UNIVERSIDADE DO ALGARVE

Faculdade de Ciências do Mar e do Ambiente

Characterization of sea bream (*Sparus aurata* L.) prolactin and control of release from pituitary gland

Lília Figueiredo Brinca

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This dissertation was submitted to the Universidade do Algarve for obtention of a PhD degree in Biological Sciences,
Area of Animal Physiology.

Declaration

I hereby declare that my dissertation contains material that has not been submitted for a degree or diploma or any other qualification at any other university. The contents of this dissertation is of the exclusive responsibility of the author

To Toni and Debbie

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Abstract

The sea bream (*Sparus aurata* L.) is an important aquaculture species along the Mediterranean coast. Prolactin (PRL) released by the pituitary gland play a central role in controlling several aspects of sea bream physiology. In this study, we examined the effect of several internal and external factors on the activity of PRL cells in cultured pituitary glands. Sea bream pituitaries were cultured during 18hrs at 21°C and the hormones present in the culture medium separated by SDS-PAGE. PRL was identified by Western blotting using antiserum directed against chum salmon PRL and quantified by optical densitometry.

A highly purified PRL was isolated from the medium of cultured pituitaries by continuous elution electrophoresis performed using a Model 491 Prep Cell. The yield of purified hormone was 3mg/g wet weight of pituitary. Sea bream PRL had an estimated molecular weight of 25kDa after SDS-PAGE. PRL did not show size heterogeneity, but multiple charge variants were detected with isoelectric points varying between 6.1 and 6.7. The partial amino acid sequence established did not reveal genetic differences between PRL molecules. Post-translational modifications were not clearly demonstrated but a potential phosphorilation at S_{158} is proposed on the basis of the present data.

Seasonal and diurnal changes of PRL release to the culture media and pituitary content were investigated in immature seabream. A marked annual cycle in the pituitary gland activity was observed. This is highlighted by the seasonal variation in the basal pituitary PRL release rates but also by a variation in the relative concentration of PRL charge variants released from the pituitary gland. Circadian changes were also observed. Temperature (but not photoperiod) influences sea bream PRL cells activity, with higher temperature increasing *in vitro* PRL secretion and lower temperature having the opposite effect.

The effect of estradiol-17β (E_2) implants on the *in vitro* secretion of PRL and its modulation by galanin (Gal) was determined. Experiments were conducted during winter and spring and it was observed that PRL cells responsiveness to E_2 varied with season. In control fish Gal caused a dose-dependent stimulation of PRL secretion *in vitro*, but on E_2 primed fish Gal had no detectable effect on the secretion of PRL.

The effect of E_2 implants on the *in vitro* secretion of PRL and its modulation by vasoactive intestinal peptide (VIP) was determined. Experiments were conducted during winter and spring and PRL cells responsiveness to E_2 varied with season. In E_2 primed fish VIP caused a dose-dependent inhibition of PRL secretion *in vitro*. VIP had no detectable effect on the secretion of

PRL from control pituitaries. Anatomical evidence of abundant VIP immunoreactive nerve fibres in neurohypophysial (NH) tissue penetrating the rostral pars distalis provide further evidence supporting an action for VIP in the regulation of PRL cells.

PRL is believed to be involved in the adaptation of fish to changes in environmental salinity. The effect of freshwater challenge on *in vitro* PRL release was studied in fish sampled 7 days after the onset of seawater dilution. Experiments were conducted during winter, spring, and autumn. Results indicate that cultured pituitaries of fish challenged with extremely low values of salinity (2ppt) released significantly more PRL that pituitary glands from seawater-adapted fish. This suggests a potential role for PRL in FW adaptation of sea bream. Moreover, the study indicates that the success of adaptation may depend on the time of year at which transfer to freshwater occurs.

Resumo

A dourada (*Sparus aurata* L.) é uma espécie importante para a aquacultura ao longo de toda a costa mediterrânea. A prolactina (PRL) é uma hormona libertada pela glândula pituitária e regula de um modo fundamental alguns aspectos da fisiologia deste teleósteo. No presente trabalho foi estudado o efeito que diferentes factores, externos e internos, exercem na actividade *in vitro* das células de PRL. Para este estudo foram feitas culturas de pituitárias de dourada com uma duração de 18 horas a uma temperatura média de 21ºC. A PRL, após separação por SDS-PAGE, foi identificada por "Western blotting" utilizando anticorpos da PRL do salmão e, posteriormente, quantificada por densidade óptica.

Por electroforese de eluição contínua e utilizando uma "Prep Cell, modelo 491", foi purificada a PRL secretada *in vitro*, tendo-se obtido um rendimento aproximado de 3mg/grama de peso fresco de pituitária. A hormona purificada tinha um peso molecular aparente de 25kDa em SDS-PAGE e formas com pontos isoeléctricos variando entre 6.1 e 6.7. A identificação da proteina purificada foi feita por "Western blotting" e pela sequência parcial de amino ácidos. Não foi possível demonstrar a existência de variantes genéticas nas moléculas de PRL isoladas, tendo sido identificado um local potencial de fosforilação no resíduo S_{158}.

A secreção *in vitro* de PRL apresenta um evidente ciclo anual, não só na quantidade de hormona secretada mas também nas formas com diferentes pontos isoeléctricos que são produzidas ciclicamente.. Também foi observado um nitido ciclo diário na secreção *in vitro* de PRL. A temperatura influencia directamene a actividade das células de PRL, mas não foi demonstrada nenhuma influência do fotoperíodo sobre a actividade daquelas células.

Foi estudado o efeito do tratamento *in vivo* com estradiol-17b (E_2) modulado *in vitro* pela galanina (Gal). As experiências decorreram durante o inverno e primavera, tendo-se verificado uma diferença estacional na resposta das células de PRL aos implantes do estrogénio. O efeito da Gal depende do nível do estrogénio no corpo dos animais, uma vez que estimula a secreção *in vitro* de PRL de pituitárias colhidas de peixes sem implantes de E_2 mas não tem qualquer efeito nas pituitárias colhidas de animais tratados com o estrogénio.

Também foi estudado o efeito do tratamento *in vivo* com E_2 modulado *in vitro* pelo peptídeo vaso-intestinal (VIP). O efeito do VIP depende do nível de E_2 no corpo dos animais, uma vez que inibe *in vitro* a secreção de PRL de pituitárias collhidas de animais tratados com o E_2 e não tem qualquer efeito nas pituitárias colhidas de animais sem implantes do estrogénio.

A PRL está relacionada com a adaptação dos teleósteos às mudanças da salinidade do meio. No presente trabalho, foi estudado o efeito sobre a secreção *in vitro* da PRL da transferência de douradas adaptadas a água salgada para água doce .(2ppt). Foram realizadas experiências no inverno, primavera e outono em que os animais foram sacrificados 7 dias após o início da mudança gradual para a água doce. Os resultados indicam que as células de PRL libertam *in vitro* quantidades significativamente maiores da hormona em animais transferidos para a água doce em comparação com as dos animais mantidos em água salgada. A PRL parece assim ter um papel importante na adaptação da dourada à diminuição da salinidade do meio, estando o sucesso da adaptação dependente da época do ano em que é feita a transferência para a água doce.

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ACTH	Adrenocorticotropic hormone
aa	Amino acid
α-MSH	α- melanophore-stimulating hormone
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAs	Catecholamines
cDNA	Complementary DNA
DA	Dopamine
E_2	Estradiol 17β
EGF	Epidermal growth factor
ETs	Endothelins
FW	Freshwater
GABA	Gamma-aminobutyric acid
Gal	Galanin
GalR	Galanin receptor
GH	Growth hormone
GH_3	Cell line (rat pituitary tumor)
GHRH	Growth hormone releasing hormone
GIP	Glucose-dependent insulin
GnRH	Gonadotropin releasing hormone
GtH	Gonadotropin
IgG-PAP	Immunoglobulin G-peroxidase-antiperoxidase
IT	Isotocin
LHRH	Luteinizing hormone releasing hormone
mRNA	Messenger RNA
MSH	Melanocyte-stimulating hormone
NGF	Nerve growth factor
NH	Neurohypophysial
NPY	Neuropeptide Y
NT	Neurotensin
OT	Oxytocin
PBS	Phosphate buffered saline
PI	*Pars intermedia*
PIF	Prolactin inhibiting factor
PL	Mammalian placental lactogen
POMC	Proopiomelanocortin
PPD	*Proximal pars distalis*
PRL	Prolactin
PRLR	Prolactin receptor
RPD	*Rostral pars distalis*
SDS	Sodium duodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SL	Somatolactin
SRIF	Somatostatin release inhibiting factor
SS	Somatostatin
SW	Seawater
TEMED	N' N' N' N' - tetramethyethylemediamine
TGFs	Transforming growth factors

TRH	Thyrotropin releasing hormone
TRIS	Tris(hidroxymethyl)aminomethane
TSH	Thyroid stimulating hormone
VIP	Vaso-intestinal peptide

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CHAPTER 1: General Introduction

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The gilthead seabream (*Sparus auratus*) is an important aquaculture species in the Mediterranean area (FAO, 2002). Its commercial production started in the early 1980s and since then production areas have spread and in southern Europe its cultivation has become predominant over other finfish species. This expansion is related to its relatively straight forward husbandry and the initial high market price of this species, although more recently with the increase in its availability prices have declined.

The gilthead sea bream is a marine teleost and belongs to the Perciformes, a group which includes a number of other commercially interesting species, such as couch's sea bream (*Pagrus pagrus)*, sea bass (*Dicentrarchus labrax)*, Nile tilapia (*Oreochromis nilotica)*, and Mozambique tilapia (*Oreochromis mossambicus*). Due to the commercial value of gilthead seabream the ecology and biology of this species has been extensively studied. The sea bream is found in both marine and brackishwater environments such as coastal lagoons and estuarine areas, in particular during the initial stages of its life cycle (Moretti *et al.,* 1999). Born in the sea during wintertime, the fingerlings typically migrate in early spring towards protected coastal waters in search for abundant food and milder temperatures, and return to the open sea in late autumn, where the adult fish breed (Moretti *et al.,* 1999).The sea bream is a protandrous hermaphrodite, i.e., individuals spawn as males during the first breeding season but may undergo sex reversal in one of the subsequent seasons. The spawning season in southern Portugal extends from October until February (Condeça, 2001).

Homeostasis of a range of physiological processes in fish, in common with other vertebrates, is regulated by the endocrine and neuroendocrine systems. The latter system regulates development and physiology in fish. Reproduction, development, immune function and environmental adaptation are regulated through the orderly release of hormones by the neuroendocrine system, which integrates information from genes and the environment. One of the most versatile hormone is prolactin (PRL) which has numerous biological actions (reviewd by Sinha, 1995 and Bole-Feysot *et al.,*1998;). Since the discovery, by Stricker and Grueter in 1928, of a pituitary factor capable of stimulating milk secretion in rabbits, a wealth of knowledge has accumulated about prolactin (PRL).

1.1 Struture and evolution of PRL

PRL belongs to a family of polypeptide hormones, which includes growth hormone (GH), mammalian placental lactogen (PL), and teleostean somatolactin (SL). Analysis of their amino acid sequences demonstrates that these hormones are highly conserved, and it has been proposed that they evolved from a common ancestral gene by duplication and subsequent divergence about $4x10^8$ years ago (Miller and Eberhardt, 1983; Nicoll *et al.,* 1986).

Mammalian PRL is a single polypeptide chain of approximately 190-200 amino acids (aa) and is synthesized as a prohormone containing a signal peptide of approximately 28 aa (Bole-Feysot *et al.*, 1998). The full-length amino acid sequence of PRL has been determined in mammals, birds, reptiles and amphibian. All PRLs identified so far in tetrapods are 197-199 aa and contain six

cysteines forming three intramolecular disulfide bonds, one at the N-terminus, one in the middle, and one at the C-terminal (Bole-Feysot *et al.*, 1998 and Manzon, 2002 for reviews). The amino acid sequence of PRL has also been characterised in a variety of teleostean and nonteleostean fish (reviewed by Manzon, 2002). Piscine PRLs are also synthesized as prohormones with a signal peptide of 23-24 aa. All teleostean PRLs lack the N-terminal disulfide bond due to the absence of 12-14 aa at the N-terminus (see Manzon, 2002 for review) (Fig. 1.1). PRL from sea bream is a protein of 212 amino acids with a putative signal peptide of 24 residues and a mature protein of 188 amino acids (Santos *et al.*, 1999).

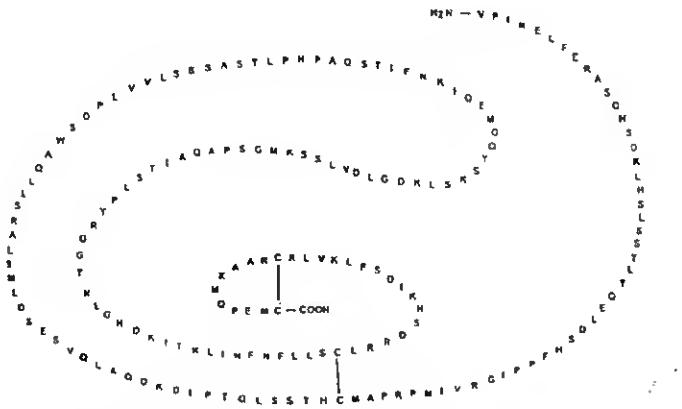

Figure 1.1 – Teleost PRLs (i.e. Nile tilapia PRL_{188}) lack the N-terminal disulfide bridge present in mammals (Manzon, 2002).

1.2 Isoforms

PRL is characterized by structural as well as functional polymorphism. The existence of both size and charge variants of PRL has been shown in mammals (Wallis *et al.*, 1980; Nyberg et al., 1982; Bollengier *et al.*, 1988; Lewis *et al.*,

1989; Martinat *et al.*, 1990; Sinha *et al.*, 1991; Briski *et al.*, 1996;), and two molecular forms of PRL have been isolated from pituitary glands of two reptiles, alligator and crocodile (Noso *et al.*, 1992), and from *Xenopus laevis* (Yamashita *et al.*, 1993). In turkeys, PRL is present in the pituitary gland as three different isoforms, a nonglycosylated form of approximately 22.5 KDa and two glycosylated forms of 24.5 KDa, which comigrate on SDS-PAGE (Corcoran and Proudman, 1991). In some teleosts, chum salmon (*Oncorhynchus keta*), common carp (*Cyprinus carpio*), Japanese eel (*Anguilla japonica*), Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*Oreochromis niloticus*), two different forms of PRL have been identified (Manzon, 2002 for review). The two salmon, carp, and eel PRLs are highly homologous, whereas the two forms of PRL secreted by tilapia pituitary share only 69% sequence identity and are designated tPRL177 and tPRL188 to indicate the number of amino acid residues in each isoform (Specker *et al.*, 1993). The biological activities of the two variants in the maintenance of the hydromineral balance are different as is their effect on growth. In addition to their differing biological activities, tPRL177 and tPRL188 are also differentially regulated during development and in response to alterations in environmental salinity (see Manzon, 2000 for review). To date only a single form of pituitary PRL has been isolated in sea bream (Santos *et al.*, 1999).

The source of prolactin variants may be by mechanisms such as alternative splicing of the primary transcripts, proteolytic cleavage of the protein,

and other posttranslational modifications, such as glycosylation and phosphorylation of the amino acid chain.

ALTERNATIVE SPLICING - Alternative splicing of PRL mRNA has been proposed as one source of the variants (Sinha, 1995). Indeed, evidence suggestive of the existence of an alternatively spliced prolactin variant has been described in the rat anterior pituitary (Emanuele *et al.*, 1992; Wilson *et al.*, 1992). However, in general alternative splicing is not considered a major source of prolactin variants.

PROTEOLYTIC CLEAVAGE - Some PRL size variants may be the products of kallikrein enzymatic activity. Studies *in vitro* have shown that kallikrein is an estrogen-induced, trypsin-like serine protease, that is found in the Golgi cisternae and secretory granules of rat lactotrophs, which cleaves the 25KDa form to a 22KDa form in a thiol-dependent manner (Powers, 1993). Thiol alters the conformation of PRL such that kallikrein recognizes it as a substrate (Anthony and Powers, 1993). These proteolytic fragments were found to be released in substantial amounts during short incubations of rat pituitaries (Anthony *et al.*, 1993).

OTHER POSTTRANSLATIONAL MODIFICATIONS - The majority of prolactin variants are probably the result of other posttranslational processing of the mature molecule in the pituitary gland or the plasma. These modifications

include dimerization and polymerization, phosphorylation, glycosylation, sulfation, and deamidation.

Dimerization and polymerization: macroprolactins.

High molecular weight forms of PRL are encountered in significant amounts both in the pituitary gland and plasma. They represent dimers, polymers and aggregates of PRL, and PRL associated with binding proteins. They arise by both covalent (disulfide linkages and linkages between the sugar moieties of the glycosylated monomer) and noncovalent bonding. In general, these forms have reduced biological activity in comparison to the monomer (Sinha, 1995).

Phosphorylation.

PRL phosphorylation occurs within the secretory vesicle of lactotrophs just before exocytosis and involves esterification of hydroxyl groups of serine and threonine residues (Freeman *et al.*, 2000 for review). Phosphorylation of serine or threonin may occur when glutamic or aspartic acid is situated two residues away C-terminally (Ser/Thr – X – acidic). Serine residues that occur C-terminally to groups of basic amino acid residues may also be phosphorylated (basic – basic – X – Ser/Thr) (Dimaline, 1988). Ocurrence of phosphorylated isoforms has been demonstrated in the case of rat, bovine, murine, and avian PRLs (Sinha, 1995 for review). Phosphorylation generally lowers the biological activity of PRL, but interaction of heterogenous hormonal forms at the level of target cell receptors may influence the magnitude of the biological effect. For example,

Wang and Walker (1993) reported that the biopotency of nonphosphorylated PRL variants is diminished as a consequence of coincubation with phosphorylated PRL, findings which suggest that posttranslational phosphorylation of the native molecule may generate a natural "antagonist" to the biological activity of non-modified variants. In rats, *in vivo* secretion of phosphorylated and nonphosphorylated PRL or their ratio, varies at different stages of the estrous cycle (Sinha, 1995 for review).

Glycosylation.

Glycosylated PRL has been found in the pituitary glands of mammalian, amphibian, and avian species. The linkage of the carbohydrate moiety may be either through nitrogen (N-glycosylation) or oxygen (O-glycosylation). The consensus sequence for N-glycosylation of aspargine is Asn – X – Thr/Ser (Dimaline, 1988). In several mammals and reptiles, in which glycosylated PRL occurs is N-glycosylation. In rat and turkey, the carbohydrate chains are attached through O-linkage (Sinha, 1995 for review). The carbohydrate residues of the oligosaccharide chain may contain varying ratios of sialic acid, fructose, mannose, and galactose that differ considerably between species and also vary with physiological and pathological states. As observed for other PRL variants, glycosylation also lowers biological activity and receptor binding, and alters the metabolic clearance rate of PRL (Freeman *et al.*, 2000 for review).

There is no published evidence in sea bream for the presence of glycosylated or phosphorylated forms of PRL. However, analysis of the primary

amino acid sequence reveals a consensus sequence for N-linked glycosylation at Asn 148 (N-I-S) and for phosphorylation at Ser 166 (R-R-D-S) (Santos *et al.*, 1999).

1.3 Sites of synthesis and secretion of PRL

1.3.1 Pituitary PRL

The principal site of production of PRL is the pituitary gland (Fig 1.2). In all vertebrates, the pituitary gland consists of two parts, separable on the bases of embryology, structure, and function. These are the neurohypophysis, a downgrowth from the floor of the diencephalon, and the adenohypophysis originating as an ectodermal upgrowth (Rathke's pouch) from the roof of the embryonic buccal cavity (Ball and Baker, 1969). The adenohypophysis is divided into the *pars distalis*, site of secretion of most adenohypophysial hormones, and the *pars intermedia* (PI).

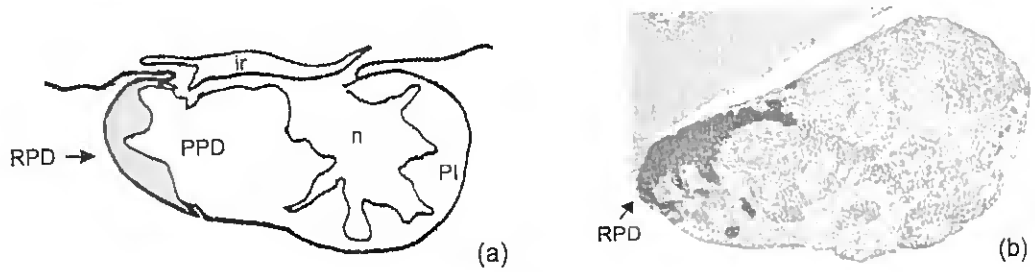

Figure 1.2 – (a) Diagram showing a sagital section through the pituitary gland of the adult sea bream with the distribution of PRL-cells indicated by a shaded area. (b) Immunohistochemistry of a sagital section of sea bream pituitary gland counterstained with haematoxylin with PRL cells revealed by anti-chum salmon prolactin (magnification x100). (n) neurohypophyseal tissue; (RPD) *rostral pars distalis*; (PPD) *proximal pars distalis*; (PI) *pars intermedia*; (ir) infudibular recess.

The adenohypophysis *pars distalis* is subdivided into *rostral pars distalis* (RPD) and *proximal pars distalis* (PPD), on the basis of cell types. The fish neurohypophysis consists essentially of a hypophysial stalk, suspending the gland from the ventral region of the diencephalon (hypothalamus) and containing an extension of the third ventricle (infudibular recess), and at the distal end of the stalk an enlargement, the neurohypophysial lobe or core (Ball and Baker, 1969). The adenohypophysis is a complex and specialized endocrine tissue that contains a functionally heterogeneous population of hormone secreting cells. At the dorsal surface of the pituitary lies the hypothalamus which is responsible for integration of a whole range of inputs from other brain centers and for the control of visceral functions, in many cases via its effects on the secretions of the pituitary gland (Chester-Jones *et al.*, 1987). In teleosts, the hypophysial stalk, which suspends the pituitary gland from the hypothalamus, do not have the median eminence-portal system of other vertrebrates. The blood to the pituitary is supplied by one (or two) hypophyseal artery (ies), and although this runs along the floor of the hypothalamus for a variable distance, no neurosecretory terminations on the vessels in this region have ever been observed (Chester-Jones *et al.*, 1987).

The hypothalamus regulates the synthesis and secretion of six major hormones: prolactin (PRL), growth hormone (GH), thyroid stimulating hormone (TSH), adrenocorticotropin (ACTH), melanocyte-stimulating hormone (MSH), and gonadotropin (GtH). In the adult sea bream, PRL cells are confined to the RPD and occupy a relatively small proportion of the pituitary (Fig. 1.2). They are oval

in shape and do not appear to be arranged in follicles (Power and Canario, 1992).

1.3.2 Extrapituitary PRL

In higher vertebrates the principal site of production of PRL is the pituitary gland, but, as reviewed by Ben-Jonathan *et al.* (1996), the PRL gene is also expressed in several PRL target tissues and PRL occurs in the mammary gland of rat, human, goat, sheep, and rabbit (Nolin and Witorsch, 1976; Fields *et al.*, 1993; Kurtz *et al.*, 1993; Le Provost *et al.*, 1994; Gabou *et al.*, 1996) and in mammalian brain and spinal cord (Emanuele *et al.*, 1992; Wilson *et al.*, 1992).

Recently, PRL transcripts were also detected at some extrapituitary sites (liver, intestine, and gonads) of the sea bream (Santos *et al.*, 1999) and in the liver, kidney, spleen, gill, muscle, gonads, and brain of goldfish (Imaoka *et al.*, 2000).

1.4 PRL receptors (PRLRs)

The effects of PRL on target tissues are mediated by the PRL receptor (PRLR), which belongs to the cytokine receptor family (Bole-Feysot, 1998). In mammals, there are long, intermediate, and short PRLR isoforms (Freeman *et al.*, 2000). All fish PRLR cDNA encode for a mature protein of approximately 600 aa in length and are most similar in appearance to the long form of mammalian PRLRs (Manzon, 2001). The isolation and characterization of fish PRLRs has revealed that several important functional domains, receptor activation

mechanisms, and signal transduction pathways have been conserved between fish and mammals. Generally, the highest PRLR expression levels were observed in the primary osmoregulatory organs (gills, kidney, and intestine). However, PRLR transcripts were also detected in other tissues such as the brain, gonad, liver, muscle, skin, spleen, head kidney, lymphocytes, and bone of some fish (Manzon, 2001 for review). In the sea bream, PRLR expression was also detected in early post-hatching stages of larvae (Santos *et al.*, 2003).

!.5 PRL actions on target tissues

Pituitary PRL acts via a classic endocrine pathway, *i. e.*, it Is secreted by the pituitary gland, transported by the circulatory system, and acts on target cells at some peripheral sites via specific receptors located on the plasma membrane. The interaction of PRL with its receptor in various target cells leads to the activation of intracellular events that ultimately promote PRL-responsive genes responsible for a biological activity in the animal.

In mammals, PRL binding to its receptor leads to dimerization and activation of an intracellular cascade mediated by JAK/STAT signal transduction pathway (Ihle, 1996). The Janus kinase JAK2 is constitutively associated with the PRLR and is activated upon PRL binding. Following activation of JAK2, tyrosine residues on the PRLR and the transcription factor STAT5 are phosphorylated (Han *et al.*, 1997). Activated Stat proteins translocate into the nucleus where they bind DNA consensus motifs to mediate activation of target genes (Bole-Feysot *et al.*, 1998).

PRL is derived primarily from the anterior pituitary and acts on tissues widely distributed through the body (endocrine action). But locally produced PRL can act on adjacent cells (paracrine action) or on the PRL-secreting cell itself (autocrine action). Intriguingly a paracrine and autocrine mechanisms could activate many of the actions associated with PRL, without affecting the circulating concentration of the hormone (Bole-Feysot *et al.*, 1998) and may explain the apparent discrepancies of some experiments where despite unchanging levels of circulating hormone specific biological activities are observed.

1.6 Regulation of PRL synthesis and secretion

The secretory cells of the anterior pituitary are influenced by a wide array of factors. The general and well-accepted view is that PRL secreting cells have a spontaneously high secretory activity. Therefore, pituitary PRL secretion is under a tonic and predominantly inhibitory control exerted by a hypothalamic PRL-inhibiting factor (PIF) that restrains *in vivo* PRL secretion (reviewed by Freeman *et al.*, 2000) (Fig. 1.3). But PRL release is also influenced by other secretagogues and gene regulators. The old notion that secretagogues act rapidly while gene regulators act slowly has been questioned when results on the induction of genes such as *c-fos* are considered. The two types of regulators may be better classified by their preferred utilization of cellular compartments, i.e. secretagogues act on the cell membrane and activate calcium-dependent exocytosis while gene regulators, directly or indirectly, utilize the nuclear compartment (Ben-Jonathan *et al.*, 1996 for review). These secretagogues and

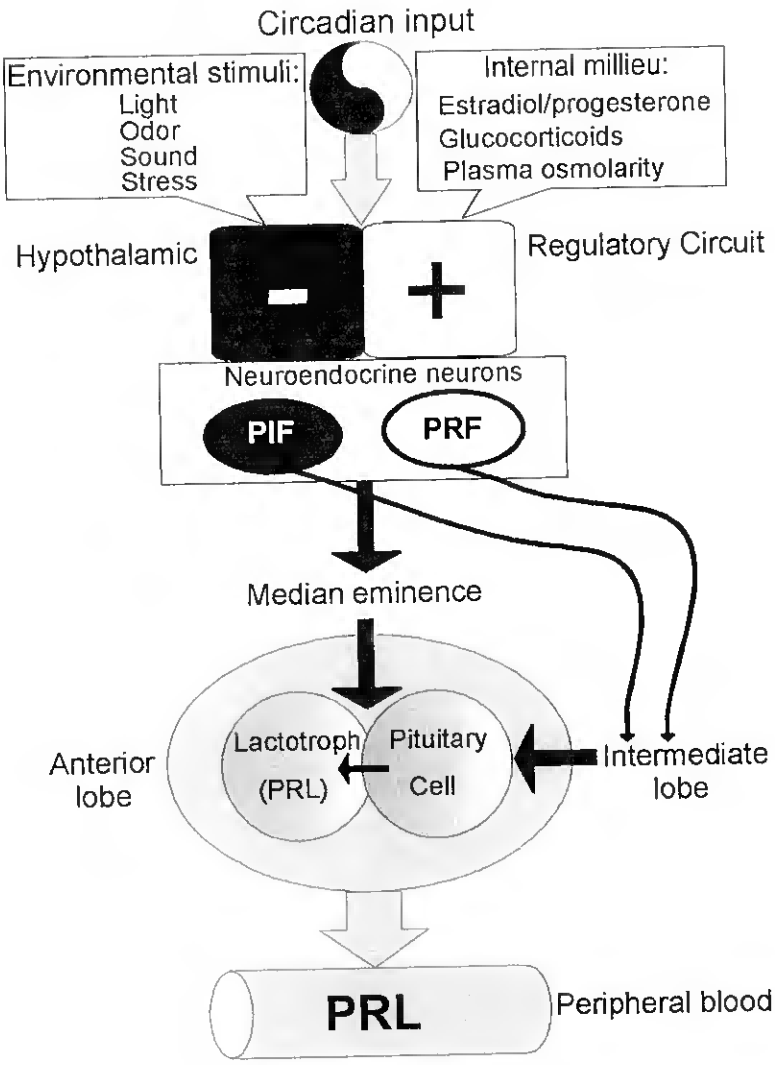

Figure 1.3 An overview of the regulation of PRL secretion in mammals. PRL secretion is paced by a light-entrained circadian rhythm, which is modified by environmental input, with the internal milieu affecting the inhibitory or stimulatory elements of the hypothalamic regulatory circuit. The final common pathways of the central stimulatory and inhibitory control of PRL secretion are the neuroendocrine neurons producing PRL inhibiting factors (PIF), or PRL releasing factors (PRF). PIF and PRF from the neuroendocrine neurons can be released either at the median eminence or at the neurointermediate lobe and reach the PRL-cells in the anterior lobe of the pituitary gland. Thus PRL-cells are regulated by blood-borne agents of central nervous system, or by factors released from neighboring cells (paracrine regulation), or from the PRL-cells themselves (autocrine regulation) (adapted from Freeman *et al.*, 2000).

gene regulators are multiple hypothalamic factors, feedback signals from the target organs of the pituitary hormones and an increasing number of factors produced and secreted within the pituitary (other hormones, particularly steroids), and environmental factors.

1.6.1 Biogenic amines

DOPAMINE (DA) - In mammals, DA is considered the major PIF. Several well-defined dopaminergic systems have been described in the mammalian brain.DA receptors have been detected in preparations of pituitary membranes by immunocytochemical methods, which include labelling the receptors with the DA antagonist haloperidol, and detection of it with an antibody against haloperidol and the peroxidase anti-peroxidase technique. Ample experimental evidence shows that DA inhibits PRL release from pituitary lactotrophs both *in vivo* and *in vitro* (reviews by Ben-Jonathan and Hnasko, 2001; Freeman *et al.*, 2000).

In avian species, DA-ir cells were detected in the developing chicken adrenal-gland (Sánchez-Montesinos *et al.*, 1996). In teleosts, the existence of dopaminergic nerve fibers innervating PRL cells has not been clearly demonstrated. However, the presence of DA in pituitary bioassays reduces PRL cell activity in some species suggesting it may also act as a PIF in some fish (reviewed by Wigham, 1992).

NOREPINEPHRINE AND EPINEPHRINE - In rats, adrenergic modulation, mediated by either norepinephrine or epinephrine, plays an important role in stress-induced PRL secretion (see Freeman *et al.*, 2000 for revision).

In the teleost fish *Poecilia latipinna*, the respective α – adrenergic and β – adrenergic agonists phenylephrine and isoproterenol inhibited PRL secretion *in vitro*, but the adrenergic blockers phentolamine and propranolol had no direct effect on their own, although they did oppose the inhibitory action of DA (Wigham, 1992). Interestingly in the saddleback wrasse (*Thalassoma duperrey*), norepinephrine stimulates both initiation and completion of sex reversal (Larson *et al.*, 2003).

SEROTONIN - In mammals, serotonin facilitates suckling-induced PRL release and regulates the estrogen-induced PRL secretion (Freeman *et al.*, 2000). Although receptors for serotonin are present in the anterior lobe of the pituitary gland, serotonin does not stimulate PRL release *in vitro*, suggesting that it functions as a neurotransmitter rather than a neurohormone (Freeman *et al.*, 2000).

In avian species, serotonin-ir was localized in the endocrine cells of the digestive and respiratory systems (Yamada *et al.*, 1985; Yamaguchi *et al.*, 1987; Adriaensen *et al.*, 1994; Lucini *et al.*, 1996). In the chicken, serotonin-ir is expressed in the sympathoadrenal system (Garcia-Arrarás and Martínez, 1990).

In some teleosts, namely clingfish (*Lepadogaster candollei*), fire eel (*Mastacembelus erythrotaenia*), goldfish (*Carassius auratus*), and turbot

(*Scophthalmus maximus*), serotonin-ir endocrine cells and fibers were observed throughout the gastro-intestinal tract, in the skin and in some nerve projections to the retina (Fasulo *et al.,* 1993; Reinecke *et al.*, 1997; Lima and Urbina , 1998). Moreover, the brain, the pituitary, and the ventral spinal cord contain a dense innervation of serotonergic fibers in sailfin molly (*Poecilia latipinna*), garfish (*Lepisosteus productus*), and European eel (*Anguilla anguilla*) (Wigham, 1992; Batten *et al.*, 1993; Chiba and Oka, 1999). Relatively few studies have been carried out with serotonin *in vitro,* in the trout serotonin produces an increase of PRL cell activity and stimulates PRL secretion but its action on the PRL cells in other species remains to be studied (Wigham, 1992). In the saddleback wrasse (*Thalassoma duperrey*), serotonin inhibits both initiation and completion of sex reversal (Larson *et al.*, 2003).

1.6.2 Acetylcholine

In GH_3 cells, the activation of the (muscarinic) acetylcholine receptor decreases PRL secretion (Freeman *et al.*, 2000). Moreover, cholinergic stimulation by administration of cholinergic agonists in rats causes a decrease in serum PRL concentrations (Freeman *et al.*, 2000).

Experiments with the teleost fish rainbow trout (*Oncorhyncus mykiss*), *in vitro*, showed that the acetylcholine agonist, carbachol, inhibits PRL synthesis and release. Moreover, there is evidence which suggests that the cholinergic inhibition of the PRL cells in teleosts operates via muscarinic receptors (Wigham, 1992).

1.6.3 Neuropeptides

A variety of peptides are known to be involved in the regulation of PRL secretion. The list of this type of PRL secretagogues is rather long, but in the present study more attention is paid to the following peptides: (1) galanin (Gal), which in terms of its potential physiological activity is one of the better characterized paracrine factors in the pituitary, (2) vasoactive intestinal peptide (VIP), which has long been characterized as a likely local factor influencing function of lactotrophs, and recently has been shown to interact with galanin, (3) somatostatin (SS) and (4) thyrotropin-releasing hormone (TRH), which have received much attention as they are respectively, a potent inhibitor and stimulator of PRL secretion in mammals.

In addition to the aforementioned neuropeptides the other putative PRL regulatory factors which will be reviewed in the introduction are those which have been studied in teleost fish and include oxytocin and isotocin, neuropeptide Y, neurotensin, substance P, and urotensin.

GALANIN (Gal) - Gal is a 29-amino acid peptide originally isolated from porcine small intestine (Tatemoto *et al.*, 1983). It shares little homology with other known peptides. It has been demonstrated that the N-terminal sequence of Gal, comprising amino acids 1-15 are highly conserved in all species from which it has been isolated as this region is probably responsible for receptor binding (Crawley, 1995). In contrast, the C-terminal region exhibits substantial

interspecies variability, suggesting that it might be responsible for the species-specific activity of Gal (Crawley, 1995) (Table 1.1).

Table 1.1 – Endogenously occurring Gal sequences (Bartfai, 2000; Chartrel *et al.*, 1995; Wang and Conlon, 1994).

	1 - 15	16 - 25	26 - 30
Man	G W T L N S A G Y L L G P H A	**V G** N H R S F **S** D K	**N** G L **T S** **
Pig	G W T L N S A G Y L L G P H A	I D N H R S F H D K	Y G L A *
Rat	G W T L N S A G Y L L G P H A	I D N H R S F **S** D K	**H** G L **T** *
Chicken	G W T L N S A G Y L L G P H A	**V** D N H R S F **N** D K	**H** G **F T** *
Frog	G W T L N S A G Y L L G P H A	I D N H R S F **N** D K	**H** G L A *
Dogfish	G W T L N S A G Y L L G P H A	**V** D N H R S F **N** D K	**H** G L A *

*C-terminal amide
**C-terminal free acid
Boldface denotes aa which were not conserved between species

Immunohistochemical studies have demonstrated that Gal-like immunoreactivity is widely distributed in the central nervous system and the gastroenteric, respiratory, urinary and reproductive system of different mammals (Crawley, 1995). Gal and its mRNA have been shown so far to be expressed prevalently in neurons, but a number of studies have colocalized Gal with PRL in at least a fraction of the lactotrophs (see Schwartz, 2000 for review). These and other studies have reported colocalization in other cells in addition to or instead of lactotrophs. One study, employing cellular immunoblotting technology with rat pituitary cells, localized Gal with PRL or ACTH and in wild type and transgenic (human GHRF-expressing) mice, immunocytochemical techniques were used to demonstrate the colocalization of Gal with GH, PRL, or TSH (see Schwartz, 2000 for review).

In humans, Gal secretion has been measured in cultures of ACTH-secreting tumours (Invitti *et al.*, 1999). Recently, using immunocytochemical

techniques, Gal has also been reported to be present in nerve fibers in monkey and canine pituitaries in close proximity to all types of secretory cells (Liu and Gao, 1998).

In reptiles and amphibians, Gal immunoreactivity was observed in brain, hypothalamus, heart, bladder, small intestine and oviducts (Crawley, 1995; Lamanna *et al.*, 1999). In avian species, Gal-ir cells and neurons were detected in the adrenal-gland of the chick embryo and the adult, in the quail brain, in the chicken medulla oblongata, and in the pancreatic islets of the bustard (Azumaya and Tsutsui, 1996; Sánchez-Montesinos *et al.*, 1996; Wang et al., 1997; Ohmori, 1998; Mensah-Brown *et al.*, 2000). Avian galanin mRNA was expressed in the quail brain, ovary, and intestine (Kohchi and Tsutsui, 2000).

Some teleost fish revealed similar extensive systems of Gal immunoreactive neurons in brain and pituitary (Moons *et al.*, 1989; Batten *et al.*, 1990; Cornbrooks and Parsons, 1991; Moons *et al.*, 1991; Olivereau and Olivereau, 1991a; Power *et al.*, 1995; Batten *et al.*, 1999). In european seabream, Gal immunoreactive fibers were observed infiltrated between growth hormone, prolactin, and adrenocorticotropin cells (Power *et al.*, 1995). Studies on seabass pituitary revealed that Gal immunoreactive fibers abutted with ACTH, PRL, TSH, GtH, and GH cells (Moons *et al.*, 1989; Batten *et al.*, 1990; Moons *et al.*, 1991), suggesting it may directly influence the activity of such cells.

In mammals, Gal receptors have been identified in localized sites in the central nervous system, pituitary, pancreas, stomach and intestine (Crawley, 1995). Effector systems linked to the Gal receptor in different tissues include

inhibition of adenylate cyclase, blockage of voltage-dependent calcium channels and activation of ATP-sensitive potassium channels (Crawley, 1995). Recently, two Gal receptors – Gal receptor-1 (GalR1) and Gal receptor-2 (GalR2) – have been characterized and shown to have different amino acid sequences, pharmacology, and second messenger signalling systems. In rats, Gal-R1 receptors have a broad role in normal synaptic transmission, while Gal-R2 receptors, in addition to a similar role in particular pathways, seem to be involved in processes prominent during the establishment and maturation of synaptic connections in developing brain and during neural damage and repair in the mature nervous system (Burazin and Gundlach, 1998; Burazin *et al.*, 2000). In three species of teleosts sailfin mollies sea bass (*Poecilia latipinna), (Dicentrarchus labrax)*, and North African catfish (*Clarias gariepinus*), the distribution of binding sites for Gal were described in the pituitary (Batten *et al.*, 1999). Moreover, in Atlantic salmon (*Salmo salar*), a specific Gal receptor was identified in the brain (Holmqvist and Carlberg, 1992) strengthening the idea that it has a direct action on the nervous system.

The regulation of Gal synthesis and secretion in pituitary cells is an area of ongoing investigation. Estrogens exert the most important influence on Gal activity in the pituitary, where estradiol positively regulates Gal-expressing cells, either increasing the amount of Gal protein and mRNA or the number of Gal-secreting cells. Pituitary Gal content is also controlled by other hormones such as thyroid hormones, vasoactive intestinal peptide (VIP) and PRL (see Schwartz, 2000 for review).

In mammalian species, the intensity of Gal innervation has been observed to be dependent on the physiological status of the animal. For example, in rats, gonadal steroids have a dramatic activational effect on the numbers of visibly stained Gal cells in the hypothalamus and pituitary gland (Bloch *et al.*, 1993; Leibowitz *et al.*, 1998; Rugarn *et al.*, 1999). Moreover, the capacity of cells located in the hypothalamus to express Gal after testosterone or estradiol exposure in ferrets shows sexual divergence (Park *et al.*, 1997). In rats, hypothalamic and pituitary levels of Gal mRNA, increased significantly after treatment with estrogen (Gabriel *et al.*, 1992; Brann *et al.*, 1993; Hyde *et al.* 1993; Crawley, 1995; Tseng *et al.*, 1997; Shen *et al.*, 1999; Degerman *et al.*, 2002). Gonadotropin-primed rat ovarian tissue cultured *in vitro* with galanin secreted significant amounts of estradiol, progesterone, and androstenedione into the medium (Fox *et al.*, 1994). Moreover, Gal mRNA levels in the rat ovary are increased by treatment with human chorionic gonadotropin (Crawley, 1995).

In rats, combined autoradiographic and immunohistochemical studies have shown colocalization of receptors for estrogens and the neuropeptide Gal (Hösli and Hösli, 1999). Studies with immortalized LHRH neurons suggest that the estrogenic control of Gal gene expression in these neurons is transduced by estrogen receptors (Shen *et al.*, 1998). Studies in the estrogen receptor alpha-knock-out mouse revealed that the estrogen receptor subtype alpha is essential to estrogen-evoked Gal gene expression in the anterior pituitary of these animals (Shen *et al.*, 1999).

Studies *in vivo* of the involvement of Gal in reproduction corroborate observations made *in vitro*. Exogenously administered Gal regulates the reproductive axis by acting as a growth regulator of the lactotrophs in rats (Wynick *et al.,* 1993) or by stimulating the lutenizing hormone in monkeys (Finn *et al.*, 2000). In female rats, Gal has both direct and indirect effects on gonadal hormone release and this response is impaired in starved animals (Baranowska *et al.*, 2001).

In reptiles, the evidence which exists does not indicate if the action of Gal on the reproductive axis is direct or indirect. Administration of 17β-estradiol to non-reproductive female lizards induced a significant increase in neurons containing Gal immunoreactivity in the oviduct (Lamanna *et al.,* 1999). Gal administration to pre-ovulatory lizard females induced premature oviposition, suggesting that Gal could be involved in the egg laying process (Lamanna *et al.*, 1999). In avian species, estradiol induces an increase in Gal binding sites in mature quail oviducts, while 17β-estradiol and progesterone induced a marked increase in Gal binding sites (Tsutsui *et al.,* 1998).

In teleost fishes Gal innervation is also dependent on the physiological status of the animal. For example, in eels treated with estradiol or methyl testosterone, increased Gal immunoreactive material was observed in some perikarya and brain fibers (Olivereau and Olivereau, 1991b). Moreover, a Gal-like peptide has been shown to have a sexually dimorphic distribution in the brain of the sailfin molly (Cornbrooks and Parsons, 1991).

In addition to its action on reproduction a myriad of other physiological functions have been attributed to Gal. One important role is related to the control of appetite. Administration of Gal has been shown to induce feeding in satiated rats and ground squirrels; the effect is dose-related, with threshold doses at approximately 0.3 mmol (Crawley, 1995). In female rats, animals showing a preference for a fat-rich diet or animals with greater body fat, independent of the diet, exhibit higher levels of hypothalamic Gal. This evidence suggests that in the female rat, Gal may contribute to the overeating and increased weight gain that is associated with a fat-rich diet (Leibowitz *et al.*, 1998). Interestingly in teleosts Gal has also been observed to influence feeding and in goldfish, where stimulation of food intake is mediated by the α_2-adrenergic system, Gal affects the central regulation of feeding, (de Pedro *et al.*, 1995).

Gal also has an important role in the mechanism of growth hormone release. Gal caused an *in vitro* increase in release of rat growth hormone (Gabriel *et al.*, 1988; Crawley, 1995) and LHRH (Lopez and Negro-Vilar, 1990), an effect proposed to be mediated via an effect on GHRH neurons (Meister *et al.*, 1987; Murakami *et al.*, 1989; Hulting *et al.*, 1991). Support for this proposal comes from *in situ* hybridisation studies which demonstrate that Gal mRNA is present in GnRH neurons (Marks et al., 1994; Selvais *et al.*, 1995). Intraventricular injections of Gal in the rat produce rapid increases of PRL (Crawley, 1995). Moreover, Gal and PRL are colocalized within secretory granules of the pituitary after estrogen treatment (Hyde *et al.*, 1991). Results from studies in mice carrying a loss-of-function mutation of the endogenous Gal

gene, support the hypothesis that Gal acts as a paracrine regulator of PRL expression (Wynick *et al.*, 1998).

Gal is a potent regulator of a number of neurotransmitters and hormones. Its actions in some systems have been shown to involve a decrease in the cytosolic Ca^{2+} concentration. This may be the consequence of the hyperpolarization brought about by opening of Gal-receptor-coupled K^+-channels or it may be a result of the Gal-receptor-mediated closure of some Ca^{2+} channels – or of a combination of both effects (Bartfai, 2000). A Gal suppressing calcium current and activating inwardly rectifying potassium current was also demonstrated in the enteric neurons of the guinea-pig small intestine. Suppression and activation of these channels was dependent on Gal concentration (Ren *et al.*, 2001). An alternative mechanism is observed for Gal inhibition of insulin secretion in a number of species (Crawley, 1995), this action is brought about by interference of Gal with adenylate cyclase activation and the activity of protein kinase C and cyclic AMP (Lindskog and Ahrén, 1991).

Several other biological activities have been attributed to Gal. In mammals, this neuropeptide produces direct effects on smooth muscle activity at several sites in the gastrointestinal tract (Botella *et al.*, 1992; Crawley, 1995). The multiple coexistence of Gal with most of the pituitary hormones during the fetal development of the rat (Cimini *et al.*, 2000), could be an indication that Gal may also have a role in cytodifferentiation. In elasmobranches, Gal causes differential vasoconstriction in vascular beds (Preston *et al.*, 1995).

VASOACTIVE INTESTINAL PEPTIDE (VIP) - VIP is a vasoactive peptide which belongs to the secretin/glucagon family, other members include glucose-dependent insulin-releasing peptide (GIP), growth hormone releasing-factor (GRF), PHI (peptide having N-terminal histidine and C-terminal isoleucine) and the reptilian peptides helodermin and helospectins. It was first isolated from porcine intestinal extracts (Said and Mutt, 1972) but has subsequently been shown to have a widespread distribution in the central and peripheral nervous systems in a range of vertebrates. The sequence of VIP appears to have been highly conserved during evolution and it is composed of 28-amino acids (Table 1.2).

VIP has a wide spectrum of biological activities in mammals (Dockray, 1987). It acts on cardiovascular, reproductive, pulmonary, immune, and gastrointestinal systems. The general physiological effects include vasodilation, bronchodilation, immunosuppression, increases in gastric motility and hormonal secretion (Brenneman *et al.*, 2000 for review). VIP is a potent stimulator of PRL release from mammalian pituitaries both *in vivo* and *in vitro* (see Freeman *et al.*, 2000 and Schwartz, 2000 for reviews).

Table 1.2 – Mammalian amino acid sequence of VIP (Brenneman *et al.*, 2000)

Pig	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N
Dog	H S D A V F T D N Y S R I R K Q M A V K K Y I N S L L A
Chicken	H S D A V F T D N Y S R F R K Q M A V K K Y L N S V L T

In reptiles, VIP-ir peptidergic nerves were detected in the pancreas (Buchan *et al.*, 1982) but PRL actions have not been reported. In amphibians, VIP has been proposed to be a PRL-releasing factor (Koiwai *et al.*, 1986). In

avian species, VIP-ir fibers are localized in the brain, hypothalamus, gut, and pancreatic islets (Mikami and Yamada, 1984; Epstein and Poulsen, 1991; Erichsen *et al.*, 1991; Mensah-Brown *et al.*, 2000). VIP is a potent stimulator of PRL release in avian species *in vivo* and *in vitro* (Hall and Chadwick, 1985a; MacNamee *et al.*, 1986). Recently VIP was also shown to be associated with a significant rise in PRL during the breeding season of birds (Youngreen *et al.*, 1994; Bédécarrats *et al.*, 1999; Maney *et al.*, 1999). In amphibians, the results from *in vitro* studies suggest VIP may be a PRL-releasing factor (Koiwai *et al.*, 1986),

In teleosts relatively few studies of the action of VIP exist. VIP-ir nerves have been detected within both the mucosa and muscularis mucosa of the swimbladder of cod (*Gadus morhua*) (Lundin and Holmgren, 1986) and it has been shown to cause a slight relaxation in strips of coeliac and swimbladder artery). In addition, endocrine cells and fibers were observed throughout the gastro-intestinal tract of the turbot (Reinecke *et al.*, 1997) and VIP influences ion and water transport in the intestine of freshwater-adapted Mozambique tilapia (*Oreochromis mossambicus*) (Mainoya and Bern, 1984). Moreover, in cod VIP induces potent and persistent inhibition of gastric acid secretion (Holstein and Humphrey, 1980). In Mozambique tilapia, VIP appears to inhibit PRL secretion (Wigham, 1992). In the sea bream, VIP modulates PRL secretion from E_2 primed pituitary glands (Brinca *et al.*, 2002 and present thesis).

SOMATOSTATIN RELEASE-INHIBITING FACTOR (SRIF) – SRIF is a multifunctional hormone which inhibits the secretion of growth hormone. SRIF is a tetradecapeptide (SRIF-14) but multiple N-terminally extended forms such as SRIF-28, SRIF-22, SRIF-25, SRIF-34, and SRIF-37 have been isolated from hypothalamus, pancreas and intestinal tissue of mammals and non-mammals (King and Miller, 1979; Conlon *et al.,* 1985; Plisetskaya *et al.*, 1986; Cutfield *et al.,* 1987) (Fig.1.4).

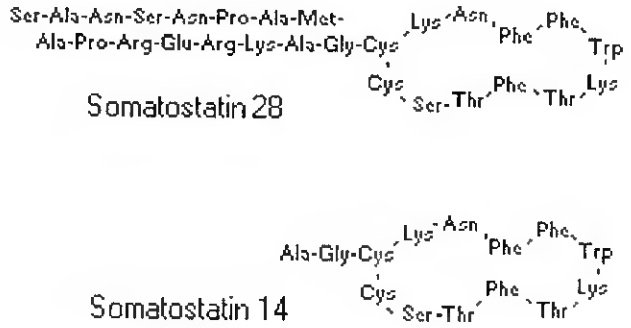

Figure 1.4 – Amino acid sequence of SS-14 and SS-18 (Rubinow *et al.*, 2000).

In mammals, *in vitro* and *in vivo* studies have shown that SRIF-14 and SRIF-28 not only inhibits GH secretion, but also secretion of PRL, TSH, and ACTH (Freeman *et al.*, 2000 for review). In rat and chick, it has also been shown to inhibit the enzyme activities of the small intestine (Taboada *et al.,* 1985).

In reptiles, SRIF immunoreactive endocrine cells have been detected in the pancreas (Buchan *et al.*, 1982). In avian species, SRIF-ir fibers and endocrine cells are localized in the brain, hypothalamus, proventriculus, gut, pancreas, and sympathoadrenal system of embryos and adults (Mikami and Yamada, 1984; Yamada *et al.*, 1985; Yamaguchi *et al.*, 1987; Garcia-Arrarás and

Martínez, 1990; Erichsen *et al.,* 1991; Epstein and Poulsen, 1991; Erichsen *et al.,* 1994; Lucini *et al.,* 1996; Sánchez-Montesinos *et al.*, 1996; Takayanagi *et al.,* 1996).

In teleosts, immunocytochemical investigations have revealed the presence of SRIF-like material in the hypothalamus, pituitary, and gastro-intestinal tract of several species (Wigham, 1992; Zupanc *et al.*, 1994; Becerra *et al.*, 1995; Groff and Youson, 1997; Reinecke *et al.*, 1997; Batten *et al.*, 1999), including the sea bream (Power *et al.,* 1996). In rainbow trout (*Oncorhynchus mykiss*), preprosomatostatin (a SRIF precursor) has been isolated and characterized (Moore *et al.*, 1995) and has been shown to be expressed in the pancreas, stomach, intestine, and brain (Kittilson *et al.*, 1999). The fruit-eating fish, the pacu (*Piaractus mesopotamicus)*, expresses two SRIF genes (de Lima, 1999). SRIF inhibits PRL synthesis and release in the trout (Wigham, 1992), and also appears to regulate the development of new neurons produced in response to injuries in the cerebellum (Zupanc, 1999).

THYROTROPIN-RELEASING HORMONE (TRH) - A hypophysiotrophic factor that stimulates thyroid-stimulating hormone (TSH) secretion from pituitary cells was first isolated in 1966 (Schally *et al.,* 1966). In 1969, a group led by Guillemin (Burgus *et al.,* 1970) and another by Schally (Boler *et al.*, 1969) announced that the hypothalamic substance that causes the anterior pituitary gland to release TSH is L-pyroglutamyL-L-histidyl-L-prolineamide (L-pGlu-L-His-

L-ProNH2) (Fig.1.5). This tripeptide is now called thyrotropin-releasing hormone (TRH) (Mason *et al.*, 2000).

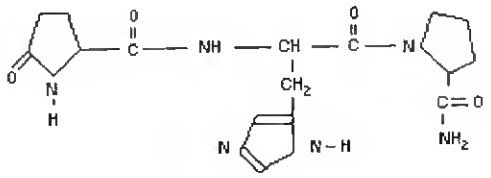

Figure 1.5 - Thyrotropin-releasing hormone (Mason *et al.*, 2000).

In mammals, TRH-like immunoreactivity is widely distributed in the CNS, and TRH receptors have been identified on lactotrophs. TRH stimulates PRL release in a dose-dependent manner both *in vitro* and *in vivo*. Pharmacological blockade of VIP receptors attenuates the PRL response to TRH, consistent with TRH acting, at least partially, via local production of VIP (see Benker *et al.*, 1990, Freeman *et al.*, 2000 and Schwartz, 2000 for reviews).

In avian species, TRH is distributed in the brain and hypothalamus (Józsa *et al.*, 1988; Geris *et al.*, 1999). In the chicken, a TRH receptor has been cloned and characterized (Sun *et al.*, 1998). TRH stimulates PRL release from pituitary glands of fowl (Hall *et al.*, 1985b), and this effect is greatly increased in turkeys treated with estradiol benzoate (Saeed and el Halawani, 1986). In amphibians, TRH also stimulates PRL release (Preece and Licht, 1987).

In some teleosts, TRH immunoreactivity is detected in the brain, pituitary, and retina (Wigham, 1992; Anadón *et al.*, 2001; Díaz *et al.*, 2002). Recently, in the brain of embryos, alevin, and juveniles of brown trout (*Salmo trutta fario*), TRH-ir neurons were observed to have a wide distribution (Díaz *et al.*, 2001). *In*

vitro studies have demonstrated that TRH plays an important role in both PRL synthesis and release in teleosts (Kagabu *et al.*, 1998; Wigham, 1992). Studies in chum salmon (*Oncorhynchus keta*) suggest TRH may be related to changes in olfactory function during migration (Hamano *et al.*, 1996).

OXYTOCIN (OT) AND ISOTOCIN (IT) - The neurohypophyseal hormone oxytocin (OT) was originally identified as a nonapeptide with an amidated C-terminus (Acher *et al.*, 1970) (Table 1.3). In mammals it is primarily associated with the contraction of uterine and mammary smooth muscle during birth and lactation. Recently, plasma hyperosmolality has been identified as a stimulus for rat pituitary OT secretion (Rinaman *et al.*, 2000). OT is also involved in the regulation of PRL secretion, and an OT receptor has been localized on lactotrophs (Freeman *et al.*, 2000; Schwartz, J., 2000). In teleosts, oxytocin-ir fibers and binding sites are observed in the pituitary (Batten *et al.*, 1999) and in rainbow trout (*Oncorhynchus mykiss)*, oxytocin increases PRL release from pituitaries *in vitro* (Wigham, 1992).

Table 1.3 – Amino acid sequence and similarities between the peptides isotocin and oxytocin

Isotocin	C Y I S N C P I G *
Oxytocin	C Y I Q N C P L G *

G*, amidated glycine residue

The functional role of isotocin the non-mammalian counterpart of OT (Table 1.3), is less well defined. The distribution of isotocin-ir fibers and binding

sites have been described in the pituitary of three species of teleost sailfin mollies (Poecilia *latipinna),* sea bass *(Dicentrarchus labrax)*, and North African catfish (*Clarias gariepinus)* (Batten *et al.*, 1999), but no physiological studies of the activity of this peptide exist in fish.

NEUROPEPTIDE Y (NPY) - NPY is a member of the pancreatic polypeptide family isolated by Tatemoto in 1982 (Table 1.4). In mammals, NPY is distributed in the CNS, particularly in the hypothalamus, and it is a substance that alters the function of several different pituitary cell types (reviewed by Freeman et al., 2000). In rats, NPY inhibits PRL secretion and attenuates both the PRL-secretory and intracellular calcium flux responses to TRH in pituitary cells. The activity of NPY varies as a function of the estrous cycle, and expression levels also change according to the steroid environment (reviewed by Schwartz, J., 2000).

Table 1.4 – NPY amino acid sequence

Y P S K P D N P G E D A P A E D L A R Y Y S A L R H Y I N L I T R Q R Y - NH_2

In avian species, NPY-ir fibers were seen in the brain and in the endocrine cells of the pancreas (Erichsen *et al.,* 1991; Erichsen *et al.,* 1994; Lucini *et al.*, 2000; Mensah-Brown *et al.*, 2000). In the chick embryo, NPY-ir cells were detected in the developing adrenal-gland (Sánchez-Montesinos *et al.*, 1996). In an immunochemical study of the pituitary gland of some teleosts sailfin mollies, (*Poecilia latipinna),* sea bass *(Dicentrarchus labrax),* and North African catfish (*Clarias gariepinus*), the distribution of numerous NPY-ir fibers and binding sites

were described in the pituitary (Batten *et al.*, 1999). In turbot, NPY-ir endocrine cells and fibers were observed throughout the gastro-.intestinal tract (Reinecke *et al.*, 1997), although no study of the brain was carried out.

NEUROTENSIN (NT) - Neurotensin (NT) is a tridecapeptide that was originally isolated from bovine hypothalamus (Carraway and Leeman, 1973) (Table 1.5) and was originally thought to be a vasoactive peptide. Subsequent studies have shown in mammals that NT is involved in a range of physiological processes, including blood flow, digestion, temperature regulation and nociception (Leeman and Carraway, 1982).

Table 1.5 – NT amino acid sequences of some mammals, avian, and fish species (Carraway and Bhatnagar, 1980; Carraway and Leeman, 1973; Hammer et al., 1980; Rodriguez-Bello *et al.*, 1993; Warner *et al.*, 1998)

Bovine, canine, human	E L Y E N K P R R P Y I L - OH
Chicken, alligator	E L **H V** N K **A** R R P Y I L - OH
Toad	E **A I V S** K **A** R R P **Y** I L - OH

Boldface denotes aa which are different from bovine sequence

Immunocytochemical studies have shown that NT is present in the mammalian brain and anterior pituitary (Uhl *et al.*, 1977; Emson *et al.*, 1982). One of the most potent central effects of NT in mammals is its analgesic action which can be blocked by thyroxine releasing hormone (Hernandez *et al.*, 1984). Intravenous injection of NT leads to an increase in circulating PRL levels while intracerebroventricular injections cause an inhibition of PRL release (Vijayan and McCann, 1979). In contrast NT increases PRL secretion in a dose-dependent manner *in vitro*. The opposite effects of NT *in vivo* and *in vitro* have been taken to

indicate that NT can affect PRL secretion at multiple levels (Freeman *et al.*, 2000).

In avian species, NT-ir endocrine cells were localized in the brain, proventriculus, and gizzard (Yamada *et al.*, 1985; Yamaguchi *et al.*, 1987; Esposito *et al.*, 1997). *In vivo* and *in vitro* studies in chickens showed that NT has an effect on the motility of the lower gut (Rawson *et al.*, 1990).

In the turbot (*Scophthalmus maximus*) and in the rosy barb (*Barbus conchonius*), NT-ir endocrine cells and fibers were observed throughout the gastro-intestinal tract (Rombout and Reinecke, 1984; Reinecke *et al.*, 1997). In the goldfish (*Carassius auratus*), extensive NT-ir was observed in the brain and pituitary (Bello *et al.*, 1994). Excitatory effects of NT on fish gut smooth muscle has been described (Holmgren, 1985).

SUBSTANCE P - Substance P was first described in 1931 by von Euler and Gaddum who demonstrated brain extracts contained substances that caused contractions of the intestinal preparations and lowered blood pressure. It was only in 1971 that the amino acid sequence of this undecapeptide was determined in extracts of bovine hypothalamus (Chang *et al.*, 1971) (Table 1.6). Substance P belongs to a family of neuropeptides known as tachykinins that share in common C-terminal sequence: Phe-X-Gly-Leu-Met-NH_2.

Table 1.6 – Amino acid sequence of substance P

Mammalian	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Substance P has a wide distribution in the nervous system of both vertebrates and invertebrates. In primates and rodents numerous immunoreactive cell bodies and fibers are present in the hypothalamus. Moreover, a high level of expression of substance P receptors has been detected in the hypothalamus and pituitary. Substance P has been shown to regulate both *in vitro* and *in vivo* PRL secretion in primates (rhesus monkey) and rats, but paradoxical effects have been obtained - it appears that the actual effect of this peptide on PRL secretion depends on the dose and route of administration (reviewed by Freeman *et al.*, 2000).

In avian species, substance P-ir fibers are localized in the hypothalamus and brain (Mikami and Yamada, 1984; Erichsen *et al.,* 1991; Erichsen *et al.,* 1994). In the teleosts, substance P-ir fibers have been described in the pituitary of sailfin mollies (*Poecilia latipinna),* sea bass (*Dicentrarchus labrax)*, and North African catfish (*Clarias gariepinus)* (Batten *et al.*, 1999). Substance P-ir endocrine cells and fibers have been observed in the gastro-intestinal tract of a range of teleosts in the elasmobranches and cyclostomes (Jensen, 1989). However, an authentic substance P has yet to be found in non-mammalian species (Severini *et al.,* 2002).

UROTENSIN - The caudal neurosecretory system of teleost fish (Fig. 1.6) which terminates in the urophysis, secretes two major regulatory peptides: urotensin I and urotensin II (Onstottk and Elde, 1986; Chester-Jones et al, 1987; Larson *et al.,* 1987), a cyclic 12-amino acid residue peptide that has some

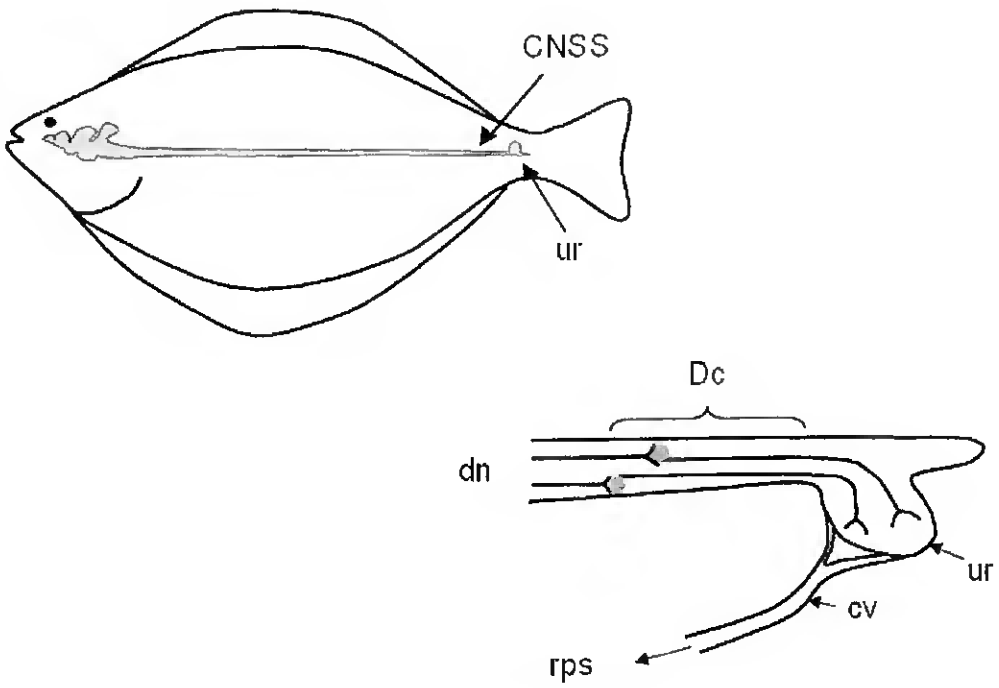

Figure 1.6 - Diagrammatic representation of the caudal neurosecretory system of fish (CNSS). Descendings neurons in the caudal spinal cord (dn) project axons to the urophysis (ur).where the neurosecretory nerve terminals link to the caudal vein (cv) of the renal portal system (rps). Dahlgren cells secrete two osmoregulatory peptides (urotensin I and urotensin II) (adapted from an original drawing by Dr. Peter Hubbard).

sequence similarity, but is not homologous, to somatostatin-14 (Pearson *et al.*, 1980). Urotensin II is a 12-amino acid peptide, and the structural characterization of this peptide from several species of fish has shown that the cyclic region of the peptide has been strongly conserved (Le Mevel *et al.*, 1996) (Table 1.7).

Urotensin II is not confined to the caudal neurosecretory system of fish, and has been identified in anterior spinal cord and brain of several species (Yulis and Lederis, 1988; Wigham, 1992; Chartrel *et al.*, 1998).

Table 1.7 – Amino acid sequence of Urotensin II from two teleosts (trout and carp) and an elasmobranch (Waugh and Conlon, 1993)

Trout	G G N S E C F W K Y C V - OH
Carp	G G N **T** E C F W K Y C V - OH
Skate	**N N** F S **D** C F W K Y C V - OH

Boldface denotes aa which are different from trout sequence

Although the precise physiological role of urotensin II remains unclear, the diverse actions of urotensin II suggest the possibility of cardiovascular and renal effects on human, rat, frog, eel and trout (Chan, 1975; Gibson *et al.*, 1986; Itoh *et al.*, 1987; Yano *et al.*, 1995; Le Mevel *et al.*, 1996; Böhm and Pernow, 2002; Zhang *et al.*, 2003), as well as a role in lipid and carbohydrate metabolism in coho salmon (*Oncorhynchus kisutch*) and dogfish (*Torpedo marmorata*) (Sheridan *et al.*, 1987; Conlon *et al.*, 1994). Moreover, urotensin II inhibited PRL release, in a dose-related manner from Mozambique tilapia (*Oreochromis mossambicus*) pituitaries cultured *in vitro* (Rivas *et al.*, 1986).

1.6.4 Hormones

OVARIAN STEROIDS - In mammals the modulation of PRL by ovarian steroids is well documented (Labrie *et al.*, 1978). In rats, estradiol-17β (E_2) increases the mitotic potency of PRL cells in the pituitary gland and has a stimulatory effect on PRL gene expression in the hypothalamo-neurohypophyseal system and a concomitant inhibitory action on PRL proteolysis at this site (Takahashi and Kawashima, 1986; Torner *et al.*, 1999). In mink, *Mustela vison*, a high systemic ratio of progesterone to E_2 has been shown to be

a prerequisite for increasing the expression of uterine PRL receptors (Rose *et al.*, 1996).

Ovarian steroids also affect the pituitary in birds, modulating *in vitro* PRL release (Knapp *et al.*, 1988). In teleost fish the regulation of PRL cells by ovarian steroids has been less extensively studied although there is evidence indicating their involvement, for example, pituitary PRL content and *in vitro* secretion is elevated by treatment with E_2 in Mozambique tilapia (Borski *et al.*, 1991; Wigham, 1992; Poh *et al.*, 1997). Moreover, preincubation of tilapia pituitary glands with E_2 *in vitro* appears to increase the sensitivity of PRL cells to stimulation by TRH and GnRH *in vitro* (Barry and Grau, 1986; Weber *et al.*, 1997). In contrast, E_2 treatment of rainbow trout pituitary cultures stimulated *in vitro* PRL synthesis but did not affect release (Wigham, 1992; Williams and Wigham, 1994).

CORTISOL – In fish, cortisol production is located in the interrenal cells. These cells do not form a compact gland comparable to the mammalian adrenal cortex, but are located in layers, strands, and cords around the walls of the posterior cardinal veins and its branches run through the head kidneys (Fig. 1.7) (Wendelaar-Bonga, 1997). Studies by Young (1993) on hypophysectomized coho salmon (*Oncorhynchus kisutch*) have indicated that the pituitary gland dominates the endocrine control of cortisol secretion. α-Melanophore-stimulating hormone (α – MSH) and ACTH are the main candidates for cortisol regulation with perhaps β – endorphin as a potentiating factor (Wendelaar-Bonga, 1997).

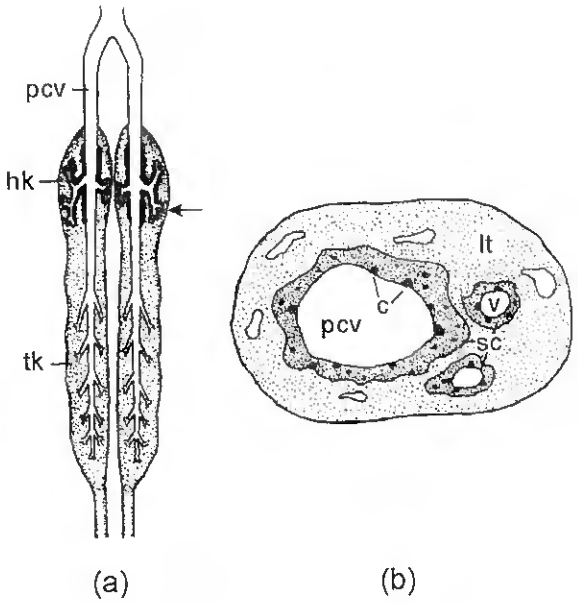

Figure 1.7 – (a) Diagrammatic representation of head (hk) and trunk (tk) kidneys of teleost fish, showing postcardinal veins (pcv) and their branches surrounded by interrenal tissue. (b) Cross section of right head kidney (at plane indicated by arrow in (a), showing steroidogenic cells (sc; adrenocortical homologue) and clusters of chromaffin cells (cc; adrenomedullary homologue) around postcardinal vein (pcv) and small blood vessels (v). Note close association of these cells with surrounding lymphomyeloid tissue (lt) (in Wendelaar Wendelaar Bonga, 1997).

Cortisol appears to be an important extrahypothalamic regulator of PRL secretion in mammals and fishes. Cortisol suppressed PRL release from GH_3 cells *in vitro*, an effect that was related to dose (Tashjian *et al.*, 1970; Melmed, 1984; Prager *et al.*, 1988). In rainbow trout (*Oncorhynchus mykiss*), experiments of chronic confinement suggest a PRL-inhibiting role for cortisol (Pottinger *et al.*, 1992). In Mozambique tilapia (*Oreochromis mossambicus*), physiological concentrations of cortisol rapidly inhibit PRL release *in vitro* and the inhibitory effect occurs through the inhibition of both the Ca^{2+} and cAMP signal

transduction pathways (Grau and Helms, 1990; Borski *et al.*, 2001, 2002; Wigham, 1992 for review).

PROLACTIN - In rats, it is well established that PRL can inhibit its own secretion by activating neuroendocrine dopaminergic neurons in the hypothalamus. However, there is evidence that PRL can also act directly at the lactotroph and inhibit its own secretion in an autocrine/paracrine manner in humans (see reviews by Benker *et al.*, 1990; Freeman *et al.*, 2000; Schwartz, 2000).

In teleosts, some studies showed that PRL may act to feed back on the secretory cells and reduce PRL secretion, *in vitro* and *in vivo* (see Wigham, 1992 for review).

1.6.5 Other pituitary and hypothalamic factors

Studies in mammals demonstrate that PRL cells are influenced by numerous factors produced and secreted within the pituitary and the hypothalamus. These include amino acids, namely gamma-aminobutyric acid (GABA), which is partially responsible for the nondopaminergic PIF activity in mammals (reviewed by Freeman *et al.*, 2000). In non-mammalian species the GABA effect on PRL secretion is less extensively studied, although GABA-ir fibers and neurons are found in the brain of the pigeon (Erichsen *et al.*, 1994) and the sea lamprey (*Petromyzon marinus*) (Meléndez-Ferro *et al.*, 2002).

Nitric oxide (NO) is a signaling molecule that seems to influence secretion of PRL in rats pituitaries (reviewed by Schwartz, 2000). In the Indian catfish (*Heteropneustes fossilis*), a dense plexus of nerve fibers containing neuronal nitric oxide synthase (nNOS) is present beneath the gill epithelium (Mauceri *et al.*, 1999). In birds, nNOS-ir neurons occurred in the pancreatic islets (Mensah-Brown *et al.*, 2000).

PRL cells are also influenced by numerous factors produced and secreted within the pituitary and the hypothalamus, such as transforming growth factors (TGFs), histamine, opioids, calcitonin, POMC fragments, nerve growth factor (NGF), epidermal growth factor (EGF), endothelins (ETs), and ATP (reviewed by Freeman *et al.*, 2000 and Schwartz, J., 2000). Very little is known about the activity of these factors in regulation of teleost PRL secretion.

1.6.6 External factors

PHOTOPERIOD AND TEMPERATURE - Annual clocks of PRL synthesis and release are synchronized with the seasons and the responses are very often a result of changes in some environmental stimulus, such as photoperiod and/or temperature. In mammals and avian species, PRL circulating levels are correlated with ambient temperature (Hooley *et al.*, 1979; Schams *et al.*, 1980; Maney *et al.*, 1999; Gahali *et al.*, 2001) and with photoperiod (Forbes *et al*, 1975; Spieler, 1979; Pijoan and Williams, 1985; Martinet *et al.*, 1992; Maney *et al*, 1999; Gahali *et al.*, 2001). In newts, PRL mRNA and plasma levels were inversely correlates with temperature (Takahashi *et al.*, 2001).

In teleosts, photoperiod and temperature influence pituitary PRL release in goldfish (*Carassius auratus*) (Mckeown and Peter, 1976), and PRL transcription in carp (*Cyprinus carpio*) (Figueroa *et al.*, 1997). In catfish (*Heteropneustes fossilis*), temperature suppresses the action of dopamine which modulates PRL release (Senthikumaran and Joy, 1995). In teleosts, temperature and photoperiod also has an effect on reproduction, although if this is associated with altered PRL release remains to be established (Zanuy *et al.*, 1986; Randall *et al.*, 1998; Koger *et al.*, 1999; Martin *et al.*, 1999; Chaube and Joy, 2002; Shimizu, 2003). In sea bass (*Dicentrarchus labrax*), sex determination is temperature-dependent (Pavlidis *et al.*, 2000b).

STRESS - In fish, the numerous stimuli stressors include sudden or extreme changes in the physical environment (temperature, turbidity, salinity), animal interactions (predation, parasites, intensive competition for space, food, or sexual partners), and human interference, including aquaculture practices (netting, handling, transport, and crowding) and water pollution (low water pH, heavy metals, and organic chemicals). The primary response to stressors is the massive release of catecholamines (CAs) that have a disturbing effect on the hydromineral balance. The rise in circulating cortisol follows more slowly and more sustained, compensating the hydromineral disturbance during stress (see Wendelaar - Bonga, 1997 for review).

Confinement stress in addition to cause a rise in cortisol levels also causes increased PRL in Nile tilapia *(Oreochromis niloticus).* Removal of stress

leads to a reduction in both cortisol and PRL (Auperin *et al.*, 1997). In contrast, experiments of chronic confinement in the rainbow trout (*Oncorhynchus mykiss*), suggest a PRL-inhibiting role on cortisol (Pottinger *et al.*, 1992), and *in vitro* experiments with Mozambique tilapia (*Oreochromis mossambicus)* demonstrate that cortisol inhibits PRL release (Grau e Helms, 1990; Wigham, 1992 for review; Borski *et al.*, 2001, 2002;). It is possible that PRL in some teleostean species is implicated in the compensation of hydromineral disturbance during stress.

1.7 Patterns of pituitary PRL release

Most animal have a circadian or/and annual clock that are usually related to the cyclic physiological processes present during the lifetime. It is clearly advantageous for a species to develop a rhythm synchronized with the biological cycle and in this way be able to anticipate the most favorable times for a certain event to occur. This anticipation can be achieved either by making the control responsive to an external factor or to an intrinsic rhythm which can be synchronized with the circadian/annual cycle by some environmental trigger. The most likely environmental trigger is day length, but temperature and other stimulus could be used (Sage and de Vlaming, 1975).

Prolactin (PRL) can elicit a variety of physiological responses in different groups of animals. Annual clocks of PRL synthesis and release are synchronized with the seasons and the responses are very often a result of changes in some environmental stimulus. In teleost fish, a correlation exists between annual environmental factors and the regulation of PRL cells. In freshwater stickleback

(*Gasterosteus aculeatus*), PRL cells form and release more secretory granules during spring (Benjamin, 1974). Moreover, PRL content of the pituitary and the functional state of PRL cells anticipate the annual migration of this species (Sage and de Vlaming, 1975). Differential expression of PRL mRNA is observed in the pituitary of carp (*Cyprinus carpio*) acclimatized to summer and to winter (Figueroa *et al.*, 1994), with temperature and photoperiod being the major factors controlling the circannual pattern of PRL transcription (Figueroa *et al.*, 1997).

Circadian clocks regulate the physiological processes that are synchronized with the environmental day/night cycle. In humans, PRL levels increase during the late hours of sleep, and pulses of secretion are superimposed on basal secretion (Benker *et al.*, 1990). In several teleost fishes, the pituitary content and plasma PRL concentrations vary significantly with the time of the day (Leatherland and Mckeown, 1973; Leatherland *et al.*, 1974, de Vlaming *et al.*,1975; Batten and Ball, 1976; Mckeown and Peter,1976).

1.8 Biological actions of PRL

PRL is a versatile hormone and multiple actions, ranging from mammary development and lactation in mammals to antimetamorphic effects in amphibians, have been described (Hirano *et al.*, 1987; Bres and Nicoll, 1993). The principal action of PRL is the maintenance of hydromineral balance in euryhaline teleosts in fresh water (Loretz and Bern, 1982; Hirano *et al.*, 1987). The function of PRL in marine teleosts is less certain and it is likely that PRL has other biological actions including a role in reproduction.

REPRODUCTION - In mammals, PRL is best known for the multiple effects it exerts on the mammary gland. However, it also exerts effects on other targets important to reproduction. In some mammals, particularly rodents, PRL is also important for the maintenance and secretory activity of the corpus luteum. It also affects other actions related to reproduction such as mating and maternal behaviours (see Freeman *et al.*, 2000 for review).

In the sea bream, PRLR transcripts are detected in spermiating gonads (Santos *et al.*, 2001), and the level of sbPRLR mRNA increases significantly after E_2 treatment of adults, although PRL expression is reduced with the same treatment (Cavaco *et al.*, 2003). In sea bream juveniles the opposite effects are observed, suggesting that the stage of maturity influences the action of PRL on both testis and ovary via its receptor (Cavaco *et al.*, 2003). Relatively few reports about the effect of PRL on fish reproduction exist. In Mozambique tilapia (*Oreochromis mossambicus*), PRL stimulates testosterone production in testicular tissue of courting males (Rubin and Specker, 1992), in vitellogenic oocytes of guppies (*Poecilia reticulata*) PRL stimulates E_2 synthesis *in vitro* (Tan *et al.*, 1988), and suppresses progesterone and E_2 in the follicular cells of rainbow trout (*Oncorhynchus mykiss*) ovaries cultured *in vitro* (Galas and Epler, 2002). In some teleosts, PRL influences parental behaviour (Slijkhuis *et al.*, 1984; de Ruiter *et al.*, 1986; Tacon *et al.*, 2000).

OSMOREGULATION - Fish interact with their environment and extract or excrete water and/or salts to maintain the ionic strength of internal fluids within

narrow limits. Stenohaline species, both freshwater and seawater, are unable to regulate their plasma ionic composition when challenged with changing environmental salinity, a process that consequently results in high mortality. In turn, euryhaline species are able to adjust their ionic and endocrine systems to cope with new environments. The maintenance within a narrow limit of circulating water and salts is under ionic and endocrine control. Endocrine factors of pituitary origin such as PRL, are believed to play key roles in the ultimate adaptation of fish to their environment. In general, PRL has been suggested to play an important role in FW osmoregulation by promoting the conservation of ions (primarily Na^+ and Cl^-) and decreasing water uptake (reviewed by Manzon 2002). Na^+/K^+ ATPase is the main Na^+ excreting mechanism in seawater-adapted teleosts, that controls not only Na^+ concentration in the plasma, but also Cl^- (Marshall 2002 for extensive review and references). In addition, other factors like cortisol, which stimulates hypotolerance in Salmonids (McCormick, 2001), atrial natriuretic peptide (Takei and Hirose 2002) or the renin-angiotensin system with importance in control of volume and water handling (Olson, 1992; Fuentes and Eddy 1997), play important roles in the osmoregulatory process in fish.

The sea bream is a marine teleost capable of some adaptation to reduction in environmental salinity which causes moderate to severe haemodilution and activation of PRL cells (Mancera *et al.*, 1993a, 2002).

OTHER ACTIONS - A large number of the reported effects of PRL are associated with growth and development, endocrinology and metabolism, and Immunoregulation and protection.

Growth and development

A large number of the reported effects of PRL are associated with growth and development. Many of these are seen in lower vertebrates, but more recent data confirm that cellular proliferation is also one of the important functions of PRL in mammals (Bole-Feysot *et al.*, 1998 for review).

The somatotropic and developmental actions of PRL have also been examined in fish. In tilapia (*Oreochromis mossambicus*), the somatotropic activities ascribed to PRL result from the binding of heterologous PRL to GH receptors (Shepherd *et al.*, 1997). In the Japanese flounder (*Paralichthys olivaceus*), PRL antagonized the stimulatory effect of T3 on the resorption of the dorsal fin rays of prometamorphic larvae *in vitro* (De Jesus *et al.*, 1994).

Endocrinology and metabolism

PRL has been reported to affect carbohydrate metabolism in several vertebrate classes and has marked effects on lipid metabolism in birds (Bole-Feysot *et al.*, 1998 for review).

In fish, PRL is also involved in the mobilization of energy substrates (Leung et al., 1991; Sheridan, 1986; Weber and Grau, 1999). In Mozambique tilapia (*Oreochromis mossambicus*), seawater transfer of food-deprived animals

results in a significant increase in plasma concentrations of glucose, total free amino acids, and in hepatic pyruvate kinase and lactate dehydrogenase activities (Vijayan *et al.*, 1996).

Immunoregulation and protection

In mammals, PRL plays a significant role in regulation of the humoral and cellular immune response. PRL stimulates mitogenesis in both normal T lymphocytes and the Nb2 lymphoma cell line. PRLRs are immunocytochemically detected on human peripheral lymphocytes, and their mRNA expression is regulated by PRL. In the thymus, spleen, lymph nodes, and bone marrow of both rats and mice mRNA encoding the short and long PRLRs isoforms is present (Freeman *et al.*, 2000). PRL is involved, directly or indirectly, in the development and maturation of immune cells in the thymus and peripheral lymphoid organs, the migration of immature lymphocytes to the periphery, and in selected T- and B-dependent cellular immune responses (Bole-Feysot *et al.*, 1998).

The immune response in fish is composed of both inherited and acquired components. For example, the mucus layer on the skin of fish acts as a physical and chemical barrier to pathogens. Immune response is adquired at a very early age in fish, and the time at antibody production is related to the temperature. In some teleosts, PRL stimulate macrophages *in vitro* and *in vivo* (Narnaware *et al.*, 1998; Sakai *et al.*, 1996), and increases blood lymphocyte counts of sea bream (Narnaware *et al.*, 1998).

1.9 Objectives of project

The aim of the project was to provide information to further investigate the different roles of PRL in sea bream physiology and development. To achieve this aim the following objectives were defined:

1) To isolate and characterize different PRL isoforms released into the medium of cultured pituitaries.

2) To study the influence of estradiol-17β, galanin, and vaso-intestinal peptide on *in vitro* PRL secretion.

3) To study the influence of photoperiod, temperature, and water salinity changes on *in vitro* PRL secretion.

4) To describe the annual and circadian variation of PRL cells activity.

CHAPTER 2: General Materials and Methods

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This chapter contains the general materials and methods utilized to generate the results presented in chapters 3, 4, 5, 6, and 7. The methods used to culture the sea bream pituitaries, to separate proteins using SDS-PAGE and isoelectric focusing, to quantify protein bands by optical densitometry, to detect proteins by immunoblotting, and to estimate protein molecular weight and isoelectric point are described in the present chapter. Additional materials and methods, specific to particular chapters are detailed in the relevant chapter.

2.1 Incubation of pituitary glands

Sea bream were anaesthetised using MS-222 (1:10000; Sigma, Madrid), sacrificed by decapitation and pituitaries collected into freshly prepared cold Kreb's Ringer bicarbonate (KRB) with sodium concentrations of 110mM or 170mM having osmotic pressure of about 230mOsm/kg (hyposmotic medium) or 320mOsm/kg (isosmotic medium), respectively. Pituitary glands were transferred individually into the wells of a sterile disposable 96 well plate (Costar, USA) containing 25µl of culture medium/well. The culture medium consisted of KRB gassed with 95% O_2/5% CO_2, with the pH adjusted to 7.8 and supplemented with 10 µl/ml of vitamins (MEM 100x Vitamins, Sigma, Madrid), 20 µl/ml essential amino acids (MEM 50x, Sigma, Madrid), 10 µl/ml non essential amino acids (MEM 100x, Sigma, Madrid), 10 µl/ml antibiotic (penicillin 10,000 IU/ml; streptomycin 10,000 UG/ml, GIBCO, Scotland) and 20 µl/ml L-glutamine (200 mM, Sigma, Madrid). Pituitary glands were incubated for 18hr at 21°C in an

atmosphere containing 95% O_2/5% CO_2. After culture pituitary glands and culture medium were stored at –70°C or -20°C until analysis. An incubation time of 18-hr was chosen on the basis of optimisation studies carried out with sea bream pituitary glands and analysis of the literature (Wigham, 1992).

2.2 Electrophoretic separation of PRL

2.2.1 SDS-PAGE

Pituitary homogenates were obtained from cultured pituitaries extracted in Tris-HCl 0.5mM pH8.6, centrifuged at 10000 rpm for 5 min at 4°C, and the supernatant removed for analysis. For each pituitary gland, culture medium (10µl) and pituitary homogenates (10µl) were mixed with an equal volume of sample buffer (6% w/v SDS, 6% v/v 2-mercaptoethanol, 40% w/v sucrose, 0.02% bromophenol blue in 0.125M Tris-HCl, pH 6.8), boiled for 5 min, centrifuged (30 sec, 12,000rpm) and run on SDS-PAGE (12.5%) gels using a discontinuous system (Laemmli, 1970). The composition of the separating and stacking gels used are included in Table 2.1. Molecular weight markers (low range, Bio-Rad, Portugal) were run on all the gels.

Table 2.1 –Composition of the separating and stacking gels (SDS-PAGE)

Material	Separating gel	Stacking gel
Acrylamide/Bis (40%)*	5.625 ml	0.68 ml
Deionized water	7.735 ml	3.82 ml
0.5 M Tris HCl pH 6.8	-	1.45 ml
1.5 M Tris HCl pH 8.6	4.46 ml	-
10% SDS	180 µl	60 µl
10% ammonium persulfate	70 µl	20 µl
TEMED	10 µl	15 µl

* Acr/bis 29:1; 3.3% C (Bio-Rad; Portugal)

2.2.2 Staining procedures for SDS-PAGE gels

Coomassie blue staining

Gels were lightly stained with Coomassie blue (0.025% w/v in 45% v/v methanol, 45% v/v distilled water and 10% v/v acetic acid) and then destained (in an aqueous solution of 7.5% v/v acetic acid and 5% v/v methanol) before analysis.

Silver staining

The procedure used for silver staining is described in Johnstone and Thorpe, 1987. Gels were rinsed in an aqueous solution of 50% v/v methanol during 1hour and soakied in this solution overnight. Gels were soaked during 15 minutes in freshly prepared staining solution (0.8g silver nitrate dissolved in 4ml deionized water and gradually added to 100ml of an aqueous solution of 1.4% v/v 14.8M ammonium hydroxide and 0.076% w/v NaOH). Gels were rinsed in deionized water for 3x10 minutes and protein bands visualized soaking the gel in a freshly prepared aqueous solution of 0.005% citric acid w/v and 0.05% formaldehyde (38%). The development of color was stopped by rinsing the gels with tap water. Gels were stored until ready to dry in an aqueous solution of 5% v/v acetic acid and 45% v/v methanol away from light.

2.2.3 Isoelectric focusing

Culture medium (10µl) and pituitary homogenates (10µl) were separated by gel isoelectric focusing, carried out on thin-layer precast polyacrylamide gels,

pH 5.5-8.5 or pH 3-10 (Amersham Pharmacia Biotech) using the LKB 2117 Multiphor II (LKB Produkter AB, Sweden). The procedure for isoelectric focusing was as described in the LKB 2117 Multiphor II Electrophoresis System User Manual (LKB Produkter AB, Sweden). The temperature of the cooling plate was maintained at 10ºC by circulating water from a MultiTemp II refrigerated cooling bath (LKB Produkter AB, Sweden). Gels were pre-run for 30 minutes at a constant current of 50 mA as the voltage increased to 500 V. Samples and a pI marker (10 µl) were applied using applicator strips and the voltage increased to 1500V or to 1600V, depending on the pH range of the gel. The isoelectric focusing was allowed to proceed for a further 1 ½ hours or to 2 ½ hours, depending on the pH range of the gel. The gel was then removed from the apparatus and immersed in a fixing solution for 30 minutes, washed in destaining solution, stained in a solution containing Coomassie Blue R 250 and destained until the background was clear. The gel was soaked in preserving solution, covered with a cellophane preserving sheet, and dried at room temperature.

2.3 Quantification of PRL

PRL bands visible in SDS-PAGE gels were quantified by densitometry (Image Master VDS system – Amersham Pharmacia Biotech, Portugal). The optical density units were transformed to micrograms/ml using calibration curves of BSA (40µg/ml, fraction V, Merck, Germany) prepared by dilution in water to the following concentrations: 0.4, 0.6, 1, 2, 4 and 8µg/ml.

2.4 Western blotting

Electrophoretic transference

Culture medium and pituitaries homogenates separated by SDS-page (section 2.2.1) were electrophoretically transferred from a gel to a nitrocellulose membrane (Hybond^{TM} – C, Amersham Pharmacia Biotech) in a semi-dry system during 1 hour at 1.5mA/cm^2 of membrane.

Diffusion transference

Culture medium and pituitaries homogenates separated by isoelectric focusing gels (section 2.2.3) were transferred to a nitrocellulose membrane (Hybond^{TM} – C, Amersham Pharmacia Biotech) by capillary blotting using phosphate buffered saline (PBS) during 24 hours.

Protein identification

The membrane was agitated in blocking solution (Tris-HCl, 0.1M, pH 7.6, 2% milk powder and 0.05% Tween 20) for 3 hours at 22°C, rinsed in Tris-HCl (0.1M, pH 7.6) and incubated overnight at 4°C with anti-chum salmon PRL serum (1:100) or anti-sea bream GH serum (1/400). The specificity of this antisera has been previously characterised (Power and Canario, 1992). Membranes were washed with Tris-HCl (3 x 10 minutes) and then incubated with anti-rabbit IgG complexed with peroxidase (IgG-PAP, 1/1000; Sigma, Madrid) for 1h at room temperature. After rinsing in Tris-HCl, membranes were developed using 4-chloro-1-napthol (0.75 mg/ml) as the chromagen.

2.5 Estimation of PRLmolecular weight and isoelectric point

Molecular weight estimation

The molecular weight (MW) determination was made using SDS-PAGE (section 2.2.1) with both molecular weight standards (low range, Bio-Rad, Portugal) and unknowns running in a single gel system. After staining, the Rf values of the molecular weight standards are plotted on the x-axis and the corresponding log(MW) on the y-axis. The calibration curve can then be drawn. By calculating the Rf of the unknown protein, it is possible to estimate the molecular weight of the protein from the curve. Rf was calculated by the following formula (See *et al.*, 1990):

$$Rf = \frac{\text{distance of protein migration}}{\text{length after de-staining}} \times \frac{\text{length before de-staining}}{\text{distance of dye migration}}$$

Isoelectric point estimation

The isoelectric point was estimated with the help of marker proteins (Amersham Pharmacia Biotech). The markers are run in parallel with the unknown sample on the isoelectric focusing gel (section 2.2.3). After focusing and staining, the migration distances from the cathode edge of the gel to the different marker protein bands are plotted on y-axis and the corresponding pIs of the marker proteins plotted on the x-axis. The calibration curve can then be drawn. By measuring the migration distance of the unknown protein, it is possible to interpolate the isoelectric point of the protein from the curve.

CHAPTER 3: Isolation and characterization of PRL and GH

CHAPTER 3: Isolation and characterization of PRL and GH

3.1 Introduction

PRL belongs to a family of polypeptide hormones, which includes growth hormone (GH), placental lactogen (PL), and somatolactin (SL). Analysis of their amino acid sequence demonstrates that these hormones are highly conserved, and it has been proposed that they evolved from a common ancestral gene by duplication and subsequent divergence about $4x10^8$ years ago (Miller and Eberhardt, 1983; Nicoll *et al.,* 1986). PRL is a versatile hormone and multiple actions, ranging from mammary development and lactation in mammals to antimetamorphic effects in amphibians, have been described in higher vertebrates (Hirano *et al.*, 1987; Bres and Nicoll, 1993). The principal action of PRL in teleost fish is the maintenance of hydromineral balance in euryhaline teleosts in fresh water (Loretz and Bern, 1982; Hirano *et al.,* 1987). The function of PRL in marine teleosts is less certain and it is likely that PRL has other biological actions including a role in reproduction.

The functional polymorphism of PRL is probably related to the structural variability of this hormone. The existence of both size and charge variants of PRL has been shown in mammals, reptiles, amphibians and birds (Bollengier *et al.,* 1988; Briski *et al.*, 1996; Corcoran and Proudman, 1991; Lewis *et al.,* 1989; Martinat *et al.,* 1990; Noso *et al.*, 1992; Nyberg et al., 1982; Sinha *et al.,* 1991; Wallis *et al.*, 1980; Yamashita *et al.,* 1993). In some teleosts, chum salmon (*Oncorhynchus keta*), common carp (*Cyprinus carpio*), Japanese eel (*Anguilla*

japonica), Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*Oreochromis niloticus*) (Manzon, 2002 for review), two different forms of PRL have been identified. The two forms of PRL identified in salmon, carp, and eel are highly homologous (Manzon, 2002 for review), whereas the two forms of PRL secreted by tilapia pituitary share only 69% sequence identity and are designated tPRL177 and tPRL188 to indicate the number of amino acid residues in each isoform (Specker *et al.*, 1993).

The amino acid sequence of PRL has been characterised in a variety of teleostean and nonteleostean fish (reviewed by Manzon, 2002). Piscine PRLs are synthesized as prohormones with a signal peptide of 23-24 aa. All teleostean PRLs lack the N-terminal disulfide bond due to the absence of 12-14 aa at the N-terminus (see Manzon, 2002 for review). PRL from sea bream is a protein of 212 amino acids with a putative signal peptide of 24 residues and a mature protein of 188 amino acids (Santos *et al.*, 1999). However, while cDNA sequences reveal the potential secretory product arising from the precursor, it does not provide information on the actual cleavage and prosthetic modifications that can occur. Predictions of protein modifications can be made from the occurrence of consensus sequences, but confirmation is possible only by isolation and characterization of the products of post-translational processing. Analysis of the putative amino acid sequence of sea bream PRL revealed the existence of a consensus sequence for N-linked glycosylation at Asn 148 and for phosphorylation at Ser 166 (Santos *et al.*, 1999). Posttranslational modifications (chapter 1, section 1.2) of native sea bream PRL can influence the apparent

molecular weight because they can reduce protein mobility on SDS-PAGE, or may have a significant impact upon net molecular charge with minimal effects on size.

The present chapter characterizes the principle forms of PRL liberated from the sea bream pituitary gland cultured *in vitro*. The principle approaches utilized for identification and characterization of this hormone are described. Moreover, different isoforms of secreted PRL are purified on a Mini Prep Cell (Bio-Rad, Portugal), and partial amino acid sequence obtained by fingerprinting and MS/MS ions search.

3.2 Additional methods

The methodology utilized for the pituitary culture, "western blotting", and protein separation by SDS-PAGE and isoelectric focusing is described in chapter 2. The methods reported in this chapter are specific to the protein isolation by continuous elution electrophoresis, and N-terminal amino acid sequencing by fingerprinting and MS/MS ions search.

3.2.1 Identification and characterization of PRL and GH

3.2.1.1 Identification of denatured PRL and GH

Sea bream pituitaries were collected into freshly prepared culture medium and incubated for 18hrs at 21ºC in an atmosphere containing 95% O_2/5% CO_2 (chapter 2, section 2.1). After culture, pituitary glands and culture medium were stored at -20ºC until analysis (usually a week later). Denatured PRL and GH in

the culture medium and in the pituitary homogenates were separated on an SDS-PAGE system (chapter 2, section 2.2), and identity confirmed by Western blotting, using an antiserum against chum salmon PRL and an antiserum against sea bream GH (chapter 2, section 2.4). Gels not transferred to nitrocellulose membranes were stained with Coomassie blue and PRL and GH quantified by optical densitometry (chapter 2, section 2.3). Estimation of molecular weight was carried out by separation on the same gel of both molecular weight standards (low range, Bio-Rad, Portugal) and unknown samples (chapter 2, section 2.5).

3.2.1.2 Identification of native PRL and GH

Throughout one full calendar year and every two months (from September to August) at the same time in the morning (± 9hrs), sea bream samples were collected. Pituitaries were collected and incubated as described previously (chapter 2, section 2.1). After culture, pituitary glands and culture medium were stored at -20°C until analysis (usually a week later). Native PRL and GH in the culture medium and in the pituitary homogenates were separated by isoelectric focusing (chapter 2, section 2.2) for estimation of isoelectric point (chapter 2, section 2.5). Proteins were transferred by diffusion to nitrocellulose membranes and identity confirmed by Western blotting using an antiserum against chum salmon PRL and an antiserum against sea bream GH (chapter 2, section 2.4). This revealed several isoforms of PRL produced during the annual cycle and the principle forms were isolated by continuous elution electrophoresis.

3.2.2 PRL isolation

PRL was isolated from the medium where pituitaries were cultured by continuous elution electrophoresis, on a Mini Prep Cell (Fig.3.1; Bio-Rad, Portugal). During a run, the protein mixtures are electrophoresed through a cylindrical gel. As molecules migrate through the gel matrix, they separate into ring-shaped bands. Individual bands migrate off the bottom of the gel, where they pass directly into the elution chamber. The elution chamber consists of a thin polyethylene frit; a dialysis membrane, directly underneath the elution frit, traps proteins within the chamber.

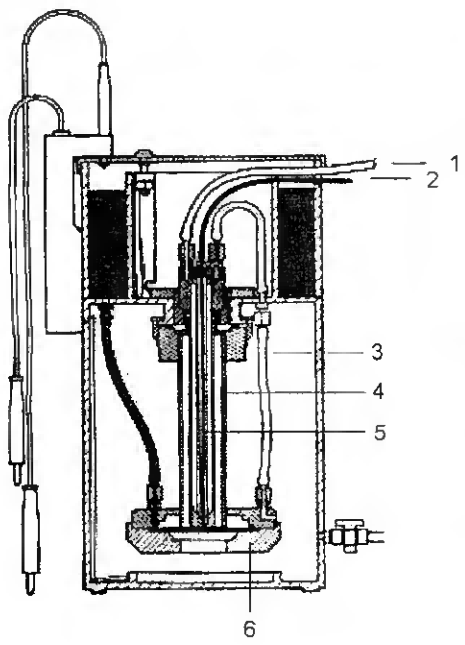


Figure 3.1- The Model 491 Prep Cell (1) cooling buffer outlet (2) elution buffer outlet (3) elution buffer feedline (4) gel assembly tube (5) cooling core (6) elution chamber.

When elution buffer enters the chamber, the proteins and the elution buffer are drawn radially inward to an elution tube by a peristaltic pump and then driven to a fraction collector. As molecules are purified, they are collected in discrete liquid fractions. The composition of the separating and stacking gels used are included in Table 3.1

A sample from each fraction collected was run in a discontinuous SDS-PAGE system. Molecular weight markers (low range, Sigma, Madrid) were run on all

the gels to assist identification of PRL and GH. The protein bands were visualized using silver staining (chapter 2, section 2.2).

Table 3.1 – Composition of the separating and stacking gels

Material	Separating gel	Stacking gel
Acrylamide/Bis (40%)*	8.475 ml	0.68 ml
Deionized water	11.565 ml	3.82 ml
0.5 M Tris HCl pH 6.8	-	1.45 ml
1.5 M Tris HCl pH 8.6	6.69 ml	-
10% ammonium persulfate	105 μl	50 μl
TEMED	15 μl	10 μl

* Acr/bis 29:1; 3.3% C (Bio-Rad; Portugal)

The fractions containing pure PRL were pooled (total volume≈20ml) in dialysis sacks that retained proteins of MW>12kDa (Sigma, Portugal), and dialyzed during 24 hours at 4ºC and against a volume of 2000ml of dialysis buffer, Tris 20mM, pH 8. The buffer was replaced 3-4 times during the time interval. The dialyzed sample was lyophilized during approximately 72 hours at -5ºC using a Serail RP2V (LabNorma, Portugal). Samples were kept at 4ºC for future utilization.

3.2.3 Identification of PRL and GH by amino acid sequencing

PRL and GH N-terminal amino acid sequences were determined by searching sequence databases using peptide molecular weights from the digestion of PRL molecule by trypsin (Peptide Mass Fingerprint) and mass spectrometry (MS) data from one or more peptide. The computer program Mascot (Perkins *et al.*, 1999) integrated the two types of search (Centro de Genómica y Proteómica, Facultad de Farmacia, Universidad Complutense de Madrid).

Peptide Mass Fingerprint

A mass spectrum of the peptide mixture resulting from the digestion of a protein by an enzyme provides a fingerprint of great specificity. Excised gel pieces containing the protein separated by SDS-PAGE, were washed in different solutions, dried and trypsin added to the dry gel pieces. The PRL mass spectrum was obtained by MALDI-TOF (matrix-assisted laser desorption/ionisation-time).

MS/MS ions search

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a relatively novel technique in which a co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse. Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule. The ionized biomolecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times. In this way each molecule yields a distinct signal. Fragments will only be detected if they carry at least one charge.

PRL identification from primary sequence databases

Database searching was performed using the program Mascot (Perkins *et al.*, 1999), which identifies the protein by searching a sequence database using

experimental data from peptide mass fingerprint integrated with tandem mass spectrometry (MS/MS) data from one or more peptides. In Mascot, the protein molecular weight is applied as a *sliding window*. That is, for each database entry, Mascot looks for the highest scoring set of peptide matches which are within a contiguous stretch of sequence less than or equal to the specific protein molecular weight. Mascot also takes into account any possible occurrence of mis-cleavages and common modifications of peptide such as oxidized methionine residues.

3.2.4 Molecular weight estimation of purified PRL and GH

Samples of isolated PRL and GH were dissolved in Tris-HCl (10mM pH8) and run on a SDS-PAGE system (chapter 2, section 2.2). Proteins were transferred by diffusion to nitrocellulose membranes and identity confirmed by Western blotting using an antiserum against chum salmon PRL and an antiserum against sea bream GH (chapter 2, section 2.4). Apparent molecular weights were estimated running both molecular weight standards (low range, Bio-Rad, Portugal) and unknowns in a single gel (chapter 2, section 2.5).

3.3 Results

Identification of denatured PRL and GH

SDS-PAGE of culture medium followed by Coomassie blue staining permitted the visualization of two predominant protein bands and their quantification by optical densitometry (Fig. 3.2). Western blotting of culture

medium and pituitary homogenates using anti-chum salmon PRL serum revealed a single immunoreactive band corresponding to a protein with an estimated molecular weight mean value of 25 kDa (Fig.3.2). Identity of GH which migrates close to PRL was also confirmed by western blotting using an antiserum against sea bream GH. A single immunoreactive band was revealed corresponding to a protein with an estimated molecular weight mean value of 22 kDa (Fig.3.2).

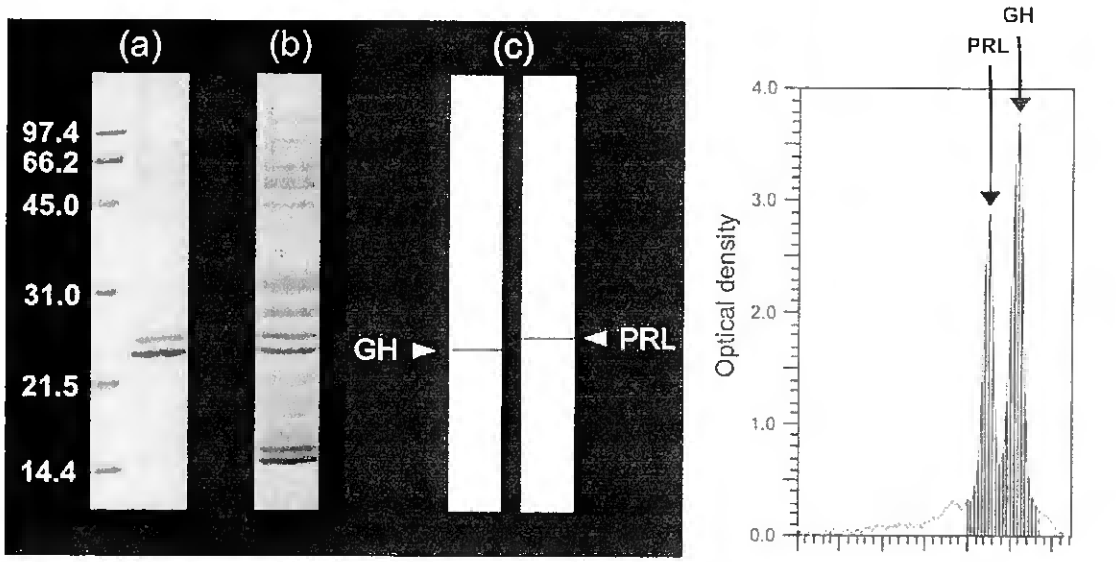


Figure 3.2 – The left hand panel shows the typical gels obtained after separation of (a) right hand lane, the culture medium and (b) the pituitary homogenate by SDS-PAGE. Molecular weight markers are indicated on the left hand side of the gel. Western blot (c) using antisera against chum salmon PRL and antisera against sea bream GH. The right hand panel is a lane profile obtained by optical densitometry after separation of the culture medium by SDS-PAGE. PRL and GH peaks are indicated by arrows.

The results confirm that the approach selected is adequate for separation of GH and PRL and substantiates the use of this method for subsequent studies in this thesis.

Identification of native PRL and GH

Native PRL and GH present in the culture medium and pituitary homogenates were separated by isoelectric focusing followed by Coomassie blue staining. Isoelectric focusing exhibited multiple intensely stained bands (Fig. 3.3). The identity of the bands was confirmed by Western blotting using an antisera against chum salmon PRL and sea bream GH. The isoelectric bands of the sea bream PRL were estimated to be between 6.1 and 6.7 (Fig. 3.3). GH bands had isoelectric points between 6.3 and 7.2 (data not shown).

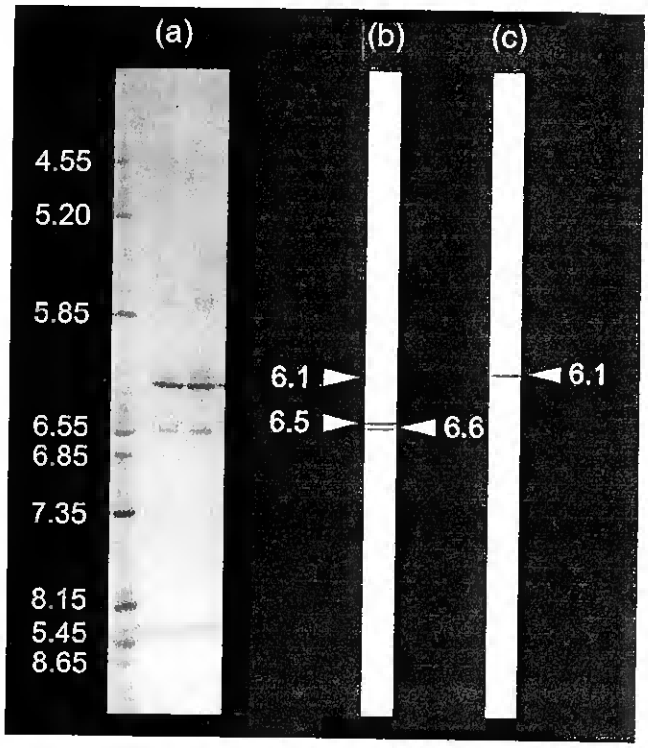


Figure 3.3 – The left panel (a) shows culture medium separated by isoelectric focusing and stained with Coomassie blue. Marker proteins are indicated on the left hand side of the gel. The right hand panel shows several charge variants of native PRL, present in the culture medium in summer (b) and winter (c), separated by isoelectric focusing and identified by Western blotting using antiserum against chum salmon PRL.

Isolation of the sea bream PRL and GH

Pituitaries were collected in winter and summer and incubated in culture medium for 48hrs at 21°C. After 24hrs the medium was collected, stored at -20°C, and replaced by freshly prepared medium. The general procedure is described in chapter 2, section 2.1. For each season, a sample of 1.5 ml of culture medium was mixed with an equal volume of sample buffer and boiled for 5 min (chapter 2, section 2.2). Denatured sea bream PRL and GH present in the medium of *in vitro* cultured pituitaries were purified on a Mini Prep Cell by continuous elution electrophoresis. The discrete fractions collected were run on a SDS-PAGE system and visualized by silver staining (Fig. 3.4). The use of the medium from 70 cultured pituitaries yielded 0.2mg of purified PRL and 0.2mg of purified GH. A yield of approximately 3mg/g wet weight of pituitary was obtained for each hormone.

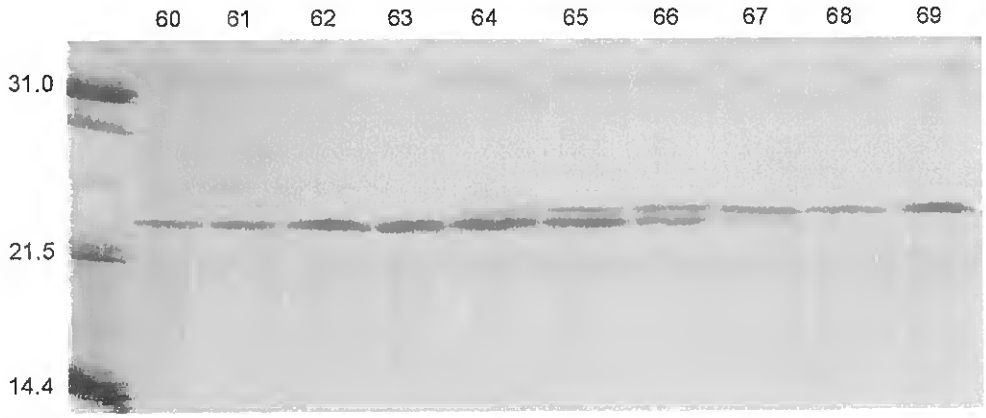


Figure 3.4 – Aliquots from the Model 491 Prep Cell fractions containing separated GH (fractions 60-63) and PRL (fractions 67-69) analyzed by SDS-PAGE and visualized by silver staining. Molecular weight size markers are indicated on the left handed side of the image.

Molecular weight estimation of purified PRL and GH

Lyophilized PRL and GH were dissolved in Tris (10nM, pH8) and separated by SDS-PAGE. The gels were stained with Coomassie blue (chapter2, section 2.2), and molecular weights of PRL and GH estimated as 25 and 22 kDa, respectively (Fig. 3.5). Isolated PRL and GH were identified by Western blotting, using anti-chum salmon PRL and anti-sea bream GH (Fig. 3.6).

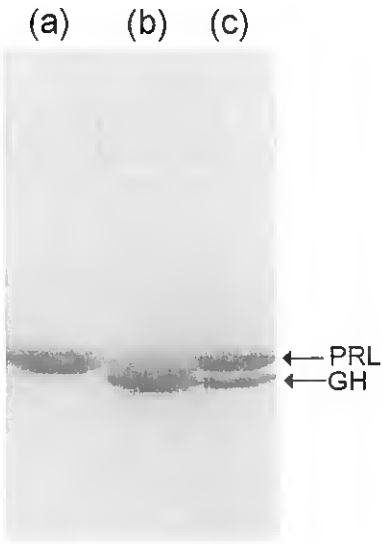


Figure 3.5 - SDS-PAGE gel stained with Coomassie, showing the electrophoretic pattern of the (a) purified sea bream PRL, (b) purified sea bream GH. Starting material is shown in lane (c).

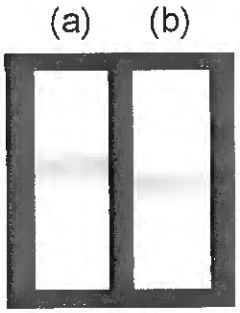


Figure 3.6 - Western blots showing (a) purified sea bream PRL detected using antisera against chum salmon PRL and (b) purified sea bream GH detected using antisera against sea bream GH.

Identification of PRL and GH by amino acid sequencing

SDS-PAGE selected bands of purified PRL and GH, obtained from pituitary cultures prepared in winter and summer, were subject to in-gel tryptic digestion. Peptides were also obtained by collision-induced fragmentation

(MS/MS ions search). The masses and charges of several peptides isolated by both methods were estimated by MALDI-TOF/TOF mass spectrometry, and amino acid sequences obtained by database searching performed using the program Mascot (Perkins *et al.*, 1999). Table 3.2 shows the amino acid sequence of peptides obtained from PRL molecules purified in winter and summer.

Table 3.2 – Comparison between peptide amino acid sequence obtained by enzymatic cleavage (fingerprint) and by MS/MS ion search of purified PRL molecule. Potential phosphorylation sites are marked in bold. One potential glycosylation site at N_{172} is marked in italic.

		Observed mass		Peptide sequence
	Start – End*	fingerprint	ions search	
"winter" PRL	25 - 33	1054.58	1054.51	VPINDLIDR
	150 - 163	1477.71	1477.70	MGPAA**QAIS**SLPYR
	189 - 198	1189.60	1189.63	DSH**KIDS**FLK
"summer" PRL	82 – 97	1790.89	1806.88	EQALQLSESDLMSLAR
	129 - 137	1139.62		IRELQEHSK
	131 - 137	870.44		ELQEHSK
	131 - 149	2026.03		ELQEHSKSLGDGLDILSGK
	138 - 149	1174.63		SLGDGLDILSGK
	150 - 163	1461.75	1477.74	MGPAA**QAIS**SLPYR
	150 - 178	2707.32		MGPAAQAISSLP**YRGS**NDIG EDN***IS***K
	164 - 175	1248.58		GSNDIGEDN***IS***K
	176 - 187	1554.80		LTNFH**FLLS**CFR
	189 - 198	1189.62		DSH**KIDS**FLK

* Note: the amino acids are numbered including the signal peptide of 24 amino acids

Comparison of PRL molecule N-terminal sequence of peptide 150-163 obtained by fingerprinting in winter and summer, which contains a potential phosphorylation site at serine 158 (basic-basic-X-S/T, Dimaline, 1988), show different mass values (1477.71 and 1461.75 in winter and summer, respectively). As the amino acid sequence is similar, the increase of mass during winter may

be an indication that some transformation occurred at site S_{158}. As a consequence of the limited sequence data obtained for PRL isolated from winter pituitaries other possible post-translational modification sites can not be comparable between the proteins isolated from winter and summer cultures.

The same analysis was made for GH molecules purified in winter and summer and results included in Table 3.3.

Table 3.3 – Comparison between peptide amino acid sequence obtained by enzymatic cleavage (fingerprint) and by MS/MS ions search of purified GH molecule. Potential phosphorylation sites are marked in bold.

		Observed mass		Peptide sequence
	Start – End*	fingerprint	ions search	
"winter" GH	26 - 33	892.54		LFS**IAVS**R
	34 - 43	1214.72		VQHLHLLAQR
	44 - 58	1815.83	1815.82	LFSDFES**SLQT**EEQR
	44 - 62	2299.11		LFSDFES**SLQT**EEQR QLNK
	63 - 80	2188.06		IFLQD**FCNS**DY**IIS**PIDK
	63 - 85	2839.34	2839.36	IFLQD**FCNS**DY**IIS**PID**KHET**QR
	91 - 97	851.52		LLSISYR
	98 - 107	1249.63	1249.61	**LVES**WEFFPSR
	108 - 116	831.45		SL**SGGS**APR
	123 - 135	1492.92		LSELKTGIHLLIR
	128 - 135	922.60		TGIHLLIR
	136 - 169	3691.66		ANEDGAEI**FPDS**SALQLAPYG N**YYQS**
	171 - 179	1144.58		TYELLACFK
	181 - 193	1534.80		DMH**KVET**YLTVAK
	181 - 193	1550.79		DMH**KVET**YLTVAK
	185 - 193	1023.58		VE**TYLT**VAK
"summer" GH	34 - 43	1214.65		VQHLHLLAQR
	44 - 56		1814.80	LFSDFES**SLQT**EEQR
	44 - 62	2299.12		LFSDFES**SLQT**EEQRQLNK
	63 - 80	2188.06		IFLQD**FCNS**DY**IIS**PIDK
	63 - 85	2839.40	2939.43	IFLQD**FCNS**DY**IIS**PID**KHET**QR
	98 - 107	1249.57		**LVES**WEFPSR
	136 - 169	3691.78		ANEDGAEI**FPDS**SALQLAPYG N**YYQS**
	136 – 170	3847.95		ANEDGAEI**FPDS**SALQLAPYG N**YYQS** L
	181 - 193	1534.75		DMH**KVET**YLTVAK
	181 - 193	1550.74		DMH**KVET**YLTVAK

GH molecule purified in winter and summer shows multiple potential phosphorylation sites (basic-basic-X-S/T, Dimaline, 1988), but no consensus sequence for glycocylation sites (N-X-T/S, Dimaline, 1988). In winter and summer, two peptides 181-193 with different masses resulted from the fragmentation of GH molecule, which may be an indication that a pos-translational modification occurs at the T_{187} or T_{190}.

The peptide amino acid sequences were aligned with the deduced amino acid sequence from sea bream PRL cDNA (Santos *et al.*, 1999). Table 3.4 shows the amino acid sequence of peptides obtained by fingerprinting and MS/MS ions search of PRL purified from pituitary cultures prepared in winter and summer.

In winter, 18% of the PRL molecule was sequenced, while 46% of the PRL isolated in summer was sequenced. Both amino acid sequences were 100% identical to the deduced amino acid sequence of sea bream cDNA (Santos *et al.*, 1999). In winter, the N-terminal sequence of the first peptide started at V_{25}, the first amino acid of the mature protein (Santos *et al.*, 1999).

The peptide amino acid sequences were aligned with the deduced amino acid sequence from sea bream GH cDNA (Almuly *et al.*, 2000). Table 3.5 shows the amino acid sequence of peptides obtained by fingerprinting and MS/MS ions search of GH purified from pituitary cultures prepared in winter and summer.

Table 3.4 – Comparison between amino acid sequence deduced from sea bream PRL cDNA (Santos *et al.*, 1999) and "winter" and "summer" PRL peptides (marked in bold), obtained by MALDI-TOF/TOF mass spectrometry. Note that amino acids are numbered including the signal peptide of 24 amino acids, and that in the "winter" PRL the 7th residue at the N-terminal of the first peptide sequenced (isoleucine) was different from the one deduced from PRL cDNA (leucine).

"winter" PRL

1	M	A	H	R	E	T	N	G	S	K	L	F	I	T	V	L
17	C	M	V	A	A	C	S	A	**V**	**P**	**I**	**N**	**D**	**L**	**I**	**D**
33	**R**	A	S	Q	R	S	D	M	L	H	S	L	S	T	T	L
49	T	K	D	L	S	N	H	V	P	P	V	G	W	T	M	M
65	P	R	P	P	L	C	H	T	S	S	L	Q	T	P	N	D
81	K	E	Q	A	L	Q	L	S	E	S	D	L	M	S	L	A
97	R	S	L	L	Q	A	W	Q	D	P	L	V	D	L	S	N
113	S	A	N	S	L	L	H	P	S	Q	S	S	I	S	N	K
129	I	R	E	L	Q	E	H	S	K	S	L	G	D	G	L	D
145	I	L	S	G	K	**M**	**G**	**P**	**A**	**A**	**Q**	**A**	**I**	**S**	**S**	**L**
161	**P**	**Y**	**R**	G	S	N	D	I	G	E	D	N	I	S	K	L
177	T	N	F	H	F	L	L	S	C	F	R	R	**D**	**S**	**H**	**K**
193	**I**	**D**	**S**	**F**	**L**	**K**	V	L	R	C	R	A	A	K	V	Q
209	P	E	M	C												

"summer" PRL

1	M	A	H	R	E	T	N	G	S	K	L	F	I	T	V	L
17	C	M	V	A	A	C	S	A	V	P	I	N	D	I	I	D
33	R	A	S	Q	R	S	D	M	L	H	S	L	S	T	T	L
49	T	K	D	L	S	N	H	V	P	P	V	G	W	T	M	M
65	P	R	P	P	L	C	H	T	S	S	L	Q	T	P	N	D
81	K	**E**	**Q**	**A**	**L**	**Q**	**L**	**S**	**E**	**S**	**D**	**L**	**M**	**S**	**L**	**A**
97	**R**	S	L	L	Q	A	W	Q	D	P	L	V	D	L	S	N
113	S	A	N	S	L	L	H	P	S	Q	S	S	I	S	N	K
129	**I**	**R**	**E**	**L**	**Q**	**E**	**H**	**S**	**K**	**S**	**L**	**G**	**D**	**G**	**L**	**D**
145	**I**	**L**	**S**	**G**	**K**	**M**	**G**	**P**	**A**	**A**	**Q**	**A**	**I**	**S**	**S**	**L**
161	**P**	**Y**	**R**	**G**	**S**	**N**	**D**	**I**	**G**	**E**	**D**	**N**	**I**	**S**	**K**	**L**
177	**T**	**N**	**F**	**H**	**F**	**L**	**L**	**S**	**C**	**F**	**R**	R	D	S	H	K
193	I	D	S	F	L	K	V	L	R	C	R	A	A	K	V	Q
209	P	E	M	C												

In winter, 75% of the GH molecule was sequenced, while only 53% of the GH isolated in summer was sequenced. Both amino acid sequences were 100% identical to the deduced amino acid sequence of sea bream cDNA (Almuly *et al.*, 2000).

Table 3.5 – Comparison between amino acid sequence deduced from sea bream GH cDNA (Almuly *et al.*, 2000) and "winter" and "summer" GH peptides (marked in bold), obtained by MALDI-TOF/TOF mass spectrometry.

"winter" GH	1	M	D	R	V	V	L	M	L	S	V	M	S	L	G	V	S
	17	S	Q	P	I	T	D	G	Q	R	L	**F**	**S**	**I**	**A**	**V**	**S**
	33	**R**	**V**	**Q**	**H**	**L**	**H**	**L**	**L**	**A**	**Q**	**R**	**L**	**F**	**S**	**D**	**F**
	49	**E**	**S**	**S**	**L**	**Q**	**T**	**E**	**E**	**Q**	**R**	**Q**	**L**	**N**	**K**	**I**	**F**
	65	**L**	**Q**	**D**	**F**	**C**	**N**	**S**	**D**	**Y**	**I**	**I**	**S**	**P**	**I**	**D**	**K**
	81	**H**	**E**	**T**	**Q**	**R**	S	S	V	L	K	**L**	**L**	**S**	**I**	**S**	**Y**
	97	**R**	**L**	**V**	**E**	**S**	**W**	**E**	**F**	**P**	**S**	**R**	**S**	**L**	**S**	**G**	**G**
	113	**S**	**A**	**P**	**R**	N	Q	I	S	P	K	**L**	**S**	**E**	**L**	**K**	**T**
	129	**G**	**I**	**H**	**L**	**L**	**I**	**R**	**A**	**N**	**E**	**D**	**G**	**A**	**E**	**I**	**F**
	145	**P**	**D**	**S**	**S**	**A**	**L**	**Q**	**L**	**A**	**P**	**Y**	**G**	**N**	**Y**	**Y**	**Q**
	161	**S**	**L**	**G**	**T**	**D**	**E**	**S**	**L**	**R**	R	**T**	**Y**	**E**	**L**	**L**	**A**
	177	**C**	**F**	**K**	K	**D**	**M**	**H**	**K**	**V**	**E**	**T**	**Y**	**L**	**T**	**V**	**A**
	193	**K**	C	R	L	S	P	E	A	N	C	T	L				
"summer" GH	1				M	D	R	V	V	L	M	L	S	V	M	S	L
	17	G	V	S	S	Q	P	I	T	D	G	Q	R	L	F	S	I
	33	A	V	S	R	**V**	**Q**	**H**	**L**	**H**	**L**	**L**	**A**	**Q**	**R**	**L**	**F**
	49	**S**	**D**	**F**	**E**	**S**	**S**	**L**	**Q**	**T**	**E**	**E**	**Q**	**R**	**Q**	**L**	**N**
	65	**K**	**I**	**F**	**L**	**Q**	**D**	F	**C**	**N**	**S**	**D**	**Y**	**I**	**I**	**S**	**P**
	81	**I**	**D**	**K**	**H**	**E**	**T**	**Q**	**R**	S	S	V	L	K	L	L	S
	97	I	S	Y	R	**L**	**V**	**E**	**S**	**W**	**E**	**F**	**P**	**S**	**R**	S	L
	113	S	G	G	S	A	P	R	N	Q	I	S	P	K	L	S	E
	129	L	K	T	G	I	H	L	L	I	R	**A**	**N**	**E**	**D**	**G**	**A**
	145	**E**	**I**	**F**	**P**	**D**	**S**	**S**	**A**	**L**	**Q**	**L**	**A**	**P**	**Y**	**G**	**N**
	161	**Y**	**Y**	**Q**	**S**	**L**	**G**	**T**	**D**	**E**	**S**	**L**	**R**	**R**	T	Y	E
	177	L	L	A	C	F	K	K	**D**	**M**	**H**	**K**	**V**	**E**	**T**	**Y**	**L**
	193	**T**	**V**	**A**	**K**	C	R	L	S	P	E	A	N	C	T	L	
					M	D	R	V	V	L	M	L	S	V	M	S	L

3.4 Discussion

PRL and GH are the predominant components released to the medium by the cultured sea bream pituitaries and run very near in reducing systems. However, separation of the two proteins by an optimized SDS-PAGE system, followed by immunological methods to confirm their identity, and analysis of lane profiles obtained by optical densitometry, revealed that this approach is adequate for separation of PRL and GH and substantiate the use of this method for studies in the present thesis.

Human and most mammalian PRLs and GHs have revealed several bands on polyacrylamide gel electrophoresis (Wallis *et al.*, 1980; Meuris *et al.*, 1984; Bollengier et al., 1988; Shah and Hymer, 1989; Mena *et al.*, 1992; Anthony *et al.*, 1993; Warner *et al.*, 1993; Briski et al., 1996; Garcia-Barros *et al.*, 2000). Some studies in reptiles, birds, and Atlantic cod demonstrate that the same size heterogeneity exists in non-mammalian species (Noso *et al.*, 1992; Rand-Weaver *et al.*, 1989; Martinez-Coria *et al.*, 2002). In sea bream, molecular weights of PRL and GH separated by SDS-gel electrophoresis were estimated as 25 and 22 kDa, respectively, and no size heterogeneity was detected for both hormones.

Charge heterogeneity has been reported to a lesser extent in vertrebrates. Some studies in rats, amphibians, and chum salmon (Nyberg *et al.,* 1982; Kawauchi *et al.,* 1983; Yamashita *et al.*, 1993; Briski *et al.*, 1996), demonstrated the existence of PRL charge isoforms. GH charge variants were also observed in birds and chum salmon (Kawauchi *et al.,* 1986; Houston and Goddard, 1988; Aramburo *et al.*, 1989; Skibeli *et al.*, 1990; Montiel *et al.*, 1992). In the present study, there is charge heterogeneity when the annual cycle is analyzed, and multiple charge variants were detected when native sea bream PRL and GH were separated by isoelectric focusing. In birds, it was possible to correlate the release of GH size and PRL charge variants with different physiological stages during the life cycle (Bédécarrats *et al.*, 1999; Aramburo *et al.*, 2000). Different biological activities were also demonstrated for tilapia PRLs variants (Sinha, 1995 and Manzon, 2001 for reviews). The pituitary of this cichlid fish secretes two PRLs of different molecular weight and charge, and amino acid sequence

information indicated that the tilapia PRLs are distinct proteins. Genetic variants of PRL have been found in some teleosts, namely goldfish (Chan *et al.*, 1996), Japanese eel (Suzuki *et al.*, 1991), chum salmon (Kawauchi *et al.*, 1986), and striped bass (Jackson *et al.*, 2000). Sea turtle and toads also have two genetic PRL variants (Yasuda et al., 1990; Yamashita *et al.*, 1993)

In order to try to explain the charge heterogeneity observed in sea bream, highly purified PRL and GH from winter and summer were obtained by continuous elution electrophoresis performed using a Model 491 Prep Cell, followed by partial amino acid sequencing. Database searches based on peptide mass fingerprint and MS/MS ions search, provided a reasonable coverage of the entire proteins and 18 and 46% of the PRL molecule, in winter and summer respectively, while 53 and 75% of the GH molecule, in winter and summer respectively, were sequenced. Both isolated PRL and GH molecules had sequences which were in agreement with previously published data (Santos *et al.*, 1999; Almuly *et al.*, 2000). The partial amino acid sequence established in the present study does not reveal any differences between molecules isolated during winter and summer. However as only 18% of the winter PRL was sequenced, further sequence is necessary in order to establish if differences are present which can explain the charge heterogeneity.

Size and charge heterogeneity can also result from different types of post-translational modifications. Glycosylation and phosphorylation of PRL and GH are major modifications in mammals (Ray et al., 1989; Sinha, 1995; Garcia-Barros *et al.*, 2000), reptiles (Noso *et al.*, 1992), and birds (Corcoran and

Proudman, 1991; Aramburo *et al.*, 1992). In Atlantic cod, GH charge heterogeneity is due to phosphorylation of the native pituitary hormone (Skibeli *et al.*, 1989). In sea bream, these modifications were previously suggested to explain the difference detected between the predicted and the observed molecular weight of PRL (Santos *et al.*, 1999). In the present study, PRL isoelectric points were estimated between 6.1 and 6.7 compared with 7.08 calculated from amino acid sequencing, and GH had isoelectric points between 6.3 and 7.2 compared with the calculated value of 6.52. The observed isoelectric points were different from the expected based upon the number of amino acids that the molecules contain. If only one form of the protein exists than it seems likely that post-translational modifications of the mature protein may cause the charge variants detected. Using data from peptide mass fingerprint, it was not possible to clearly demonstrate the existence of such transformations, but potential phosphorilation at S_{158} for PRL and at T_{187} or T_{190} for GH, are proposed on the basis of the present data.

For each hormone, a yield of approximately 3mg/g wet weight of pituitary was obtained and this is higher than the yields obtained from salmon and Atlantic cod (Kawauchi *et al.*, 1983; Kawauchi *et al.*, 1986; Rand-Weaver *et al.*, 1989; Skibeli *et al.*, 1989).

In summary, sea bream PRL and GH gave no detectable molecular variants with different size but demonstrated charge heterogeneity, which could be accounted for by modifications due to multiple post-translational modifications. However, sea bream PRL and GH post-translational modifications were not

elucidated in the present study and deserve further investigation. Moreover, the procedure of continuous elution electrophoresis performed using a Model 491 Prep Cell, described in the present study for the purification of sea bream PRL and GH, is of general applicability because relatively small amounts of starting material are necessary for this procedure and losses are kept at the minimum as few steps are employed.

CHAPTER **4:** Circannual and circadian rhythms of PRL *in vitro* release

CHAPTER **4:** Circannual and circadian rhythms of PRL *in vitro* release

4.1 Introduction

Most of the animals develop biological and behavioral circadian and/or annual cycles that are usually related to the cyclic physiological processes present during their lifetime. Annual cycles of plasma levels of steroids (and association of these cycles with specific phases of the reproductive cycle) are observed in humans, mammals, birds, amphibians, reptiles, and crustaceans (Amey and Whittier, 2000; Andersson *et al.*, 2003; Garcia *et al.*, 2002; Quinitio *et al.*, 1991; Shelby *et al.*, 2000; Sockman and Schwabl, 1999). In teleost fish, circannual variations in serum concentrations of steroids, vitellogenin and thyroid hormones are reported in the dentex (*Serranus subligarius*), sea bass (*Dicentrarchus labrax*), the catfish (*Heteropneustes fossilis*), the bitterling (*Acheilognathus rhombea*), the jundia (*Rhamdia quelen*), and the Indian carp (*Labeo rohita*) (Lamba *et al.*, 1983; Shimizu *et al.*, 1985; Cheek *et al.*, 2000; Pavlidis *et al.*, 2000; Barcellos *et al.*, 2001; Sen et al., 2002). Changes in gonadotropin, lipid and cholesterol are observed during the annual reproductive cycle of the freshwater teleost mrigal (*Cirrhinus mrigala*) (Singh and Singh, 1984). Pronounced seasonal rhythms in plasma somatolactin levels were observed in rainbow trout (*Oncorhynchus mykiss*) (Rand-Weaver *et al.*, 1995).

In addition to annual cycles there is clear evidence that daily light-dark cycle governs rhythmic changes in the behavior and/or physiology of most vertebrates. These regularly repeated patterns require a timing mechanism, which may be controlled by stimuli external to the animal, by internal timing mechanisms (biological clocks), or a combination of both mechanisms. Mammals have an internal biological clock located in the suprachiasmatic nuclei (SCN) of the brain, and the most likely exogenous signal is day length, but temperature and other stimulus can also be used to entrain the internal clock. In diurnal mammals, photoperiodism depends on the generation of a 24-hr nocturnal melatonin signal by the pineal gland, and the decoding of this signal in specialized target organs, including the pituitary gland (for a review, see Lincoln *et al.*, 2003). In non-mammalian species there is relatively little information about these mechanisms. In amphibians, the photoreceptor cells within the retina contain a circadian clock that controls melatonin release (Zhu *et al.*, 2000; Wiechmann and Smith, 2001). In reptiles, circadian photoreceptors are present in the retinas of the lateral eyes, pineal, parietal eye, and, possibly, the SCN (Tosini *et al.*, 2001). In birds, the circadian clock is found in the pineal gland, which is sensitive to light and produces melatonin. Moreover, it was found that temperature directly influences the synthesis and release of melatonin in chicks (Barrett and Takahashi, 1995). In Atlantic salmon (*Salmo salar*), the nocturnal increase in circulating melatonin accurately reflects the duration of darkness, which potentially could be used by the animal to time daily and seasonal events (Randall *et al.,* 1995). In trout, melatonin receptors have been described in

visually related areas of the brain, suggesting that in this species the hormone is involved primarily in the processing of visual signals (Mazurais *et al.*, 1999; Mazurais *et al.*, 2000). In pike (*Esox lucius*), transcripts of melatonin receptors and binding sites are expressed in the optic tectum, retina and pituitary gland (Gaildrat and Falcón;1999; Gaildrat and Falcón, 2000; Gaildrat *et al.*, 2002), and the number and activity of melatonin binding sites are synchronized by the photoperiod (Gaildrat *et al.*, 1998). In the white sucker (*Catostomus commersoni)*, cultured pineal glands show a circadian rhythmicity on melatonin secretion, influenced by photoperiod and temperature (Zachmann et al., 1992). In pikes and trouts, arylalkylamine N-acetyltransferases (enzymes involved in melatonin synthesis) are controlled by different genes in the retina and in the pineal gland (Benyassi *et al.*, 2000). While the duration of raised melatonin synthesis is dictated by the prevailing photoperiod, studies in juveniles of Atlantic salmon have shown that the amplitude of melatonin secretion could be influenced by other environmental factors such as temperatures and light intensity (Randall et al., 1995; Porter *et al.*, 2001). In Atlantic cod (*Gadus morhua)*, body size appears to have an important influence, with the smallest fish exhibiting significantly higher levels of dark phase melatonin (Porter *et al.*, 2000). In the sea bass, photoperiod length controls the duration of the nocturnal melatonin rise, while water temperature determines the amplitude of the melatonin rhythm (Garcia-Allegue *et al.*, 2001).

The internal clock mechanism appears to be under genetic control. Clock genes have been discovered and cloned in the fruit fly (*Drosophila*

melanogaster), and in the fungus *Neurospora* (for a review, see Wager-Smith and Kay, 2000). In mammals, clock genes are expressed in the SCN (for a review, see Lincoln *et al.*, 2003). Recently, circadian rhythms have been observed in various isolated mice tissues (Yamazaki *et al.*, 2000), and restricted feeding strongly entrained the expression of circadian genes in the liver (Stokkan *et al.*, 2001). These observations indicate that the cells and tissues of the body may be capable of modulating their activity on a circadian basis without the participation of an SCN clock function or with the SCN acting as a master circadian pacemaker. In teleosts, the expression of genes associated with melatonin synthesis in pike and zebrafish (*Danio rerio*), but not the trout, is controlled by a circadian clock in the pineal organ (Bégay et al., 1998; Coon *et al.*, 1998). A homolog of the mouse clock gene has been described in rainbow trout with the highest density of clock transcripts observed in the optic tectum and the pretectal area (Mazurais *et al.*, 2000).

In several species, a nocturnal melatonin signal that reflects nightlength and daylength is produced by the pineal gland. In mammals, this signal is decoded in the melatonin target cells, calendar cells, localized principally in the *pars tuberalis* of the pituitary gland. The calendar cells are thought to use a clock gene-based mechanism to decode melatonin signal to produce a long or short day physiology (for a review, see Lincoln *et al.*, 2003). In non-mammalian species there is relatively little information about this mechanism, but *in vitro* studies show that melatonin modulates GH and PRL secretion in trout pituitaries (Falcón *et al.*, 2003).

The pituitary hormone, PRL, has been observed to elicit a variety of physiological responses in different groups of animals in response to environmental stimulus. In mammalian and avian species, PRL circulating levels are correlated with ambient temperature (Gahali et al., 2001; Hooley *et al.*, 1979; Maney *et al.*, 1999; Schams *et al.*, 1980) and with photoperiod (Gahali et al., 2001; Forbes et al.,1975; Maney *et al.*, 1999; Martinet *et al.*, 1992; Pijoan and Williams, 1985; Spieler, 1979). In newts, PRL mRNA and plasma levels are inversely correlated with temperature Takahashi *et al.*, 2001). In several teleost fishes, the pituitary content and plasma PRL concentrations varied significantly with the time of the day (Leatherland and Mckeown, 1973; Leatherland *et al.*, 1974, de Vlaming *et al.*,1975; Batten and Ball, 1976; Mckeown and Peter,1976).

The effect of annual environmental factors on the regulation of PRL cells has been less extensively studied but there is evidence indicating that some correlation exists. In freshwater stickleback (*Gasterosteus aculeatus*), PRL cells formed and released more secretory granules during spring (Benjamin, 1974). Moreover, PRL content of the pituitary and the functional state of the PRL cells anticipate the annual migration of this species (Sage and de Vlaming, 1975). Differential expression of PRL mRNA was observed in the pituitary of carp (*Cyprinus carpio*) acclimatized to summer and winter (Figueroa *et al.*, 1994), and temperature and photoperiod appear to be the major factors controlling the circannual pattern of PRL transcription (Figueroa *et al.*, 1997). In goldfish (*Carassius aurata*), longer photoperiods and higher temperatures cause higher levels of pituitary PRL release (Mckeown and Peter,1976).

It has also been suggested that PRL might participate in regulation of the seasonal cycle of growth and metabolism. In mammals, the active immunization against PRL suppresses food intake and gain in body weight. Infusion of PRL increases nitrogen retention (see Curlewis, 1992 for review). In the golden hamster, hyperprolactinaemia induced by ectopic pituitary homographs has also been shown to prevent the increase in brown fat mass, protein content and thermogenic capacity which is induced by transfer to short daylenght (Kott, 1989). In teleosts, the somatotropic activities ascribed to PRL result from the binding of heterologous PRL to GH receptors in tilapia (*Oreochromis mossambicus*) (Shepherd *et al.*, 1997). In the prometamorphic larvae of Japanese flounder (*Paralichthys olivaceus*), PRL antagonized the stimulatory effect of T3 on the resorption of the dorsal fin rays *in vitro* (De Jesus *et al.*, 1994).

In the sea bream no studies exist describing the effect of season/photoperiod on the release of pituitary hormones. The purpose of the present study was to investigate the influence of temperature and photoperiod on *in vitro* PRL release, describe the annual and circadian variation of PRL cells activity, and characterize the PRL isoforms seasonally secreted into the medium of cultured pituitaries.

4.2 Additional methods

The methodology concerning the pituitary culture, the identification, separation and quantification of PRL is described in chapter 2. The specific

methods reported in this chapter describe the characteristics of the animals and experimental design used in the experiments.

Animals

All studies were carried out on immature sea bream purchased from commercial suppliers who reared the fish in aquaculture ponds, under normal photoperiod and temperature in the Southern Iberian Peninsula (MARESA, Ayamonte, Spain and TIMAR, Algarve, Portugal). Prior to experiments fish were maintained in 5000 liter tanks with a through-flow of aerated sea water (7.0±0.5 mg/l oxygen) in the experimental facility of the Centre of Marine Sciences, Algarve. During the adaptation period salinity was 36±4‰ and the ambient water temperature was 24±3°C and 14±0.5°C in summer and winter, respectively. The average photoperiod for the summer experiment was 14hours light/10hours dark and for winter experiments was 10hours light/14hours dark. The fish were fed to satiation on dry pellets nº3 formulated for marine fish (Provimi, Portugal). The summer experiment was conducted in the first week of August and temperature and photoperiod were the same as those during the adaptation period. The winter experiment was conducted in the last week of December and first week of January and fish were acclimated to photoperiod and temperature as outlined below. The feeding regime during the experiments was the same as those during the adaptation period.

Experiment 1: Circannual cycle of PRL retained in the cultured pituitaries and characterization of PRL released into the culture medium

Throughout one full calendar year and every two months (from September to August) at the same time in the morning (±9hours), sea bream samples (from the same commercial lot) were collected from MARESA, Spain. A sample of 20 specimens was taken, and individual standard length and body weight registered. Fish were sacrificed by decapitation and 8 whole pituitaries were frozen in liquid nitrogen and stored at -80ºC for later analysis. Twelve pituitaries were collected into freshly prepared culture medium as described previously (chapter 2, section 2.1). Pituitary glands were incubated for 18hr at 21ºC in an atmosphere containing 95% O_2/5% CO_2. After culture, pituitary glands and culture medium were stored at –20°C until analysis (usually a week later). Native PRL, in the culture medium and in the pituitary homogenates, was separated by isoelectric focusing for estimation of isoelectric point (chapter 2, sections 2.2 and 2.5) and identity confirmed by Western blotting using an antiserum against chum salmon (chapter 2, section 2.4). Proteins in culture medium and pituitary homogenates were separated by SDS-PAGE, the gels were stained with Coomassie blue and PRL quantified by optical densitometry (chapter 2, section 2.3). The optical density units were transformed to micrograms/ml using calibration curves of BSA. The estimation of PRL molecular weight was made running molecular markers on the same gel as the unknown samples (chapter 2, section 2.5) (see Fig. 4.1 for a schematic representation of the sampling plan).

Experiment 2: Circadian cycle of PRL secreted by cultured pituitaries

The objective of the present study was to determine if a circadian cycle exists for PRL secretion on the seabream. A sample interval of 4 hours was selected on the basis of previously published experiments (Leatherland *et al.*,1974; Leatherland and McKeown,1973; McKeown and Peter, 1976; Spieler, 1979;).

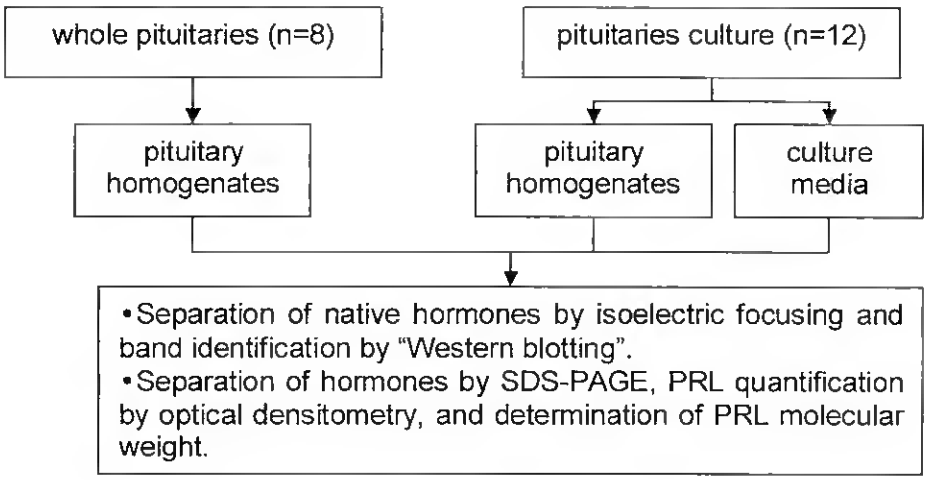


Figure 4.1 – *Experiment 1*: Schematic representation of the sampling plan used every two months to study the circannual cycle of PRL pituitary content and PRL released into the culture medium.

In order to minimize the effects of handling the same group of fish repeatedly, fish were sampled alternately from the two experimental tanks ensuring an interval of 8 hours between each sample. At the start of the experiment sea bream (± 50g) were randomly allocated into two 500 liter (n=18 per tank) through-flow sea water tanks. The experiment was conducted at the beginning of August and fish were acclimated to natural temperature (24±3ºC)

and photoperiod (14hours light/10hours dark), and fed once daily (2-3% of body weight) on commercial sea bream pellets (Provimi, Faro, Portugal). After an initial adaptation period to the experimental tanks of 8 days three samples were collected per tank at a pre-determined time (Table 4.1).

Table 4.1 – *Experiment 2*: Sampling regime carried out over the 3 days of the experiment. To minimize stress, tanks were sampled alternately.

Day of experiment	Time (hours)	Tank number
1	09.00	1
1	21.00	2
2	13.00	1
3	01.00	2
3	05.00	1
3	17.00	2

Pituitary samples were collected from 6 fish at 4 hour intervals over a period of 3 days, according to the sequence in Table 4.1. Fish were sacrificed by decapitation and pituitaries were collected into freshly prepared culture medium as described in the methods. After separation by SDS-PAGE of the hormones present in the culture medium, the gels were stained with Coomassie blue and PRL band quantified by optical densitometry. The optical density units were transformed to micrograms/ml using calibration curves of BSA (chapter 2, section 2.3).

Experiment 3: Photoperiod and temperature influences on growth and in vitro PRL secretion in the presence and absence of galanin

Sea bream (n=84;± 89g) were randomly allocated into four 500 liter through-flow sea water tanks. The experiment was conducted during December/January and fish were pre-adapted to experimental tanks for 8 days in natural winter temperature (14±0.5ºC) and photoperiod (10hours light/14hours dark). After the adaptation period, a two-level factorial experimental design was defined with the factors photoperiod and temperature set to partially simulate different seasons of the year in the Iberian peninsula (Table 4.2). To simulate summer, natural water temperature was increased during 3 days at a ratio of 1ºC/day. Fish were exposed to the experimental conditions for 3 weeks after which 21 fish from each of the different experimental regimes were sampled at the same time in the morning (±9hours). Individual weights and lengths were recorded for all specimens. No mortality was recorded during the experiment and fish were fed to satiation twice daily on commercial sea bream pellets (Provimi, Faro, Portugal).

Fish were sacrificed by decapitation and pituitaries were collected into freshly prepared culture medium as previously described (chapter 2, section 2.1). Pituitary glands were incubated for 18hr at 21ºC in an atmosphere containing 95% O_2/5% CO_2. Six pituitaries per experimental group were individually incubated with galanin. Galanin is a peptide which has been shown to affect PRL secretion in sea bream (chapter 5, sections 5.3 and 5.4). In the present experiment in which winter and summer conditions were simulated, it was made

a first attempt to study the seasonal effect of galanin on *in vitro* PRL secretion. An aqueous stock solution of galanin (1mg/ml; Sigma, Madrid) was diluted with culture medium to a final concentration of 150nM. After incubation, culture media was stored at –20°C until analysis (generally a week later).

Table 4.2 – *Experiment 3*: The different experimental regimes of photoperiod and temperature utilized and their designation.

Regimes	Photoperiod	Temperature °C	Approximately seasonal simulation
1 (short/hot)	10hours light/14hours dark	24±3°C	(Autumn)
2 (short/cold)	10hours light/14hours dark	14±0.5°C	(Winter)
3 (long/hot)	14hours light/10hours dark	24±3°C	(Summer)
4 (long/cold)	14hours light/10hours dark	14±0.5°C	(Spring)

After separation by SDS-PAGE of the hormones present in the culture medium, the gels were stained with Coomassie blue and destained. Time of staining and destaining was kept constant between experiments to be as comparable as possible. PRL was quantified by optical densitometry with the optical density units transformed to micrograms/ml using calibration curves of BSA (chapter 2; section 2.3).

Statistical analysis:

For each sample, variance was checked for homogeneity using the Levene test. Data were statistically evaluated by one-way analysis of variance (experiments 1 and 2) or by a two-way analysis of variance (experiment 3). Significantly different means were identified by Duncan's multiple range test. The

statistical package SPSS version 10.0 (SPSS Inc., USA) was used for analysis of the data.

4.3 Results

Experiment 1: Circannual cycle of PRL retained in the cultured pituitaries and PRL released into the culture medium

PRL release into the culture media displayed a distinct circannual cycle (Table 4.3; Fig. 4.2), with a significantly higher level ($P<<0.001$) occurring in the middle of winter. After this peak, a significant decline in PRL release occurred during February followed in the months from spring to summer by a steady and slow increase of PRL release, with another significant ($P<<0.001$) but smaller peak of PRL release during spring.

Table 4.3 – *Experiment 1*: Seasonal pattern of PRL pituitary content and PRL released (mean ± SEM) by the pituitary glands incubated *in vitro*.

	n° of pituitaries	PRL pituitary content (μg/ml)	PRL release (μg/ml)
September	5	1.90 ± 0.06	0
November	6	1.34 ± 0.06	0
January	6	7.30 ± 0.37	0
February	6	1.40 ± 0.23	4.22 ± 0.31
April	5	4.41 ± 0.25	2.47 ± 0.11
June	5	4.66 ± 0.44	2.37 ± 0.03
August	5	1.28 ± 0.08	3.01 ± 0.28

An annual cycle in the PRL content of the pituitary was also observed (Table 4.3; Fig. 4.2). During autumn and winter, very low levels of PRL were observed to be retained in the pituitary. The pituitary PRL contents increased

dramatically during February (P<<0.001) and subsequently decreased gradually during spring when PRL release from the pituitary surged.

In August a significant (P<0.05) but smaller increase in the PRL pituitary content was observed. The secretion pattern observed in February and August is similar, with the quantity of PRL retained in the pituitary being 2-3 fold the quantity of PRL released to the culture medium.

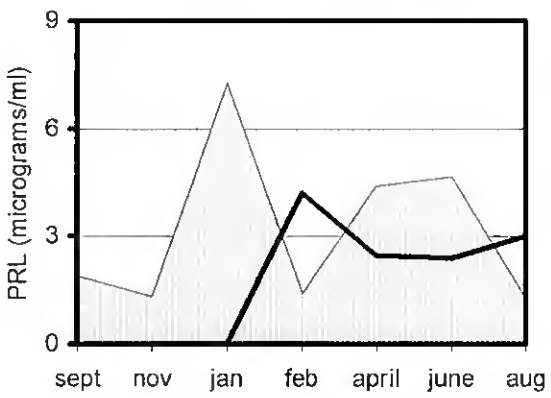


Figure 4.2 – Comparison of the concentrations of *in vitro* PRL release and content of the pituitary at different times of the year, measured by optical densitometry after separation by SDS-PAGE.

There was a seasonal variation in the isoforms of PRL secreted from the pituitary gland and a number of different charge variants were identified using chum salmon anti-PRL serum. Fig. 4.3 presents a schematic representation of the PRL isoforms identified after separation by isoelectric focusing. During autumn and winter, an acidic isoform (pI=6.1) of PRL was released into the medium. In spring and summer, concomitant with the decrease of this variant there was a marked increase in the release of multiple less acidic variants (pI=6.3-6.7).

The charge variants of PRL showed very similar apparent molecular weights as assessed by SDS-PAGE (25± 0.6 kDa), with the exception of a form detected during February which was 22 kDa. It was not possible to determine the reason for the apparent decrease in molecular weight. Although it is unlikely to be due to loss of carbohydrate moieties as treatment of the predominant 25kDa form with N- and O-glycosidase (Roche, Portugal) failed to decrease its molecular weight.

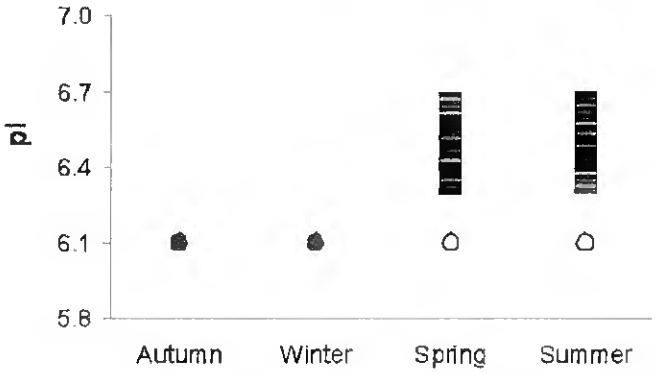


Figure 4.3 – Seasonal variation in PRL isoforms released into the medium by cultured pituitaries. The solid bars represent the multiple bands of PRL occurring during spring and summer (pI = 6.3-6.7). The most acidic form (pI = 6.1) was always present in the culture medium but higher levels were observed during Autumn and Winter.

Experiment 2: Circadian cycle of PRL secreted by cultured pituitaries

In fish acclimated to natural summer temperature (24±3°C) and photoperiod (14light:10dark), PRL was secreted into the culture media with a marked circadian rhythm – significant higher values were observed during daylight at 9a.m. ($P<0.01$) and 5p.m. ($P<0.001$). A nadir occurred during the dark phase (Fig. 4.4).

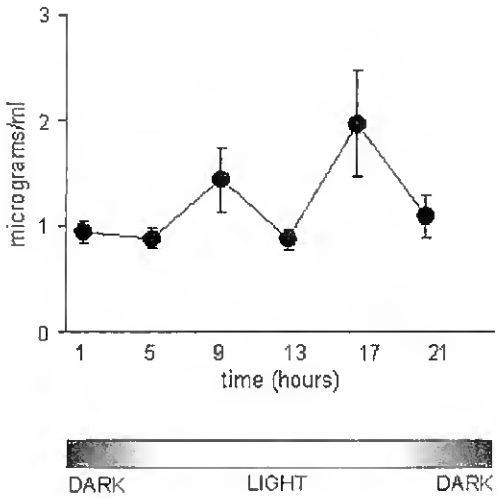


Figure 4.4 – PRL release (μg/ml) at 4 hours intervals over a 24 hours period. The abscissa is the time of day samples were taken and the underlying horizontal bar represents the14light:10dark photoperiod. Each point on the graph represents the mean±SEM of PRL secreted by the pituitaries of 6 fishes.

Experiment 3: Photoperiod and temperature influences on growth and in vitro PRL secretion in the presence and absence of galanin

A two-way analysis of variance showed that the winter and summer photoperiods imposed during the experiment did not affect PRL release. In contrast temperature significantly increased PRL release ($P<<0.001$) during short and long days (Fig. 4.5a). During hot days, either with short or long photoperiod, significantly ($P<<0.001$) more PRL was released. In all experimental groups, PRL released into the culture medium was always much higher (>4μg/ml) than the PRL retained in the pituitary (<3μg/ml). A significantly ($P<<0.001$) higher pituitary PRL content was observed during hot days and long photoperiod, which simulates summer conditions (Fig. 4.5b).

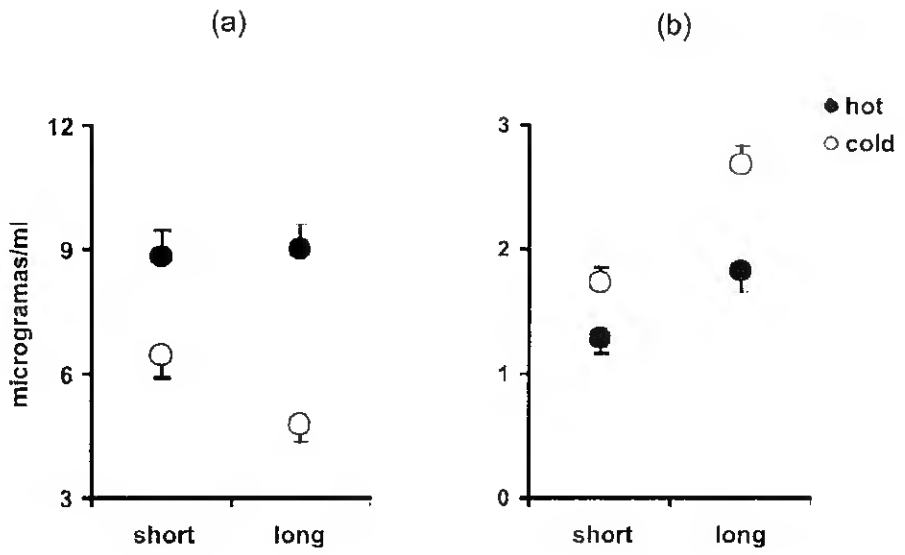


Figure 4.5 - The effects of long (15light:9dark) and short (10light:14dark) photoperiods, and hot (23±2°C) and cold (13±1°C) temperatures on (a) PRL release from the pituitary gland (µg/ml) and (b) the PRL pituitary content (µg/ml). Each point represents the mean±SEM of the results obtained from 15 fishes.

The amount PRL released by the fish acclimated to short days dropped significantly ($P<0.01$ and $P<0.05$ for hot and cold temperature, respectively) when 150 nM galanin was included in *in vitro* pituitary cultures (Fig. 4.6). The only pituitaries in which Gal appears to have a stimulatory effect on PRL release are those from fish maintained under conditions that simulate summer (long/hot) (Fig. 4.6).

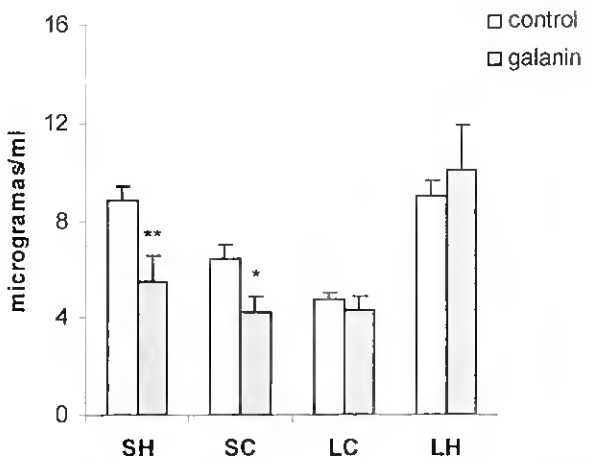


Figure 4.6 – The effects of long (L) and short (S) photoperiods, hot (H) and cold (C) temperatures on *in vitro* PRL release. Each point represents the mean±SEM of PRL secreted (µg/ml) by the pituitary of control fish (n=14, 15, 15, and 15, respectively) and by the pituitary incubated with 150 nM of Gal (n=6, 6, 6, and 4, respectively). (*) significantly different from control value for P<0.05;.(**) significantly different from control value for P<0.01.

Deploying the same factorial design the effect of temperature and photoperiod on growth performance of fish was examined. At the end of the experiment, mean individual body weight differed between treatments (Table 4.4). The variation in individual body weight was significantly correlated with both water temperature (P<<0.001) and, to a lesser extent, to change in daylength (P<0.05), but no interaction between the two factors was found.

Table 4.4 – *Experiment 3*: The effects of long (L) and short (S) photoperiods, hot (H) and cold (C) temperatures on total body weight. Each interval represents the mean±SEM of 21 animals.

Environmental regime	Total body weight (g)
SH	112.96 ± 2.39
SC	92.52 ± 4.01
LC	89.68 ± 3.31
LH	101.69 ± 3.15

Animals experiencing the higher temperature (24±3°C) exhibited significantly ($P<0.05$) higher gains in mean individual body weights (101.69g and 112.96g for long and short days, respectively) over the 3 weeks of the experiment, compared to the other treatment groups. Fish that grew more and had a larger body weight values showed a positive correlation ($r = 0.998$) with PRL secretion (Fig. 4.7).

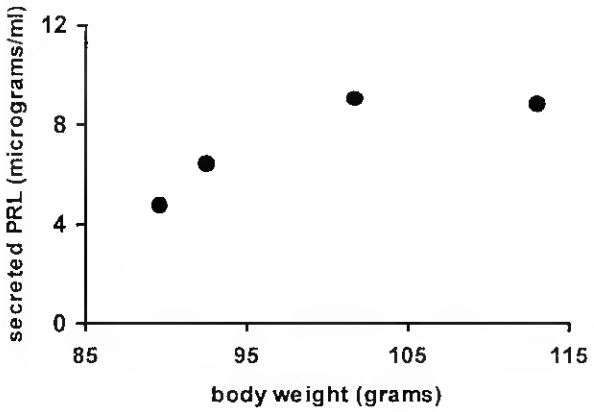


Figure 4.7 – Positive correlation between the mean value of total body weight in each tank sampled and the mean value of PRL released by the cultured pituitaries of the same fish.

4.4 Discussion

Seasonal differences in pituitary activity and isoforms of PRL release may explain the pluripotent nature of PRL activity. Circadian change was also observed. The annual cycle in the PRL release and pituitary content observed is an indication of the cyclic physiologic stages of the animal during the different seasons of the year. Because turnover rates of PRL in the blood of fish and in particular in the seabream are unknown it is not possible to establish a direct link

between the quantity of PRL released and the amount of hormone utilized and catabolized by the animal. However, because the PRL release and pituitary content are dependent respectively on the rates of release and synthesis, by simultaneously measuring the concentration of both components they give a picture of the physiological state of the pituitary and through inference the animal.

During autumn and winter PRL release and pituitary content show low values, suggesting a low activity of release and synthesis. As temperature and day length rapidly decrease during December, PRL secretion and pituitary content of PRL decline to a minimum. The low level of pituitary PRL content concomitant with the sudden increase in PRL release observed in January (which coincides with spawning) suggest that there must be a surge in PRL synthesis by the pituitary gland in order to meet secretory needs during this month. In February, there was a significant increase in pituitary PRL concentration and a decrease in release indicating that the pituitary was releasing PRL more slowly than it was being synthesized. Interestingly it has previously been shown that the sensitivity of sea bream PRL cells to E_2 change during this month as well (Brinca *et al.*, 2003) and are much less sensitive to this steroid than during winter. As day length and temperature slowly increases during spring there is another combination of changes with a steady decrease in the pituitary PRL concentration and an increase in the PRL secretion, again suggesting that there must be a surge in PRL synthesis by the pituitary gland in order to meet secretory needs during this period (related to growth? related to migration of juveniles from coastal waters to more deep waters?). In August the

pituitary physiologic state was similar to February suggesting another "less sensitive period" after a peak of PRL release.

A pattern of seasonal change in PRL cell response to the presence of Gal in the culture medium was also suggested by the results of the experiment with galanin. The general tendency was that Gal (150 nM) significantly reduced or had no effect on PRL release, although during the summer simulation (long days and hot temperature) Gal tend to stimulate PRL release from pituitaries *in vitro* (Fig. 4.6). In vertebrates, the involvement of Gal on the reproductive axis has been extensively studied (Baranowska *et al.*, 2001; Cornbrooks and Parsons, 1991; Finn *et al.*, 2000; Lamanna *et al.*, 1999; Olivereau and Olivereau, 1991b; Tsutsui *et al.,* 1998; Wynick *et al.,* 1993), although other physiological functions have also been attributed to this peptide (such as control of appetite) in mammals and goldfish (Crawley, 1995; de Pedro *et al.*, 1995; Leibowitz *et al.*, 1998). In the present study, the physiological significance, if any, of Gal stimulated PRL release from pituitaries collected from fish maintained under a regime simulating summer (Fig. 4.6) remains to be established.

Somatotropic and developmental actions of PRL have been characterized in mammals (Bole-Feysot *et al.*, 1998 for review), although studies on tilapia and Japanese flounder and the early expression of PRL and its receptor in sea bream (Santos *et al.*, 2003) suggest PRL may have similar action in fish (de Jesus *et al.*, 1994; Shepherd *et al.,* 1997). In the present study, higher temperatures and feeding *ad libidum* significantly increased growth rate (Fig. 4.7) and PRL release was highly correlated (see discussion of experiment 3). However, complementary

studies on the contribution of GH to this growth effect still remain to be established in order to clarify this important aspect of the sea bream physiology.

A seasonal variation was also observed in PRL forms contained in the culture medium (Fig. 4.3). During autumn and winter, a single and more acidic charge variant (pI=6.1) was detected (related to spawning?). In spring and summer, concomitant with the decrease of this variant there was a marked increase in the release of multiple variants (pI=6.3-6.7) that showed very similar apparent molecular weights by SDS-PAGE (25± 0.6 kDa). The hypothesis of the possible involvement of PRL in several physiological events during spring, including increase of weight and/or migration of fingerlings towards coastal waters, is consistent with the observed peak of PRL release, concomitant with the presence of multiple forms secreted *in vitro* by the sea bream pituitaries.

An increasing number of teleosts exhibit a circadian rhythm of PRL cell activity and/or plasma concentration. However, the diel PRL rhythm does not appear to be consistent among different studies or species to the daily light-dark cycle. In several species the PRL pituitary activity or plasma levels were highest during the period of darkness and fell to a minimum in the middle to the end of the light period (Leatherland and Mckeown, 1973; Leatherland *et al.*, 1974; de Vlaming *et al.*,1975; McKeown and Peter,1976). In freshwater sailfin mollies (*Poecilia latipinna*) the period of highest synthetic activity of the PRL cells was from midday to evening (Batten and Ball, 1976). In the present study seabream PRL secretion was lower during the period of darkness and the highest value (2μg/ml) was obtained by the end of the light phase; a small peak (1.4μg/ml) was

present in the beginning of the photophase. One possible explanation for the discrepancies between different studies could be that the diel rhythm of activity of the PRL cells is not directly locked-in to some environmental factor but is linked to endogenous changes and its release is modulated in response to random daily changes in the environment. The presence of a clear annual cycle in seabream and also in goldfish, carp, and freshwater stickleback (Benjamin, 1974; Sage and de Vlaming, 1975; Mckeown and Peter,1976; Figueroa *et al.*, 1994; Figueroa *et al.*, 1997) will also probably have a significant impact on the dial cycle detected. Therefore, depending on the time of the year that samples are taken and the physiologic state of the animal, different results are possible even for the same species. The results of the present study demonstrated that the activity and responsiveness of the sea bream pituitary changes during the year.

Experiment 3 showed that PRL release was positively stimulated by temperature. Previous studies have shown that temperature can modulate directly the PRL controlling mechanism; in catfish, environmental factors suppressed the dopamine mechanism (Senthikumaran and Joy, 1995) and in some mammals there is evidence of changes of pituitary sensitivity to dopamine related to changes in environmental factors (Curlewis, 1992). Data from experiment 3 is compatible with the circannual cycle observed in the present study – as temperature slowly increases there is a steady increase in the PRL secretion. Conversely, when the temperature decreased the PRL release also decreased until minimum levels were observed during winter. But temperature can also act as a signal to the onset of several biological processes like

spawning for instance. In sea bass, spawning time can be advanced or delayed by changing water temperature and photoperiod (Zanuy *et al.,* 1986). A rapid decrease of temperature during winter may act as a cue for spawning and this could explain the sudden increase of PRL release observed in the present study in January after a minimum temperature value (=11ºC) was attained during December. In the present study temperature also had a significant effect on PRL pituitary content. Increasing the temperature when the fish are on either a short or a long photoperiod decreased the PRL pituitary content, which is in accordance with the stimulation of PRL release observed with high temperature. The relative decrease in the pituitary concentration and the relative increase in PRL release probably indicates a faster release rate than the synthetic rate. The process under laboratory conditions is in accordance to the changes observed in seabream reared in natural conditions.

Changes in photoperiod are more difficult to interpret. Photoperiod had no significant effect on PRL release and positively modulated pituitary content. For a range of different animals – lambs, sheep, minks, sparrows, and turkeys - photoperiod has been correlated with PRL serum levels release and/or pituitary content (Forbes *et al.*, 1975; Pijoan and Williams, 1985; Martinet *et al.*, 1992; Maney *et al.*, 1999; Gahali et al., 2001). In goldfish, a change in photoperiod caused both serum and pituitary PRL levels to change at least at low temperatures (10ºC). In fish acclimated to 20ºC the photoperiod influenced the pituitary content but no effect was observed in serum PRL levels (Mckeown and Peter, 1976). In the present study the photoperiod information was not clearly

translated into changes in PRL cell activity. One possible explanation is that the laboratory conditions did not simulate the natural progressive changes of photoperiod. It is possible that the gradual changes in these two parameters provide a signal to the internal calendar about the specific time of the year and thus the season.

In summary, a marked annual cycle in the pituitary gland activity was observed. This is highlighted by the seasonal variation in the basal pituitary PRL release rates but also by a variation in the relative concentration of PRL charge variants released from the pituitary gland. Circadian changes were also observed. Results all suggest that in common with other species sea bream has circannual or/and circadian clocks but the evidence is still insufficient for any definitive conclusion to be made. Moreover, temperature (but not photoperiod) influences sea bream PRL cells activity, with higher temperature increasing *in vitro* PRL secretion and lower temperature having the opposite effect. More information is needed to increase our knowledge of how sea bream translate photoperiod information into endocrine signals.

CHAPTER **5:** Effect of Gal on PRL *in vitro* release

CHAPTER **5**: Effect of Gal on PRL *in vitro* release

5.1 Introduction

Galanin (Gal) is a neuropeptide that has been detected in the central nervous system and the gastroenteric, respiratory, urinary, and reproductive system of mammals, reptiles, amphibians, birds, and teleost fish (Moons *et al.*, 1989; Batten *et al.*, 1990; Cornbrooks and Parsons, 1991; Moons *et al.*, 1991; Olivereau and Olivereau, 1991a; Crawley, 1995; Power *et al.*, 1995; Azumaya and Tsutsui, 1996; Sánchez-Montesinos *et al.*, 1996; Wang et al., 1997; Ohmori, 1998; Batten *et al.*, 1999; Kohchi and Tsutsui, 2000; Mensah-Brown *et al.*, 2000). In mammals, co-expression of Gal and PRL within secretory granules have been reported (Schwartz, 2000 for review) and in the pituitary gland of european seabream and seabass. Gal immunoreactive fibers have also been detected and appear to infiltrate between ACTH, PRL, TSH, GtH, and GH cells. The presence of Gal immunoreactive fibres in the pituitary gland suggest it may directly influence the activity of such cells (Moons *et al.*, 1989; Batten *et al.*, 1990; Moons *et al.*, 1991; Power *et al.*, 1995).

The function of Gal in vertebrates remains to be established but numerous studies have shown that Gal may have a direct or indirect effect on the reproductive axis. For example, in mammals, Gal regulates the number of lactotrophs and stimulates gonadal hormones release (Wynick *et al.*, 1993; Finn *et al.*, 2000; Baranowska *et al.*, 2001). Gal also strongly stimulate the release of PRL in humans and rats (Bartfai, 2000). In reptiles and avian species, Gal may

contribute as a neurotransmitter or a neuromodulator to oviposition (Li *et al.*, 1996; Lamana *et al.*, 1999).

Estrogens exert an important influence on Gal activity in the pituitary. In mammals, gonadal estrogens positively regulate Gal-expressing cells either increasing the amount of Gal protein and mRNA (Gabriel *et al.*, 1992; Brann *et al.*, 1993; Hyde *et al.* 1993; Crawley, 1995; Tseng *et al.*, 1997; Shen *et al.*, 1999; Degerman *et al.*, 2002) or the number of Gal-secreting cells (Bloch *et al.*, 1993; Leibowitz *et al.*, 1998; Rugarn *et al.*, 1999). Estrogens also induced an increase in Gal immunoreactive material in the pituitary of lizards, quails, and eels (Olivereau and Olivereau, 1991b; Tsutsui *et al.*, 1998; Lamanna *et al.*, 1999).

Data from transgenic mice deficient in Gal support the hypothesis that this peptide acts as a paracrine regulator of PRL expression and lactotroph growth and mediates estrogen action on the lactotroph. These mutants have reduced PRL mRNA and serum PRL levels, and females fail to lactate. Their lactotrophs do not proliferate in response to high doses of estrogen, nor do they up-regulate PRL gene expression and release (Wynick *et al.*, 1998).

On the whole there are relatively few studies of the function of Gal in teleosts. Moreover, although studies exist demonstrating the distribution in the brain and pituitary gland of this peptide (Moons *et al.*, 1989; Batten *et al.*, 1990;; Cornbrooks and Parsons, 1991; Moons *et al.*, 1991; Olivereau and Olivereau, 1991a; Power *et al.*, 1995; Batten *et al.*, 1999) relatively few studies have demonstrated the action of this peptide on pituitary PRL cells. In the present

study, sea bream pituitary glands have been incubated *in vitro* to study the response of PRL cells to Gal, together with an assessment of the effect of E_2 priming in intact fish on the response of PRL cells to Gal.

5.2 Additional methods

The methodology utilized for the pituitary culture, the identification, separation and quantification of PRL is described in Chapter 2. The methods reported in this chapter are specific to the present experiments and describe the characteristics of the animals and the experimental design.

Animals

All studies were carried out on immature sea bream purchased from commercial suppliers who reared the fish in aquaculture ponds, under normal photoperiod and temperature in the Southern Iberian Peninsula (MARESA, Ayamonte, Spain and TIMAR, Algarve, Portugal). After transportation to the experimental facility of the Centre of Marine Sciences, Algarve, the fish were maintained in 5000 liter tanks with a constant through-flow (16 liter/hour) of aerated sea water (7.0±0.5 mg/l oxygen) and temperature and salinity for winter in the Algarve. The fish were fed once daily to satiation on dry pellets nº2 formulated for marine fish (Provimi, Portugal).

Experiment 1: Effect of E_2 and Gal on in vitro PRL release during winter

Sea bream (n = 36) were randomly allocated into two 500 liter through-flow sea water tanks. The experiment was conducted during February under natural temperature (15±1°C) and photoperiod (10hours light/14hours dark). After an adaptation period of 7 days, one group of fish (n=18, with an average weight and length of 47.2g and 13.8cm, respectively) received an implant of coconut oil alone, and the other group (n=18, with an average weight and length of 49.0g and 12.9cm, respectively) received 10mg/kg of E_2 (Sigma, Madrid) in coconut oil.

From each tank, 18 fish were sacrificed 4 days after implant by anaesthesia with a sub-lethal dose of MS-222 (1:10000; Sigma, Madrid), followed by decapitation and pituitaries were collected into freshly prepared culture medium (chapter 2, section 2.1). An aqueous stock solution of porcine Gal (1mg/ml, Sigma, Madrid) was diluted with culture medium to the appropriate dilution. Pituitaries from fish with coconut oil implants (control, n=18) were collected and cultured in 0 and 150 nM Gal (concentration selected based on dose/response curve) for 18hours at 21°C in an atmosphere containing 95%O_2/5% CO_2. Pituitaries from the fish implanted with E_2 (n=18) were also collected and cultured in 0 and 150nM. After culture, pituitary glands and culture medium were stored at -20°C until analysis (usually a week later).

Experiment 2: Effect of E_2 and Gal on in vitro PRL release during summer with manipulated short photoperiod and low temperature

Sea bream were randomly allocated into a 500 liter through-flow sea water tank (n = 75, with an average weight and length of 84.3g and 16.8cm, respectively), and into a 200 liter tank (n = 30, with an average weight and length of 79.7g and 16.7cm, respectively) equipped with a recirculating cooling system. A greater number of control fish were utilized in order to carry out a dose/response study with Gal *in vitro*. The experiment was conducted during June and fish were gradually adapted over 8 days to a reduction in temperature from the ambient temperature (23°C) to the mean spring value (18°C). Photoperiod was also adjusted from the ambient photoperiod for June (14hours light/10hours dark) to that of the spring photoperiod (12hours light/12hours dark). After an adaptation period of 3 weeks, the larger group received an implant of coconut oil alone and the other one received 10mg/kg of E_2 (Sigma, Madrid) in coconut oil.

Fish were sacrificed 7 days after implants by anaesthesia with a sub-lethal dose of MS-222 (1:10000; Sigma, Madrid), followed by decapitation and pituitaries were collected into freshly prepared culture medium as described elsewhere. An aqueous stock solution of porcine Gal (1mg/ml, Sigma, Madrid) was diluted with culture medium. Pituitaries removed from the fish which received the coconut oil implant alone were cultured in the following range of Gal concentrations: 0, 25, 100, 300, and 600 nM. Pituitaries from the fish which received an E_2 implant (10mg/kg wet weight) were cultured in the following Gal

concentrations: 0 and 100 nM. Fifteen glands were cultured individually per experimental group.

Statistical analysis

For each sample, variance was checked for homogeneity using the Levene test. Data were statistically evaluated by one-way analysis of variance or by a two-way analysis of variance, according to the sampling plan. Significantly different means were identified by Duncan's multiple range test. The statistical package SPSS version 10.0 (SPSS Inc., USA) was used for analysis of the data.

5.3 Results

Dose response of pituitary glands to Gal

During spring and in fish not primed with E_2, the effect of Gal became significant and there was a concentration-dependent response with 0-600 nM Table 5.1; Fig. 5.1).

Table 5.1 – The effect of *in vitro* Gal treatment on PRL release (μg/ml) from pituitaries of fish not treated *in vivo* with E_2. Results are given as means±SEM and sample size for each treatment group is indicated in brackets.

Gal dose (nM)	0	25	100	300	600
	0.45 ± 0.03 (13)	0.49 ± 0.06 (14)	0.60 ± 0.06 (11)	0.61 ± 0.06 (15)	0.52 ± 0.03 (14)

The amount of PRL released into the medium increased significantly ($P<0.05$) in the presence of 100nM and 300nM of Gal. However, PRL secretion decreased to the basal control level when 600nM of Gal were included in the

culture medium presumably because of pharmacological effects (Table 5.1 and Fig. 5.1).

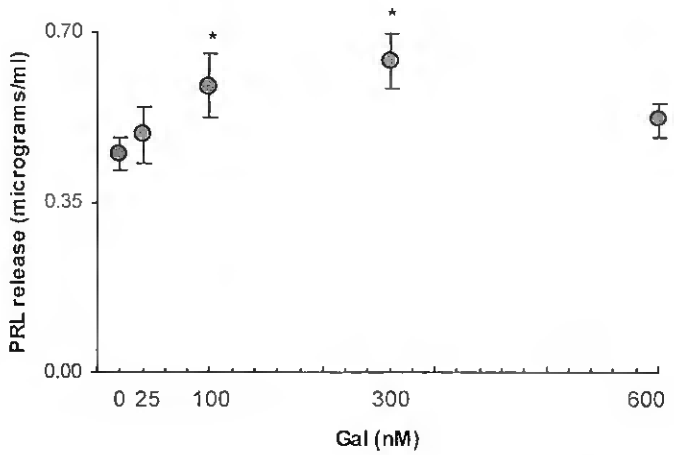
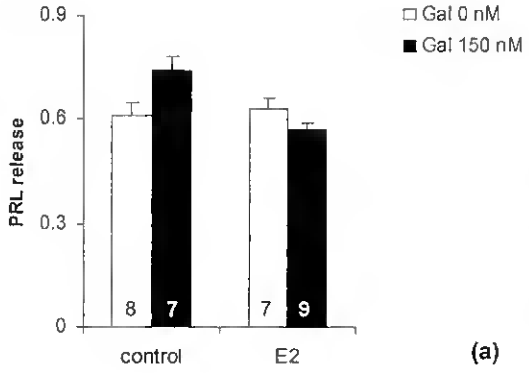


Figure 5.1 – The effect of Gal (0, 25, 100, 300 and 600nM) on *in vitro* PRL release (μg/ml) from fish not primed with E_2. Each point represents the mean±SEM. (*) Significantly different from control value, P<0.05.

Modulation of PRL secretion by Gal

In both experiments, *in vitro* Gal treatment of pituitaries from control fish significantly stimulated (P<0.05) PRL secretion (Fig. 5.2). In contrast, in fish primed with E_2, PRL secretion when Gal was added to the culture medium was not significantly different from secretion in the absence of Gal (Fig. 5.2). Circulating E_2 seems to "inhibit" the stimulatory action of Gal on PRL-cells. Because the effect of E_2 on basal pituitary PRL secretion depends on season - no effect in winter and a significant increase (P<0.05) of PRL secretion in spring - the "level" of PRL secretion from fish treated *in vivo* with E_2 and *in vitro* with Gal show the same seasonal variation (Fig. 5.2).



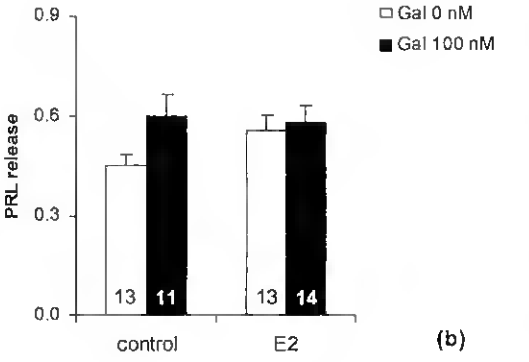


Figure 5.2 – Comparison of PRL secretion (µg/ml) from pituitary glands collected in winter (a) and spring (b) from control fish and fish primed with E_2. In both experiments, *in vitro* Gal treatment of pituitaries from fish not primed with E_2, significantly stimulated ($P<0.05$) PRL secretion. PRL released from pituitary glands of fish treated *in vivo* with E_2 did not show differences when incubated with or without Gal. Each bar represents mean±SEM and numbers inside bars indicate the sample size in each treatment group.

5.4 Discussion

The neuroendocrine control of PRL secretion is a multifactorial process that involves both stimulatory and inhibitory molecules. Pituitary PRL cells maintain a high rate of active basal secretion in the absence of an inhibitory signal. Numerous factors are capable of stimulating PRL release and gene expression. Studies in some teleosts have shown that E_2 has a stimulatory effect on the activity of PRL cells (Barry and Grau, 1986; Borski *et al.*, 1991; Wigham, 1992; Williams and Wigham, 1994; Poh *et al.*, 1997; Weber et al., 1997). The results from the present study demonstrate that E_2 modulates PRL secretion and that the responsiveness of PRL cells in sea bream varies with season and probably with age. During the winter experiment, E_2 had no effect on PRL

secretion, a result which is in conflict with results obtained in other experiments (see chapter 6, sections 6.3 and 6.4). One possible explanation is that the experiments included in chapter 6 were done in the middle of the spawning season, while the present study was carried out by the end of the spawning season, when a refractoriness of PRL cells to *in vivo* E_2 treatment has previously been observed (unpublished results). Another explanation could be related to differences in size/age of fish. Smaller fish (50-70g) was used in the experiments discussed in chapter 6, compared with fish used in the present study (70-100 g).

Previous studies have shown in juvenile sea bream and during the breeding season, PRL expression increased significantly after E_2 treatment (Cavaco *et al.*, 2003). The identification of estrogen receptors in the pituitary gland of the sea bream (Socorro *et al.*, 2000) suggests that E_2 may act directly on PRL cells via its receptors. This action may be similar to that observed in mammals, where estrogens may act in both a direct and indirect manner. The direct effect of E2 is shown by the up-regulation it induces in mammalian pituitary PRL gene expression which is also associated with a subsequent increase in PRL secretion (Rose *et al.*, 1996; Takahashi and Kawashima, 1986; Torner *et al.*, 1999). The indirect effects of E2 appear to be its effect on neurotransmitters and neuropeptides which rapidly stimulate PRL release from storage granules.

One of the peptides proposed to be of physiological significance in the neuroendocrine regulation of PRL cells is Gal. Gal is a 29-amino acid peptide

originally isolated from porcine small intestine (Tatemoto *et al.*, 1982) which shares little homology with other known peptides. In mammals, exogenously administered Gal regulates the reproductive axis by acting as a growth regulator of the lactotrophs (Wynick *et al.*, 1993) or by having both direct and indirect effects on gonadal hormones release (Finn *et al.*, 2000; Baranoska *et al.*, 2001). In reptiles, Gal administration in pre-ovulatory lizard females induced premature oviposition (Lamanna *et al.*, 1999). In the present study on the marine teleost sea bream, Gal significantly stimulated PRL secretion ($P<0.05$) from pituitary glands cultured *in vitro* in a dose-dependent manner. The mechanism by which Gal affects PRL secretion in this study, either by direct stimulation of PRL cells via a receptor or indirectly by increasing the number of PRL cells (Ottlecz et al., 1988; Wynick *et al.*, 1998), remains to be clarified.

In contrast, in fish primed with E_2 PRL secretion when Gal was added to the culture medium was not significantly different from secretion in the absence of Gal. Circulating E_2 seems to "inhibit" the stimulatory action of Gal on PRL-cells. This is in contrast with what was observed in other species. For example, on rats Gal mRNA increased significantly after treatment with estrogen (Gabriel *et al.*, 1992; Brann *et al.*, 1993; Hyde *et al.* 1993; Crawley, 1995; Tseng *et al.*, 1997; Shen *et al.*, 1999; Degerman *et al.*, 2002), and gonadal steroids have a dramatic activational effect on the numbers of visibly stained Gal cells in the hypothalamus and pituitary gland with a concomitant increase in basal PRL release (Bloch *et al.*, 1993; Wynick *et al.*, 1993; Leibowitz *et al.*, 1998; Rugarn *et al.*, 1999). In teleost fishes, the intensity of Gal innervation is also dependent

on circulating levels of estrogens. For example, in eels treated with estradiol or methyl testosterone, increased Gal immunoreactive material was observed in some perikarya and brain fibers (Olivereau and Olivereau, 1991b). The reason for the discrepancy reported between the observed results in the sea bream and the action described for Gal on E_2-primed animals in previous studies is not clarified and needs further investigation.

In conclusion, the results confirm that the sensitivity of PRL cells to E_2 varies with season. Moreover, in immature sea bream Gal acts as a potent stimulator of *in vitro* PRL secretion except when fish is primed with E_2. So that, changes in hormonal status accompanying reproduction in sea bream change the sensitivity of PRL cells to Gal. One possible explanation is that the responsiveness of PRL cells to Gal is linked to the sexual maturity of this species. To clarify this issue it will be of interest to carry out a more detailed analysis of the response of PRL cells to Gal using fish at different stages of their life cycle.

CHAPTER 6: Effect of E_2 and VIP on PRL *in vitro* release

CHAPTER 6: Effect of E_2 and VIP on PRL *in vitro* release

6.1 Introduction

The pituitary hormone prolactin (PRL) is a versatile hormone and more than 300 different actions have been described in vertebrates and many of these actions in mammals are associated with reproduction (Bole-Feysot *et al.*, 1998). In teleost fish the principal action of PRL is the maintenance of hydromineral balance in euryhaline species in fresh water (Loretz and Bern, 1982; Hirano *et al.*, 1987). Relatively few reports about the effect of PRL on fish reproduction exist. In tilapia (*Oreochromis mossambicus*) homologous PRL stimulates testosterone production in courting males (Rubin and Specker, 1992) and PRL may influence parental behaviour (de Ruiter *et al.*, 1986; Slijkhuis *et al.*, 1984).

In mammals the modulation of PRL by ovarian steroids is well documented (Labrie *et al.*, 1978). In rats, estradiol-17β (E_2) increases the mitotic potency of PRL cells in the pituitary gland and has a stimulatory effect on PRL gene expression in the hypothalamo-neurohypophyseal system and a concomitant inhibitory action on PRL proteolysis at this site (Takahashi and Kawashima, 1986; Torner *et al.*, 1999). In mink, *Mustela vison*, a high systemic ratio of progesterone to E_2 has been shown to be a prerequisite for increasing the expression of uterine PRL receptors (Rose *et al.*, 1996).

Ovarian steroids also affect the pituitary in birds, modulating *in vitro* PRL release (Knapp *et al.*, 1988). In teleost fish the regulation of PRL cells by ovarian steroids has been less extensively studied but there is evidence indicating their involvement, for example, in tilapia, *Oreochromis*

mossambicus, pituitary PRL content and *in vitro* secretion is elevated by treatment with E_2 (Borski *et al.*, 1991; Wigham, 1992; Poh *et al.*, 1997). Moreover, preincubation of tilapia pituitary glands with E_2 *in vitro* appears to increase the sensitivity of PRL cells to stimulation by TRH and GnRH *in vitro* (Barry and Grau, 1986; Weber *et al.*, 1997). In contrast, E_2 treatment of rainbow trout pituitary cultures stimulated *in vitro* PRL synthesis but did not affect release (Wigham, 1992; Williams and Wigham, 1994).

The way in which peptides regulate E_2 stimulated PRL release in fish has not been studied, despite the anatomical evidence demonstrating peptidergic innervation of the rostral and proximal pars distalis (Batten *et al.*, 1983). One of the peptides proposed to be of physiological significance in neuroendocrine regulation of PRL cells in mammals and birds, is vasoactive intestinal peptide (VIP). This peptide was first isolated from porcine intestinal extracts (Said and Mutt, 1972) and was subsequently detected in the brain. In mammals, VIP has been identified in the hypothalamus in neurones projecting to the median eminence and neurohypophysis and has also been identified in the hypophysial portal blood (Said and Porter, 1979; Okamura *et al.*, 1986). Hypophysial VIP is a potent stimulator of PRL release from mammalian pituitaries both *in vivo* and *in vitro* (Rostene *et al.*, 1982; Abe *et al.*, 1985). Similar observations have also been made in the bantam hen *in vivo* and in pituitary cultures of turkey, broiler and bantam chickens (Hall and Chadwick, 1985; MacNamee *et al.*, 1986). Recently VIP was also shown to be associated with a significant rise in PRL during the breeding season of birds (Youngreen *et al.*, 1994; Bédécarrats *et al.*, 1999; Maney *et al.*, 1999). In amphibians and fish relatively few studies of the effect of VIP on PRL

secretion exist. In *Rana catesbeiana*, VIP has been proposed to be a PRL-releasing factor (Koiwai *et al.*, 1986). However in tilapia, *O. mossambicus*, the only teleost in which the effect of VIP on secretion of pituitary hormones has been determined, it appears to inhibit PRL secretion (Wigham, 1992).

The purpose of the present study was to investigate the influence of ovarian steroid exposure (E_2) on basal PRL release from *in vitro* pituitary gland cultures of sea bream (*Sparus aurata*) during winter and spring. The modulation by VIP of E_2 stimulated PRL secretion in sea bream was also studied.

6.2 Additional methods

The methodology concerning the pituitaries culture, and the PRL separation and quantification of PRL, is described in chapter 2. The specific methods reported in this chapter describe the characteristics of the animals, the experimental design used in the experiments, and the immunochemistry technique used on pituitary sections mounted in glass slides.

Animals

All studies were carried out on immature sea bream (body weight 50-70g) purchased from commercial suppliers who reared the fish in aquaculture ponds, under normal photoperiod and temperature in the Southern Iberian Peninsula (MARESA, Ayamonte, Spain). Prior to experiments fish were maintained for 1 month in a 1000 litre tank with a throughflow of aerated sea water (7.0±0.5mg/l oxygen) in the experimental facility of the Centre of Marine Sciences, Algarve. Salinity was 36°/oo and the water temperature was

14°C±0.5°C and 18°C±0.5°C in winter and spring respectively. The average photoperiod for the winter experiment was 10h 30min light/13h 30min dark and for spring experiments was 13h 30min light/10h 30min dark. Fish were fed to satiation twice daily on dry pellets formulated for marine fish (Provimi, Portugal). Winter experiments were conducted in the first week of February and spring experiments were conducted in the last week of April as outlined below. Temperature and salinity of water, photoperiod and the feeding regime during the experiments were the same as those during the adaptation period.

Experimental design

Experiment 1 – Effect of E_2 and VIP on in vitro PRL release during winter

Sea bream (n = 130) were randomly allocated into two 500 litre through-flow sea water tanks, one group received an implant of coconut oil alone and the other one received 10mg/kg of E_2 (Sigma, Madrid) in coconut oil. Fish were sacrificed 7 days after implants by anaesthesia with a sub-lethal dose of MS-222 (1:10000; Sigma, Madrid), followed by decapitation and pituitaries were collected into freshly prepared culture medium as described above. An aqueous stock solution of porcine VIP1-38 (1mg/ml, Sigma, Madrid) was diluted with culture medium and pituitaries (n = 13 per treatment) were cultured in the following VIP concentrations: 0, 25, 50, 100 and 200 nM. Thirteen glands were cultured individually per experimental group. The concentrations of VIP used in the experiments were based upon results from previous experiments in which a wider range of VIP concentrations (25-1000nM) was used (results not shown).

Experiment 2 – Effect of E_2 on in vitro PRL release during spring

Sea bream (n = 20) were randomly allocated into 2 groups, one group of fish (n = 10) received an implant of coconut oil alone and the other group (n = 10) received an implant of coconut oil containing 10mg/kg of E_2 for 7 days. At the end of the experiment pituitaries were collected as described above and cultured individually *in vitro* with 10 glands per experimental group.

A small-scale study of the effect of VIP on *in vitro* pituitary cultures in sea bream treated with E_2 showed that in spring VIP had no effect on PRL production *in vitro*. For this reason no further studies were carried out with VIP in spring.

Immunohistochemistry

Brains with pituitary glands attached were collected from fish anaesthetised in MS-222 (1:10000, Sigma, Madrid). Tissue was fixed in Bouin-Hollande sublimate for several days, rinsed in PBS and dehydrated, embedded in wax and serial sagittal sections (6μm) cut and mounted on poly-L-lysine coated slides for subsequent immunohistochemical studies.

Immunohistochemistry was carried out using a modification of the unlabeled antibody peroxidase-antiperoxidase (PAP) method (Sternberger, 1986). Briefly sections with both brain and pituitary visible were dewaxed, rehydrated through graded alcohols and non-specific background staining blocked by immersion in 4% normal goat serum in phosphate buffered saline (PBS), containing 1% bovine serum albumin (PBS/BSA) for 60 minutes. Excess blocking solution was removed and sections were incubated for 3

hours at room temperature with primary antisera specific for the N-terminus of porcine VIP (L25, 1/2000, provided by Dr R. Dimaline, University of Liverpool, UK) or C-terminus dogfish VIP (L311, 1/2000, previously characterised in Dimaline *et al.*, 1986) or anti-chum salmon prolactin (1/3000, previously characterised in Power and Canario, 1992). After washing in PBS (2 x 5 minutes) sections were incubated for 1hr with anti-rabbit IgG (1/50, Dako, Sweden) and then 45 minutes with rabbit peroxidase antiperoxidase (1/100, Dako, Sweden) and colour developed by reaction with diaminobenzidine and hydrogen peroxide. Sections were then counterstained with Meyers haematoxylin.

Method specificity was checked on sections adjacent to those, which were positively stained. Several controls were carried out; substitution of primary antisera by normal rabbit sera; sequential omission of reagents from the various steps of the staining procedure; or pre-absorption of the primary VIP antisera with porcine VIP. All these procedures abolished staining.

Statistical analysis

In each experiment results were obtained for 13 (experiment 1) or 10 (experiment 2) individuals/group. Variance was checked for homogeneity using the Levene test. Results from experiment 1 were analysed with the data classified in two-ways, by treatment with E2 and by treatment with VIP (two-way analysis of variance). In experiment 2 a one-way analysis of variance was used to determine the statistical significance of differences between the means of the control group and the group of fish treated *in vivo* with E_2. The

statistical package SPSS version 10.0 (SPSS Inc., USA) was used for analysis of the data.

Table 6.1 - The effect of VIP treatment on PRL secretion from pituitaries of control or E2 primed fish in winter. Results are given as the mean ± SEM; sample size for each treatment group is indicated in brackets.

VIP dose (nM)	E_2 primed	Control
0	1.66 ± 0.22 (13)	1.08 ± 0.14 (13)
25	1.52 ± 0.24 (13)	0.94 ± 0.14 (11)
50	1.28 ± 0.45 (10)	1.22 ± 0.17 (12)
100	1.15 ± 0.21 (10)	0.92 ± 0.12 (13)
200	1.05 ± 0.12 (12)	1.10 ± 0.17 (12)

6.3 Results

Effect of E_2 on basal pituitary PRL secretion

In vivo implants of E_2 for 7 days in sea bream during winter (experiment 1) significantly increased above control levels *in vitro* PRL release ($P<0.05$; Fig.6.1). During spring (experiment 2), the basal secretion of PRL by pituitary glands was significantly lower than that observed during winter ($P<0.01$, Fig. 6.1). Moreover, *in vivo* implants of E_2 significantly lowered *in vitro* PRL release to a level 15% less than that observed in control pituitaries ($P<0.01$, Fig.6.1).

Modulation of E_2 stimulated PRL secretion by VIP

In control fish during winter, co-incubation *in vitro* of pituitary glands with increasing doses of VIP (0 - 200 nM) had no effect on PRL secretion at any of the doses used (Table 6.1). However, E_2 stimulated pituitary PRL secretion in winter was modulated by VIP which reduced PRL release in a

dose-dependent manner (Table 6.1). The inclusion of 100 nM and 200nM VIP in *in vitro* cultures of pituitary glands from E_2 primed fish caused a significant reduction ($P<0.05$, Fig. 6.2) in PRL secreted into the medium compared to control pituitaries collected from E_2 primed fish.

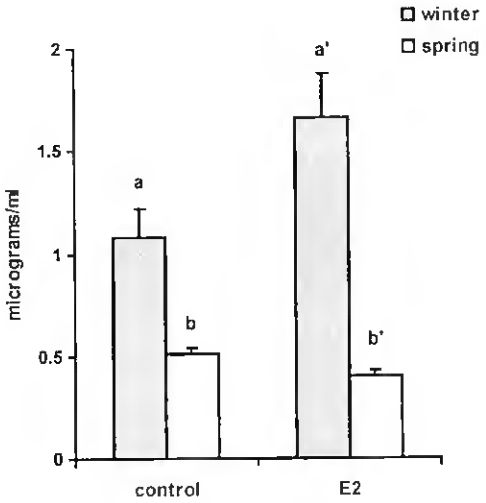


Figure 6.1 Comparison of the basal secretion of PRL from pituitary glands collected during spring and winter. Note that the basal secretion of PRL is significantly higher in pituitaries collected in winter ($P<0.01$) compared to pituitary glands collected during spring (**a** and **b,** respectively). *In vivo* implants of E_2 for 7 days during winter, in sea bream significantly increased *in vitro* PRL release above control levels (**a** and **a'**, $P<0.05$). In contrast, during spring, *in vivo* implants of E_2 significantly lowered *in vitro* PRL release compared to the control (**b** and **b'**, $P<0.01$). Each bar represents mean ± SEM of PRL secreted (μg/ml) by the pituitary of control (n=13) and E_2 stimulated fish (n=13) in winter and control (n=10) and E_2 stimulated fish (n=10) in spring.

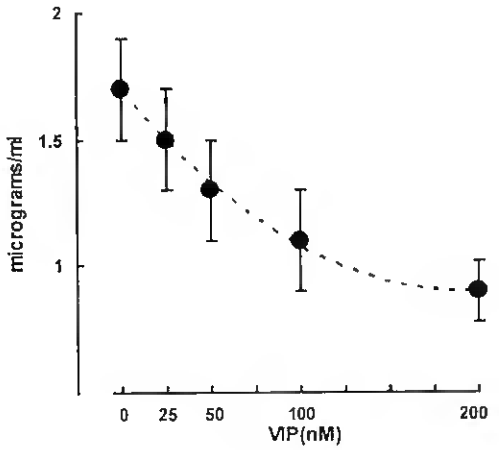


Figure 6.2 *Winter experiment* – A linear trend of decreasing PRL secretion by pituitary glands *in vitro* was found with increasing doses of VIP (0, 25, 50, 100 and 200 nM) subsequent to prestimulation *in vivo* with E_2 (10mg/kg). The amount of PRL secreted into the medium dropped significantly ($P<0.05$) in the presence of 100 nM and 200nM VIP when compared with control fish. Each point represents the mean ± SEM of PRL secreted by pituitaries from E_2 stimulated fish in the presence of increasing concentrations, 0, 25, 50, 100 and 200nM of VIP (n = 13, 11, 12, 13 and 12, respectively).

Immunohistochemistry (kindly carried out by Dr. Deborah Power)

Intense VIP-immunoreactivity (irVIP) was present in the neurohypophysial (NH) tissue penetrating the rostral pars distalis (RPD) and proximal pars distalis (PPD) of the pituitary gland (Fig. 6.3b-e). PRL immunoreactive cells were observed to be in close proximity with the irVIP axons (Fig. 6.3a). The distribution of irVIP in the neurohypophysis differed slightly when L311 and L25 were compared. The antiserum against the C-terminus of dogfish VIP (L311) stained principally nerve fibres and the antiserum against the N-terminus of VIP stained nerve fibres but the cytoplasm of some pituitary cells in the PPD and PI was also stained, although in the present study it was not possible to establish colocalisation with other pituitary hormones.

6.4 Discussion

The role of PRL in fish reproduction is largely unstudied. Recently PRL receptors were reported to be present in sea bream gonads and their expression found to be up-regulated by E_2 which also increased pituitary PRL expression (Cavaco *et al.*, 2003). The results from the present study demonstrate that ovarian steroids may also modulate pituitary PRL secretion, although whether this effect was direct or indirect was not established. The responsiveness of PRL cells in sea bream varied with season and in winter secretion of PRL was stimulated by E_2 but in spring this steroid inhibited PRL secretion. These results do not agree with data for pituitary PRL secretion *in vitro* obtained in other experiments (see chapter 5, section 5.4 for discussion

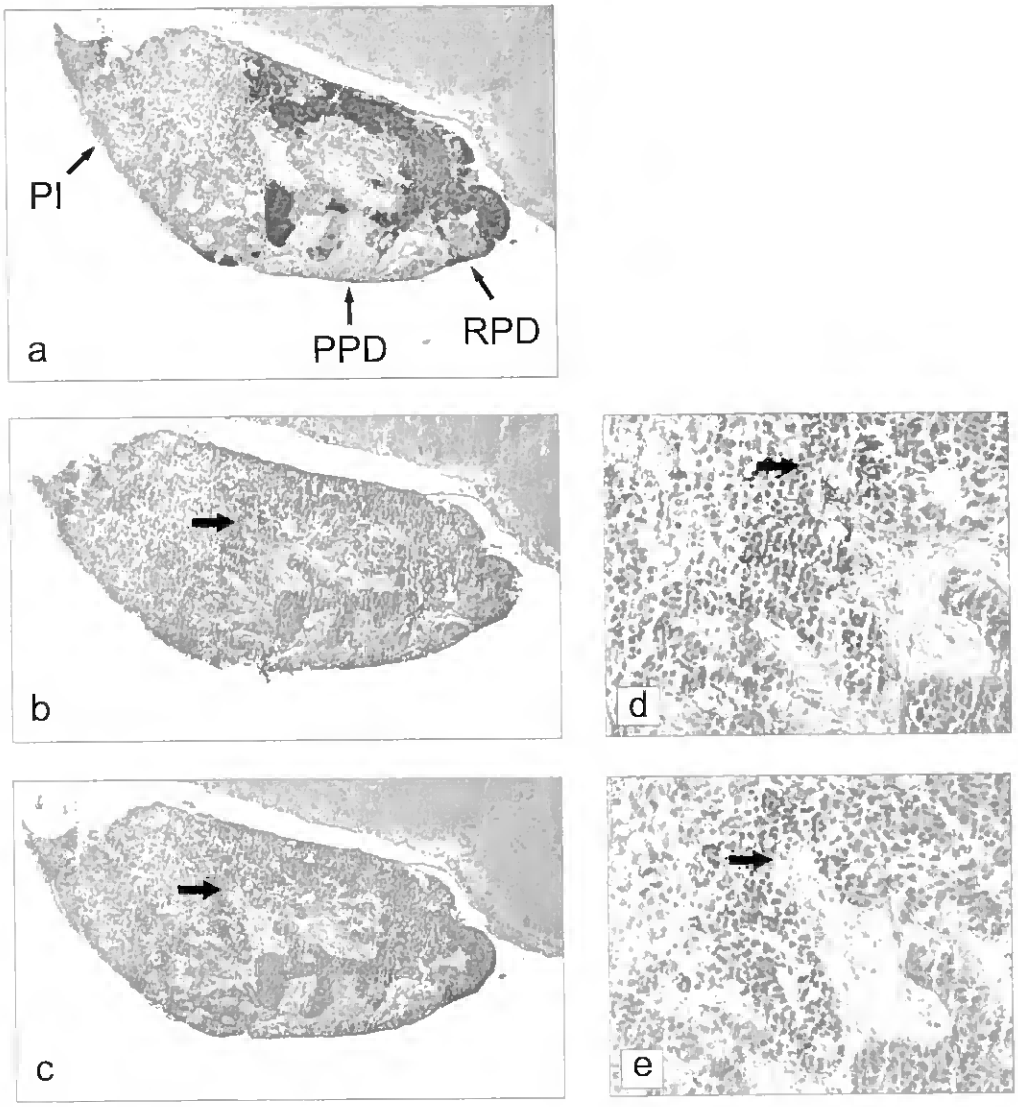


Figure 6.3 Immunohistochemistry of consecutive sagittal sections of sea bream pituitary gland counterstained with haematoxylin. **a**) intensely staining PRL cells revealed by anti-chum salmon prolactin in the rostral pars distalis (RPD,), **b**) VIP-immunoreactivity (irVIP) detected in nerve fibers in the neurohypophysis using antisera specific for the N-terminus of porcine VIP (L25) and **c**) a similar pattern of immunoreactivity was found with antisera specific for the C-terminus of dogfish VIP (L311). Note that ir-VIP nerve fibers penetrated the proximal pars distalis (PPD) and the rostral pars distalis (RPD). **a**, **b** and **c** are amplified X100. **d**) and **e**) are higher magnifications (x400) of the sections in **b**) and **c**) respectively. Note that at this magnification both nerve fibers and nerve terminals of axons appear to stain intensely in the neurohypophysial tissue and that the surrounding cells do not stained.

of results). Immunohistochemical studies with both N- and C-terminal VIP antisera showed that the neuropeptide VIP was present in neurohypophysial tissue projecting into the RPD of the pituitary gland, suggesting it might be involved in the regulation of PRL cells. In vitro pituitary cultures revealed that VIP significantly inhibited PRL secretion ($p<0.05$) from pituitary glands of E_2 primed fish in winter. VIP had no effect on PRL secretion from pituitary glands in vitro in the absence of E_2 pre-treatment. The results from the present study with the sea bream show that ovarian steroids can modulate PRL cell secretory activity and may change the sensitivity of PRL cells to VIP, it also provides further evidence supporting a role for PRL in fish reproduction.

It has been proposed that the biological versatility of PRL in vertebrates may be a reflection of its molecular heterogeneity. For example, PRL variants which differ in both size and charge, regulate osmoregulatory processes in tilapia (Borski *et al.*, 1992). A glycosylated isoform of turkey PRL was recently shown to be important in the reproductive cycle (Bédécarrats *et al.*, 1999) and in rats a range of glycosylated forms of PRL have been characterised (Bollengier *et al.*, 1988). The size of sbPRL estimated by SDS-PAGE (26,000) was larger than the size predicted from the cDNA sequence (Santos *et al.*, 1999) suggesting posttranslational modifications of this hormone may have occurred. In fact a consensus sequence for N-linked glycosylation (Asn 148) and phosphorylation (Ser 166) exist in sbPRL (Santos *et al.*, 1999). It will be of interest to determine if different isoforms of sbPRL occur and to establish their biological function.

Studies in other teleosts, with the exception of the platyfish (*Xiphophorus maculatus*, Kim *et al.*, 1979), have shown that E_2 has a

stimulatory effect on the activity of PRL cells (Barry and Grau, 1986; Borski *et al.*, 1991; Wigham, 1992; Williams and Wigham, 1994; Poh *et al.*, 1997; Weber *et al.*, 1997), although there are no previous reports about the effect of season on pituitary responsiveness. In common with observations in other teleosts E_2 influences PRL cell function directly in the sea bream although it remains to be established if it also acts via the hypothalamus in vivo (Barry and Grau, 1986; Borski *et al.*, 1991; Wigham, 1992). The seasonal differences observed in basal PRL cell activity and E_2 sensitivity in the sea bream is intriguing. In the present study PRL cells were most sensitive to E_2 during winter and this heightened sensitivity coincided with the natural reproductive peak in the sea bream. In contrast, during spring when gonads were regressing PRL secretion was inhibited by E_2, suggesting PRL may be involved in reproduction although its function remains to be established.

The variation in sea bream PRL cell responsiveness with season raises question about whether this also occurs in other teleost fish. The discrepancy reported between the action of E_2 on PRL secretion in platyfish compared to other teleost species may be due to experimental factors, such as season and hormonal status of the fish and highlights the need to take into consideration these factors when planning experiments. The factors underlying the differing seasonal responsiveness of PRL cells in the sea bream remain to be established but factors such as photoperiod, temperature, age and general hormonal status are strong candidates. It will be of interest to determine if the activity of other pituitary cell types in the sea bream can also be modulated by season.

The use of E_2 primed sea bream in the present study overcame many of the problems reported when E_2 treatment is carried out in vitro (Wigham et al., 1977; Barry and Grau, 1986; Borski *et al.*, 1991; Wigham, 1992). Although the mechanism by which elevated circulatory levels of steroid hormones bring about the increase in secretion of PRL noted in this and previous studies on teleosts remain unclear. In tilapia it has been suggested that E_2 may stimulate PRL synthesis and that this leads indirectly to enhanced secretion (Wigham *et al.*, 1977; Barry and Grau, 1986). The recent identification of estrogen receptors in the pituitary gland of the sea bream (Soccorro *et al.*, 2000) and the observation that E_2 increases transcription of the gene encoding PRL in this species (Cavaco *et al.*, 2003), suggests that E_2 acts directly on PRL cells via its receptor. It seems likely that in vivo circulating E_2 also indirectly stimulates PRL release through its action on neurotransmitter and neuropeptide systems which normally regulate PRL cell function. Similar observations have been made in rats where E_2 is reported to enhance PRL release via several different mechanisms. It increases the transcription of the gene encoding PRL in the pituitary lactotrophs and this increases PRL release (Maurer, 1982) and it also decreases the potency of dopamine by uncoupling the dopamine D2 receptor (Munemura *et al.*, 1989).

The neurotransmitters and neuropeptides, which regulate PRL secretion in teleosts remain to be clearly identified but existing studies suggest a range of substances may be involved and that regulation may be species specific (Batten *et al.*, 1983). In general two inhibitory factors appear to have been identified, SRIF and dopamine (Le Goff *et al.*, 1992; Williams and Wigham, 1994). The available anatomical evidence further supports a

role for SRIF and dopamine in the regulation of PRL secretion in fish. A detailed immunohistochemical study of the brain and pituitary gland in the green molly (*Poecilia latipinna*, Batten *et al.,* 1983) demonstrated that numerous neuropeptides innervate the pituitary gland. The role of these peptides in regulation of pituitary function in teleost fish remains to be established. The identification by immunohistochemistry of VIP in the brain and neurohypophysial tissue penetrating the RPD in sea bream (Power and Ingleton, 1998) and in vitro studies with tilapia pituitaries demonstrating a function for this peptide (Kelley *et al.,* 1988), make it a candidate pituitary cell regulating peptide in fish.

In the present study using immature sea bream, in the absence of E2 priming VIP had no effect in vitro on PRL secretion. This is in contrast to the inhibitory effect of VIP on PRL secretion from untreated adult male tilapia pituitaries in vitro (Kelley *et al.,* 1988). The differing effect of VIP in these two species is more likely to be a consequence of the age and associated hormonal status of the experimental animals used, than species-specific differences. This idea is supported by other studies in tilapia showing that both testosterone and E_2 increase the sensitivity of PRL cells to GnRH (Weber *et al.*, 1997). It would appear that E_2 priming changes pituitary PRL release in two ways in the sea bream, it directly stimulates PRL release in winter (Fig. 6.1), but it also enhances the sensitivity of PRL-secreting tissue to VIP (Fig. 6.2). In sea bream relatively low concentrations of VIP blocked the stimulatory action of E_2 on pituitary PRL release in a dose dependent manner. The results are in contrast to observations in mammals, birds and amphibians where VIP generally has a stimulatory action in sexually mature animals

(MacNamee *et al.*, 1986; Koiwai *et al.*, 1986; Youngreen *et al.*, 1994; Balsa *et al.*, 1998; Maney *et al.*, 1999; van der Beek *et al.*, 1999). The results from the sea bream and those previously obtained with tilapia suggest that reproductive status may alter the regulation of PRL cells by VIP (Kelley *et al.*, 1988). So that in sea bream VIP negatively regulates elevated PRL levels induced by E_2 implants. One possible explanation is that the negative feedback action of VIP counterbalances the effect of E_2 on PRL release, thus limiting the increase in circulating PRL levels. This negative feedback may be important in preventing uncontrolled E_2-stimulated PRL release during the breeding season in the sea bream.

In birds, VIP stimulates PRL release and in turkeys, a VIP pulse generator appears to be located within the hypothalamus (Chaiseha *et al.*, 1998). In the turkey, VIP stimulates PRL expression by up-regulating the transcription rate of PRL and by enhancing PRL mRNA stability (Tong *et al.*, 1998). VIP released by electrical stimulation in turkeys increases PRL secretion and is involved in the regulation of pituitary PRL mRNA expression (Youngreen *et al.*, 1994). The responsiveness of PRL cells to VIP in some passerine species has been shown to be dependent on season. A rapid increase in plasma PRL is induced by VIP during the breeding season, but PRL cells subsequently become refractory after termination of this season (Maney *et al.*, 1999). It remains to be established in the sea bream the factors which control VIP release in the pituitary gland. Moreover, it will be of interest to carry out a more detailed analysis of the response of PRL cells to VIP at different stages of the reproductive cycle in sea bream and also using fish at different stages of their life cycle.

In conclusion, the present study is the first on a nontetrapod species, the immature sea bream, in which the interaction between E_2 and VIP on PRL secretion has been studied. The results from experiments carried out in spring and winter suggest that the sensitivity of PRL cells to E_2 varies with season. Interestingly PRL cells are most sensitive to E_2 during the reproductive season (winter). These observations suggest that changes in hormonal status accompanying reproduction in sea bream, change the sensitivity of PRL cells to E_2 and that VIP may function as a physiological hypophysiotropic-inhibiting factor of PRL cells in sea bream primed with E_2. The latter suggestion is substantiated by the identification of VIP immunoreactive fibres in the neurohypophysis penetrating the RPD and the negative regulation of PRL secretion by low concentrations of VIP in vitro. The identification of VIP receptors in PRL cells would further substantiate this idea. Overall the present study supports the proposal that the fundamental control mechanisms of PRL secretion may change with the reproductive status of the animal (Barry and Grau, 1986; Weber *et al.*, 1997).

CHAPTER 7: PRL release in response to freshwater challenge

CHAPTER 7: PRL release in response to freshwater challenge

7.1 Introduction

Fish interact with their environment and extract or excrete water and/or salts to maintain the ionic strength of their internal fluids within narrow limits. Stenohaline species born in either freshwater (FW) or seawater (SW) are unable to regulate their plasma ionic composition when challenged with changing environmental salinity, a process that consequently results in high mortality. In contrast, euryhaline species can adjust to changing environmental salinity through adjustment of their ionic and endocrine systems to cope with new environments. The maintenance within narrow limits of circulating water and salts is under ionic and endocrine control and in teleosts, PRL has been viewed as "the" FW adapting hormone, as a consequence of its ability to regulate hydromineral balance (reviewed by Manzon 2002). Cortisol, under some conditions, may promote ion uptake and interacts with PRL during acclimation to FW (Eckert et al., 2001; McCormick, 2001).

Ultrastructural and immunocytochemical data demonstrated that in *Gambusia* the activity of PRL cells was stimulated when fish were transferred to FW (Olivereau, 1986). In killifish (*Fundulus heteroclitus*), the volume occupied by the PRL cell mass was larger in FW-adapted than in SW-adapted specimens and contained larger PRL cells (Betchaku and Douglas, 1980). In the sea bream adapted to hypoosmotic environments, ultrastructural evidence suggest an activation of synthesis and release of PRL (Mancera *et al.*,

1993b). In Mozambique tilapia, both forms of PRL increased after transfer to FW (Yada *et al.*, 1994; Shepherd *et al.*, 1999). Moreover, PRL cells from larvae hatched and maintained in FW or in those transferred from SW to FW had a stronger immunoreaction, a significantly larger PRL cell size, and the area occupied by PRL cells was larger (Ayson *et al.,* 1994). In species that are dependent on PRL for FW osmoregulation, pituitary and plasma PRL levels increase during adaptation to FW to regulate both the permeability of the osmoregulatory surfaces and the ion transport mechanisms. By decreasing water uptake and increasing ion retention PRL regulates the hydromineral balance (reviewed by Manzon, 2002). In the sea bream, *in vivo* treatments with PRL induced an increase in plasma osmorality and ion concentration in fish transferred to brackish water (5ppt salinity) (Mancera *et al.*, 2002).

Another link of PRL with osmoregulation is observed from studies on PRLR gene expression. In Nile tilapia (*Oreochromis niloticus*), trout (*Oncorhynchus mykiss*), Japanese flounder (*Paralichthys olivaceus*), and sea bream, high levels of PRLR gene expression were detected in the osmoregulatory organs, such as gills, kidney, and intestine (Sandra *et al.*, 2000; Prunet *et al.*, 2000; Higashimoto *et al.,* 2001; Santos *et al.*, 2001). An increase in environmental salinity resulted in a decrease in the expression of PRLR in the gills of Nile tilapia (Sandra *et al.*,2000) but did not change the abundance of PRLRs (Sandra *et al.*, 2001), suggesting a down-regulation of the PRLR protein may occur in the presence of elevated PRL levels associated with transfer to FW.

Pioneering studies in various teleosts identified an inverse relationship between environmental salinity and PRL cell activity *in vivo*, and between medium osmorality and PRL release *in vitro* (for reviews Clarke and Bern, 1980 and Wigham, 1992). These findings seem to indicate that the release of PRL is a direct response to changes in extracellular osmorality during adaptation to different salinities. However, studies *in vivo* with the Mozambique tilapia (*Oreochromis mossambicus*) suggested that plasma osmolarity was not the predominant factor in the control of PRL cell activity *in situ*. Wendelaar-Bonga *et al.* (1985) proposed that PRL cell activity could be controlled principally by the hypothalamus, rather than any direct effect of plasma osmorality.

The sea bream is a marine teleost capable of a certain degree of adaptation to changes in environmental salinity. The challenge of low salinity for short periods of time has been reported to result in the activation of PRL cells (Mancera *et al.*, 1993b, 2002). The aim of the present work was to evaluate the ability of the sea bream to survive and adapt to low salinity environments and to test the endocrine response of PRL in isolated pituitary glands collected from fish maintained at normal (SW 36 ppt) or challenged with low salinity for up to 7 days (FW 2 ppt).

7.2 Additional methods

The methodology concerning the pituitary culture, the identification, separation and quantification of PRL is described in chapter 2. The specific methods reported in this chapter describe the characteristics of the animals and experimental design used in the experiments.

Animals

All studies were carried out on immature sea bream (body weight 50 to 70g) purchased from local commercial suppliers in the Algarve (South Portugal). Fish were transferred and maintained at Ramalhete Marine Station (University of Algarve) under natural annual conditions of water temperature, photoperiod, and salinity in 1000L tanks with flowing SW and fed once daily (1.5%, 2.8%, and 1.8% of body weight during late winter, spring and autumn, respectively) on commercial sea bream pellets (Provimi, Faro, Portugal).

Salinity challenge

Fish were stocked in 500L tanks (density 5kg/m^3) for at least 2 weeks before salinity challenge. After the pre-adaptation period to tanks, water entering the tanks was SW (control fish) or switched from SW to FW (salinity challenged fish) without disturbing the fish. The group of control fish was exposed to the natural value of SW salinity of the season (late winter, spring and autumn) when the experiment was carried out. The other group was exposed to a steady decrease in salinity over 3 days by running FW into the tank until a value of 2ppt (4mOsm/kg) was reached. Water salinity was checked twice daily and corrected when necessary. In all experiments fish were sampled 7 days after the onset of seawater dilution.

Experiment 1: Effect of low salinity challenge on in vitro PRL release during late winter

A total of 32 specimens (n=16 from each salinity group) were reared during March under natural conditions of temperature (16± 2°C), photoperiod

(12hrs), and salinity (37± 1ppt). Fish were sacrificed 7 days after the onset of salinity challenge by anaesthesia with a sub-lethal dose of MS-222 (1:10000; Sigma, Madrid) followed by decapitation and pituitaries were collected into freshly prepared culture medium. Two different culture media were tested: hypotonic and isotonic (chapter 2, section 2.1). Half of the pituitaries collected from FW-adapted fish were cultured in hypotonic medium (n=8) and another half in isotonic medium (n=8). The same scheme was followed for SW-adapted fish. Pituitary glands were incubated for 18hr at 21°C. Individual pituitaries and culture medium were stored at -20°C and were analyzed approximately 1 week later.

Experiment 2: Effect of low salinity challenge on in vitro PRL release during spring

This experiment was performed as described for experiment 1 with the exception that a total of 60 specimens were sampled (n=30 from each salinity group), and the values of temperature (21± 3°C), photoperiod (14hrs), and salinity (37± 1ppt), corresponded to natural conditions in May.

Experiment 3: Effect of low salinity challenge on in vitro PRL release during autumn

This experiment was performed as described for experiment 2 with the exception that the values of temperature (17± 2°C), photoperiod (10hrs), and salinity (35± 1ppt), were the values that corresponded to natural conditions in November. A total of 60 specimens (n=30 from each salinity group) were sampled.

Statistical analysis:

For each experiment, variance was checked for homogeneity using the Levene test. Data were statistically evaluated by a one-way or two-way analysis of variance. Significantly different means were identified by Duncan's multiple range test. The statistical package SPSS version 10.0 (SPSS Inc., USA) was used for analysis of the data.

7.3. Results

Gradual adaptation of juvenile sea bream to FW in winter, spring, and autumn resulted in 40%, 20%, and 20%, respectively of cumulative mortality after 7 days of onset of salinity challenge. Total deaths occurred between the 3rd and the 4th days, when salinity reaches its lowest level (2ppt) corresponding to freshwater.

In spring and autumn (results not available for winter), higher quantity of PRL was released compared to the level of pituitary gland content (Table 7.1). In all experiments, PRL released from pituitaries incubated in isotonic medium show significant differences between fish reared in SW and fish transferred to FW (Tables 7.1 and 7.2; Fig. 7.1), In contrast, very similar results were obtained between FW and SW fish which pituitaries were incubated in hypotonic medium, except during autumn. In this season, the significant difference between PRL released by pituitaries of FW and SW fish may be partly explained by the high variability encountered between the values of FW adapted fish (Tables 7.1 and 7.2; Fig. 7.1).

The results indicate that sea bream PRL cells are directly responsive to the culture medium osmorality, although again in autumn the differences were

not significant, probably related with the high variability between the values obtained in this experiment. Moreover, pituitaries from SW-adapted fish in winter secreted the same quantity of PRL in both types of culture medium (Tables 7.1 and 7.2; Fig. 7.1).

A seasonal variation was observed on *in vitro* PRL release. Interestingly, pituitaries collected from FW fish and cultured in isotonic medium released similar levels of PRL in all seasons (2-3μg/ml) (Table 7.3; Fig. 7.1).

Table 7.1 – PRL release and pituitary gland PRL content (mean± SEM) after incubation in isotonic medium and hypotonic medium. The number of pituitary glands analysed are shown in brackets.

Isotonic medium		PRL release (μg/ml)	PRL pituitary content (μg/ml)
Experiment 1 (winter)	FW	2.16 ± 0.27 (8)	-
	SW	0.45 ± 0.01 (8)	-
Experiment 2 (spring)	FW	2.43 ± 0.24 (13)	0.26 ± 0.07 (14)
	SW	1.85 ± 0.11 (15)	0.27 ± 0.07 (14)
Experiment 3 (autumn)	FW	2.58 ± 1.74 (13)	0.33 ± 0.08 (14)
	SW	0.94 ± 0.48 (13)	0.27 ± 0.03 (15)
Hypotonic medium			
Experiment 1 (winter)	FW	0.48 ± 0.03 (8)	-
	SW	0.48 ± 0.02 (8)	-
Experiment 2 (spring)	FW	1.53 ± 0.24 (14)	0.26 ± 0.07 (15)
	SW	1.15 ± 0.12 (14)	0.28 ± 0.07 (15)
Experiment 3 (autumn)	FW	1.97 ± 1.24 (14)	0.37 ± 0.12 (13)
	SW	0.75 ± 0.45 (14)	0.35 ± 0.11 (15)

When fish are transferred from SW to FW there is an initial rapid adaptative period (± 24hr) with disturbances of osmotic and ionic balance and a period of chronic regulation when the preceding parameters achieve homeostasis. In the present study, a more detailed analysis was made of *in vitro* PRL release from pituitaries cultured in isotonic medium because fish were sacrificed 7 days after the onset of salinity challenge and survivors had

already achieved osmotic and ionic balance homeostasis. Moreover, incubation of pituitaries from SW-adapted fish in hypotonic medium could indicate how osmotic and ionic environment might alter *in vitro* PRL cells activity. In all the experiments a more detailed analysis of these two aspects were carried out.

Experiment 1: Effect of low salinity challenge on in vitro PRL release during late winter

In isotonic culture medium, cultured pituitary glands obtained from FW-adapted fish released significantly ($P<0.001$) more PRL that the pituitaries collected from SW-adapted fish (Tables 7.1 and 7.2; Fig 7.1). Under these conditions, the level of PRL secretion in FW-challenged sea bream was about 4–fold higher to that shown by SW-adapted fish.

Pituitaries glands from SW-adapted fish incubated in hypotonic and isotonic culture medium released the same amount of PRL (Tables 7.1 and 7.2; Fig 7.1).

Table 7.2 – Significant differences between means of PRL released a) by pituitary glands incubated in isotonic and hypotonic medium, and b) by pituitary glands collected from FW and SW fish. (*), (**), and (***) means significantly different at $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

	Isotonic medium	Hypotonic medium
Experiment 1 (winter)		
FW	a) ***	
SW	a) ns	
	b) ***	b) ns
Experiment 2 (spring)		
FW	a) **	
SW	a) ***	
	b) *	b) ns
Experiment 3 (autumn)		
FW	a) ns	
SW	a) ns	
	b) **	b) **

Experiment 2: Effect of low salinity challenge on in vitro PRL release during spring

In isotonic culture medium, cultured pituitary glands obtained from FW-adapted fish released significantly ($P<0.05$) more PRL that the pituitaries collected from SW-adapted fish (Tables 7.1 and 7.2; Fig 7.1). Under these conditions, the level of PRL secretion in FW-challenged sea bream was about 3–fold higher than that of SW-adapted fish.

Pituitary glands from SW-adapted fish incubated in hypotonic culture medium, secreted significantly ($P<0.001$) less PRL compared to the levels obtained in isotonic cultures (Tables 7.1 and 7.2; Fig 7.b).

Table 7.3 – Seasonal variation of *in vitro* PRL release expressed as significance level of difference between means. (*), (**), and (***) significantly different at $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

	Isotonic medium
FW	ns
SW	***
	Hypotonic medium
FW	*
SW	**

Experiment 3: Effect of low salinity challenge on in vitro PRL release during autumn

In isotonic culture medium, cultured pituitary glands obtained from FW-adapted fish released significantly ($P<0.01$) more PRL than the pituitaries collected from SW-adapted fish (Tables 7.1 and 7.1; Fig 7.1). In isotonic culture, the level of PRL secretion in FW-challenged sea bream was about 1–fold higher than that of SW-adapted fish.

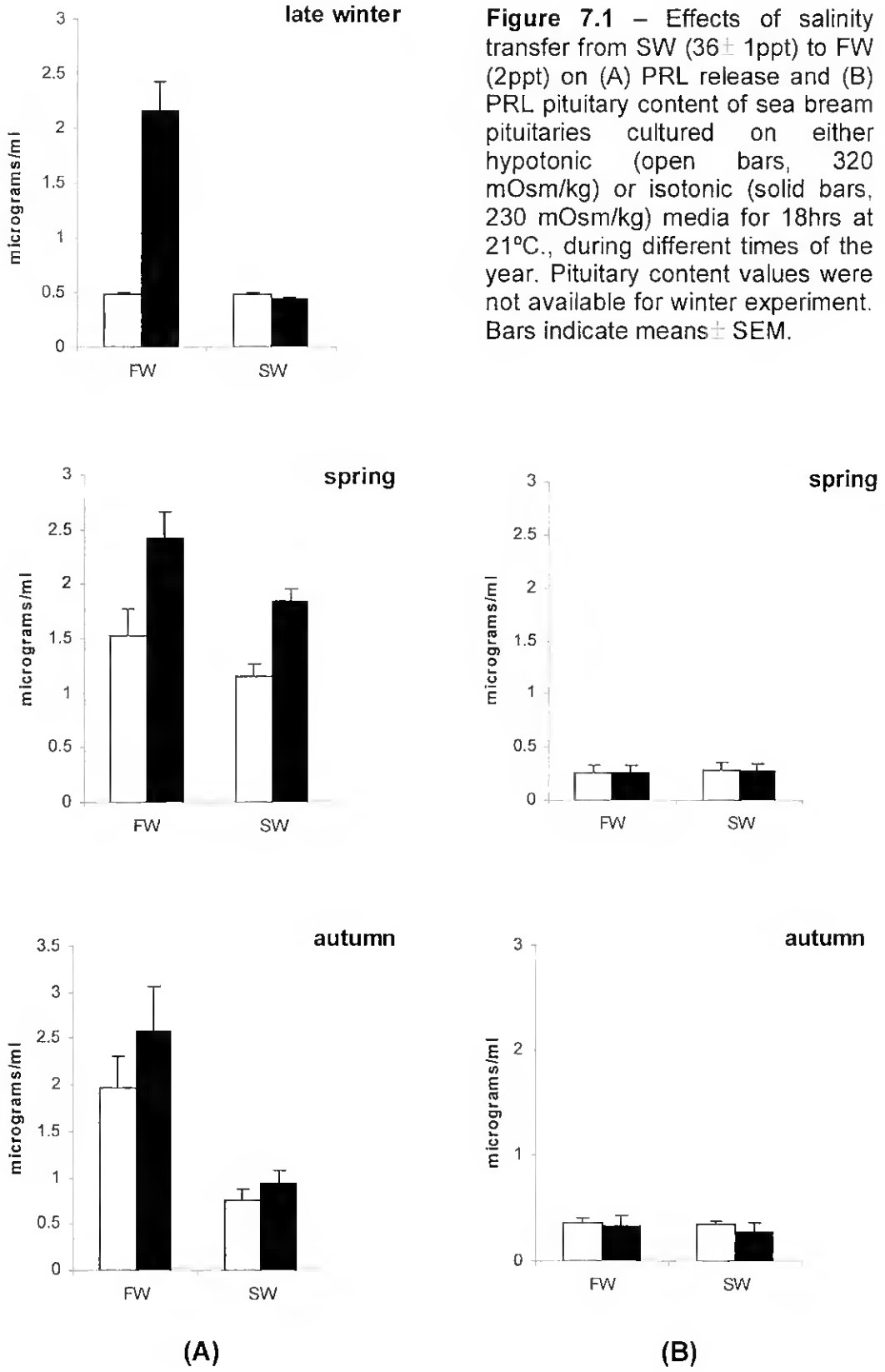


Figure 7.1 – Effects of salinity transfer from SW (36± 1ppt) to FW (2ppt) on (A) PRL release and (B) PRL pituitary content of sea bream pituitaries cultured on either hypotonic (open bars, 320 mOsm/kg) or isotonic (solid bars, 230 mOsm/kg) media for 18hrs at 21°C., during different times of the year. Pituitary content values were not available for winter experiment. Bars indicate means± SEM.

Pituitary glands from SW-adapted fish incubated in hypotonic culture medium, secreted quantities of PRL which were not significantly different from the levels secreted by pituitaries maintained in isotonic cultures, although the lack of a significant difference may be partly explained by the high variability encountered between the values obtained in this experiment (Tables 7.1 and 7.2; Fig 7.1).

7.4 Discussion

The present results suggest that PRL has an important role in the survival of juvenile sea bream transferred to FW. In late winter, the cumulative mortalities of up to 40%, demonstrated at this time of year sea bream had a poor osmoregulatory capacity. A clear improvement in efficiency of the osmoregulatory mechanism was observed in spring and autumn, when the cumulative mortality decreased to about 20%. A similar seasonal change in the osmoregulatory ability has previously been reported for brook charr (*Salvelinus fontinalis*) and Atlantic cod (*Gadus morhua*) (Dutil *et al.*, 1992; Claireaux and Audet, 2000). Moreover, the time needed to restore osmotic and ionic balance after transfer was also dependent on season. Establishing the existence of a dynamic response to low salinity was beyond the scope of the present study, but it has been reported in Mozambique tilapia (*Oreochromis mossambicus*), zander (*Stizostedion lucioperca*), brook charr (*Salvelinus fontinalis*), and sea bream that the adaptation to changes in external salinity takes place in two different phases (Mancera *et al.*, 1993a; Morgan *et al.*, 1997; Kelly and Woo, 1999; Claireaux and Audet, 2000; Brown *et al.*, 2001; Seale *et al.*, 2002). There is an initial rapid adaptative period

(± 24hr), with disturbances of osmotic and ionic balance, and a subsequent period of chronic regulation, when the preceeding parameters achieve homeostasis. The length of the second period is 4, 5, 6 or 7 days and is species dependent (Mancera *et al.*, 1993a; Morgan *et al.*, 1997; Kelly and Woo, 1999; Claireaux and Audet, 2000; Brown *et al.*, 2001; Seale *et al.*, 2002) and also on the time of the year that transfer takes place (Dutil *et al.*, 1992; Claireaux and Audet, 2000).

It is possible that there is seasonal variation in the physiological response of sea bream to low salinity. The seasonal differences in pituitary activity and the differential secretion of PRL isoforms (chapter 4, figures 4.2 and 4.3) may explain the different results obtained when sea bream are exposed to changes in salinity (2ppt) during late winter.

In sea bream, Mancera *et al.* (1993b) describe an activation of the PRL cells in the pituitary gland after challenge with brackish water. The preceding results are in good agreement with the results obtained in the present study where in all experiments PRL secretion was stimulated in pituitaries from FW-adapted fish cultured in .isotonic medium (320 mOsm/kg, Na^+ 170 mM). Isotonic medium was utilized for this analysis because it was considered that after 7 days of salinity challenge, the survivors had achieved full acclimation and plasma osmorality was not significantly different from SW-adapted fish (Mancera *et al.*, 1993a; Kelly and Woo, 1999; Brown *et al.*, 2001). In previous studies with sea bream a short adaptative period of 24-48hrs exists when a significant plasma hypoosmorality and reduction in ion levels occurs. This is then followed by a chronic regulatory period in which osmotic and ionic balance are restored to near those prior to transfer (Mancera *et al.*, 1993a). In

the present study the amount of PRL release in isotonic culture medium by FW-adapted fish reached similar levels in all seasons (2-3μg/ml) and a significant increase compared with the amount secreted by SW fish. The increment of PRL release from SW- to FW-adapted fish was smaller in spring (31%), when the basal secretion of PRL is relatively high. In contrast, the increment obtained in late winter (380%) was significantly higher as it coincides with a period when an abrupt decrease in basal PRL secretion is observed after the spawning season (chapter 4, figure 4.2).

Low osmolarity challenge by itself *in vitro* has been used as a model to study the control of pituitary secretion and synthesis, but the capacity of isolated PRL cells to respond directly to changes in osmolarity of the culture medium is unclear. Earlier studies in several different teleost fish identified an inverse relationship between medium osmolarity and PRL release *in vitro* (for reviews Clarke and Bern, 1980 and Wigham, 1992). In the present study, the results indicate that sea bream PRL cells are directly responsive to the culture medium osmorality, although in autumn the differences were not significant which was probably due to the high variability between the values obtained in that time of the year. Moreover, pituitaries from SW-adapted fish in winter secreted the same quantity of PRL in both types of culture medium. There are conflicting results in the literature about the effects of osmolarity on *in vitro* PRL release (see Wigham, 1992 for review). One explanation given for the variable results (Wendelaar-Bonga *et al.*, 1985) was that PRL cell activity *in vitro* was related to the ambient osmotic pressure previously experienced *in vivo,* and such an explanation seems to apply to the results of the present study. This means that the mechanism which stimulated PRL release in FW-

adapted fish was developed *in vivo* when the salinity challenge took place and was retained and reflected *in vitro* when the pituitary glands were cultured.

In conclusion, the present results indicate that cultured pituitaries of fish challenged with water with extremely low values of salinity (2ppt), release significantly more PRL that pituitary glands from SW-adapted fish. This suggests a potential role for PRL in FW adaptation of sea bream. Moreover, the study indicates that the success of adaptation may depend on the time of year at which transfer to FW occurs.

8. General Discussion

Chapter 8: General Discussion

PRL released *in vitro* by cultured pituitaries during winter and summer had different isoelectric points and was isolated by continuous elution electrophoresis. Database searches using the partial amino acid sequence of the principal fragments obtained by enzymatic cleavage of the winter and summer form of PRL revealed they are similar. The existence of PRL genetic variants, observed in some teleosts (see Mazon, 2002 for review), was not clearly defined in the sea bream. Only 18% of winter PRL molecule was sequenced, while it was possible to sequence 46% of the PRL isolated in summer. The amino acid sequences obtained from winter and summer PRL were nearly identical to each other and 99.5% identical to the deduced amino acid sequence of sea bream PRL cDNA (Santos *et al.*, 1999). The seasonal charge variants of PRL identified by isoelectric focusing may arise as a consequence of post-translational modifications. In summary, sea bream PRL gave no detectable variants which differed in molecular weight although charge variants were found, which could be accounted for by modifications due to multiple post-translational modifications. The present study is the first report to clearly demonstrate that modified forms of PRL exist in fish although a brief reference was already made for chum salmon PRL by Kawauchi *et al.* (1983). Although in the present study PRL modifications were not elucidated this important aspect of the PRL characterization is an issue that still remains to be clarified and needs further investigation.

Although teleosts do not possess a hypothalamo-hypophyseal portal system, depending instead on more-or-less direct contacts between hypothalamic neurosecretory neurons and the endocrine cells of the pituitary, accumulating evidence suggests that the regulatory hypothalamic factors in these animals are similar to those in other vertebrate which have a well defined portal blood system (chapter 1, section 1.6). The list of PRL secretagogues is rather long but in the present study attention was paid to galanin (Gal), which in terms of its potential physiological activity is one of the better characterized paracrine factors in the pituitary, and vasoactive intestinal peptide (VIP), which has long been characterized as a likely local factor influencing function of PRL-cells, and recently has been shown to interact with galanin. The action of a non-hypothalamic factor, the steroid E_2, was also studied. It is now well established that estrogens in addition to having an important role in the mammalian reproductive axis also have a modulatory effect on PRL release. (see Falkenstein, 2000 for review). A stimulatory effect of E_2 on the activity of PRL cells has also been reported in birds (Knapp *et al.*, 1988) and teleosts (Barry and Grau, 1986; Borski *et al.*, 1991; Wigham, 1992; Williams and Wigham, 1994; Poh *et al.*, 1997; Weber *et al.*, 1997). In sea bream, previous studies have shown that during the breeding season, PRL expression increases significantly after E_2 treatment in juveniles. However, PRL expression was significantly reduced by E_2 treatment in adults (Cavaco *et al.*, 2003), suggesting that the response of PRL-cells to E_2 is not a direct action on the releasing mechanism and is more likely to be part of an overall enhancement of the secretory process. In the present study

the responsiveness of PRL cells to E_2 is not clear cut because results from experiments carried out in the same season (but in different years) do not give entirely consistent results. The variation in sea bream PRL cell responsiveness to E_2 raises question about whether this also occurs in other teleost fish, and explains the different responsiveness of fish to E_2, e.g. the platyfish (*Xiphophorus maculatus*), where E_2 had no effect on PRL cells activity (Kim *et al.*, 1979). If an environmental cue such as changing temperature can accurately be used to alter the responsiveness of PRL cells to E_2, the comparison between experiments carried out in the same month but in different years, which may have significant fluctuations on environmental factors, should be analyzed with particular care. The identification and characterization of interactions between internal and external factors certainly represent a useful approach to understanding the complex responsiveness of PRL cells to estrogens.

The interaction between a range of factors within the pituitary gland, although likely to be of a subtle nature, almost certainly temper or potentiate the responses to signals both internal and external of the animal. For example, intrapituitary Gal plays a key role in modulating the effect of estrogens on mammalian lactotrophs and also seems to be implicated in the response of lactotrophs to VIP (see Falkenstein, 2000 for review). Estrogens also induced an increase in Gal immunoreactive material in the pituitary of lizards, quails, and eels (Olivereau and Olivereau, 1991b; Tsutsui *et al.*, 1998; Lamanna *et al.*, 1999). The interaction between E_2 and Gal on the *in vitro* release of PRL from the sea bream pituitary gland has been demonstrated in the present study (Fig. 8.1).

In experiments carried out both during winter and spring, Gal stimulated pituitary PRL secretion *in vitro* when the fish from which the pituitary gland was obtained had not previously been primed with E_2. However, when pituitary glands are obtained from fish primed with the E_2, Gal had no effect on *in vitro* PRL secretion. VIP has no effect on *in vitro* PRL release from pituitaries of control fish but blocks the stimulatory action of E_2 on pituitary PRL release (Fig. 8.1). In general E_2 seems to enhance the sensitivity of PRL cells to both VIP and Gal suggesting these peptides may be involved in modulating PRL release during the reproductive cycle as has been shown in birds and mammals (Hyde et al., 1991; Bloch *et al.*, 1993; Youngreen *et al.*, 1994; Wynick *et al.*, 1998; Leibowitz *et al.*, 1998; Bédécarrats *et al.*, 1999; Maney *et al.*, 1999; Rugam *et al.*, 1999).

Our results seem to confirm that there is an underlying physiological difference in the role of peptides in fish and tetrapods. For example, in the present study, the effect of VIP on pituitary PRL secretion in sea bream differs from that observed in mammals, amphibians, and avian species, where the peptide is a potent stimulator of PRL release (Hall and Chadwick, 1985; Koiwai *et al.*, 1986; MacNamee *et al.*, 1986; Youngreen *et al.*, 1994; Bédécarrats *et al.*, 1999; Maney *et al.*, 1999; Schwartz, 2000). But VIP in tilapia has an inhibitory effect on PRL secretion (Kelley *et al.*, 1988) and the results of sea bream and tilapia taken together may be indicative of a difference in the role of VIP in fish. The same rationale could be applied to Gal since estrogens positively regulate this peptide in mammals, lizards, quails, and eels (Olivereau and Olivereau, 1991b; Gabriel *et al.*, 1992; Bloch *et al.*, 1993; Brann *et al.*, 1993; Hyde *et al.*

1993; Crawley, 1995; Tseng *et al.*, 1997; Leibowitz *et al.*, 1998; Tsutsui *et al.*, 1998; Lamanna *et al.*, 1999; Rugarn *et al.*, 1999; Shen *et al.*, 1999; Degerman *et al.*, 2002), and, in the sea bream Gal inhibited or had no effect on PRL release in E_2 primed animals.

In addition to the involvement of E_2, Gal and VIP in modulating PRL release during the sea bream reproductive cycle, PRL also plays a role in water and electrolyte balance. In most euryhaline teleosts it is generally accepted that PRL is the FW-adapting hormone, although this is not the case for teleosts in general (see Manzon, 2002 for review). Sea bream eggs hatch during the winter generally in full sea water, the larvae migrate in early spring towards protected coastal waters, and return to the open sea in late autumn, where the adult fish breed (Moretti *et al.*, 1999). The lagoon and estuarine systems where sea bream inhabit during the juvenile period usually suffer wide variations in salinity, and fish must adapt at different stages of their life cycle to different salinity challenges. A fine endocrine control enabling the fish to cope with such variations of external salinity is essential for their survival. In the present study, the fact that extreme low salinity challenge stimulated *in vitro* PRL release (Fig. 8.1), suggests that the hormone, PRL, may be the main candidate for the role of a FW-adapting hormone in sea bream. Moreover, studies of the distribution of PRL receptors have shown abundant transcripts in sea bream osmoregulatory tissues (Santos *et al.*, 2001).

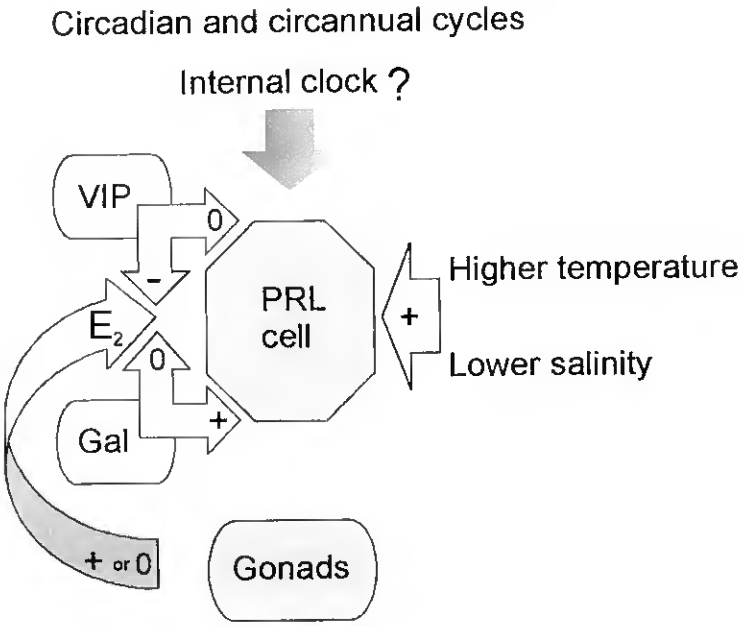


Figure 8.1 – An overview of the regulation of *in vitro* PRL secretion in sea bream. Circulating E_2 either stimulates or has no effect on PRL secretion but, in general, seems to enhance the sensitivity of PRL cells to both VIP and Gal. In pituitary glands obtained from control fish Gal stimulates PRL secretion, but on fish primed with E_2, Gal has no effect on PRL secretion (0). VIP has no effect on *in vitro* PRL release from pituitaries of control fish (0) but blocks the stimulatory action of E_2 on pituitary PRL release (-). PRL secretion show a circadian and circannual pattern which may be controlled by an internal biological clock, entrained by temperature increase. Lower salinity is also a stimulatory exogenous signal of sea bream *in vitro* PRL secretion.

Temperature (but not photoperiod) is another external factor that influenced sea bream PRL cells activity, with higher temperature increasing *in vitro* PRL secretion and lower temperature having the opposite effect (Fig. 8.1). In addition, a seasonal variation was observed in the *in vitro* responsiveness of PRL cells to external (water osmolarity) and internal stimuli (E_2, Gal and VIP), and a clear annual cycle of pituitary gland activity. This is highlighted by the seasonal variation in the basal pituitary PRL release rates but also by a variation in the relative concentration of PRL charge variants released from the pituitary gland.

For example, during autumn and winter, a single acidic variant of PRL is detected (related to spawning?). In contrast, during spring and summer there is a decrease in the concentration of the dominant winter PRL variant and a concomitant rise in the release of multiple charge variants (related to the abundance of food during spring, and/or migrations from the open sea to coastal waters?).

In mammals, some external factors induce a melatonin signal produced by the pineal gland, which is decoded by a gene-based clock mechanism in specialized target organs, including the pituitary gland (for a review, see Lincoln *et al.*, 2003). In some teleosts, melatonin secretion from the pineal gland shows a rhythmicity and is influenced by external factors such as temperature, photoperiod, and light intensity (Zachmann *et al.*, 1992; Randall *et al.*, 1995; Garcia-Allegue *et al.*, 2001; Porter *et al.*, 2001). The expression of genes associated with melatonin production have been proposed to be controlled by a circadian clock in the pineal gland (Bégay *et al.*, 1998; Coon *et al.*, 1998). In trout, melatonin modulates *in vitro* PRL secretion in this species (Falcón *et al.*, 2003). If a similar clock exists in the sea bream, it could be a factor responsible for both the cyclic variation in activity and reponsiveness of the pituitary prolactin cells identified in the present thesis.

In summary, PRL cell regulation in the sea bream have a multifactorial control, involving the interaction between internal secretagogues and external factors. In the present study, higher temperatures and low salinity stimulate *in vitro* PRL release. There is a physiological difference in the role of VIP and Gal in

sea bream compared with tetrapods. Moreover, E_2 seems to enhance the sensitivity of PRL cells to both VIP and Gal, suggesting that these peptides may be involved in modulating PRL release during the reproductive cycle. The temporal pattern of pituitary activity and release of PRL charge variants combined with the widespread distribution of PRLR in the sea bream reproductive and osmoregulatory tissues (Santos *et al.*, 1999; Santos et al., 2001; Cavaco *et al.*, 2003), suggests that PRL may have an important function in reproduction and osmoregulation in this species

Future Work

Future work

It has been recognized that PRL is a highly polymorphic hormone. In the present study sea bream PRL heterogeneity has begun to be defined in structural terms. The results suggested that sea bream PRL is not transcribed in more than one genetic form, although after translation a variety of chemical modifications may be introduced into the hormone and a potential phosphorilation at S_{158} was proposed based on present data. Moreover, the diversity of charge variants detected in the present study gives an indication of the high PRL heterogeneity. This important aspect of the sea bream PRL characterization is an issue that still remains to be clarified. Further investigation to a better understanding of structural modifications in sea bream PRL and their biological consequences, would allow us to understand exactly how the hormone is produced and how it regulates several physiological functions in sea bream.

The results from the present thesis into sea bream PRL cell regulation confirm that, as for all vertebrate species, a multifactorial control involving at least E_2, Gal, VIP, and environmental factors exists. During the reproductive season, E_2 plays a role of a primary secretagogue and Gal and VIP act as modulators of sea bream PRL secretion. But under different physiological circumstances Gal and VIP change their mode of action and become primary secretagogues. One possible explanation is that the responsiveness of PRL cells to Gal and VIP is linked to the sexual maturity of this species. To clarify this issue it will be of interest to carry out a more detailed analysis of the response of PRL cells to Gal and VIP using fish at different stages of their life cycle. Moreover, the

identification of Gal immunoreactive fibres in the pituitary gland and the identification of Gal and VIP receptors in PRL cells would further substantiate the results from control mechanisms of *in vitro* PRL release. The seasonal variation of PRL cells responsiveness to E_2 observed in the present study also needs a more systematic investigation.

Results all suggest that in common with other species sea bream has circannual or/and circadian clocks but the evidence is still insufficient for any definitive conclusion to be made. If a circadian clock exists in the sea bream, it could be a factor responsible for both the cyclic variation in activity and reponsiveness of the pituitary prolactin cells identified in the present thesis. More information is needed to increase our knowledge of how sea bream translate environmental information into endocrine signals.

For the future, both a more comprehensive investigation of sea bream PRL cell regulation and a broadening of the research to discover the biological roles of PRL in the sea bream are required.

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