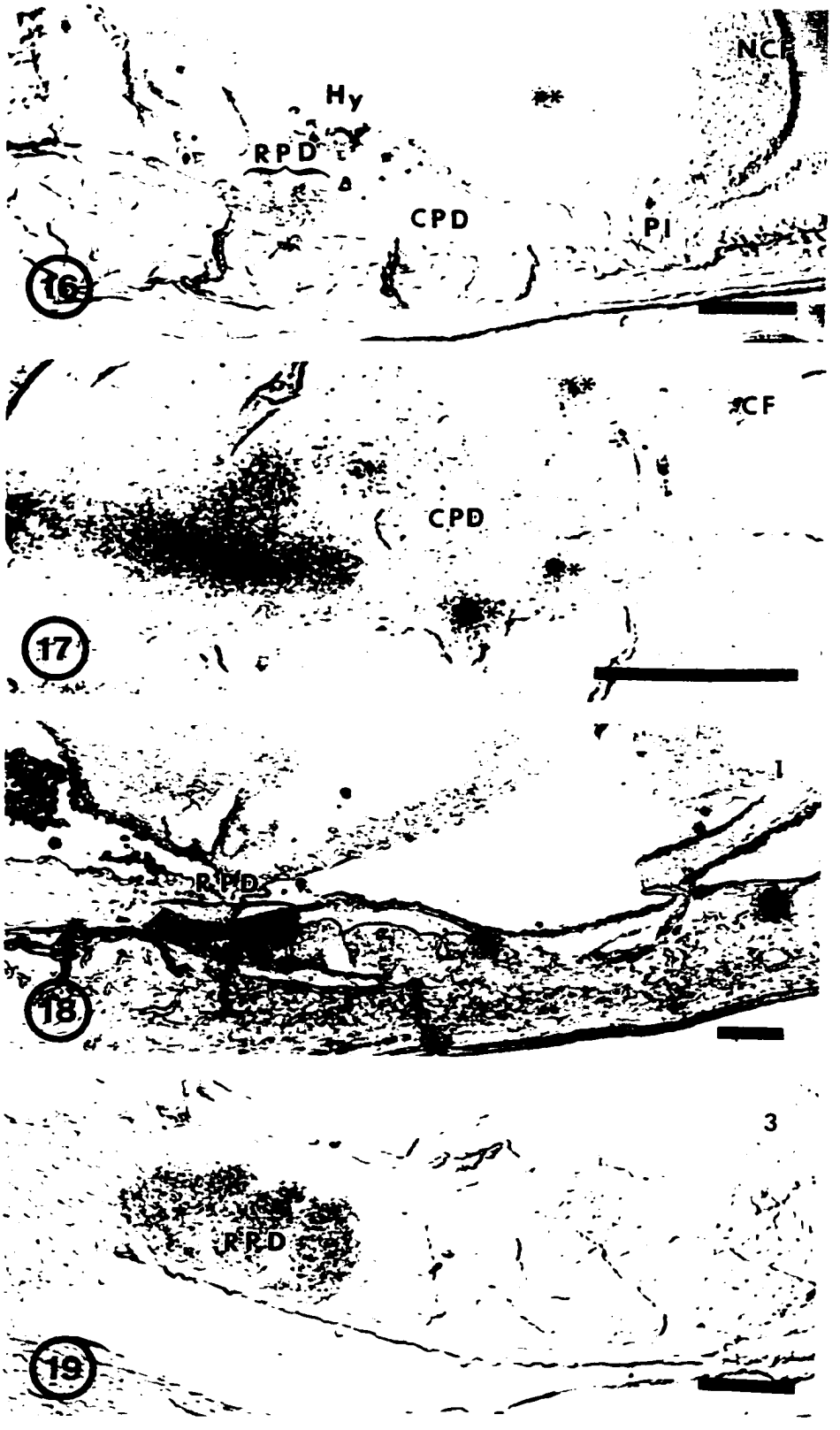
3.2 Proopiocortin (POC)

3.2.1 Qualitative observations

No specific hybridization of POC probe could be determined above background levels in young larvae (n=2, 60 and 70 day) (results not shown) and therefore it was not possible to observe POC expression at this early stage. The expression of POC was examined during all other stages of development: NCF larval (n=4), CF larval (n=3), metamorphic stages 1 (n=2), 3 (n=3), 4 (n=3), 5, 6,7 (n=4), juvenile parasitic adult (n=5), prespawners (n=5, 3 male and 2 females), and spawners (n=3, 1 male and 2 females). Expression of POC is seen to be confined to most cells of the RPD in NCF larvae and at all other stages of the lamprey life cycle (Figs. 3.16 to 3.26). Although it appeared that all cells of the RPD express POC, scattering of radioactivity from areas of high expression can create adjacent non-specific signal making it difficult to determine if all cells express POC.

Proopiocortin appears to be expressed at low levels in the RPD of NCF and CF larvae

Figures 3.16 to 3.19. Proopiocortin (POC) expression in sagittal sections of the rostral and caudal pars distalis (RPD and CPD) during development. Caudal pars distalis, CPD; Rostral pars distalis, RPD; Pars intermedia, PI; Hypopthalamus, Hy. ' * ' depicts non-specific hybridization. Bar = 100 μm.
Figure 3.16. Noncondition factor (NCF) larva; **Figure 3.17.** Condition factor (CF) larva; **Figure 3.18.** Metamorphic stage (1); **Figure 3.19.** Metamorphic stage (3).


Hy
RPD
CPD
PI
NCF
16
CF
CPD
17
1
RPD
18
3
RPD
19

Figures 3.20 to 3.22. Proopiocortin (POC) expression in sagittal sections of the rostral and caudal pars distalis (RPD and CPD) during development. Arrows and arrowheads indicate expression signal in the CPD which begins at stage 5 of metamorphosis and continues until the end of the life cycle. Caudal pars distalis, CPD; Rostral pars distalis, RPD; Pars intermedia, PI. ' * ' depicts non-specific hybridization. Bar = 100 μm. **Figure 3.20.** Metamorphic stage 4 (4); **Figure 3.21 A.** Metamorphic stage 5 (5); **Figure 3.21 B.** Higher magnification of CPD of metamorphic stage 5 animal; **Figure 3.22.** Metamorphic stage 6 (6).


4
CPD
CPD
20
5
21 A
5
CPD
21 B
6
22

Figures 3.23 to 3.25. Proopiocortin (POC) expression in sagittal sections of the rostral and caudal pars distalis (RPD and CPD) during development. Arrows and arrowheads indicate expression signal in the CPD which begins at stage 5 of metamorphosis and continues until the end of the life cycle. Caudal pars distalis, CPD; Rostral pars distalis, RPD; Pars intermedia, PI; Third ventricle of the brain III. ' * ' depicts non-specific hybridization. Bar = 100 μm.
Figure 3.23. Metamorphic stage 7 (7); **Figure 3.24.** Juvenile parasitic stage (P); **Figure 3.25 A.** Spawner (S); **Figure 3.25 B.** Higher magnification of CPD of a spawner.

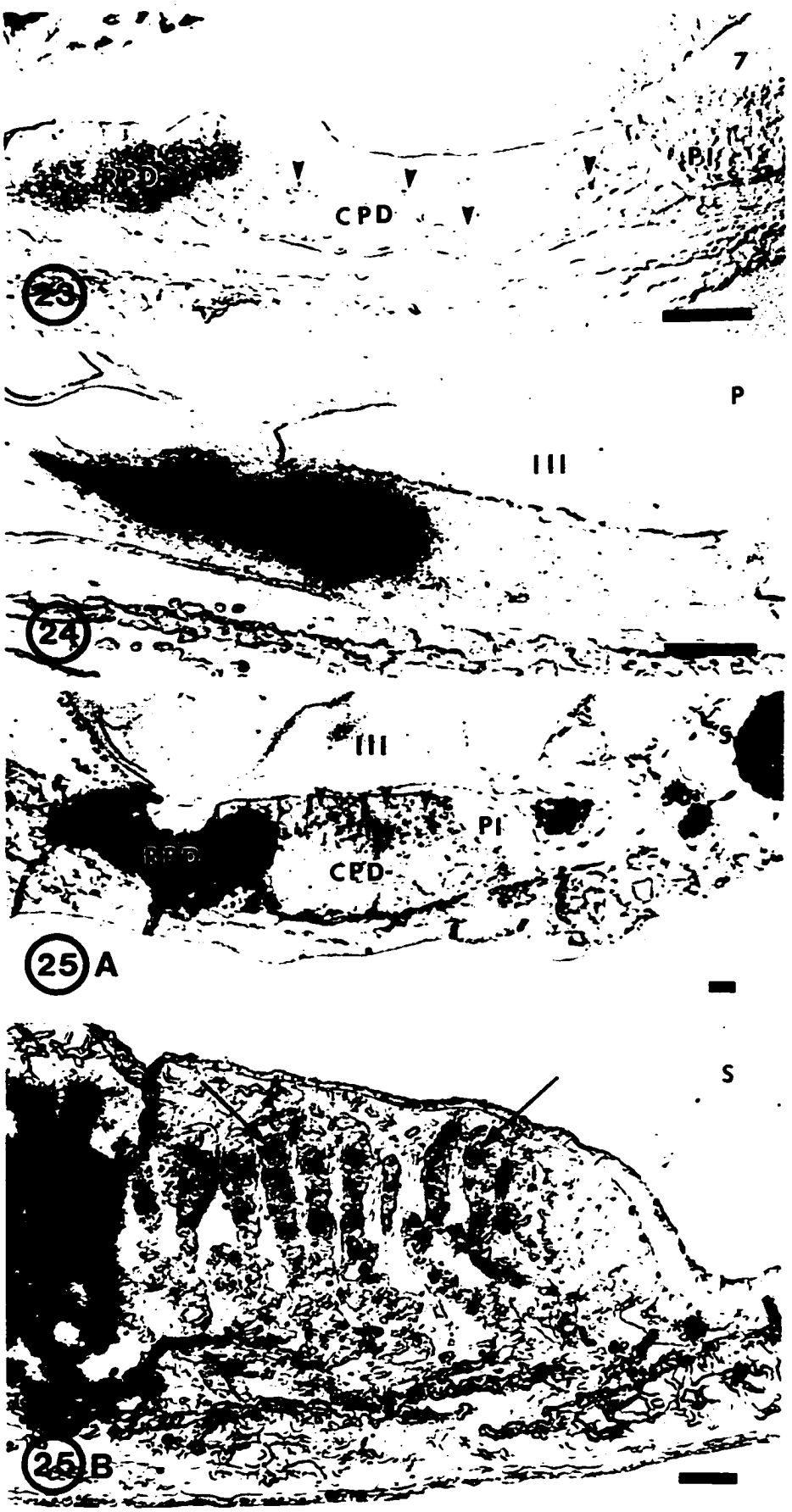
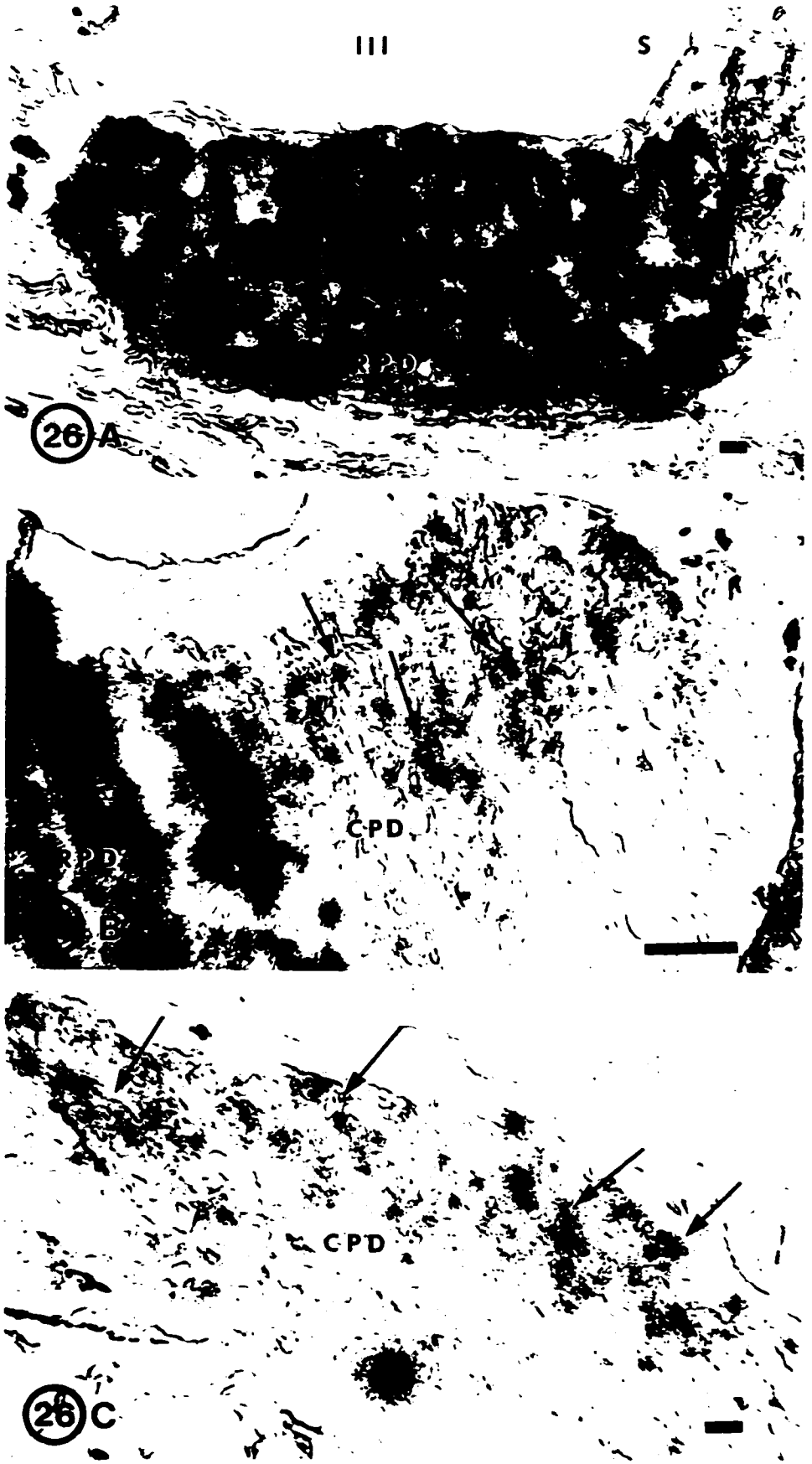

7
RPD
CPD
PI
23
P
III
24
III
S
PI
RPD
CPD
25 A
S
25 B

Figure 3.26. POC expression in transverse sections of a spawning animal (S): the RPD (**A**); the RPD and CPD (**B**) and; the CPD (**C**). Note the dorsal distribution of POC expression in the CPD. Arrows indicate isolated clusters of POC expressing cells. Caudal pars distalis, CPD; Rostral pars distalis RPD; Third ventricle of the brain, III. ' * ' denotes non-specific hybridization.


III
S
CPD
26 A
CPD
B
CPD
26 C

as observed by the low signal intensities (Figs. 3.16, 3.17). The signal intensity in stage 1 animals appeared to be slightly greater than that of larvae (NCF and CF) and stage 3 metamorphic animals (compare Figs. 3.18 and 3.19). The intensity of signal observed in early and mid-metamorphic animals appears to be similar and it is difficult to visually determine differences in levels of expression between these stages. However, it appears that expression and signal intensity increases by at least stages 6 and 7 (Figs. 3.22, 3.23) as compared to stages 3, 4 and 5 (Figs. 3.19, 3.20, 3.21A). During stages 5 and 6, (Figs. 3.21A, 3.22) there appears to be greater signal intensity in the dorsal aspect of the RPD than the remainder of the lobe. In general, the intensity of POC expression seems to gradually increase proceeding into and throughout metamorphosis, and the greatest amount of signal occurs during the spawning stages (Fig. 3.25).

Specific hybridization signal can be first visualized above background levels in scattered isolated cells throughout the CPD during stage 5 (Fig.21 A, B) and becomes more prominent throughout the remainder of the life cycle. The number of cells expressing POC in the CPD appears to increase gradually from stage 5 to the spawning stages. The intensity of the POC signal in the CPD is the greatest by the spawning stages (Fig. 3.25 B and 3.26 C). In the spawning stage, POC expression in the CPD is mainly distributed in cells scattered within the dorsal aspect of the lobe (Figs. 3.25). The spatial distribution of POC expression was also examined in transverse sections of a spawner pars distalis. POC is expressed in most cells throughout the RPD (Figs. 3.26 A and B) and within cells mainly scattered throughout the dorsal aspect of the CPD (Fig. 3.26 B and C).

3.2.2 Quantification of POC expression

3.2.2.1 Expression of POC in the RPD

To corroborate the observed intensities of POC expression throughout development, computer assisted image analysis was performed as per quantification of POM expression. As described previously (see 3.1.2. Quantification of POM expression), experimental limitations could not permit simultaneous analysis of every stage of development in any individual trial and the data from all trials could not be grouped together.

The area fraction (AF) data of signal density for POC expression in each of the nine trials in the RPD showed significant difference within each trial ($p<0.05$) (Table 3.2). A general pattern of expression was interpreted from histogram plots of mean AF values generated from one-way-analysis-of-variance similar to that done for POM expression (Fig. 3.27). These histograms illustrate the general pattern of signal density of POC signal in the RPD. A histogram of all the data from all nine trial is illustrated in figure 3.28. Trials 1 and 3 (Fig.3.27.A and C) illustrate the low density of expression in the larval stages which increases gradually in early and mid-metamorphic stages (Fig. 3.27, C and E). Trials 7 and 8 (Fig. 3.27.G and H) illustrate the latter end of metamorphosis (stages 6 and 7) and the completion of the life cycle with the highest signal densities occurring at the spawning stages as observed qualitatively.

Statistical significance between signal densities throughout development (as determined by Fisher's pairwise comparisons) varied between individual trials. In general, signal density is at its lowest in NCF and CF larvae and was not significantly greater than any other stage. It appears that signal densities increase during the early stages of

Table 3.2. Mean area fraction values of POC expression in the RPD.

	Stage of development											
Trial	NCF	CF	1	3	4	5	6	7	P	PS	S	p-value
1	0.0646			0.3228					0.2802			0.0000
2		0.3716		0.4210		0.2869						0.0190
3	0.3681		0.4128		0.5580	0.4141				0.8592	0.7230	0.0000
4	0.2576			0.2300	0.3236	0.3415		0.1927	0.2165	0.6065		0.0000
5				0.2638	0.4158	0.4187		0.4548		0.7676		0.0000
6	0.4501	0.4401	0.5973	0.5685			0.6838					0.0040
7				0.5701		0.4140	0.4650	0.6722	0.6991			0.0000
8						0.4758	0.5141	0.6751			0.9226	0.0000
9	0.5245	0.4546			0.5183	0.4818			0.7230			0.0000

Figure 3.27. Histograms of each of the nine trials representing mean area fraction values of the signal density of POC expression in the RPD throughout development. Values were generated by computer assisted quantification and one-way ANOVA. Noncondition factor (NCF) and condition factor larvae (CF); metamorphic stages 1, 3 to 7; juvenile parasitic adult (P); prespawners (PS) and spawners (S).

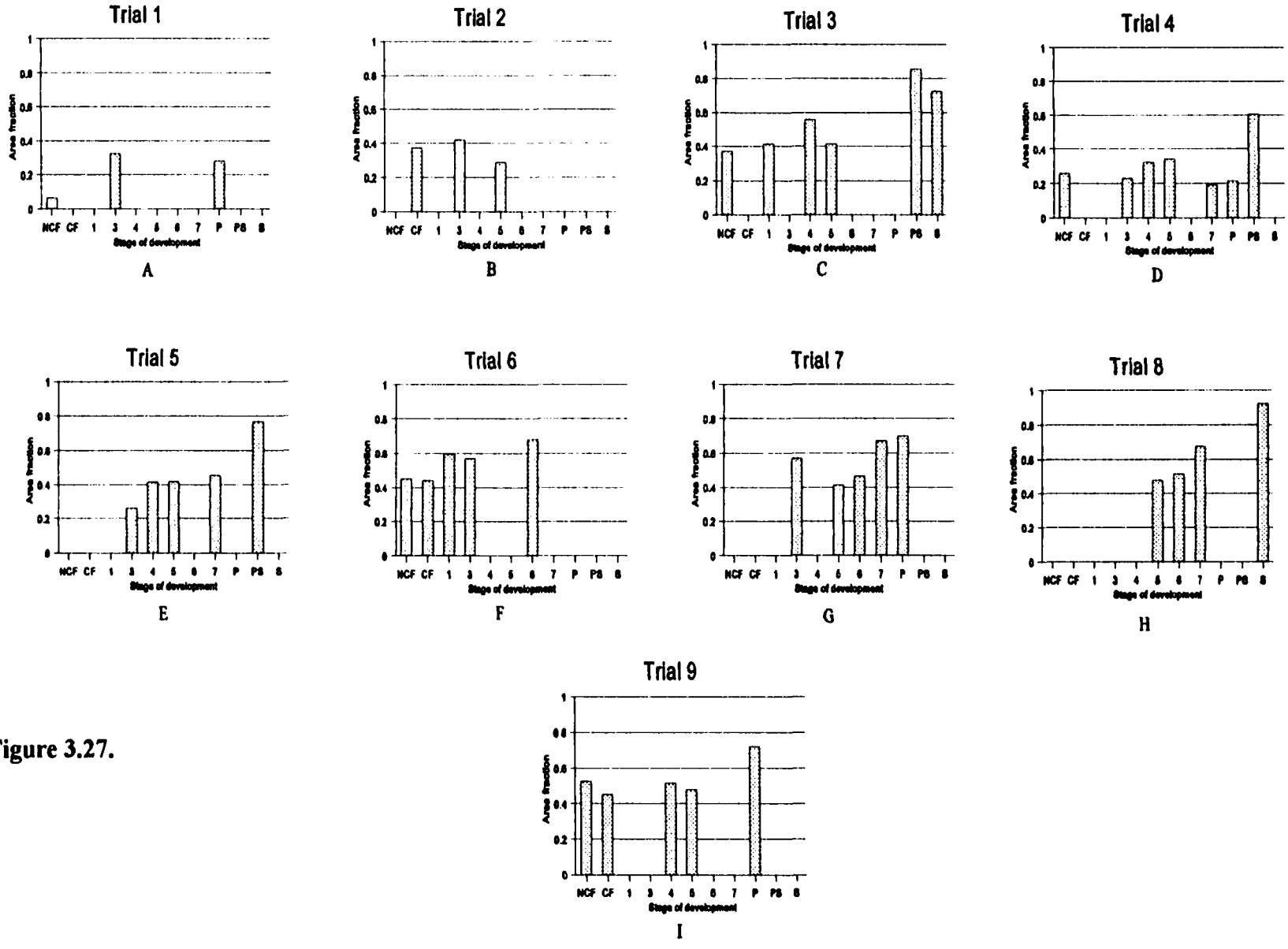

Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Trial 7
Trial 8
Trial 9
Area fraction
Stage of development
NCF CF 1 3 4 5 6 7 P PS S
A
B
C
D
E
F
G
H
I

Figure 3.27.

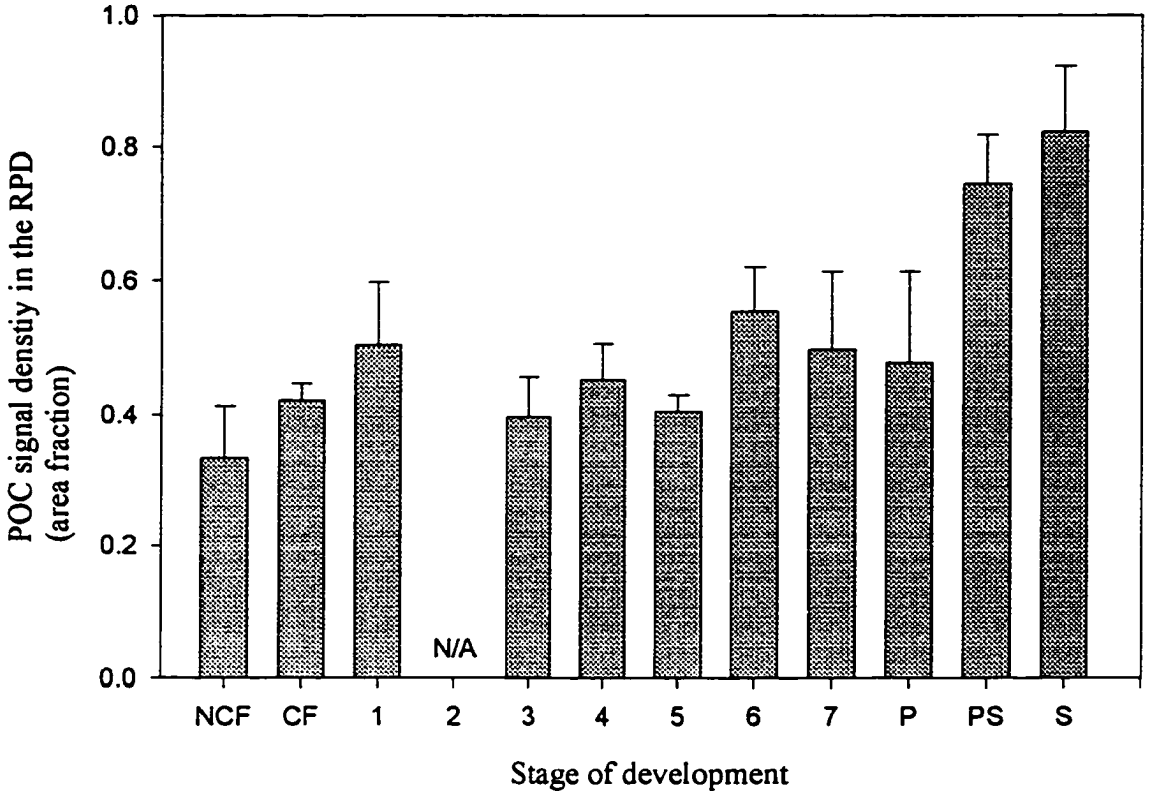


Figure. 3.28. Histogram of the mean values from all nine trials of the signal density of POC expression in the RPD during development. Bars represent S.E. mean values. Nonconditon factor (NCF) n=4 and condition factor larvae (CF) n=3; metamorphic stages 1 n=2; 3 and 4 n=3; 5, 6, and 7 n=4; juvenile parasitic adult (P) n=5; prespawner (PS) n=5 and spawners (S) n=3. Stage 2 metamorphic animals were not available for this study.

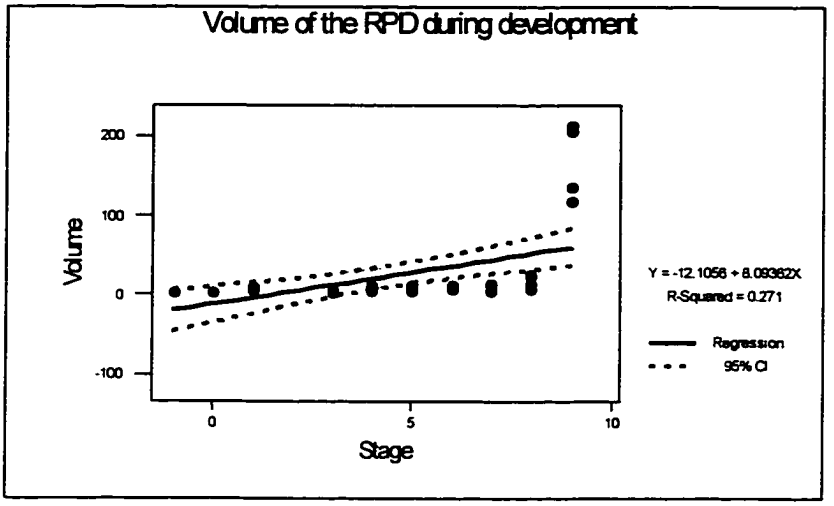
development and that stage 1 animals express at significantly higher levels than larvae. The increase at stage 1 however, is followed by a decrease in signal density by stage 3 (Fig. 3.28). A general conclusive statement of which stages of development exhibit a greater signal density than others is not possible because the results of a few trials were conflicting. For example, in trial 2 (Fig. 3.27 B), POC signal density in the RPD of a stage 3 animal was significantly greater than that of a stage 5 animal. These results conflict with trials 4 and 5 (Figs. 3.27,D and E) where the POC signal densities of stages 5 and 4 are significantly greater than stage 3 animals. Analysis of trial 4, revealed that the density of expression at stages 4 and 5 is significantly greater than during stage 7 and juvenile parasitic adults (Fig. 3.27. D). No other trials revealed that signal densities of stages 4 and 5 were significantly greater than that during stage 7. Fluctuations in stages 3 to 7 of metamorphosis make it difficult to make accurate statements of statistically significant difference of levels of expression within these stages (Fig. 3.28). However, in general, the histograms suggest a trend of increasing signal density. By stage 7 and later, expression levels reach their maximum. The signal densities of stage 7 and parasitic adults are comparable (not significantly different from each other) and only significantly less than that of spawning animals. The density of expression is at its greatest during the spawning stage.

Changes in the total volume of the RPD, and volume of expression within the lobe, were examined as they were for POM expression in the PI described earlier. Two separate regression analyses of data sets including and excluding the much larger volume data of spawner stages were done ($p<0.001$). As described earlier, the volume of the lobes of the pituitary of the spawning animal is much greater than that of all other stages and therefore

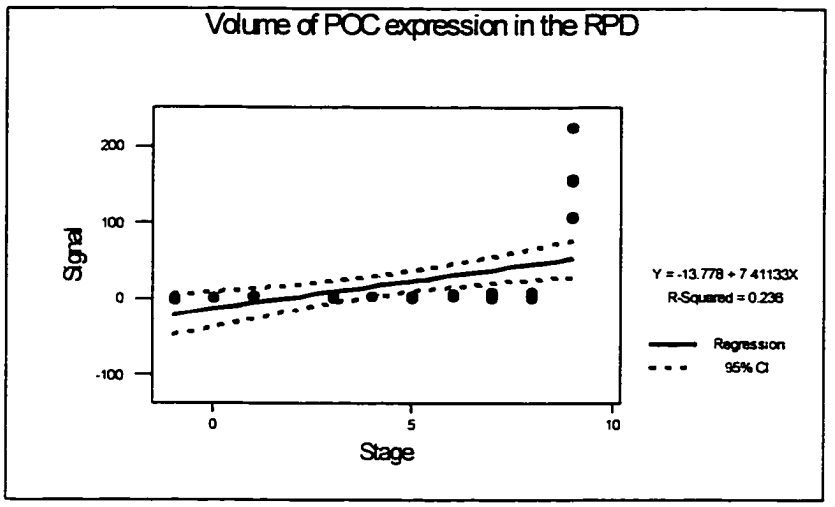
creates an outlier in the data shifting the regression lines to the left thereby creating non-real negative predicted volume numbers (Fig. 3.29 C). The data set including the outlier spawning data indicates that the volume of cells expressing POC is very close to the total volume of the lobe as indicated by their similar slopes (m= 8.09 in Fig. 3.29 A, and m =7.411in Fig. 3.29 B). The plot illustrating both fitted regression lines (Fig. 3.29 C) also displays that there is not very much difference in these volume data. That is, as the size of the lobe increases with age, the number of cells expressing POC increases accordingly.

The data set without the outlier spawning data also indicates that the total volume of the RPD and volume of cells expressing POC are similarly correlated with development ($r=0.734$ in Fig. 3.30 A to $r=0.740$ in Fig. 3.30 B) ($p<0.001$). The correlation of volume of expression in this data set however, is much greater than the data set containing the spawning data (compare $r=0.740$, Fig. 3.30 B and $r= 0.486$, Fig. 3.29 B). The difference in correlation coefficients illustrates the skewing effect that the large spawner data has on the smaller volume data. The slopes of the linear regression of the data without the spawning data ($m=1.340$ in Fig. 3.30 A for volume of RPD, and $m=0.593$ in Fig. 3.30 B for volume of POC) are not as close as the slopes with spawning data (Fig. 3.29 A, m= 8.09 and Fig. 3.29 B, m =7.411). The difference in slopes indicates that there is a greater difference in the volume of the RPD and the volume of POC not observed with spawner data (compare fitted regression graphs Fig. 3.29 C with Fig. 3.30 C). The linear regression of the data set not including the spawning animal stage indicates more gradual and subtle change of expression than that indicated by the linear regression containing the spawning data. The fitted linear regression plot of data up to and including the juvenile parasitic adult stage (Fig. 3.30 C)

A



B



C

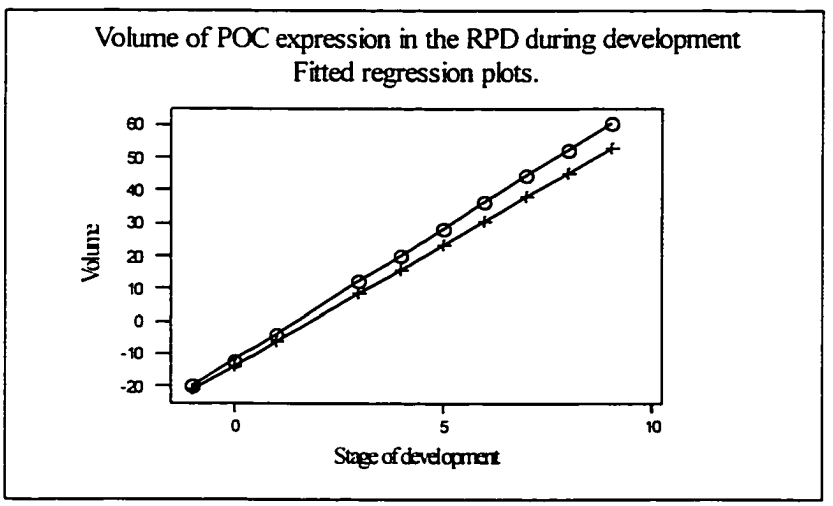
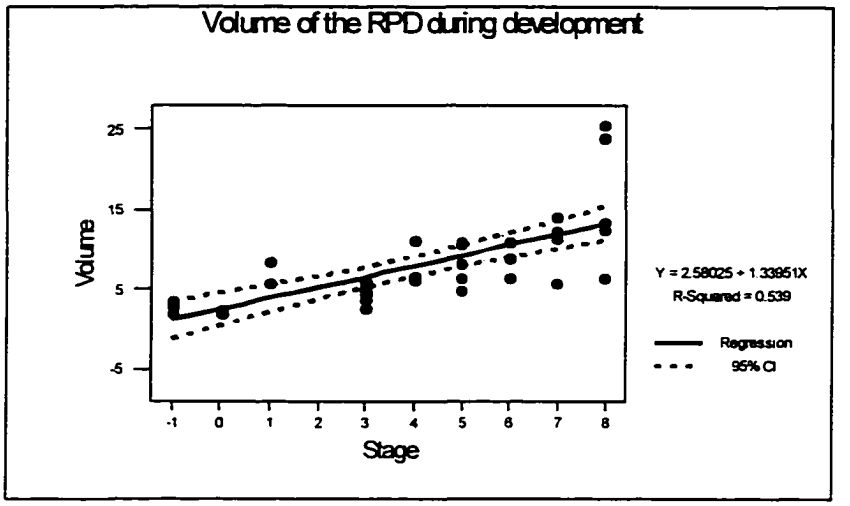


Figure 3.29. A. Scatter plot of total volume (μm^3) of the RPD. **B.** Scatter plot of volume of signal (cells expressing POC) during development. Dashed line represents the 95% confidence interval of the linear regression (solid line) of volume data. **C.** Fitted regression plots of figure A (line with "o") and B (line with "+"). Stages -1, 0: NCF and CF; 1 to 7: metamorphic stages; 8 and 9: parasitic and spawning stages.

A



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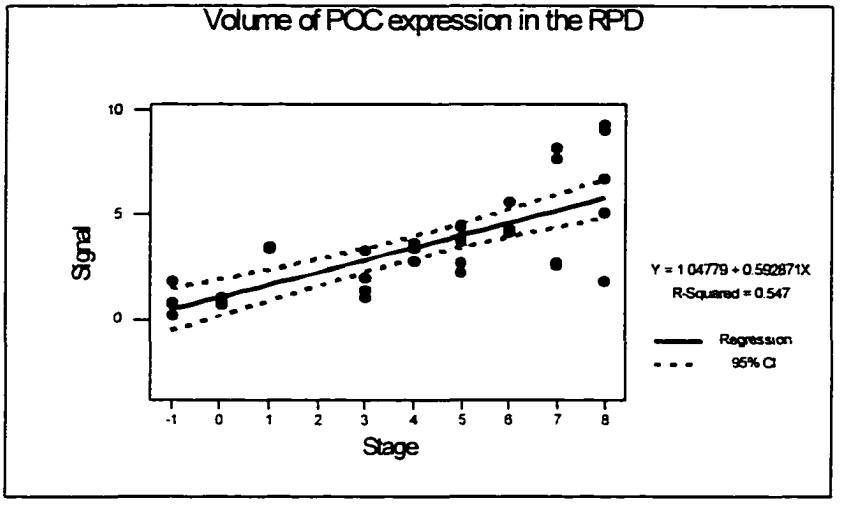


C

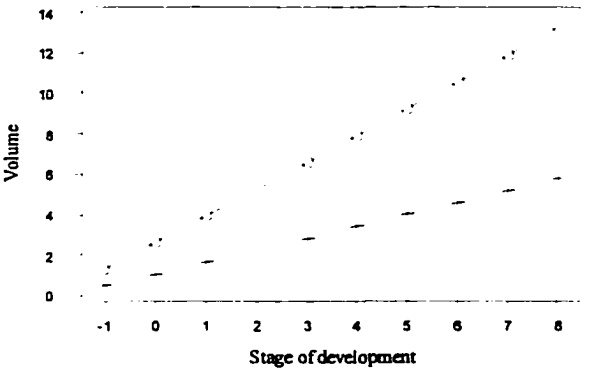


Figure 3.30. **A**. Scatter plot of total volume (μm^3) of the RPD. **B.** Scatter plot of volume of signal (cells expressing POC) during development not including the spawning stage. Dashed line represents the 95% confidence interval of the linear regression (solid line) of volume data. **C**. Fitted regression plots of figure A (line with "o") and B (line with "+"). Stages -1, 0: NCF and CF; 1 to 7: metamorphic stages; 8: parasitic stage.

reflects the general increasing pattern of POC expression in the RPD. The volume of cells expressing POC is less than the total volume of the lobe throughout development (not including spawners). The volume of cells increases gradually and the greatest proportion of cells expressing POC occurs during the spawning stages. The increasing amounts of POC expression throughout development illustrated by volumetric analyses correlates and supports the qualitative and AF (signal density) quantification described earlier.

3.2.2.2 Expression of POC in the CPD

Expression of POC in the CPD was examined in 6 different trials comparing stage 5 metamorphic animals and older. Analysis of area fraction data (AF) revealed that the mean AF values were statistically significantly different ($p<0.05$) in all but two trials (Table 3.3). The expression density pattern in the CPD was interpreted from histograms of mean AF values (Fig. 3.31) and a histogram of the data from all six trials (Fig. 3.32) as described for expression of POM in the PI and POC in the RPD. In general, the amount of signal density (expression) in the CPD increased throughout the latter part of metamorphic development (stages 5 to 7) and reached significantly higher levels by the parasitic stage. The highest signal density occurred during the spawning stage (Fig. 3.31 E). The signal densities of POC expression in the CPD of stage 6 animals and older was significantly higher than levels of stage 5 animals. No statistically significant difference was found in signal densities between stage 6 and 7 animals and levels of expression in the CPD was greatest in spawning animals.

The volume of the CPD and volume of cells expressing POC in this lobe, from stage 5 metamorphic animals to spawning animals, were also analysed. As described earlier two

Table 3.3. Mean area fraction values of POC expression in the CPD.

	Stage of development						
Trial	5	6	7	P	PS	S	p-value
1	0.0139				0.0986	0.1217	0.0000
2	0.0535		0.0357	0.0456	0.0793		0.1670 *
3	0.0449		0.0615		0.0868		0.0860 *
4	0.0372	0.0521	0.0479	0.1214			0.0100
5	0.0165	0.0457	0.0455			0.2112	0.0000
6	0.0573			0.1633	0.2880		0.0030

Asterisks '*' denote non-significant data (trial 2 and 3).

Figure 3.31. Histograms of each of the six trials representing the mean area fraction values of the signal density of POC expression in the CPD from stage 5 to the spawner stage. Values were generated by computer assisted quantification and one-way ANOVA.

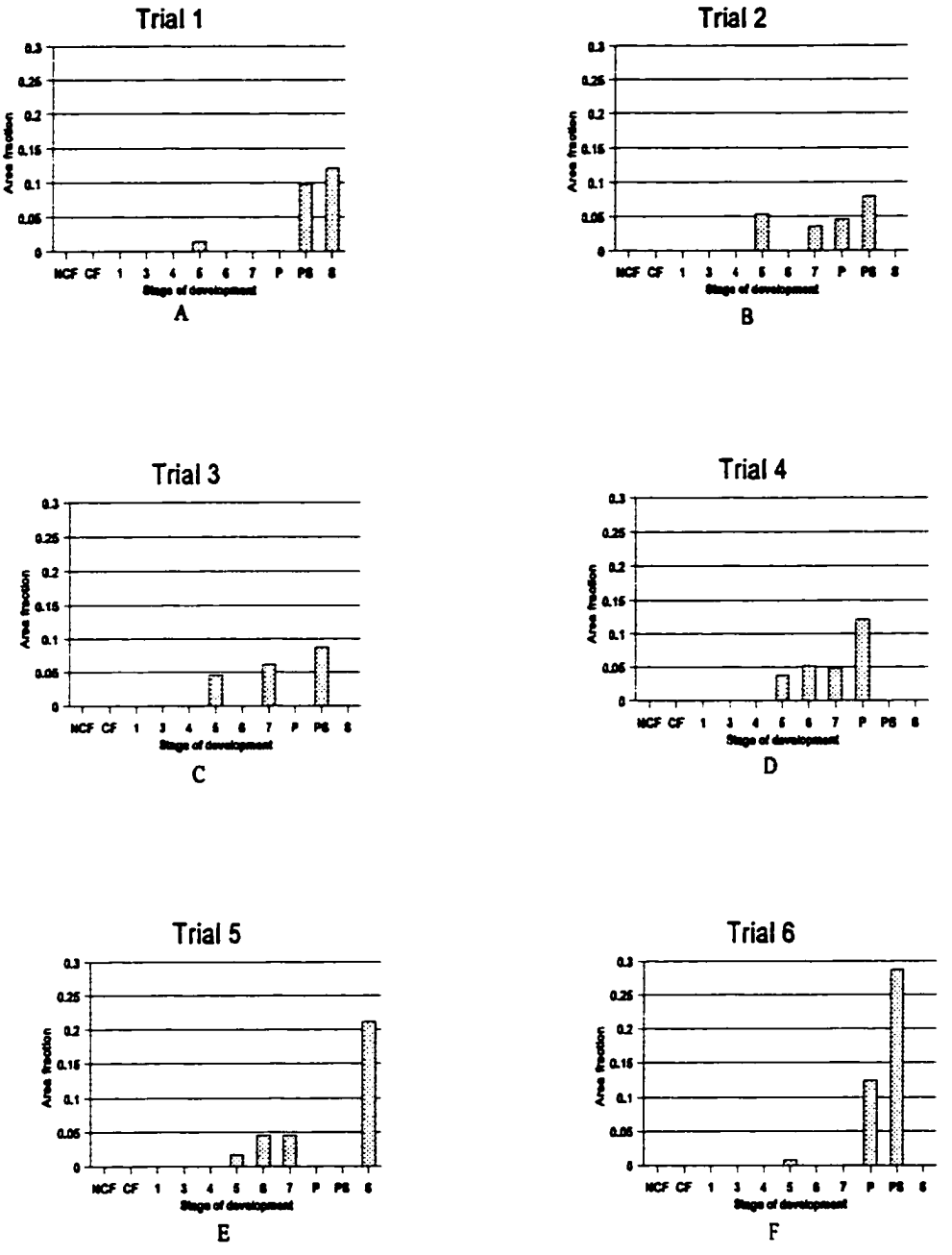

Trial 1
Area fraction
0.3
0.25
0.2
0.15
0.1
0.05
0
NCF CF 1 3 4 5 6 7 P PS S
Stage of development
A
Trial 2
Area fraction
0.3
0.25
0.2
0.15
0.1
0.05
0
NCF CF 1 3 4 5 6 7 P PS S
Stage of development
B
Trial 3
Area fraction
0.3
0.25
0.2
0.15
0.1
0.05
0
NCF CF 1 3 4 5 6 7 P PS S
Stage of development
C
Trial 4
Area fraction
0.3
0.25
0.2
0.15
0.1
0.05
0
NCF CF 1 3 4 5 6 7 P PS S
Stage of development
D
Trial 5
Area fraction
0.3
0.25
0.2
0.15
0.1
0.05
0
NCF CF 1 3 4 5 6 7 P PS S
Stage of development
E
Trial 6
Area fraction
0.3
0.25
0.2
0.15
0.1
0.05
0
NCF CF 1 3 4 5 6 7 P PS S
Stage of development
F

Figure 3.31.

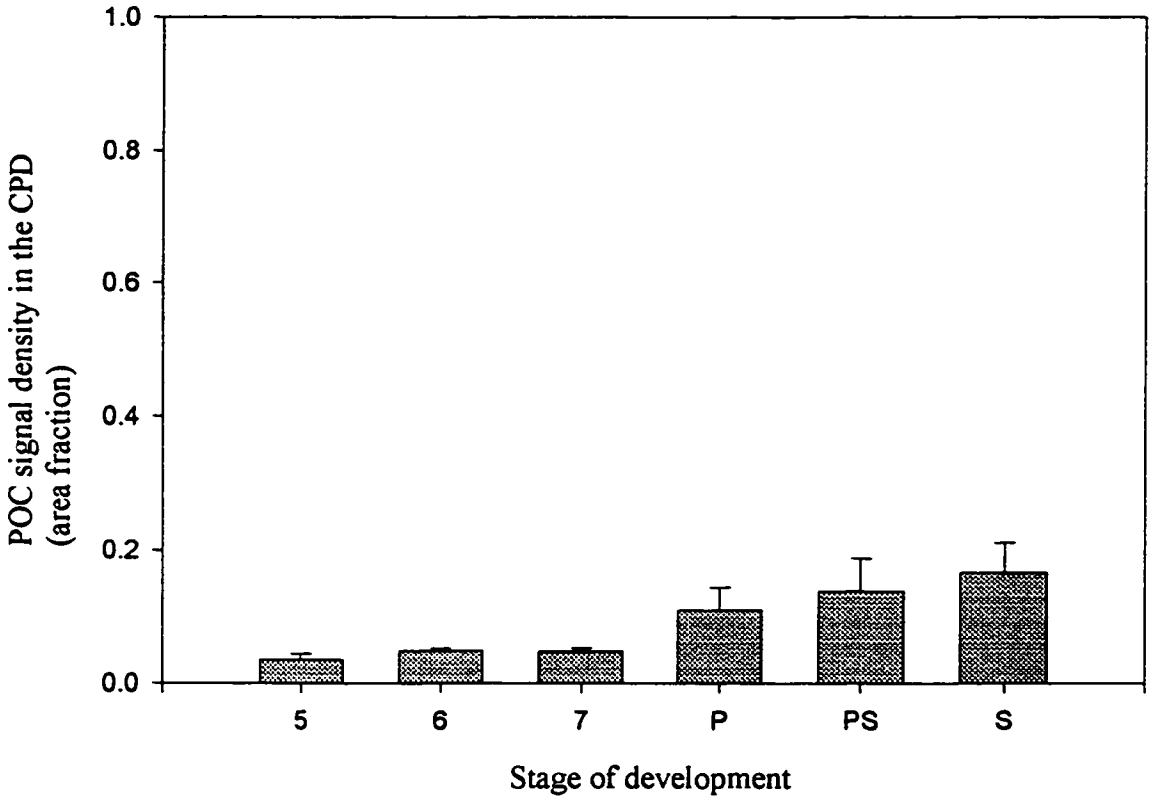
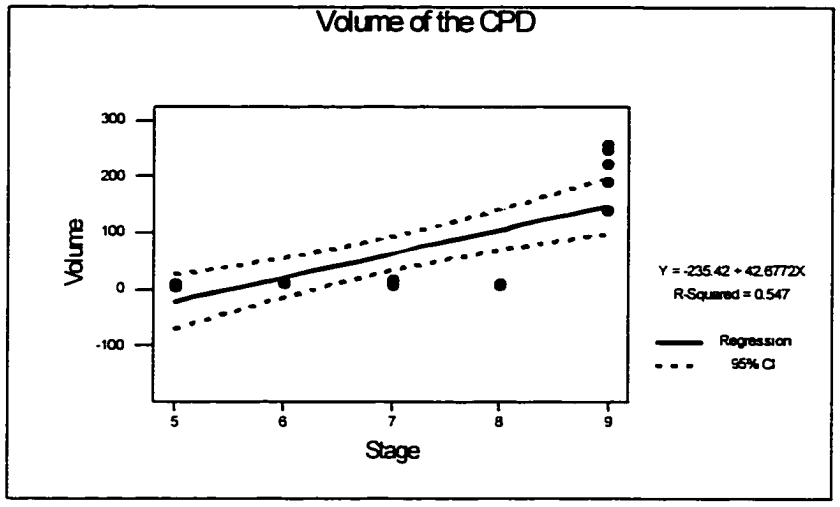


Figure 3.32. Histogram of the mean values from all six trials of the signal density of POC expression in the CPD during development. Bars represent S.E. mean values. Metamorphic stages 5 to 7 n=4;juvenile parasitic adult (P) n=5; prespawner (PS) n=5; and spawners (S) n=3. There is no expression of POC in the CPD prior to stage 5 of metamorphosis.

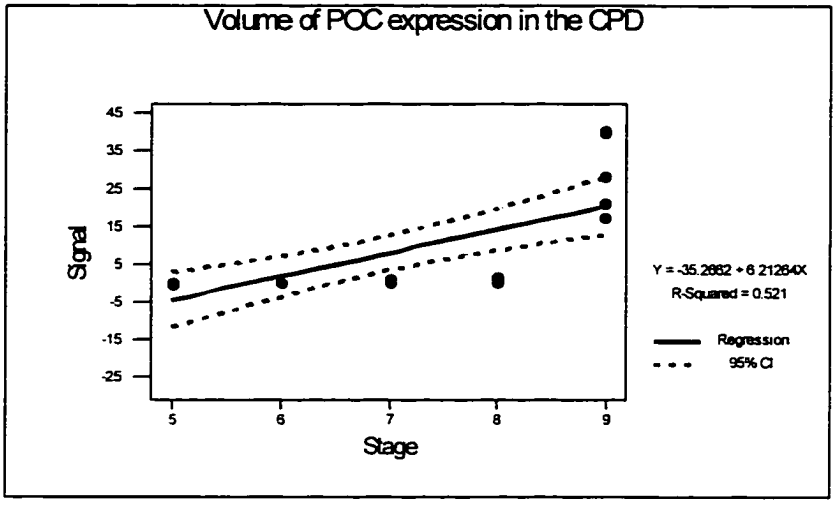
separate regression analyses were performed, one with the spawner data (Fig. 3.33) and one without the spawner data (Fig. 3.34). Regression analysis including spawning data shifted the linear regression to the left creating non-real negative predicted volume numbers. As described earlier, the predicted negative values are a result of compensating for very large outlier data values for spawner animals. The slopes of the linear regression lines of total volume of the lobe and the volume of cells expressing POC in the lobe differed substantially when spawner data were included (m= 42.68 in Fig. 3.33 A, and m= 6.21 Fig. 3.33 B respectively). The large difference indicates that very few cells express POC in the CPD. Note that in the plot of both fitted linear regressions (Fig 3.33 C) the CPD total volume line begins as a negative value, and at that point (stage 5) the volume of cells expressing POC is greater than the total volume of the lobe (which is non-sensical).

When the outlier spawning data are removed (i.e. comparing stage 5 to parasitic adults only) the slopes of the regression lines of the total volume of the CPD and the volume of expression within the lobe differ considerably (m=0.684, Fig. 3.34 A, and m=0.256, Fig. 3.34 B respectively). More importantly, regression analysis of the total volume of the CPD (Fig. 3.34 A) was not statistically significant (p=0.341) indicating that the volume of the CPD does not change considerably between stage 5 and juvenile parasitic adults. The volume of cells expressing POC in the CPD, however, does increase with statistical significance between these stages (p=0.013) (Fig. 3.34 B). The fitted regression plots of both regression analyses (Fig. 3.34 C) indicate that the volume of cells expressing POC in the CPD increase gradually. This volume is still very small when compared to the total volume of the lobe. Volumetric analyses of POC expression in the CPD correlates with qualitative

A



B



C

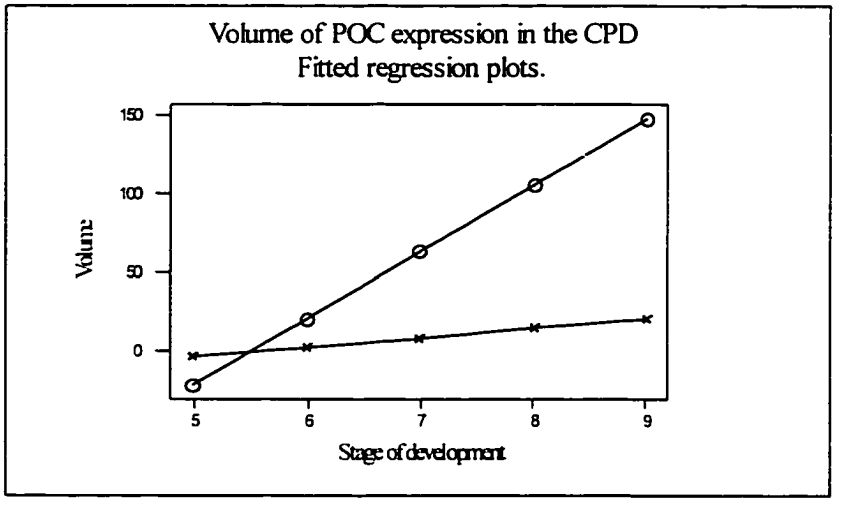
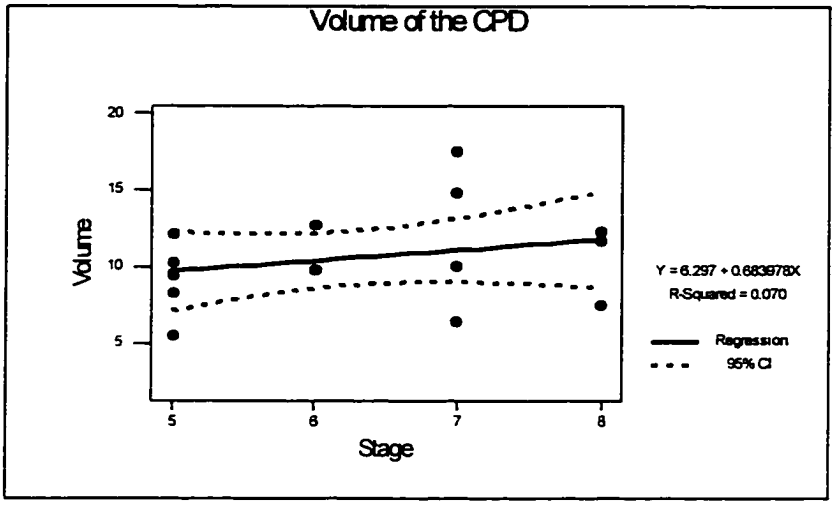
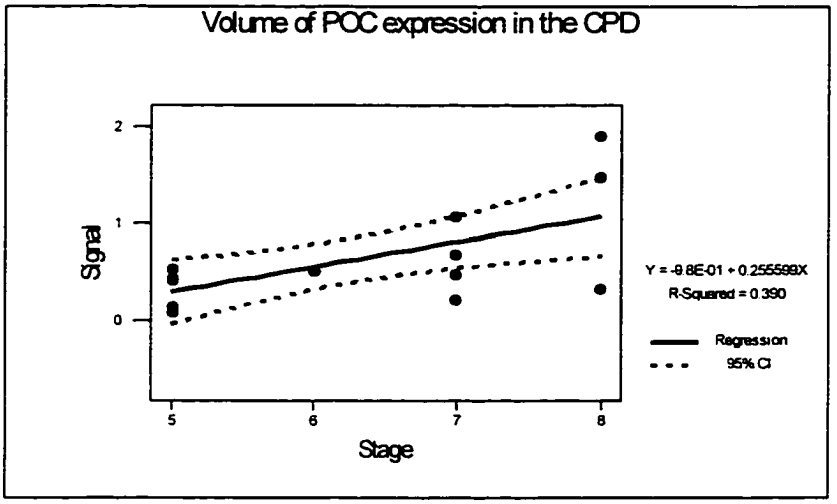


Figure 3.33. A. Scatter plot of total volume (μm^3) of the CPD. **B.** Scatter plot of volume of signal (cells expressing POC) during development. Dashed line represents the 95% confidence interval of the linear regression (solid line) of volume data. **C.** Fitted regression plots of figure A (line with "o") and B (line with "x"). Stages 5 to 7: metamorphic stages; 8 and 9: parasitic and spawning stages.

A



B



C

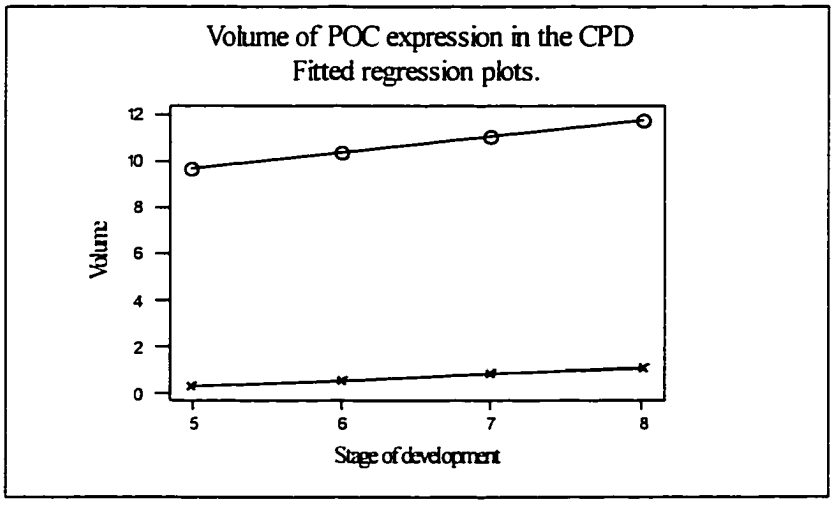


Figure 3.34. **A.** Scatter plot of total volume (μm^3) of the CPD. **B.** Scatter plot of volume of signal (cells expressing POC) during development not including the spawning stage. Dashed line represents the 95% confidence interval of the linear regression (solid line) of volume data. **C.** Fitted regression plots of figure A (line with "o") and B (line with "x"). Stages 5 to 7: metamorphic stages; 8: parasitic stage.

and AF (signal density) quantification described earlier.

The spatial and temporal distributions of POM and POC are summarized diagrammatically in figure 3.35.

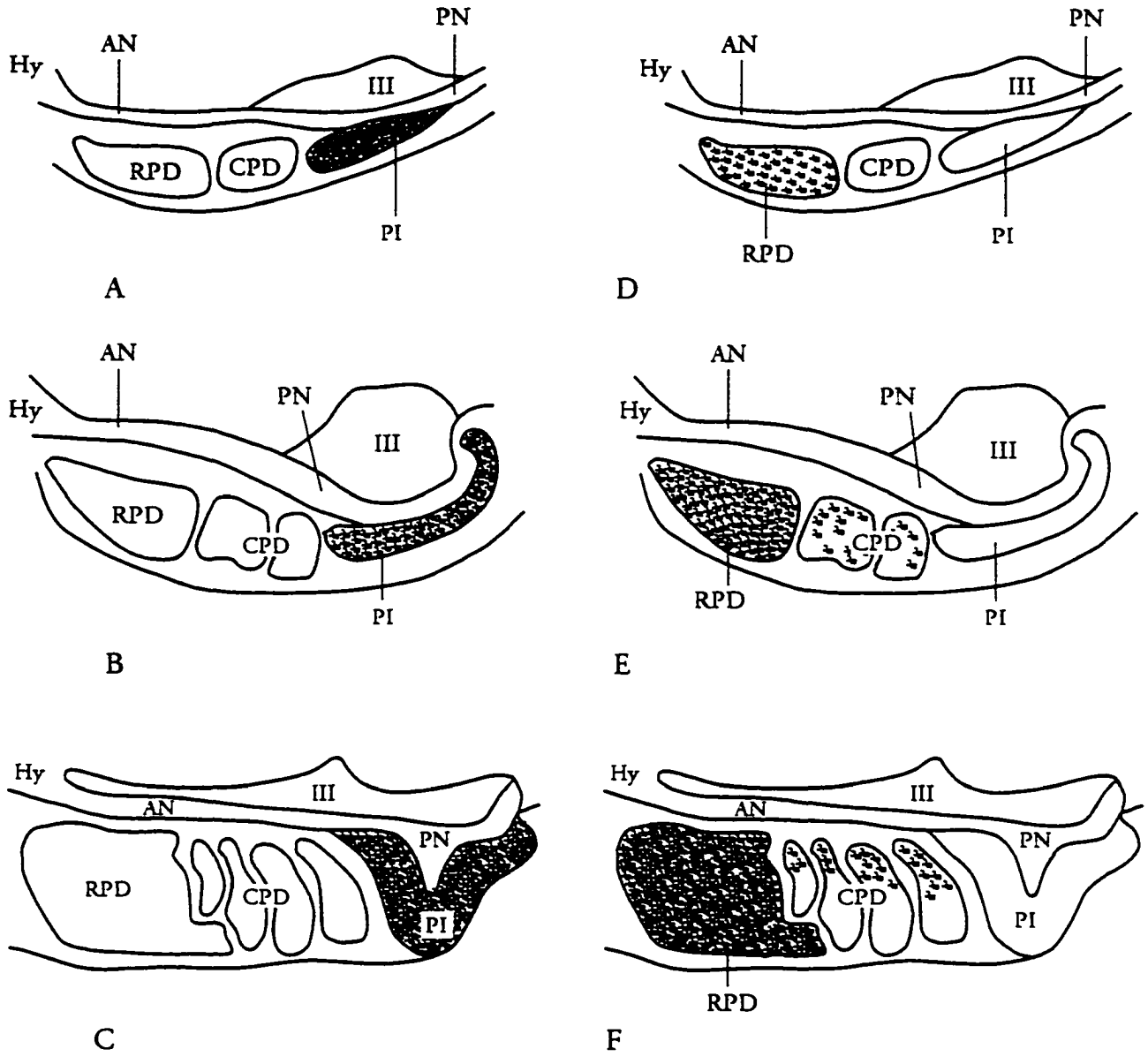


Figure 3.35. Diagrammatic representation of the spatial and temporal distributions of POM and POC expression during development and relative signal intensities represented by amount of shading. Figures A to C: POM expression in the PI. Figures D to F: POC expression in the PD. Figures A and D: larval animals; figs. B and E: mid-metamorphic animals; and figs. C and F: typical spawners. Anterior neurohypophysis, AN; Posterior neurohypophysis, PN; Hypothalamus, Hy; Rostral pars distalis, RPD; Caudal pars distalis, CPD; Pars intermedia, PI; Third ventricle of the brain, III. Diagram not to scale.

4. DISCUSSION

4.1 POM and POC distribution

In situ hybridization (ISH) using cRNA probes of fragments of the MSH-B region of POM and the NHF region of POC mRNAs were used to qualitatively and quantitatively determine variations of the expression of these prohormones during development of the sea lamprey. POM and POC expression in the head region of the lamprey is localized within the pituitary with POM expression restricted to the PI and POC expression restricted to the PD. This is unlike POMC expression in gnathostomes which is localized in the pituitary (Naito *et al.*, 1984), hypothalamus and other parts of the brain (Ma *et al.*, 1994).

In the pituitary of gnathostomes, the major POMC end products α-MSH and β-END are localized in the PI; and ACTH, β-END and β-LPH are found in the PD (Hanneman *et al.*, 1989). Numerous immunocytochemical studies of POMC-end products have demonstrated the distribution of ACTH-like immunoreactivity in the corticotrophs of the PD, and α-MSH-like and β-END-like immunoreactivity in the melanotrophs of the PI in primitive teleosts (Joss *et al.*, 1990b) and higher vertebrates (Douglass *et al.*, 1984; Naito *et al.*, 1984; Soma *et al.*, 1984; Martens *et al.*, 1985; Hilario *et al.*, 1990). Like their POMC counterpart, both POM and POC are multi-domain hormone precursors. POM encodes the domains of MSH-A, MSH-B and β-END, and POC encodes the domains of NHF, an MSH (not α), ACTH and a different β-END (Takahashi *et al.*, 1995a). Dores *et al.*, (1984) have described ACTH-like and met-enkephalin-like immunoreactivity in the RPD, and α-MSH-like and met-enkephalin-like immunoreactivity in the PI of adult brook lamprey. Recently, antisera generated against synthetic *P. marinus* ACTH, MSH-A and MSH-B have demonstrated

ACTH and MSH-A immunoreactivity in the RPD and CPD and MSH-A and MSH-B immunoreactivity in the PI of adult lamprey (Nozaki *et al.*, 1995). However, preabsorption of anti-MSH-A with MSH-B abolished the MSH-A positive reaction in the PD but not the PI indicating that the MSH-positive material in the PD has a lower antigenic specificity than that in the PI and that its immunocytochemical determinants may be different between the PD and the PI. Lamprey ACTH, MSH-A and MSH-B share a similar amino acid sequence as part of their primary sequence and cross reactivity of anti-ACTH and anti-MSH-A may be related to a shared protein structure. Therefore, further study is needed to clarify MSH-A material in the PD (Nozaki *et al.*, 1995). This study specifically localizes POC mRNA in the cells of the PD only and POM mRNA in cells of the PI only. Analysis of the expression of the POM and POC prohormones of this study details the presence of the mRNA and does not suggest which cell types are translating the mRNA and producing specific POM and POC end-products. However, the expression of POC in the PD correlates with that of ACTH immunoreactivity in the PD and the localization of POM mRNA in the PI correlates with immunoreactivity studies of POMC MSHs in the PI of gnathostomes (Hanneman *et al.*, 1989).

4.2 POM

4.2.1 Quantification

Signal intensities (qualitative observations) and signal densities (quantitative measurements of area fraction of signal in a defined area of adenohypophysial region) of POM expression in the PI appear to be very high in NCF and CF larvae prior to metamorphosis and comparably high in parasitic and spawning animals. The high levels of POM expression seen in NCF larvae reach a climax in CF larvae and decrease upon initiation of metamorphosis (stage 1). By metamorphic stages

3 and 4, the signal intensities of POM expression appear to be at their lowest. POM signal densities then increase gradually throughout the remainder of metamorphosis with the greatest signal densities occurring during the spawning stages.

Recent Northern blot analyses of POM expression differ from the results of this study in that POM expression in larval lamprey appeared low, and remained low until about stage 4 (J. Heinig, personal communication). In light of these differences it must be recalled that *in situ* hybridization and Northern blots are similar as they both depend on hybridization of a labelled nucleic acid probe. However, in a Northern blot, the cellular relationships are lost and the mRNA levels in an aliquot represent the average amount of mRNA from all the cells of a tissue digest from more than one animal (Wilcox, 1993). On the other hand, ISH is extremely sensitive and can detect the amount of mRNA contained in a single specimen while still maintaining cellular relationships (Wilcox, 1993). Therefore, the ISH analysis of POM and POC expression in this study may detail subtle variations in expression not possible by Northern blots.

The amount of mRNA present in a cell represents a balance between the transcriptional activity of the gene and intracellular degradation processes (Ayoubi *et al.*, 1992). The very high levels of POM mRNA signal density in larval animals may suggest that POM is expressed and translated at very high levels, constantly producing end-products that may play some role during this period. The lower signal densities apparent during early metamorphic stage 1, 3 and 4 may be due to a decrease in transcriptional activity.

High signal densities during larval stages may also be due to the storage of POM mRNA in translationally inactive form until it is subsequently required and translated during metamorphic stages 1, 3 and 4 when there are decreased signal densities. If POM transcripts are stored in

translationally inactive form until they are used in metamorphic animals, this may imply that considerable amounts of POM end-products may be necessary for the initiation of metamorphosis. Translational regulation has been shown to play a role in programming early development in the amphibian, *Xenopus laevis* and the surf clam, *Spisula solidissima* oocytes, where various mRNAs are associated with protein components believed to act as repressors of translation and are stored as messenger ribonucleoprotein particles (Kwon and Hecht, 1993). *Cis*-acting elements (specific RNA-protein binding sites) present in the 3' untranslated region (poly-A tail) of the mRNAs determine whether the mRNA is stored or immediately translated (Kwon and Hecht, 1993). Considering the possibility that most of the POM mRNA is present in NCF and CF larvae in a translationally inactive form, the mechanisms by which the mRNA is translationally regulated or stored do not appear to impede hybridization as very high levels of hybridization were detected during these stages. To determine whether or not POM is stored, an immunocytochemical correlation of type and quantities of end-products during these stages is necessary.

In light of these two possible scenarios, POMC storage has not been previously reported and in general, most mRNAs have a very short half-life of less than 5 hours due to an array of exogenous factors such as mRNases (for review see Ross, 1995). A decrease in the level of POMC mRNA due to a decrease of POMC gene transcription, or to an increased degradation of POMC mRNA or both has been reported to occur when the amphibian *Xenopus laevis* is transferred from a black to white background. This process is rapid and occurs within hours (Ayoubi *et al.*, 1992). Based on these observations, it would seem most likely that POM mRNA is not being stored. Alternately, the high signal densities in larval animals and decreased signal densities during early metamorphic stages are likely due to transcriptional activity alone.

The fluctuations of POM area fraction signal densities early in development are more clearly interpreted from volumetric measurements which do not reveal a decreased volume of POM at metamorphic stages 1, 3 and 4. Therefore, it is likely that the decreased area fraction (signal density) in stage 1, 3 and 4 animals is attributed to an increased area of PI and a proportionately less increased area of POM expressing cells, which contributes to a smaller area fraction but overall greater volume of POM expression. When considering all data, it appears that the net amount of POM expression is lowest in larval animals and increases with development. However, the fact that expression appears maximized in larval animals, that is, a very large proportion of the cells present are expressing POM, implies its importance in NCF and CF larval stages just prior to metamorphosis. Again, to determine the importance of POM expression it may be necessary to immunocytochemically correlate the types and quantities of the final end-products of POM with those of POM mRNA expression during these stages.

Regression analysis of the total volume of the PI and net amount of cells expressing POM reveal a linear relationship and any fluctuations, or variations in discrete points along the stages of development may be trial dependent. The PI and POM volume linear regressions depict a gradual linear relationship. More importantly, the two linear regressions appear to intersect early in development suggesting that POM transcription is being maximised in larval animals in what appears to be most, if not all, of the cells of the PI. The linear regression lines diverge with development indicating the presence of other differentiated cells not expressing POM.

4.2.2 Importance of POM-derived products

The mechanism by which POMC-derived peptides influence development is poorly

understood. However, the main activities of the largest peptides (ACTH, MSH, and β-END) are most well known. The role of MSH in melanin dispersion in melanophores of amphibia is well established (Smith-Gill and Carver, 1981). Of the three melanocyte stimulating hormones (α, β, and γ), α-MSH is the most potent involved in the dispersal of pigment granules in the cytoplasmic processes of dermal melanophores in amphibia (Hanneman *et al.*, 1989).

The role of α-MSH as a regulator of melanin dispersion, as seen in amphibians, is not as clear in teleosts. A few teleost species such as the rainbow trout, *Onchorhynchus mykiss*, European eel, *Anguilla anguilla*, and the yellow bullhead catfish, *Ictalurus natalis*, respond to MSH (Baker *et al.*, 1984). However, the involvement of α-MSH in melanin dispersion in the sailfin molly, *Poecilia latipinna* (Lamers *et al.*, 1992), and the flounder, *Pleuronectes flesus* (Baker *et al.*,1984), has not been demonstrated. In fact, no difference between the hormone contents, and secretory activity of the melanotrophs of pituitaries of flounder in different shaded backgrounds occurs even after three weeks. The melanophores of these and most teleosts, including trout, are under strong neural control which usually overrides the effects of MSH and controls pigmentation (Baker *et al.*, 1984).

To date, no α-MSH has been identified in the lamprey (Baker and Buckingham, 1983; Takahashi *et al.*, 1995a). However, *in vitro* assay of melanin-dispersing activities (assessed visually) of lamprey MSH-A and B and salmon α-MSH on frog skin (*Rhacophorus shlegelii*) revealed that MSH-B was 10 and 100 times more potent than salmon α-MSH and MSH-A respectively (Takahashi *et al.*, 1995b) suggesting that this POM end-product may be an important factor in pigment changes during lamprey transformation.

Pallor and concentration of melanin in larval lamprey caused by extirpation of the pars intermedia suggests that an MSH is secreted by the PI prior to metamorphosis (Young, 1935;

Barrington and Sage, 1966). The hybridization of the POM riboprobe in this study is specific for a fragment of the MSH-B region of the POM cDNA. The specific localization of the POM riboprobe in this study is the first to demonstrate the transcription and presence of the MSH-B mRNA fragment which implies the presence of the entire POM mRNA in the pars intermedia as early as the larval stages and throughout the entire development of the sea lamprey. It is at stage 5 of metamorphosis that the skin colouration of the sea lamprey starts to change from the larval to adult condition as noted by a progressive blue-black darkening of the dorsal surface and a silvering of the ventral surface (Youson and Potter, 1979). This change in pigmentation has been attributed to an increase in the secretory activity of the pre-existing granulated PI cells of the ammocoete as well as an overall increase in numbers of these cells as the region grows during this stage of the life cycle (Wright, 1983). Stage 6 of metamorphosis marks the period where POM signal density is greater than all younger metamorphic stages and the density of expression increases further, later reaching its highest levels during the upstream migration and spawning stages.

ACTH and α-MSH have been shown to exert corticosteroidogenic activity in the adrenal cortex of mammals and also in the interrenal gland of frogs, suggesting that the interrenal tissues of frogs can be considered homologs of the adrenal cortex of mammals (Leboulenger *et al.*, 1986). Also, α-MSH producing cells appear activated and levels of α-MSH are elevated during stress in fish indicating that α-MSH may be involved in adaptation to stress. Elevated levels of α-MSH during stress may indicate a role of the pars intermedia-interrenal axis in fish similar to that of corticotrophs and ACTH production of higher vertebrates in response to stressful stimuli (Lamers *et al.*, 1992). However, synthetic α-MSH has very low corticosteroidogenic potency in the frog as compared to synthetic ACTH (Leboulenger *et al.*, 1986), and is more than 100 times less potent than human

ACTH in stimulating cortisol release *in vitro* during a stress (low pH water) response in fish (Lamers *et al.*, 1992). These differences may imply that the interrenal tissue of lower vertebrates have varying sensitivities to α-MSH and perhaps there could be species differences among vertebrates with regard to the function of this POMC-derived peptide (Leboulenger *et al.*, 1986).

4.3 POC

4.3.1 Quantification

The expression of POC was first observed at low signal intensities in the RPD of larval animals and gradually increased throughout the remainder of the life cycle, with the greatest signal intensity observed during the spawning stages. Analyses of signal density and volume measurements of POC signal within the RPD correlates with qualitative observations, i.e. there is an increasing amount of signal and cells expressing POC throughout development. POC signal intensity and density are at their lowest during the larval stages. Upon initiation of metamorphosis at stage 1, POC signal density is significantly greater than that of the larval animals. This is in direct contrast to the very high POM expression in larvae followed by a decrease in stage 1 animals. The increase of POC signal density and apparent intensity may reflect an up regulation of this precursor upon initiation of metamorphosis.

Fluctuations of signal densities from stages 3 to 7 of metamorphosis made it difficult to conclude which if any stages express POC at significantly different levels. The signal densities of expression in earlier metamorphic animals sometimes appeared to be greater than those of later metamorphic animals. These variations in levels of expression may be animal or trial dependent. Variations in expression may also be sex dependent, however no attempt was made to determine sex

in any but spawning animals and variations in expression between sexes in spawners were not significant. POC signal densities increase gradually throughout metamorphosis and by stage 7, expression within the RPD reaches very high levels, the highest occurring during the spawning stage. The increase in expression may relate to the amount of differentiation that occurs in the RPD during metamorphosis. Through stages 1-5 of metamorphosis, the RPD is similar to that of the larval AH in that there is only one granulated and one non-granulated cell type. However, by stages 6 and 7, a second type of granulated cell appears (Wright, 1989). POC expression is confined to most cells of the RPD during all stages and by stage 5 of metamorphosis, expression signal is also evident in cells scattered within the CPD. The expression of POC in the CPD continues until the end of the life cycle when the animal reaches its spawning stage.

Volumetric analysis of expression also indicates a generally increasing amount of POC expressing cells. The linear regressions of total volume of the RPD and the total volume of POC expressing cells diverge during the course of development indicating that a greater volume of the region is not hybridized. This indicates the presence of other as yet undefined cell types.

The CPD displays the most pronounced morphological changes throughout metamorphosis. At the onset of metamorphosis the cells of the caudal pars distalis appear inactive and non-granulated cells predominate. During stage 3 and by stage 4 almost all cells of the CPD appear more synthetically active and granulated (Wright, 1989).

The signal densities of POC expression in the CPD animals older than stage 6 was significantly higher than those of stage 5 animals and no difference was noted between stage 6 through to juvenile parasitic adults. Expression in the CPD in spawning animals was significantly greater than all other stages. Volumetric analysis of the CPD showed that the volume of this lobe

did not increase significantly between stages 5 and juvenile adults. However, the volume of cells expressing POC in this lobe did increase significantly. The greatest volume of CPD and POC signal in the CPD was in spawning animals.

Northern blots of POC expression during development show low expression in the larval stages through metamorphosis and then an increase in expression in young adults and prespawners (J. Heinig, personal communication). This ISH study further details the results of these Northern blot analyses and shows that the levels of POC expression are constantly and steadily increasing throughout development (significantly higher by stages 6 and 7), reaching a maximum at the spawning stage.

4.3.2. Importance of POC-derived products

One of the major end products of POC is ACTH. The primary physiological function of ACTH in various species including dogfish, sheep, cow, pig and man, is to stimulate the steroidogenic activity and secretion of cortisol from the corticosteroidogenic tissue (McDonald and Pineda, 1989). In addition, Lowry *et al.* (1983) showed that peptides from the N-terminal of rat ACTH precursor proopiocortin (N-POC), not containing the γ-melanocyte-stimulating hormone (γ-MSH) can stimulate adrenal mitogenesis.

Bioassays using lamprey pars distalis extracts have stimulated bioactivity similar to the effects of mammalian ACTH (Larsen and Rothwell, 1972; Eastman and Portanova, 1982; Hardisty and Baker, 1982; Baker and Buckingham, 1983). More recently, a bioassay using synthetic lamprey ACTH incubated with spawning lamprey interrenal tissue, demonstrated steroidogenic activity

assessed by the ability of the ACTH to stimulate the conversion of radiolabelled 11-deoxycortisol to 11-deoxycorticosterone and deoxycorticosterone (Takahashi *et al.*, 1995b).

ACTH may be a major POC end product in the lamprey and this study has demonstrated that the levels of POC expression in lampreys increase during metamorphosis. Thus there may be a correlated increase in plasma levels of ACTH during lamprey metamorphosis. Yu *et al.*, (1985) have demonstrated the presence of an ACTH-like substance in tadpoles (*Rana catesbeiana*) that slowly increased early in development, and rose rapidly at the end of metamorphosis suggesting a possible correlation between the synthesis of ACTH and amphibian metamorphosis. The increasing levels of POC expression may indirectly imply a role of ACTH in lamprey metamorphosis as assumed for amphibian metamorphosis.

A large proportion of α-MSH producing cells have been identified in the pars distalis of fetal and neonate mammals (Hanneman *et al.*, 1989) and in larval, neotene (metamorphosed animal which has retained larval characteristics) and nonreproductive metamorphosed amphibians (Joss *et al.*, 1990a). In both mammals and the amphibians there is an increased production of ACTH and decreased production of α-MSH by the pars distalis cells as the animals approach maturity (Joss *et al.*, 1990a). It follows that the presence of a large population of α-MSH-producing cells in the pars distalis of adult lungfish (*Neoceratodus forsteri*) signifies the retention of a juvenile feature (neoteny) (Joss *et al.*, 1990a). Similarly, although no α-MSH has been identified in the lamprey, the increasing levels of POC expression in this study indirectly indicate the presence of ACTH and may be necessary for lamprey to achieve maturity.

The importance of PD products in lamprey metamorphosis has been described by Joss (1985) who determined by hypophysectomy that the RPD is required to initiate metamorphosis and

that without the CPD, metamorphosis only proceeds to stage 3. Hence the expression of POC in the CPD by stage 5 and the increased expression of POC in this lobe indicate that it may be required to sustain this process.

An immunocytochemical study using anti-sera directed towards rat prolactin (PRL) on upstream migrant *P. marinus* revealed a population of PRL-like cells in the dorsal region of the caudal pars distalis (Wright, 1984). The rat PRL antibodies however showed no reaction with cells in the RPD and the PI (Wright, 1984). Interestingly, in a histological study, the RPD of the marine alewife, *Alosa pseudoharengus* during its migration to fresh water was found to contain PRL, ACTH and TSH cells (Betchaku and Douglas, 1981). These three cell types are intermingled throughout the RPD suggesting some functional relation between PRL and ACTH cell types at a time when reproductive development and behaviour is associated with large changes of ambient salinity (Betchaku and Douglas, 1981). Betchaku and Douglas (1981) suggested that the position of the ACTH cells could expose them to the secretory product of the PRL cells. The fact that PRL-like cells, and POC expressing cells (this study) have been described as being similarly localized in the dorsal aspect of the CPD, may suggest a similar spatial and functional relationship in the lamprey pituitary. In most teleosts PRL is considered to be important in maintaining water and electrolyte balance (Gorbman *et al.*, 1983). The timing of the appearance of POC expressing cells in the CPD during the middle of metamorphosis occurs just prior to the lampreys' downstream migration to the sea from a fresh water habitat. The presence of PRL in the lamprey may suggest a similar function as the osmoregulatory mechanisms for lampreys are similar to those of teleosts (Hardisty, 1979).

A portion of the N-terminus domain of POMC has been reported to act synergistically with ACTH to stimulate adrenosteroidogenesis, adrenal DNA synthesis (Hanneman *et al.*, 1989) and

subsequent mitogenesis (Lowry *et al.*, 1983). Also, Tilemans *et al.* (1994), found an 11 kDa peptide, the N-terminal fragment of rat POMC to be a paracrine growth factor during postnatal development of the rat pars distalis. The fragment was isolated and purified by HPLC and SDS-PAGE from gonadotroph rich cell aggregates and the 10 peptides sequenced were identical to the first 10 residues of rat POMC. The POMC N-terminal mimics the effect of LHRH which stimulates *in vitro* development of lactotrophs in cell cultures of rat pituitary by selectively targeting and stimulating the number of lactotrophs entering or progressing through the S-phase of their cell cycle (Tilemans *et al.*, 1994).

Tilemans *et al.* (1994) suggest that the rat N-terminal POMC fragment is not only generated by corticotrophs as a product of POMC proteolytic cleavage but also by gonadotrophs. This assumption was based on the considerable amounts of lactotroph growth stimulating-activity assumed not to be contributed solely by the presence of a small number of corticotrophs present in a highly enriched medium of gonadotrophs. The fact that POMC is generated in gonadotrophs is also supported by other findings of ACTH-immunoreactivity in LH and FSH-containing cells in rat (Moriarty and Garner, 1977; Childs *et al.*, 1982). In postnatal rats the proportion of ACTH-immunoreactive gonadotrophs is high and decreases in older rats (Tilemans *et al.*, 1994).

In the lamprey, the N-terminus signal peptide of POC is followed by a polypeptide designated as nasohypophysial factor (NHF) (Heinig *et al.*, 1995; Sower *et al.*, 1995). NHF has no sequence homology to any other pituitary hormone and its function is not known (Sower *et al.*, 1995). It is interesting that NHF immunoreactivity in the majority of the cells of the RPD, and a few cells of the CPD of upstream migrant adult spawners (Sower *et al.*, 1995) is similar to the distribution of POC expression in metamorphic stages 5 to 7, parasitic and spawning lamprey. Hybridization to a 115

base pair fragment of the NHF domain of POC specifically localizes the expression of POC within the RPD and CPD. Although many RPD and CPD cells express POC, ACTH and NHF may be produced by either the same or different cell types. More importantly, in light of co-localization of ACTH in gonadotrophs of other vertebrates, it is possible that POC is expressed in gonadotrophs as well as corticotrophs of the lamprey PD. Whether NHF acts similarly to the rat N-terminus of POMC in stimulation of lactotrophs remains to be investigated.

4.4. Lipolytic potency of POM and POC end-products

The increasing levels of POM and POC during metamorphosis and very high levels in spawning animals suggests their involvement in lamprey development and sexual maturation. In mammals, α- and β-MSH have been proposed to mediate a variety of biological and behavioural activities including lipolysis, hypocalcemia, interaction with sex steroids, and inhibition of growth hormone release (LaHoste *et al.*, 1980). ACTH stimulates lipolysis and mobilizes fat from adipose tissue of rats and rabbits and is one of four lipolytic centres which has been suggested to be present in the POMC precursor (Richter and Schwandt, 1987). The remaining three include the N-terminal portion, the middle portion of the β-lipotropin (β-MSH) and in the C-terminal portion of β-lipotropin (β-END) (Richter and Schwandt, 1987). Adrenocorticotropin amino acids 4-11, and residues 1-13 corresponding to the sequence of α-MSH, also have strong lipolytic potency. The entire sequence (ACTH 1-39), has the greatest lipolytic potency. POM and POC domains may have similar lipolytic characteristics as those of POMC, however the lipolytic potencies of POM and POC derived products have not been assessed.

Lipolysis appears to be a very important physiological process in the life cycle of the lamprey. Lipids in the form of triglycerides are stored in the bodies of ammocoetes and adult lampreys during periods of intense feeding and are later utilized during periods of starvation, especially during metamorphosis and sexual maturation. Lipid accumulation in the whole body and in muscles is high prior to metamorphosis, declines during metamorphosis and is at its lowest at the end of metamorphosis. Then it increases during the feeding period and again declines during the period of migration and spawning (Hardisty and Rovainen, 1982).

Metamorphosis begins following a larval period of 3 to 5 years. In the last year, lipid is accumulated in order to sustain the animal through a non-trophic period of transformation (Holmes and Youson, 1993). This period is marked by a loss of weight but little or no reduction in length (Hardisty and Potter, 1971). The lipid of the musculature, consisting mainly of triacylglycerols can be obtained directly from fatty acids assimilated from the diet or created from non-lipid components of the diet by the muscle cells themselves (Cake *et al.*, 1995). It is known that corticotrophin inhibits basal and insulin-stimulated lipogenesis and in general, lipolytic hormones and insulin act antagonistically on the processes of lipolysis and lipogenesis (Ng, 1990). The low levels of POC expression in larvae seen in this study may indirectly implicate low levels of corticotrophin which would allow insulin-stimulated lipogenesis to occur during feeding. Also, β-END (a possible end-product of both POC and POM) has been reported to stimulate the secretion of insulin in humans (Hanneman *et al.*, 1989). The relatively high expression of POM and possibly high levels of β-END in larvae may implicate its role in stimulation of insulin secretion and lipogenesis.

Insulin levels in *P. marinus* do not change much in feeding larval animals and throughout most of the non-trophic metamorphic stages (when lipid stores are catabolized). They only increase

by stage 6, which may be due to the development of the endocrine pancreas (Youson *et al.*, 1994). It is interesting to note that insulin levels increase late in metamorphosis, prior to the lamprey's parasitic feeding stage. It is also by stages 6, 7 and the juvenile parasitic adult stage that POC is expressed at significantly higher levels than all other younger stages. These increased levels of insulin, POC and POM during stages 6 and 7 may be an important to the transition of the larval animal to the adult. It would seem inefficient however that the antagonistic roles of these hormones, insulin in lipogenesis and ACTH in stimulation of lipolysis would occur simultaneously. Therefore, lamprey corticotrophin may not be involved in inhibition of insulin-stimulated lipogenesis during the later stages of metamorphosis and the juvenile parasitic stage of the lamprey when lipid stores increase (Hardisty and Rovainen, 1982). If POM and POC end-products are involved in lipolysis, the increasing levels of their expression during the non-trophic metamorphic period and the very high levels during the spawning stages would correlate with the greater energetic demands during these periods.

During the parasitic stage at sea, female lampreys undergo vitellogenesis and males undergo spermatogonia proliferation with development of primary and secondary spermatocytes (Linville *et al.*, 1987). Following this parasitic period, the lampreys migrate to streams and undergo their final sexual maturational and spawning phase over a period of 2-3 months. During the lampreys' upstream migration, there are many degenerative changes which occur, including cessation of feeding, a shrinkage in length and weight, atrophy of the intestine and closure of the foregut, shedding of the functional sharp cusps, degeneration of the eyes, and the loss of marine osmoregulatory mechanisms with replacement by adaptations to freshwater life (Hardisty and Potter, 1971). Again, the lamprey relies to a great extent on internal lipid and protein substrates as the

principle source of energy to develop the gonads or to sustain migratory activity and eventually spawning (Hardisty and Baker, 1982).

The importance of lipid metabolism is reflected by the high levels of plasma free fatty acids in the migratory lamprey (Hardisty and Baker, 1982). Lipolysis frees fatty acids to maintain muscle activity, leaving glycerol to be used in gluconeogenesis for maintenance of adequate muscle glycogen content (Hardisty and Baker, 1982). The energetic demands of the upstream migration must be met by catabolism of stored carbohydrate, fat and protein. Little is known about the roles of the anabolic and catabolic hormones in lamprey during this period. However, the fact that both POM and POC are expressed at very high levels during the spawning stages may invoke their role in this process, either in lipolysis and maintenance of energetic demands or in some other homeostatic process.

4.5. Sexual maturation

The gonads of larval lampreys are immature at the initiation of metamorphosis (Hardisty, 1971). Reports of corticosteroids, male and female sex steroids, and progesterone present in the blood of *P. marinus* larvae with undifferentiated gonads and poorly developed adrenal cortex equivalents may originate from tissue not commonly considered major sources of steroids or a dietary uptake of these steroids (Dashow *et al.*, 1984). There have been no studies correlating plasma sex steroid concentration with the different reproductive stages and the functional role of various sex steroids cannot be resolved until lamprey steroids have been identified and correlated with biological actions during reproductive processes (Sower *et al.*, 1985). Youson and Sower (1991) suggested that in *P. marinus*, metamorphosis is essential before the final phases of maturation

of gonads can occur. In parasitic lamprey species, gonad maturation occurs following metamorphosis, and is spread out over the feeding period (several months up to 2 years) but the stimulus for this development in sea lampreys likely occurs before or immediately after metamorphosis is complete (Youson and Sower, 1991; Wright *et al.*, 1994).

Accelerated oocyte growth during metamorphosis of the lamprey, *Geotria australis*, led Hardisty *et al.* (1986) to believe that pituitary gonadotrophins or other endocrine factors are involved in sexual maturation. Immunocytochemical studies of GnRH in lamprey demonstrated low intensity of immunostain in the cells and fibres of the brain and neurohypophysis in larvae, but a marked increase by stage 5 of metamorphosis and a consistent positive reaction until the end of the life cycle, the greatest immunostain intensity occurs in juveniles and prespawning adults (Wright *et al.*, 1994). The changes in GnRH immunoreactivity correlate with increased concentration of brain GnRH determined by RIA measurements of HPLC fraction of lamprey brain during development (Youson and Sower, 1991). These increases in GnRH may indicate that there have been either marked morphological changes in the brain or that existing cells within the brain have been stimulated to synthesize the hormone (Youson and Sower, 1991). The increased concentrations of brain GnRH also correlate with the timing of presumed changes in the activity of cells in the adenohypophysis during metamorphosis (Wright, 1989) and as well as the timing of increased POC expression in the CPD at stage 5.

In fact POC may be a modulator in brain development as ACTH-related peptides have been found to be neurotrophic, as they regulate astrocyte proliferation and morphological differentiation *in vitro* (Zohar and Salomon, 1992). Although no POC expression (and presumably ACTH) has been localized in the brain, systemic ACTH may also target the brain and exert a stimulatory effect similar

to the neurotrophic effects described in gnathostomes.

4.6 Summary

In vertebrates the melanotrophins, corticotrophins and endorphins are encoded on a single precursor gene, POMC. In lampreys however, two separate genes encode these hormones, POM encoding melanotrophins and endorphin, and POC encoding corticotrophin, endorphins and a novel nasohypophysial factor (NHF). This study is the first to describe spatial, temporal and quantitative analysis of the expression of any lamprey pituitary hormone mRNA throughout its life cycle.

The localization of POC or POM mRNA within the cells of the lamprey adenohypophysis does not provide identification of the final end-products of the cells. It will be necessary to generate specific antisera towards the many possible translational end-products of POC and POM to corroborate their temporal and spatial distributions with those of POM and POC transcripts found in this study.

Immunocytochemical studies of possible POC (ACTH) and POM end-products (MSH-A and MSH-B) has been examined in upstream migrant spawning lamprey (Nozaki *et al.*, 1995). However, immunocytochemical localization of these and other end-products including NHF and β-END must be expanded to include all or as many stages of development as possible. It would seem especially important to define both qualitatively and quantitatively the pituitary hormone(s) produced just prior to metamorphosis in larval animals and during early metamorphic development. The final end-product(s) of the POM and POC expressing cells localized in this study may be different in different cells. Variations in quantities of POM and POC end-products may implicate possible roles of these products during different stages of development.

With what is known of the functions of POMC-derived products in gnathostomes and the expression patterns described in this study, we can only speculate on the possible roles of POM and POC end-products during development of the lamprey. Previous bioassays demonstrating the well known roles of ACTH in stimulation of corticosteroid production and MSH stimulation of melanin dispersion have been described. However, bioassays exploring other possible roles of POC and POM end-product, as discussed in this study, need to be examined. These include the role of lamprey MSHs on the interrenals, ACTH and NHF stimulation of lactotroph development, the role of lamprey ACTH in insulin-stimulated lipogenesis, and the role of POC and POM end-products in lipolysis and sexual maturation.

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6. APPENDIX A

The stringency at which an *in situ* hybridization experiment is carried out determines the approximate percentage of nucleotides that are correctly matched in the probe and target nucleic acid molecule, calculated using the equation (Leitch *et al.*, 1994):

Stringency (%) = 100 - M_f (t_m - t_a)

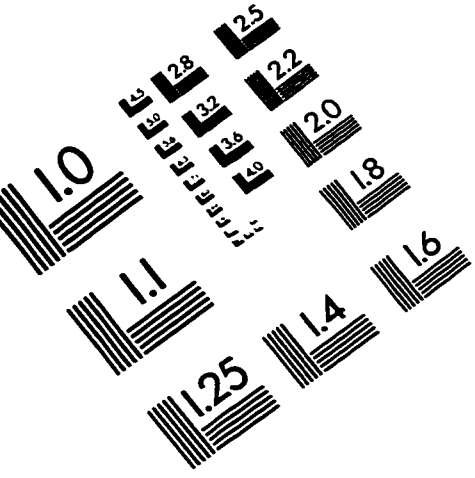
where M_f = mismatch factor (1 for probes longer than 150 bp, 5 for probes shorter than 20 bp).
t_m = calculated melting temperature (°C; T_m)

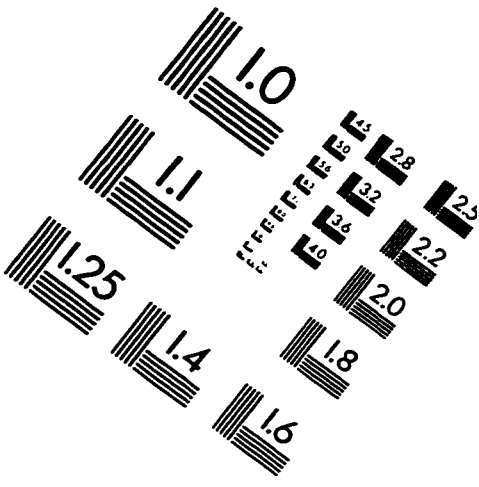
$$T_m = 0.41\ (\%GC) + 16.6 \log M - (500 / n) - 0.61\ (\%formamide) + 81.5$$

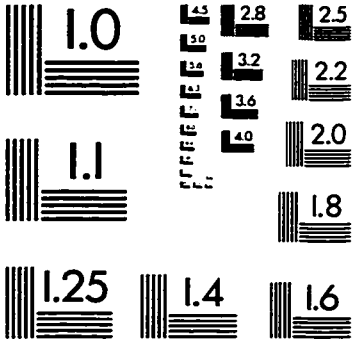
where T_m= melting temperature (°C), (%GC) = percentage of guanine and cytosine in the probe sequence, M = concentration of monovalent cations (Na^+) (1 / mol) in the hybridization solution, n = probe length in base pairs, % formamide = concentration of formamide expressed as a (v/v) percentage. T_m for RNA:DNA hybrids is 10 - 15 °C higher. T_m for RNA:RNA hybrids is 20 - 25 °C higher.

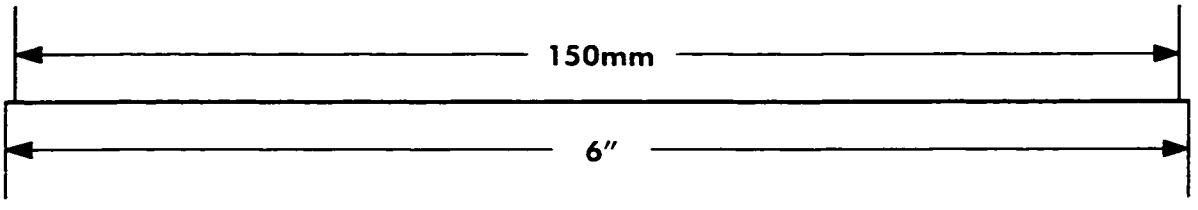
t_a = temperature (°C) at which the *in situ* hybridization mixture or washing conditions were used.

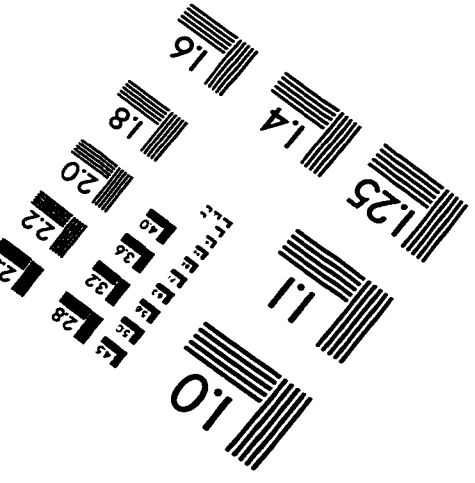
IMAGE EVALUATION
TEST TARGET (QA-3)

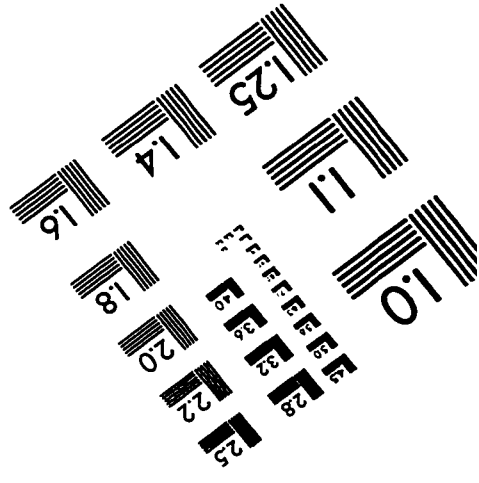












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