BIOCHEMICAL AND PHYSIOLOGICAL STUDIES WITH SALMON PROLACTIN

by

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Biochemical and Physiological Studies with Salmon Prolactin

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ABSTRACT

The objectives of this thesis were 1) to isolate a biologically active prolactin (sPRL) from chum salmon (*Oncorhynchus keta*); 2) to develop a homologous radioimmunoassay (RIA) for the quantification of prolactin in salmon; 3) to describe the response of plasma prolactin and ions in juvenile coho salmon (*O. kisutch*) upon transfer from seawater (SW) to fresh water (FW); 4) to determine if environmental calcium is involved in the hydromineral regulatory function of sPRL; 5) to establish whether salmon prolactin acts as a hypercalcaemic hormone in salmon.

sPRL was purified from chum pituitary glands by acid–acetone extraction, iso–electric precipitation, cation–exchange HPLC, and gel filtration. Purity was established by detection of a single band in acid–urea PAGE, SDS PAGE, 2–dimensional PAGE and Western blots; its molecular weight was estimated to be 25,000 daltons. The amino acid composition and sequence of the first ten residues were determined. Only one N–terminal amino acid (isoleucine) was detected.

sPRL showed dose–dependent activity in the hypophysectomized mummichog bioassay. sPRL antisera showed specific immunocytochemical staining of pituitary lactotropes; such staining was blocked by sPRL. A homologous RIA was developed and validated for use in measuring sPRL in plasma and tissues of coho salmon.

Transfer of SW–adapted juvenile coho salmon to FW induced a size–dependent response of plasma prolactin with post–smolts showing a pronounced and prolonged increase in plasma prolactin, an associated hypercalcaemia and little disturbance of plasma sodium. Smaller smolts, upon transfer to FW, showed little change in plasma prolactin but had depressed levels of plasma calcium, and sodium, and decreased osmotic pressure.

There was no clear correspondence between environmental calcium levels and plasma prolactin: transfer to high calcium FW resulted in higher levels of prolactin, a brief hypercalcaemia and decreases in plasma sodium and osmotic pressure; transfer to calcium–free SW caused a brief rapid elevation then
depression of plasma prolactin levels with lowered plasma sodium and osmotic pressure levels.

Salmon prolactin was shown to be hypercalcaemic in coho salmon. Lowering of plasma calcium by
the calcium chelator EGTA caused an elevation of plasma prolactin; injections of calcium caused a depres-
sion of plasma prolactin. Injection of sPRL elevated both plasma calcium and sodium.
I would like to thank my supervisor, Dr. Brian McKeown, for his advice, assistance and encouragement during the course of my Ph.D. research. I could not have asked for a better supervisor. I would also like to thank my Examining Committee members, Dr. Nancy Sherwood, Dr. Ed Donaldson, and Dr. Howard Bern for their kind comments and constructive criticism of my thesis.

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I would like to dedicate this thesis to my parents, Ron and Pat Fargher, for making it all worthwhile.
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GENERAL INTRODUCTION

The pituitary gland of vertebrates is the site of production and release of at least six, and possibly more, hormones. The pituitary gland is also the site of release into the circulation of some hormones produced in the hypothalamus. Prolactin (PRL) is a protein hormone that is synthesized in, and released from, the adenohypophysis of the pituitary glands of vertebrates (Clarke and Bern, 1980). Prolactin may also be present in invertebrates as prolactinergic neurons have been detected in the cerebral ganglion of an ascidian (Pestinaro, 1983).

Biological, chemical and immunocytochemical studies on the PRLs and growth hormones (GH) of vertebrates from chondrichthyans to mammals strongly suggest that PRL and GH evolved from a common ancestral molecule (Farmer and Papkoff, 1979, Kawauchi et al., 1986). This family grouping also includes human placental somatomammotropin (Gorbman et al., 1983) and proliferin (Linzer and Nathans, 1985). It is thought that there has been more structural evolution of PRL than GH and that GH more nearly represents the ancestral molecule (Farmer and Papkoff, 1979; Nicoll et al., 1987). In chum salmon (*Oncorhynchus keta*), PRL and GH show 26% identity but no common conserved core region (Kawauchi et al., 1986); they are distinct hormones in teleosts.

This family grouping is also shown in the overlapping functions of PRL and GH in some species. For example, in amphibians, PRL seems to be the larval (tadpole) growth hormone while post-metamorphic growth is dependent upon GH (Gorbman et al., 1983).

Unlike the well defined functions of other hormones (e.g. insulin, progesterone, or thyroid stimulating hormone), the role of PRL is extremely varied. Nicoll (1974) stated that there were 85 clearly-established functions known for PRL in vertebrates; he classified these functions into five groups: (1) actions related to reproduction, (2) somatotropic effects, (3) osmoregulatory effects, (4) actions on the integument and its derivatives, and (5) steroid hormone synergism. The relative importance of each of these functional groups varies from one vertebrate class to another but PRL's most primitive function is
probably in osmoregulation (see Hirano, 1986), as this hormone is required by some teleosts for survival in a fresh water (FW) environment. This function can be briefly summarized as preventing the loss of salts to the hypotonic environment, by increasing sodium absorption from urine stored in the bladder and by reducing the branchial sodium efflux. It also has a role in preventing dilution of body fluids by the osmotic uptake of water in that it reduces branchial water permeability (Ogasawara and Hirano, 1984) and increases urinary water elimination (Clarke and Bern, 1980). This role, however, is not universal in teleosts. Some species, such as the catadromous eel (*Anguilla*) and the stenohaline FW goldfish (*Carassius*), can regulate blood electrolytes independently of PRL (Clarke and Bern, 1980). Others, such as the mummichog (*Fundulus*) have an absolute requirement for PRL in FW (Pickford and Phillips, 1959).

Homeostatic control of blood calcium is essential for vertebrate life and evolution has provided endocrine control over blood calcium. There seem to be two different methods of controlling blood calcium, one characteristic of terrestrial vertebrates and a second characteristic of bony fishes.

In terrestrial vertebrates (such as birds and mammals) parathormone, produced by the parathyroid glands, elevates blood calcium, and calcitonin, produced by thyroid C cells in mammals and ultimobranchial glands in other vertebrates, lowers blood calcium. These organisms obtain calcium from their diet, by a mechanism involving vitamin D and use bone tissue as a calcium reservoir. Knowledge of calcium homeostasis in bony fish is less perfectly known but has great differences from that of terrestrial vertebrates. Parathyroid glands and parathormone are absent from fish, first appearing, in evolution, in amphibians. The other two terrestrial vertebrate endocrine controls, calcitonin and vitamin D, are present in fish, often in large quantities, but do not appear to be involved in calcium homeostasis (Björnsson *et al.*, 1987, Feinblatt, 1982, Taylor, 1985). These teleost hormones are capable of exerting effects in mammals: salmon calcitonin is used in treatment of Paget's disease and cod liver oil has been used as a dietary supplement of vitamin D for prophylaxis of rickets, both being pathologies of calcium regulation.

Fish obtain calcium from their FW environment via branchial mechanisms and environmental calcium seems to be their calcium reservoir as fish bone is mostly acellular (Dacke, 1979). Two hormones
have been suggested to be involved in calcium homeostasis in teleosts; teleocalcin (or hypocalcin), from the corpuscles of Stannius and prolactin, from the pituitary gland. Teleocalcin acts to inhibit gill calcium uptake (Wagner et al., 1986), leading to hypocalcaemia. Prolactin had been thought to be a hypercalcaemic hormone in teleosts since mammalian prolactin can correct the hypocalcaemia produced by hypophysectomy (Pang, 1981).

The functions of prolactin in teleosts have been derived from studies employing mammalian prolactin, i.e., a heterologous prolactin, from a source evolutionarily distant from teleosts. This has led to criticisms (e.g. Hirano, 1986, Nicoll, 1974) of the validity of conclusions drawn from heterologous studies. Thus the objectives of this thesis were to purify prolactin from chum salmon for studies on functions of homologous prolactin in salmon, to develop a homologous radioimmunoassay for the measurement of prolactin in Pacific salmon, to quantify responses of prolactin in salmon upon transfer to fresh water, to determine if environmental calcium affects the plasma prolactin of salmon, and to establish if teleost prolactin has a hypercalcaemic function.
CHAPTER I
ISOLATION AND CHARACTERIZATION OF CHUM SALMON PROLACTIN AND
DEVELOPMENT OF THE RADIOIMMUNOASSAY

Introduction

Anadromous teleosts such as salmon (*Oncorhynchus*), whose PRL physiology is imperfectly known, are extremely interesting as they encounter two salinity challenges in their life cycle; firstly as smolts when they migrate downstream from their FW nursery areas to the ocean where they spend the majority of their life and secondly, on their spawning migration back into FW. The first, and until recently the only, report on prolactin in salmon is that of McKeown and van Overbeeke (1972) who measured PRL in serum and pituitary glands of sockeye salmon (*Oncorhynchus nerka*) prior to, and during, their spawning migration. Using a heterologous radioimmunoassay (RIA), they found that entry into FW resulted in a drastic reduction in prolactin levels in both serum and pituitary glands. As the salmon migrated to the spawning grounds, both levels increased. Due to the absence of purified salmon PRL at that time, McKeown and van Overbeeke (1972) were unable to quantify absolute levels of PRL; instead deviations from a reference standard were measured.

However, this study and others (Nicoll, 1975, Nicoll *et al.*, 1977) employing a heterologous RIA have had their validity questioned (Nicoll, 1974). The overwhelming majority of studies on the physiology of prolactin in teleosts have used heterologous PRL (generally ovine or bovine) or fish pituitary homogenates, as homologous PRL had not been available. Hirano (1986) criticized this early work as many investigators used large doses of heterologous PRL with the concomitant problem that a minor contaminant from another pituitary hormone in the PRL preparation may be delivered in substantial quantities. Also these studies may be obfuscated by the difficulty in establishing a physiological dose for a mammalian hormone in a species phylogenetically distant; in a specific bioassay for salmon PRL, ovine PRL showed only 1% of the activity of salmon PRL (Grau *et al.*, 1984; Hirano, 1986). Similar results have
been reported for tilapia (*Oreochromis mossambicus*) PRL (Farmer *et al*., 1977) which was 160 times more active than ovine PRL. Another problem results from the fact that some studies have shown that the response to teleost PRL is parabolic in some species, i.e., some lower doses are more effective than some higher doses (Grau *et al*.; 1984, Hasegawa *et al*., 1986). While these earlier studies were invaluable, particularly in that they established the existence of PRL in teleosts, the fact that non-homologous PRL was used means that the results must be viewed with caution, i.e., some of the effects attributed to PRL may be pharmacological.

Prolactin has been recently purified from a number of teleosts. The first reported purification of salmon prolactin was that of Idler *et al*. (1978) whose preparation showed some biological activity although not in a dose-dependent fashion. Due to our inability, and that of a number of other workers, to reproduce Idler *et al*.'s (1977) purification and due to some anomalous characteristics of the PRL of Idler *et al*. (1977), it is generally accepted that they did not succeed in isolating salmon PRL (see Prunet and Houdebine, 1984). Prolactin has been successfully isolated from tilapia (*Oreochromis mossambicus*, Farmer *et al*., 1977), carp (*Cyprinus carpio*, Yasuda *et al*., 1987), chum salmon (*Oncorhynchus keta*, Kawauchi *et al*., 1983, Yasuda *et al*., 1986) and chinook salmon (*Oncorhynchus tshawytscha*, Prunet and Houdebine, 1984). Due to their simplicity, sensitivity, and specificity; RIA's are the usual technique of choice for the measurement of hormones in physiological investigations and RIA's for the above PRL's have also been developed recently (Nicoll *et al*., 1981; Hirano *et al*., 1985; Prunet *et al*., 1985). A RIA for the PRL of Idler *et al*. (1977) has never been reported.

It should be kept in mind that a RIA measures only the immunological, not the biological, activity of an antigen in a sample. Biological activity can be measured in a bioassay and receptor binding can be measured in a radioreceptor assay (RRA). Bioassays are usually tedious, require the use of large numbers of animals and are generally insensitive to the levels of hormone in plasma samples; they are best used in following the course of hormone purification and in establishing the biological activity of a purified hormone. RRA's are analogous to RIA's, substituting a purified hormone receptor for the specific antibody. While PRL receptors have not yet been isolated from teleosts, Edery *et al*. (1984) showed that iodinated
ovine PRL did bind to membrane fractions of tissues of tilapia and such binding was displacable by unlabelled PRL (either tilapia or ovine). A heterologous RRA (for ovine PRL) has been used in studies on fish PRL (Prunet et al., 1977, 1979; Prunet and Houdebine, 1984); chinook salmon PRL in this assay showed non-identity with the ovine PRL standard. A homologous RRA for teleost GH has been reported (Fryer, 1979).

At the commencement of this study, purified salmon PRL was not readily available for physiological studies as Idler was not distributing his material and no homologous salmon RIA was available. Thus the objective of this part of the study was to purify PRL from chum salmon for physiological experiments on salmon and to develop a homologous RIA for the measurement of PRL in samples obtained from such experiments.
Materials and Methods

Purification

Pituitary glands were collected from *Oncorhynchus keta* at BC Packers, Steveston B.C. These were ocean fish, caught by commercial fishermen in local tidal waters prior to entering FW on their spawning migration. The fish were stored on ice prior to arrival at BC Packers for up to three days. Heads from these fish were obtained while they were being processed for the fresh fish market. The top of the cranium was sawn off with a bandsaw and the brain was reflected rostrally, exposing the pituitary gland in the *sella turcica*. Due to the length of time post mortem before dissection, many of the pituitary glands obtained from these fish had undergone considerable decomposition. Immediately upon removal from the fish, the glands were frozen on dry ice and stored at -20 C.

The protocol for the purification of chum salmon PRL is modified from that of Kawauchi *et al.* (1983). Fifty g of pituitary glands were homogenized for one minute, on ice, with a Brinkman Polytron in acid-acetone (150 ml 11.6N HCl:acetone, 1:28, v/v). Following homogenization, the pituitary glands were left on ice for one hour, after which they were centrifuged at 14,000 g for 30 min at 4 C. The pellet was re-extracted with 100 ml 80% acetone, on ice, for one hour. The second extraction was centrifuged at 14,000 g for 30 min. and the supernatants from the two extractions combined, and added to 3 l of acetone at 4 C for 30 min. The mixture was centrifuged at 14,000 g for 30 minutes at 4 C and the supernatant discarded. The precipitate was dissolved in a minimal volume (about 25 ml) of 0.1N acetic acid and applied to a 5 x 60 cm Sephadex G-25 column. The column was developed with 0.1N acetic acid and the void volume was collected and lyophilized. The lyophilized protein was dissolved in double distilled water (100 ml, pH 3). The pH of the solution was raised to 4.4 with 0.1N NaOH. The mixture was then centrifuged for 30 min. at 4 C; the supernatant was collected and lyophilized. This lyophilate was dissolved in 10 ml 25 mM sodium phosphate buffer, pH 6.4 (Buffer A; all phosphate buffers were made by titrating monobasic sodium phosphate with dibasic sodium phosphate to desired pH) and applied to a high pressure liquid chromatography (HPLC) cation exchange column (Waters SP5W Protein-Pak), previously equilibrated to
Buffer A. A linear gradient of Buffer A to Buffer B (25 mM sodium phosphate buffer, pH 6.4, 250 mM sodium acetate) was run at 1 ml/min. for 30 min., using a Waters Model 440 HPLC. The optical density of the effluent was monitored at 280 nm. The major peak was lyophilized and dissolved in 50 mM ammonium acetate, pH 6.8. This was then chromatographed on a 2 x 45 cm Sephadex G–100 column using 50mM ammonium acetate at pH 6.4 to develop the column. The retained material (salmon prolactin) eluted as a single peak and was lyophilized.

Electrophoresis

The purity of the isolated prolactin was checked by electrophoresis. Three different methods were employed: acid–urea polyacrylamide gel electrophoresis (PAGE) (Panyim and Chalkley, 1969), discontinuous sodium dodecyl sulphate (SDS) (Laemmli, 1970) and two–dimensional (2–D) PAGE modified from O'Farrell et al. (1977).

For the 2–D gels, the first dimension gels were the non–equilibrium pH gradient electrophoresis system (NEPHGE) and were run in 1.5 mm. i.d. glass tubes. These tubes were first cleaned in chromic acid, soaked in a dilute solution of Photoflo (Kodak), then dried in air. The gel was composed of 6.6% acrylamide containing 6.67% Ampholyte 5/7 and 1.67% Ampholyte 3/10 (LKB). The gels were polymerized with ammonium persulphate and water was overlayed. The protein to be analysed in this system was weighed on a microbalance and dissolved in 15% sucrose, 6.67% Ampholyte 5/7, 1.67% Ampholyte 3/10, to a concentration of 1 μg/μl. The gels were loaded into a water–jacketed tube gel apparatus connected to a chilling unit. The lower (cathode) chamber contained 10 mM ethylene diamine and the upper (anode) chamber contained 10 mM phosphoric acid. The top of the gels was rinsed with anolyte and the sample was applied with a Hamilton syringe. Unlike isoelectric focussing gels, NEPHAGE gels are not pre–run to establish the pH gradient so that immediately following sample application, power was applied.

Electrophoresis continued for two hours at 750 V constant voltage (1500 V–hr). During electrophoresis, the catholyte was constantly stirred with a magnetic stir bar and cooled to 2°C. After electrophoresis, the gels were removed from the tubes by injecting water between the gel and the glass wall of the tube; the
gels were then forced from the tubes by air pressure. The cathode end of the gel was marked by the inser-
tion of a small piece of wire into the gel.

The NEPHAGE gels were placed into screwcap tubes and incubated for 30 min. at 37 C on a shak-
ing water bath. The incubation buffer was 62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5%
2-mercaptoethanol. Following incubation, the gel was loaded on top of the second dimension gel (SDS
PAGE) by filling the top of the SDS gel with molten 1% agarose (BDH) containing 0.125 M Tris–HCl, pH
6.8, 0.1% SDS, 1.25 x 10^{-5}% bromophenol blue. After the agarose had solidified, the gel was run at 90V
until the bromophenol blue tracking dye ran off the end of the gel. Following electrophoresis, all gels
were stained either tinctorially using the standard Coomassie stain (Pharmacia) or by immunological stain-
ing methods.

The immunological staining was done by Western blotting (Renart and Sandoval, 1984). The pro-
cedure followed, in short, was to incubate the gel in 3 half-hour washes of 50 mM sodium phosphate buf-
fer, pH 6.5, 0.1% SDS, to remove glycine from the gel which would interfere with the transfer procedure.
Following incubation, the gel was laid against freshly prepared diazothiophenol (DTP) paper, sandwiched
between foam pads to form a cassette, and mounted in a Bio-Rad Trans-blot cell. The cassette was orien-
ted such that the gel faced the cathode side of the cell and the DTP paper faced the anode side of the cell.
The transfer buffer was 50 mM sodium phosphate buffer, pH 6.5. The cell was packed in ice and con-
nected to a Bio-Rad Destaining power supply (25V) for three hours. Following transfer, the paper replica
of the gel was incubated at room temperature for 15 min. in 0.25% gelatin, 10% ethanolamine, 100 mM
Tris–HCl, pH 9. This served to block or destroy any remaining diazo groups. The replica was washed in
water and placed in a heat-sealable bag (Sears Seal and Save). Ten ml 1:40,000 antiserum against salmon
prolactin in 0.25% gelatin, 50 mM Tris–HCl pH 7.5, 5.0 mM ethylene diamine tetraacetic acid (EDTA), 150
mM NaCl, 0.05% Nonidet P40 (Sigma) (Buffer 1) were added to the bag. The bag was sealed and left to
incubate overnight at 37 C on a shaking water bath. Following the specific immunological reaction, the
paper replica was washed for 2 hours in Buffer 1 at 37 C in the shaking water bath.
Protein A (Sigma), a cell wall protein from *Staphylococcus aureus* which binds specifically to immunoglobulins (Langone, 1982), was radio-iodinated by the lactoperoxidase technique (see below). Iodinated Protein A (10–20 x 10⁶ cpm) in 10 ml Buffer 1 was added to the paper replica, following the 2 hour wash, in a sealable bag. This was incubated for 30 min. at 37 °C in the shaking water bath. The final wash, after the radio-labelling, was for 2 hours in Buffer 2 (0.25% gelatin, 50 mM Tris–HCl pH 7.5, 5.0 mM EDTA, 1.0 M NaCl, 0.4% sodium n-lauroysarcosine (Sigma)). The replica was dried in air and mounted for autoradiography. Autoradiography of the paper replica was done using Kodak XAR-5 film and fluorescent screen (Dupont Cronex Lightening Plus FC) at −80 °C. Processing of the film was done by standard developing procedures.

*Amino Acid Analysis and Sequence*

Samples of the purified prolactin were submitted to the Tripartite Microsequencing Centre, University of Victoria, for amino acid residue analysis and sequencing. The amino acid analysis was done on a Beckman Amino Acid Analyser Model 119CL using the single column, 90 minute protocol suggested by Beckman. Hydrolyses were performed *in vacuo* in 6N HCl at 110 °C for 18 hours. The values for the amino acid residue content were converted to numbers of residues based on a total of 187 residues. Sequencing was done on an Applied Biosystems 470A gas phase protein sequencer using the standard program supplied by the manufacturer.

*Generation of Antiserum*

Three young New Zealand White rabbits were immunized against the purified prolactin by the method of Vaitukaitus *et al.* (1971). Prolactin was dissolved in 150 mM NaCl and this solution was mixed with an equal volume of complete Freund's adjuvant (Difco). The dosage was adjusted so that each animal received 50 µg prolactin in 2 ml. Prior to injection, the back and hindquarters of each animal were thoroughly wetted with soapy water so that the skin could be easily seen for injection. This obviated the need to shave the animals for the injection. The animals were injected with the immunogen in multiple intradermal sites on the back and hindquarters. The animals were re-injected with the immunogen every
two months and blood was taken one week following the second, third and fourth injections.

Blood was taken from the central ear artery in restrained animals. Prior to the bleeding, the ear was shaved with clippers and swabbed with xylene to induce vasodilation. A needle (23-gauge) was inserted into the artery and blood was collected either by allowing the free-flowing blood to drip into a hand-held test tube or withdrawn by syringe. Routinely, 10 to 20 ml of blood were collected. The blood was transferred to centrifuge tubes and allowed to clot overnight at 4 C. The clot was loosened from the side of the tube with a wooden applicator stick and then centrifuged. The serum was removed with a Pasteur pipette, divided into 100 µl aliquots and stored frozen at -20 C.

Estimation of the titre of the antiserum was performed by binding studies whereby the binding of radio-iodinated salmon prolactin (see below) to various dilutions of the antiserum was measured. The antiserum was diluted, in RIA buffer (see below), from 1:100 to 1:100,000 and 100 µl aliquots of these dilutions were added to 100 µl (10,000–15,000 cpm) of the labelled prolactin. These tubes were incubated overnight at 4 C following which 100 µl each of 2% non-immune rabbit serum (NRS) and goat anti-rabbit gamma globulin (GARGG, Calbiochem) were added to each tube. The NRS and GARGG had been previously titrated against each other such that the added quantities resulted in maximal precipitation of rabbit immunoglobulin. After the addition of the NRS and the GARGG, the tubes were counted with a Beckman Gamma 4000 solid scintillation spectrophotometer to determine the total counts added to each tube and then left overnight at room temperature. The separation of free label from the antibody bound label was accomplished by adding 3 ml cold RIA buffer to each tube followed by centrifugation for 30 min. at 3000g. The supernatant, containing the unreacted PRL was decanted and the antibody-bound PRL in the pellet was counted. The amount bound was expressed as a percentage of the counts added initially.

**Immunocytochemistry**

The specificity of the antiserum was examined by immunocytochemical means. Pituitary glands from sockeye salmon, *Oncorhynchus nerka*, European eels, *Anguilla anguilla* and mummichogs, *Fundulus heteroclitus*, were fixed in Hollande–Bouin’s solution, a general fixative (Humason, 1972). After
dehydration in increasing concentrations of ethanol and embedding in paraffin, 5 μm sections were mounted on glass slides. Sectioning was done in the sagittal plane. Paraffin was removed with xylene and the sections were hydrated in decreasing concentrations of ethanol. Prolactin cells were localized by using the peroxidase–antiperoxidase staining technique (Sternberger, 1974). The hydrated slides were equilibrated with Tris–buffered saline (TBS; 5 mM Tris-HCl pH 7.5, 0.15 M NaCl). The sections were incubated overnight in various dilutions of the rabbit antiserum to salmon prolactin (1:500 – 1:1000 in TBS). The sections were washed 3 times for 10 min. each in TBS. A solution of 1:10 GARGG in TBS was applied for ten minutes and the slides were washed again 3 times for 10 min. each in TBS. This was followed by incubating the slides in a 1:50 dilution of horseradish peroxidase–rabbit antiperoxidase complex (Miles–Yeda) in TBS, again followed by 3 washes for 10 min. each in TBS. The specific immunological binding was made visible by staining the slides in 0.01% hydrogen peroxide containing 0.025% 3,3-diaminobenzidine (Sigma). The process of staining was monitored and when sufficient intensity of staining was obtained (generally within 10 – 15 min.), the slides were transferred to distilled water, dehydrated and mounted.

Control slides were treated identically as above, except that either the specific rabbit anti–prolactin was replaced by NRS or the specific antiserum was presaturated by prior incubation with pure chum salmon prolactin or chum salmon growth hormone.

**Bioassay**

The purified salmon prolactin was tested for biological activity in the hypophysectomized mummichog, *Fundulus heteroclitus*, bioassay (Grau et al., 1984). Mummichogs were collected from the wild in New Brunswick and shipped to Vancouver by air freight. Upon arrival, they were maintained in seawater tanks at room temperature, and fed either frozen brine shrimp or flakes of tropical fish food, for at least one week prior to experimentation.

The animals were anaesthetized in 0.4% tricaine methane sulphonate (Syndel Labs) and hypophysectomized by the opercular approach wherein the anaesthetized fish was placed ventral side up
and fine forceps were inserted between the operculum and the last gill arch. The gill arch and the operculum were gently spread apart exposing the roof of the mouth. A fine scalpel was used to make an incision along the ventral ridge of the parasphenoid bone. The skin and underlying musculature were teased away with a dissecting needle. The pituitary gland was clearly visible, through the parasphenoid bone, immediately caudal to the optic chiasma. A small hole was drilled with a dentist's drill through the parasphenoid bone directly over the pituitary gland. The gland was removed by gentle suction with a Pasteur pipette connected to a water aspirator and completeness of removal was checked visually. The fish were placed back in their seawater holding tanks for recovery for at least one week prior to their use in the bioassay. Post-operative survival was about 95% and operated fish fed the day following surgery; only seemingly healthy post-operative fish were used in the bioassay.

Thirty-five μg of prolactin was dissolved in 0.75 ml of ice cold 0.1N acetic acid. After dissolution, 8 ml of 150 mM NaCl were added so that the concentration was 40 ng prolactin/10 μl. Further dilution with 150 mM NaCl made concentrations of 20, 10, 4 and 0.25 ng/10 μl.

Each fish was individually weighed and injected intraperitoneally with either one of the above solutions (experimental fish) or the 150 mM NaCl vehicle (control fish) using a 100 μl Hamilton syringe with a 27-gauge needle. Five fish were injected in each treatment group. The volumes injected were adjusted so that each fish received an injection of 10 μl/gram of body weight. Fish were not anaesthetized for the injection; they were restrained in a fish net and injections given through the net.

Following the injections, the seawater-adapted mummichogs were transferred to plastic shoebox cages containing 9 l of fresh water. Preliminary trials had shown that it was essential that the fresh water be adjusted to 0.5 °/oo NaCl, 5 mM CaCl₂ to ensure the survival of the hypophysectomized fish for the duration of the experiment. The cages were aerated and covered. Thirty-one hr following the injection, the fish were netted and blotted briefly on paper towels. A blood sample was taken from the severed caudal peduncle with an ammonium–heparinized haematocrit tube. The fish were killed by decapitation. The haematocrit tube was sealed and centrifuged. After removal of the cellular fraction, the plasma was stored
frozen at -20 °C until analyzed.

For analysis, the thawed plasma was diluted 1:1000 with double distilled water and the sodium concentration in the plasma measured by flame emission spectrophotometry on a Pye Unicam SP 191 Atomic Absorption Spectrophotometer at 589.6 nm, calibrated to read out directly in mEq/l. The results were statistically analysed by analysis of variance and t-tests (BMDP PlV, 1983).

Development of the Radioimmunoassay

Labelled prolactin for the radioimmunoassay was iodinated by the lactoperoxidase technique using ¹²⁵I (Bolton, 1977). Cow's milk lactoperoxidase (Boehringer Mannheim) was supplied as a 5 mg/ml suspension in 3.2 M ammonium sulphate, 0.1 M potassium phosphate buffer, pH ca. 7. Prior to use, this suspension was mixed and 10 µl were removed and added to 40 µl of double distilled water, resulting in a final concentration of 10 µg per 10 µl.

Ten µg of purified chum salmon prolactin in 50 mM sodium phosphate buffer pH 7.6 (1 µg/µl) were added to 50 µl of 500 mM sodium phosphate buffer pH 7.6 in a 6x50 glass tube. To this was added 10 µl of the lactoperoxidase preparation (10 µg), 1 mCi Na¹²⁵I (15 mCi/µg I, 100 mCi/ml, Amersham) and 10 µl of 0.005% hydrogen peroxide. The tube was capped with parafilm and mixed for one min. To stop the reaction, an excess of RIA buffer (10 mM sodium phosphate pH 7.6, 150 mM NaCl, 0.5% BSA, 2.5 mM EDTA, 15 mM sodium azide, 0.1% Triton X-100) was added. Azide is an inhibitor of lactoperoxidase.

The reaction mixture was transferred in a Pasteur pipette to a small G-15 desalting column that had been previously saturated with BSA (500 µl 30% BSA) and regenerated with RIA buffer. After the mixture had drained onto the column, several washes of RIA buffer were applied, and the column was developed with RIA buffer. Thirty tubes of 20-drop fractions each were collected using a Gilson fraction collector. Five µl aliquots of each tube were counted and the peak tubes from the protein peak combined. The label was divided in aliquots containing approximately 2 X 10⁷ CPM. The label was stored at -80 °C.
For use in the assay, the necessary number of aliquots of the label were thawed and re-purified on a 1 x 60 cm Sephadex G-100 column. The column was developed with RIA buffer and 80 tubes of 25 drops each were collected using the Gilson fraction collector. Ten μl from each fraction were counted and the labelled hormone peak identified by the talc–resin–TCA test (Tower et. al., 1980). In this test, which distinguishes aggregated labelled hormone and free 125I from usable labelled hormone, three 100 μl aliquots from each peak seen on the repurification profile were incubated with either 100 mg talc (Fisher), 150 mg resin (anion exchange resin, Dowex 2, Sigma), or 1 ml of 10% trichloroacetic acid (TCA). The results are expressed as the percentage of radioactivity initially added to each tube that binds to the talc or resin or is precipitated by TCA. The peak tube of the hormone peak and sufficient tubes on the downslope of the peak were combined and diluted with RIA buffer to 10,000 – 15,000 cpm/100 μl.

The protocol of the radioimmunoassay was as follows. On the first day, the standards and samples were prepared and added to the antibody. The standards were prepared by thawing a tube of 1 μg/ml of purified unlabelled hormone in RIA buffer; this reference was diluted with RIA buffer to 100, 50, 20, 10, 5, 2, 1, and 0.5 ng/ml. The zero standard was RIA buffer with no added prolactin. As 100 μl of each standard were used per tube, the masses of prolactin at each standard were: 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng. Not all standards were used in every assay, the range was adjusted to suit the desired sensitivity of the particular assay. All standards were done in triplicate. The non-specific binding (NSB) of the labelled hormone was assessed in each assay by the inclusion of tubes in which specific antiserum was omitted and substituted by RIA buffer (standard NSB). Each of the types of samples, either plasma or pituitary, also had its own blank (sample NSB).

After the preparation of the standards, the samples were prepared. The samples, either plasma or pituitary, were pipetted into numbered 12x75 disposable glass tubes (as were the standards) and sufficient RIA buffer added so that the final volume of each sample was 100 μl. All tubes, except the various NSBs, then had 100 μl of the specific antiserum (1:80,000 – 1:140,000 initial dilution in RIA buffer) added so that the volume at this point was 200 μl in every tube. The tubes were then covered with aluminum foil and incubated overnight at 4 C. Each sample was assayed at least in duplicate.
The following day, the labelled hormone was re-purified as above and 100 μl containing 10,000 – 15,000 cpm were added to every tube. Tubes were covered with foil and left to incubate for 2 days at 4 C. Following this step, each tube had 100 μl of 2% NRS and 100 μl of GARGG added. The NRS and GARGG had been previously titrated to provide maximal precipitation at the concentrations used. The assay tubes were left at room temperature overnight. Generally, at this time the tubes were counted to determine the total counts added to each tube. The tubes were placed on ice and 3 ml ice-cold RIA buffer were added to every tube. The tubes were centrifuged at 3000g for 30 min., the supernatant (containing the unbound labelled hormone) was decanted and tubes left to drain, briefly, upside down on absorbant paper. Prior to counting, the small amount of supernatant that collected on the rim of the inverted tube was swabbed away with Q-Tips. The tubes were counted for 10 minutes or 10,000 counts each.

In all cases the plasma blank, and in a few cases the pituitary blank, showed lower NSB levels than did the standard blank. Thus the standard curve was corrected for the lower NSB levels of the samples when appropriate.

Data were analysed using the SIGMOID program of Rodbard et al. (1980). This program uses weighted regression to a four-parameter logistic model. The model is based on the mass-action law and provides the theoretical best fit to the standard curve as well as providing potency estimates for each of the samples.

To determine the within- and between-assay variation, a control plasma was used from a large sample of plasma obtained from an adult coho salmon that had returned to Capilano Hatchery to spawn. Ten ml of plasma from this fish was divided into 300 μl aliquots and stored frozen at -20 C. The values measured from this pool in six separate assays were used to determine the within- and between-assay variation. This was calculated using the QUALITY program of Munson and Rodbard (1977). This program uses analysis of variation methods to calculate the variation within assays; the variation not accounted for by the within-assay variation is considered to be the variation between assays.
Parallelism of my purified hormone compared to plasma, pituitary rostral pars distalis (RPD) extract and pure hormone donated by Dr. Kawauchi was determined in the RIA. Varying volumes of coho plasma (10, 20, 50, 100, and 200 μl) and of chum salmon RPD extract (10, 20, 50, 100 and 200 μl from a preparation of 135.4 mg chum salmon RPDs homogenized in RIA buffer and diluted to an equivalent of 400 litres; i.e., 336 ng RPD/ml) were assayed. Kawauchi's material was prepared identically to the standards. The resulting curves for the standards, the plasma, the RPD extract and Kawauchi's material were linearized by the log-logit transformation (Rodbard, 1974) and tested for parallelism using analysis of covariance (BMDP P1V, 1983).

The only hormone that would be expected to cross-react in the assay is GH. Prolactin and GH are related hormones and are similar in some respects; they are thought to have evolved through gene duplication (Farmer and Papkoff, 1974). Thus the susceptibility of the assay to interference from GH was measured. Pure chum salmon GH (Wagner et al., 1985) at masses of 10, 20, 50, 100, 200, 500, and 1000 ng were measured in the assay. The curves for prolactin and GH were again linearized by the log-logit transformation and parallelism checked by analysis of covariance.

The extent of interference in the assay by plasma constituents was determined by adding 0, 0.5, 1.0 and 2.0 ng of purified hormone (in 50 μl volumes) to 50 μl of plasma and determining the recovery of the added hormone.

The sensitivity of the assay to changes in sample volume was measured. The normal assay volume is 300 μl: 100 μl each of sample or standard, labelled hormone and antiserum. In this experiment, the standard was diluted in the antiserum instead of RIA buffer. Thus the volume was 200 μl: 100 μl each of labelled hormone and the standard-antiserum mixture. Five standard curves were done with 0, 20, 50, 100 or 200 μl of RIA buffer added to each tube.
Results

**Purification and Characterization**

By the method described, PRL from chum salmon was purified to homogeneity. The final yield was approximately 10 mg hormone from 50 g of pituitary glands.

The purity of the hormone was examined by electrophoresis. Figure 1a shows the behaviour of the hormone in acid-urea PAGE. Two μg of sample was applied to a 10.77% gel, pH 2.7. As can be seen, it ran as a single band in this system. Figure 1b shows the single band pattern obtained in a discontinuous 12% SDS gel when a two μg sample was run. Both Fig. 1a and b show gels stained by Coomassie stain, and Fig. 1c is an autoradiograph of an immunologically stained paper replica (Western blot) of 0.4 μg of purified hormone run in an SDS gel. Fig. 2 is a photograph of a 2-D gel (NEPHGE in the first dimension, SDS–PAGE in the second dimension) where 42 μg of purified material was run in the first dimension. Only one spot was detected.

A comparison of my preparation and purified hormone donated by Dr. Kawauchi can be seen in Fig. 3. In this western blot, my hormone and both preparations from Kawauchi co–migrated and ran as single bands in all the various sample concentrations. Fig. 4 shows my purified PRL in western blot when run with 2 dilutions of a RPD extract. The main band in the RPD extract co–migrated with my purified material. The more rapidly migrating band obviously shares an antigenic site with PRL; this band ran at the dye front, in the SDS gel. In urea–SDS PAGE, this pituitary band ran as two bands of molecular weight 8400 and 5700. However, this material is not detectable in plasma. Upon G–25 gel filtration of coho plasma, all immunoreactivity appeared in the void volume; none occurred at lower molecular weights.

The molecular weight of the hormone was estimated to be 25 kilodaltons by SDS PAGE. Radiolabelled PRL elutes from a G–100 column as a single peak with a Kav of 0.44, characteristic of a protein of this size (Fig. 5).
Figure 1: Electrophoretograph of chum salmon prolactin

In each case, "+" indicates the anode and "−" indicates the cathode. In all case, the direction of migration is downward.

a: Two $\mu$g of sPRL in a 10.77% pH 2.7 acid-urea PAGE gel, stained with Coomassie stain.
b: Two $\mu$g of sPRL in a 12% discontinuous SDS PAGE gel. Marker proteins were run in the lane next to sPRL: Phosphorylase b (94 kDa.), Albumin (67 kDa.), Ovalbumin (43 kDa.), Carbonic anhydrase (30 kDa.), Trypsin inhibitor (20.1 kDa.), a-Lactalbumin (14.4 kDa.). Stained with Coomassie stain.
c: Western blot of 0.4 $\mu$g sPRL run in a 12% discontinuous SDS PAGE gel (20 min exposure).
Figure 2: Electrophoretograph of chum salmon prolactin in a 2-D system.

42 µg of sPRL were applied to the anode of the NEPHAGE pH 3-10 tube gel in the first dimension. The second dimension was a 12% discontinuous SDS gel. "+" indicates the anode and "-" indicates the cathode of each dimension; the arrows indicate the direction of migration in each dimension.
Figure 3: Western blot of my chum salmon prolactin and two isohormones of chum salmon prolactin from Kawauchi.

From the left, the first four lanes are Kawauchi’s first isohormone, the second four are my chum salmon prolactin and the last four are Kawauchi’s second isohormone. The masses for each group are: 50, 25, 12.5 and 6.25 ng protein. Autoradiographic exposure was five hours. "+" and "-" indicate, respectively, anodic and cathodic ends; arrow indicates direction of migration in a 12% SDS gel.

Figure 4: Western blot of chum salmon prolactin and chum salmon RPD extract.

The left and right lanes are two dilutions of an extract of RPD lobes from spawning chum salmon (40 and 20 μl of 67 μg/ml RPD lobes). By densitometry, these samples contained 14 and 5 ng (respectively) of PRL. Between are serial dilutions of my sPRL (50, 25, 12.5, 6.25 and 3 ng). Autoradiographic exposure was five hours. "+" and "-" indicate, respectively, anodic and cathodic ends; arrow indicates direction of migration in a 12% SDS gel.
Figure 5: Profile of $^{125}$I–PRL on a G–100 column.

The peak fraction from the immunoreactive peak (Fig. 20) of $^{125}$I–sPRL after gel filtration of a 1x60 cm column of G–100 was re-chromatographed on the same column. sPRL eluted at a $K_v$ of 0.44. The minor peaks at fractions 10 and 50 represent contamination from non-immunoreactive material (see Fig. 20) that eluted in the same fractions in the first gel filtration.
Fraction Number (25 drop fractions)
Amino Acid Analysis and Sequence

Two different samples were analysed for the amino acid composition. The residue estimates were obtained by converting the percentage composition of each amino acid given by the amino acid analyser to numbers of residues based on a composition of 187 residues as given by Kawauchi et al. (1984). The results of this analysis are presented in Table 1 along with the residue results reported by Kawauchi et al. (1984), and by Idler et al. (1978) (expressed as 187 total residues). My hormone is similar to that of Kawauchi et al. (1984). However, the chum salmon 'PRL' of Idler et al. (1978) shows distinct differences (especially Ser, Glx, Pro, Ala, Val, Met, Tyr, and Arg).

The sequence of the first ten N-terminal residues was:


This is identical to the sequence for salmon PRL reported by Yasuda et al. (1986). Only one N-terminal amino acid was detected (See Appendix A for the sequence printout).

Generation of Antiserum

Three animals were immunized with PRL; the animal that generated the highest titre was chosen. Figure 6 shows the results of the binding studies with the serum taken from this animal. The titre of antibodies to salmon PRL increased between the second injection of the immunogen (first bleeding) and the third injection (second bleeding). The titre dropped off between the third and fourth (third bleeding) injections. Thus the antiserum used in the RIA was from the second bleeding from this single rabbit.
Figure 6: Binding of $^{125}$I–sPRL to antisera.
Percent tracer bound reflects the amount of tracer (equal in every tube) that was bound by the various dilutions of antiserum. The three antisera used were the three bleedings of the one rabbit (#75) that showed the best response. The second bleeding had the highest titre.
First Bleeding of #75

Second Bleeding of #75

Third Bleeding of #75

Percent Tracer Bound

Antiserum Dilution

24b
Table I

Amino Acid Residue Analysis

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**Immunocytochemistry**

The immunocytochemical localization of PRL cells in the pituitary gland of sockeye salmon can be seen in Fig. 7. This is a low magnification (25X) photomicrograph of the rostral and caudal pars distalis (CPD) stained with a 1:1000 dilution of specific antiserum. The primary antibody reacts specifically only in the follicular PRL cells of the RPD. Scattered follicles and isolated cells can be seen outside the RPD but the growth hormone cells in the CPD are unstained. Fig. 8 is the same section (200X) showing an isolated follicle of the RPD where staining is cytoplasmic with both the nuclei and follicle lumen unstained; isolated unstained cells are gonadotropes. Figure 9 is a control section (25X) from the same block where the primary, specific, antibody was replaced by a 1:100 dilution of NRS; this completely abolished staining. Another control section is shown in Figs. 10 (25X) and 11 (200X) where the specific antiserum (1:1000) was incubated with salmon PRL prior to staining; again, staining is abolished in the low magnification picture of the pars distalis and in the high magnification picture of PRL follicles. However, staining is not affected by prior incubation of the specific antiserum with pure salmon growth hormone [Figs. 12 (25X) and 13 (200X)].

The antiserum against salmon PRL also stains PRL cells in species other than those from the same genus *Oncorhynchus*. In Figs. 14 (25X) and 15 (200X) cross reaction in PRL cells was observed from *Fundulus heteroclitus* and in Fig. 16 from eels, *Anguilla anguilla*. Non-isospondylyous fish, such as *Fundulus*, do not show the follicular arrangement of PRL cells in their pituitary.

**Bioassay**

One of the main functions of PRL in certain fish is in osmoregulation; specifically, it is thought to be a fresh water survival hormone maintaining plasma sodium concentrations in the physiological range. Hypophysectomized mummichogs die in fresh water due to homeostatic failure. The results of the bioassay of PRL are shown in Fig. 17. PRL was bioactive in hypophysectomized mummichogs in that it prevented the decline of plasma sodium concentrations of these animals upon transfer to fresh water. The plasma sodium concentration in the control animals transferred to fresh water (112.9 ± 4.7 mEq/l, mean ±
Figure 7: Immunocytochemical localization of lactotrops in salmon pituitary gland. Sagittal section of a sockeye salmon pituitary gland immunologically stained with antiserum to chum salmon prolactin. 25X magnification. Both sections in this figure and in Fig.10 were from the same block.

Figure 8: Individual RPD follicle immunocytochemically stained. Higher magnification (200X) of a RPD follicle from the same section as in Fig. 7. Staining is seen to be cytoplasmic with neither the follicle lumen nor the lactotrop nuclei being stained.
Figure 9: Non-specific immunocytochemical staining of salmon pituitary gland. Sagittal section of sockeye salmon pituitary gland RPD and CPD. In this section, anti-salmon prolactin was replaced by NRS which produced no immune staining of lactotrops. 25X magnification.

Figure 10: Immunocytochemical staining of salmon pituitary gland after preincubation of specific antiserum with sPRL. Sagittal section of a sockeye salmon pituitary gland. Prior to specific staining with anti-salmon prolactin, the antiserum was pre-incubated with pure sPRL, resulting in abolition of specific staining. (25X magnification).
Figure 11: Single RPD follicle stained with specific antiserum pre-incubated with sPRL. Same section as Fig. 10 under higher magnification (200X). No cytoplasmic staining of follicular lactotrops is seen after staining is blocked by prior incubation of specific antiserum with sPRL.

Figure 12: Immunocytochemical staining of sockeye salmon pituitary gland after pre-incubation of specific antiserum with growth hormone. Sagittal section of sockeye salmon pituitary gland (25X magnification). Prior to staining, the anti-salmon prolactin was pre-incubated with pure chum salmon growth hormone. Staining of lactotrops was not blocked.
Figure 13: Single RPD follicles stained with specific antiserum pre-incubated with growth hormone.
Higher magnification (200X) of same section as Fig. 12. Pre-incubation of anti-salmon prolactin with pure chum salmon growth hormone did not block cytoplasmic staining of lactotrops.

Figure 14: Cross-reaction of anti-salmon prolactin with lactotrops of *Fundulus.*
Mid-sagittal section of hypothalamus and pituitary gland of *Fundulus.* Anti-salmon prolactin cross-reacted with cells in the RPD only. 25X magnification.
Figure 15: Cytoplasmic staining of *Fundulus* lactotrops.
Higher magnification (200X) of same section of *Fundulus* pituitary gland as Fig. 14. Staining is seen to be cytoplasmic in the RPD lactotrops. Light grey cells above the stained lactotrops are unstained ACTH cells.

Figure 16: Cross-reaction of anti-salmon prolactin with lactotrops of *Anguilla*.
Sagittal section of pituitary gland and hypothalamus of *Anguilla*. Anti-salmon prolactin cross-reacted with the follicular lactotrops in the RPD.
Figure 17: Bioassay of sPRL in hypophysectomized *Fundulus*.

The response of plasma sodium concentrations of SW-adapted hypophysectomized *Fundulus* to administration of sPRL 31 hours after transfer to FW. Both the 20 and 40 ng/gm doses of sPRL resulted in significant (p<0.05) elevation of plasma sodium from controls. Each point represents the mean ± 1 SEM of 5 animals. (Note: The point for the 0.25 ng/gm dose has been offset slightly for clarity.)
1 S.E.M.) was depressed below what would be considered normal in an intact fish (approximately 150 mEq/l). The plasma sodium elevation in the experimental fish responded to PRL in a dose-dependent fashion. None of the low dose groups (0.25 ng/g, 1 ng/gm, 4 ng/g, or 10 ng/g) was significantly different from the controls while both the 20 ng/gm and 40 ng/gm dose groups were significantly different (p<0.05). Also, the 20 and 40 ng/g dose groups were significantly different from each other (p<0.05).

**Development of the Radioimmunoassay**

The results of a typical iodination of 10 µg PRL with 1 mCi $^{125}$I is shown in Fig. 18. Fractions 8 – 12 were combined and stored as frozen aliquots for use as labelled hormone. The percentage of incorporation of iodine into protein was 84%, giving a specific activity of 84 µCi/µg. However, it was not possible to determine what proportion of the iodinated protein was lactoperoxidase. Thus, a blank iodination identical to a normal one, excepting that no PRL was present, was done to get an upper estimate of the incorporation of iodine into lactoperoxidase. This showed a 44.2 percentage incorporation of $^{125}$I into protein. Thus the specific activity of the labelled PRL was likely between 39.8 to 84 µCi/µg.

The repurification of the labelled PRL on a G–100 column is shown in Fig. 19. The column profile shows three peaks: the first peak elutes at a Kav of 0.15 and is non-immunoreactive to anti-salmon PRL, the second peak elutes at a Kav of 0.44 and is immunoreactive, the third peak elutes at a Kav of 0.98 and is not immunoreactive (Fig. 20). The values for the talc–resin–TCA test are given in Table 2. This test discriminates between monomeric iodohormone, aggregated iodohormone and $^{125}$I–labelled salts (or free $^{125}$I)(Tower et al., 1980). Based on its immunoreactivity and performance in the talc–resin–TCA test, the second peak was used as the labelled hormone in the RIA. The third peak represented free $^{125}$I or $^{125}$I–labelled salts; with storage at –80 C, the relative height of this peak increased while the labelled PRL peak declined. It was found that a label was useful for approximately one month, yielding a NSB of 5.8% (mean of 9 assays, SD = 2.1). When the label taken from the second peak was re-run on the G–100 column, it eluted as a symmetrical peak with minor contamination from the other two peaks (Fig. 5).
Figure 18: Purification of $^{131}$-sPRL after iodination.

Following iodination, the reaction mixture was separated on a 1x10 cm G-15 column and eluted with RIA buffer. 30 fractions of 20 drops each were collected and the radioactivity in a 5 µl aliquot of each fraction determined. Fractions 8-12 from this iodination were combined and aliquots of 2 X 10⁷ cpm stored at -80 C. The radioactive peak at fraction 21 represents non-incorporated $^{131}$I.
Figure 19: Re-purification of $^{125}\text{I}$-sPRL for use as RIA label.
An aliquot of $^{125}\text{I}$-sPRL (Fig. 18 separated on a 1 x 60 cm column of G-100 developed with RIA buffer. The first peak (Fraction 15, Kav 0.14) represents non-immunoreactive aggregates of $^{125}\text{I}$-sPRL and lactoperoxidase, the second peak (Fraction 33, Kav 0.44) is $^{125}\text{I}$-sPRL used as the label in the RIA and the third peak (Fraction 68, Kav 0.98) represents free $^{125}\text{I}$ or $^{125}\text{I}$-labelled salts. Fraction 33 and fractions of the downslope from this re-purification were combined for use as RIA label.
Figure 20: Immunoreactivity of $^{125}$I–sPRL after repurification.

An aliquot of $^{125}$I–sPRL (Fig. 18 separated on a 1 x 60 cm column of G-100 developed with RIA buffer. Fractions (20 drops) were collected after the void volume eluted and the radioactivity in an aliquot (20 μl) from each fraction determined. The immunoreactivity of each fraction (broken line) was determined by adding 100 μl of each fraction to a constant amount of anti–salmon prolactin and measuring the percentage of radioactivity from each fraction that was bound by the antiserum.
CPM per 20 µl

Fraction Number (20 drop fractions)
Table II

Talc–Resin–TCA Test of $^{125}$I-labelled Prolactin

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<th>Resin (% bound)</th>
<th>TCA (% ppt)</th>
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</table>

The peak numbers represent peaks seen on repurification of $^{125}$I-PRL as in Fig. 20. This test is supposed to distinguish between aggregated iodohormone (Peak 1), monomeric iodohormone (Peak 2) and $^{125}$I-labelled salts or free $^{125}$I (Peak 3) (Tower et al., 1980). Although peaks one and two were not differentiated in this test, based on the immunoreactivity, peak two was used as the label in the RIA.
The sensitivity of the assay is defined as the minimum detectable dose or "the smallest dose having a predicted or expected response which would be different from the response at zero dose when a one-sided t-test is used to compare the mean response of the replicates of the unknown with the best estimate of the response for zero dose" (Rodbard, 1978). The minimum detectable dose in the PRL assay is a $B/Bo$ of 96.27 ($B/Bo$ represents the fraction of label bound (B) referred to the zero binding level (Bo)). This represents 120 pg of PRL with a 95% confidence interval for the zero dose when samples are analysed in triplicate; i.e., samples containing less than 120 pg PRL are not distinguishable from the zero dose.

The co-efficient of variation (CV) for the within (or intra-) assay variation based on six assays using the control sample was 2.76%. The CV for the between (or inter-) assay variation is 16.45%.

The results of the parallelism studies between the purified PRL, plasma and RPD extract are shown in Figs. 21, 22 and 23, respectively. The slopes for plasma and PRL are not significantly different, nor are the slopes for RPD extract and PRL ($p>0.05$). The cross-reaction of growth hormone in the assay is shown in Fig. 24 and was calculated to be 0.62%. The GH slope (100 to 1000 ng range) was not significantly different from that of PRL (0.2 to 5 ng range; $p>0.05$).

The mean recovery of 0.5, 1.0 and 2.0 ng of PRL added to 50 µl of plasma was 106%, showing minimal interference by plasma constituents.

The effect of sample volume on the assay is shown in Fig. 25. As variation in sample volume did have an effect on the assay (by lowering the zero binding level), assays were conducted at a constant sample volume of 100 µl, routinely by diluting 20 µl of plasma with RIA buffer.
Figure 21: Parallelism of sPRL and Kawauchi’s PRL.

RIA standard curves obtained using either sPRL or PRL from Kawauchi as standards. B/Bo represents the fraction of label bound (B) referred to the zero binding level (Bo). The slopes of the two curves obtained were not significantly different (P>0.05).
Kawauchi

PRL

- sPRL
- Kawauchi PRL

B/Bo

Nanograms Hormone Added
Figure 22 Parallelism between sPRL and coho salmon plasma.

RIA standard curve obtained with sPRL plotted as B/Bo vs logarithm mass of sPRL. The immunoreactivity of 50, 100, and 200 μl volumes of plasma from coho salmon produced a line the slope of which was not significantly different from sPRL (p>0.05).
Figure 23: Parallelism between sPRL, coho salmon plasma and chum salmon RPD extract.
The logit-log transformation used in this figure is a linearizing transformation for RIA data.
The slopes of the sPRL standards, coho plasma, and chum salmon RPD extract (338 ng
RPD/ml) were not significantly different (p>0.05).
μl RPD Extract or Plasma

Logit B/Bo vs ng. sPRL

- sPRL
- RPD Extract
- Plasma
Figure 24: Cross-reaction of growth hormone in the RIA.
Standard curves were obtained using either sPRL or pure chum salmon growth hormone. The slopes of the two lines were not significantly different (p>0.05) over the range of 0.2–5 ng. The cross-reactivity of growth hormone was calculated to be 0.62%.
Nanograms Hormone Added

- Growth Hormone
- sPRL
Figure 25: Effect of sample volume on the RIA.

Standard curves obtained using sPRL as the standard with varying volumes (0–200 μl) of extra RIA buffer added. As this resulted in a lowering of binding of tracer to the antiserum, a constant sample volume was employed in the RIA.
0 µl added volume
20 µl added volume
50 µl added volume
100 µl added volume
200 µl added volume
Discussion

The first reported purification of salmon prolaction was by Idler et al. (1977). Their purification protocol, which differs substantially from the one reported here, produced a protein of MW 20.5 kDa with an isoelectric point of 6 that showed "essentially one band" upon electrophoretic analysis (Idler et al., 1977), and had some significant bioactivity in a bioassay using hypophysectomized mollies (Poecilia), although the response was not dose dependent. In my initial attempts to purify salmon prolactin, I tried many times to reproduce Idler et al.'s procedure and was unsuccessful. Similarly, others have been unable to reproduce Idler et al.'s purification (Prunet, pers. comm.; see Prunet and Houdebine, 1984).

Salmon prolactin was successfully purified by Kawauchi et al. (1983) who reported an isoelectric point of 10.3 and a MW of 22.3 kDa. This prolactin also was bioactive. My purification of salmon prolactin involved a modification of the Kawauchi et al. (1983) protocol. I found it essential to include a cation exchange HPLC step in the purification. As Kawauchi et al. used fresh fish as a source of pituitary glands for the isolation of prolactin and I used pituitary glands from commercially caught fish, the post-mortem autolysis that occurred in my source probably was responsible for the difference. By using the unmodified Kawauchi protocol, I was never able to achieve greater than 90–95% purity (as detected electrophoretically). My yield, 10 mg/50 g pituitary glands, was only 20% of that reported by Kawauchi et al. (1983), (which, in view of the condition of the pituitary glands at the time of collection, I consider a good yield).

Comparing the amino acid residue analysis of my material with that of Kawauchi et al. (1983), a good match is seen. Also, the sequence of the first ten amino acids from my material is identical to that of Kawauchi et al. as reported by Yasuda et al. (1986). In contrast, Idler et al.'s 'prolactin', when converted to 187 amino acid residues, does not show a close match, although there are some similarities.

That my material is pure is shown by electrophoresis where only one species was detected in any buffer system whether stained tinctorially or immunologically. Also, in SDS electrophoresis, my material
co-migrated with Kawauchi et al.'s (1983) material and a specific antiserum raised against my material recognized both mine and Kawauchi et al.'s prolactins. My preparation was also shown to be homogeneous in the sequence analysis where only one N-terminal amino acid, isoleucine, was detected.

Later work by Yasuda et al. (1986) on chum salmon prolactin and by Specker et al. (1985) on tilapia prolactin have shown that PRL is not a unique molecular species. Chum salmon PRL exists as two isohormones that have identical molecular weights and isoelectric points but differ by replacement of four amino acid residues at positions 25, 53, 63, and 122 (Yasuda et al., 1986). Tilapia PRL also occurs in two forms that differ substantially: molecular weights of 20 and 25 kDa and isoelectric points of 6.7 and 8.7. In both cases, the variants were present in approximately equal amounts: on reverse phase HPLC, the abundance of the two forms of chum salmon PRL was 54 and 46% (Yasuda et al., 1986) while in pulse chase experiments, the two forms of tilapia PRL were synthesized and released at similar rates, with similar immunological and biological activities (Specker et al., 1985).

As Yasuda et al. (1986) found that the two isohormones of chum PRL could only be separated by reverse phase HPLC, and as I did not have such a step in my purification, I would think that my PRL contains both isohormones. In salmon, duplication of prolactin, and other pituitary hormones such as α-MSH, CLIP, β-MSH, endorphin, the N-terminal peptide of proopiocortin (Kawauchi, 1983; Yasuda et al., 1986) and GH (Kawauchi et al., 1986), as well as several enzymes and haemoglobin (Ohno et al., 1968) has been found. As salmonids are thought to have evolved by tetraploidization from a diploid ancestor (Ohno et al., 1968), gene duplication by tetraploidy is thought to explain this phenotypic duplication (Kawauchi et al., 1986; Yasuda, et al., 1986).

Immunologic staining of sections of sockeye salmon (Oncorhynchus nerka) pituitary glands showed that the η cells (erythrosinophilic, follicular cells) of the RPD were the only cell type stained. These cells have been previously identified as lactotropes (Ball, 1969; McKeeown, 1970). The specificity of this procedure was established: firstly, staining could be abolished by prior incubation of the antiserum with prolactin; secondly, staining was not affected by prior incubation of the antiserum with salmon growth
hormone.

Also, antiserum to salmon prolactin specifically bound to the RPD of both eels (*Anguilla anguilla*) and mummichogs (*Fundulus heteroclitus*). In contrast to this, Nagahama *et al.*, (1981) found that antisera raised against tilapia (*Oreochromis mossambicus*, Family Cichlidae) PRL did not stain the RPD cells of eels and stained both PRL and GH cells in salmon. Thus there seem to be distinct differences in the prolactin molecules of various teleost species. Naito *et al.* (1983), using an antiserum prepared against the PRL of Kawauchi *et al.*, also investigated the immunological staining of anti-salmon prolactin in a wide variety of teleosts (including tilapia). In this study, the antiserum against salmon prolactin showed no species specificity; the RPD cells of all species tested were stained although staining was strongest in salmon and closely related species.

Although a multitude of functions have been ascribed to prolactin, in fish one of the roles seems to be as a fresh water osmoregulatory hormone. The now classic demonstration by Pickford and Phillips in 1959 that hypophysectomized mummichogs (*Fundulus heteroclitus*) could only survive in fresh water (FW) by the administration of exogenous mammalian prolactin provided the first evidence that there was a prolactin in fish and that it had an osmoregulatory function. The specific response seen was that without a source of prolactin, plasma sodium levels declined in FW and that prolactin was effective in maintenance of plasma sodium in FW. Grau *et al.* (1984) developed a sensitive and specific bioassay for teleost (specifically salmon) prolactin using hypophysectomized mummichogs. They showed that the plasma sodium concentrations of seawater (SW) adapted hypophysectomized *Fundulus* when transferred to FW were dose responsive to injections of chinook salmon (*Oncorhynchus tschawytscha*) and ovine PRL. The dose response curve they obtained was parabolic, wherein some of the higher doses were less effective than some of the lowest doses. A similar response was seen by Hasegawa *et al.* (1986) upon administration of chum salmon prolactin to hypophysectomized mummichogs and to intact ayu (*Plecoglossus altivelis*: Suborder Salmonoidei), but not in rainbow trout (*Salmo gairdneri*) which showed a log-linear, not parabolic, response to doses up to 2 μg/g body weight. In the original bioassay of Grau *et al.*, chinook prolactin was most effective at a dose of 4 ng/g body weight. Chum salmon prolactin showed lower activity in this
assay; the most effective dose being either 20 ng/g (Naito et al., 1983) or 40 ng/g, (the highest dose tested in the present study). Prolactin has also been purified from tilapia (Farmer et al., 1977; Specker et al., 1985). When this prolactin was bioassayed in hypophysectomized juvenile FW tilapia, a log-linear, not parabolic, dose response is seen at doses up to 1 μg/g body weight. Thus there seems to be distinct differences in the responses shown by different fish species to administration of exogenous prolactin from various teleosts.

With the antiserum to chum salmon prolactin, I was able to develop a radioimmunoassay that was capable of measuring the concentrations of prolactin in biological samples. This method was validated by several criteria. Firstly, serial dilutions of chum salmon prolactin obtained from Kawauchi produced an inhibition curve parallel to a curve obtained from my material. While parallelism does not establish identity, it is essential that the standards and the unknowns 'dose out' in a parallel fashion (Rodbard, 1974) as non-parallelism indicates non-identity. The parallelism demonstrated between my material and that of Kawauchi's confirms the results of the immunological staining of the electrophoresis gels. Also, serial dilutions of coho salmon plasma and pituitary extracts were parallel (Figs. 22 and 23) to the pure standard.

A second criterion for validation was assessing interference in the assay by sample components or by possible crossreacting species. The recovery study illustrated negligible interference by plasma constituents. As growth hormone and prolactin show many similarities and are thought to have arisen by gene duplication (Farmer and Papkoff, 1974), it would be expected that growth hormone would have the greatest probability of interfering in the prolactin RIA. The measured extent of cross-reaction was only 0.6%. As the detection level (i.e., the minimum detectable dose) in the assay is 120 pg, and standard sample volume is 20 μl, GH would not significantly interfere unless the GH concentration exceeded 1000 ng/ml, at which point it would be measured as 1.2 ng/ml PRL. Reported levels of growth hormone from salmon range from 16 to 20 ng/ml in SW fish. Growth hormone levels were elevated to 60 ng/ml in SW to SW transferred fish but were not affected by SW to FW transfer (Bolton et al., 1986). Similarly, Wagner (1985) reported that GH levels varied from 23 ng/ml to 66.1 ng/ml over a six week period. However, Wagner (1985) also reported that starved fish showed a much higher titre of GH, up to 765 ng/ml. This is still
only 76% of the minimal concentration that would interfere in the assay. No cross-reaction of the antiserum with somatotropes was seen in the immunocytochemical (which is reportedly more sensitive than RIA) study and specific staining of the lactotropes could not be abolished by prior incubation of the antiserum with GH. Thus, routinely, growth hormone will not significantly interfere in the assay. McKeown (pers. comm) has found GH levels exceeding 1 μg/ml in the medium from in vitro organ culture of pituitary glands. If PRL was to be assayed in such studies, it is possible that GH may interfere with the measurement of PRL (unless the samples were diluted).

When I assayed plasma from hypophysectomized salmon for prolactin, I obtained lower, but not zero, concentrations than were measured in sham operated fish. Normally, one would expect that plasma samples from hypophysectomized fish would show zero levels of pituitary hormones. This has proved problematical in salmonids. Hirano et al. (1985) reported significant levels of prolactin in both hypophysectomized rainbow trout and hypophysectomized coho salmon. Although prolactin levels from hypophysectomized fish were lower than those from intact fish, they did not detect significant depression of prolactin levels in hypophysectomized fish compared to sham operated controls. As autopsy verified completeness of hypophysectomy, the possibility exists for an extra-pituitary source of prolactin. Prolactin has been detected in the hypothalamus of rats (Emanuele et al., 1986; Barbanel et al., 1986), fish (Calamoichthys calabaricus, Hansen and Hansen, 1982) and lampreys (Wright, 1986); also, Schachter et al. (1984) reported prolactin messenger RNA from rat hypothalamus. Although hypophysectomy of rats decreases plasma prolactin to undetectable levels, neither hypothalamic prolactin (Emanuele et al., 1986) nor cerebrospinal fluid prolactin levels (Barbanel et al., 1986) were affected by hypophysectomy. Many other pituitary hormones (e.g. GH) have also been detected in brains and when cells from the amygdaloid nucleus of rats are grown in tissue culture, a progressively increasing release of immunoreactive GH occurs, even when the medium is completely replenished every 3 to 5 days (Krieger and Liotta, 1979).

Thus the detection of immunoreactive prolactin in plasma from hypophysectomized fish needs further investigation. If there is an extra-pituitary source of PRL in salmon, the contribution to plasma levels from such a source needs to be investigated. There has been one study of plasma clearance kinetics of
PRL in teleosts. Brewer and McKeown (1980) acclimated coho salmon parr to SW for 4 days, then transferred these fish to either SW or FW. $^{125}$I-ovine PRL, which had been injected into the parr prior to transfer, showed a 44% decrease in the plasma of the FW group at 4 hours post-transfer but only a further 1% drop after 52 hours post-transfer. Virtually none of the injected labelled PRL had been removed from the plasma of the SW fish at these times. Thus they showed that the ovine PRL molecule remains in the plasma of coho salmon for long periods of time. As Brewer and McKeown (1980) used ovine PRL, these results must be viewed with caution but they do suggest that it is possible that a significant fraction of endogenous plasma prolactin (or an immunologically detectable form) may be long lived and it is this fraction that is detected in plasma from hypophysectomized animals.

Thus, in summary, I have prepared a salmon prolactin which shows the appropriate biological activity. A sensitive and specific antiserum produced against the PRL was used to develop a sensitive homologous RIA to salmon PRL which was validated for use in measuring immunoreactive PRL levels in samples of salmon plasma and pituitary glands. Both of these, the hormone and the RIA, will be essential in further investigations into the physiology of PRL in salmon.
CHAPTER II
EFFECTS OF TRANSFER OF SW-ADAPTED COHO SALMON TO FW AND CALCIUM-ALTERED ENVIRONMENTS

Introduction

During the life cycle of Pacific salmon (Oncorhynchus) two major osmoregulatory challenges must be faced. The first occurs when the juvenile salmon undergo the parr-smolt metamorphosis, leave their fresh water (FW) nursery areas and undertake the downstream migration to the ocean. The second occurs when the maturing salmon return from the ocean to their FW natal streams during the spawning migration.

These environmental changes cause osmoregulatory problems in that teleost fish plasma has an osmotic pressure of approximately 1/3 seawater (SW). The gills, organs specialized for molecular exchanges between the internal and external environments, have a surface area ten times the rest of the body surface and have a constant flow of water across them that is always of a different osmotic and ionic composition than the plasma. Thus, in FW, salmon are faced with a loss of internal electrolytes to the environment and dilution of the blood by the osmotic uptake of water. In SW, the reverse problems are encountered.

In broad outline, the mechanisms employed by teleosts to counteract these physiological problems have been known since 1932 (Smith, 1932). Fish in FW do not drink and excrete a copious urine to balance the osmotic influx of water. Specialized cells in the gills of teleosts act as salt pumps, actively transporting electrolytes from the environment to the plasma. Also, the kidneys and bladder of teleosts are capable of resorbing some electrolytes from the urine, thus reducing the excretory electrolyte loss. In SW, fish do drink and absorb both water and electrolytes across the gut. Excess monovalent ions are excreted via the gills and divalent ions are excreted in the urine.
Several hormones are known to be involved in controlling the osmoregulatory mechanisms. In many teleosts, prolactin is thought to be a major hormone involved in FW adaptation – many teleosts cannot survive in FW without prolactin (see Clarke and Bern, 1980) although they survive in SW without this hormone. This function of prolactin, plasma sodium conservation in hypophysectomized Fundulus in FW, is the basis for bioassay of this hormone (Grau et al., 1984). Mammalian prolactin has been shown to be hypercalcaemic in intact Fundulus in SW or in calcium deficient SW (Pang, 1981) and corrected the hypocalcaemia of hypophysectomized Fundulus in calcium deficient SW (Pang, 1978). The osmoregulatory actions of mammalian prolactin in different FW-acclimated teleost species were summarized by Hirano (1986) and are given below.

1. Survival of hypophysectomized fish in FW.
2. Decreased gill Na⁺ efflux.
3. Decreased gill osmotic water permeability.
4. Restoration of plasma Na⁺ in hypophysectomized eels when given with cortisol.
6. Increased skin and gill mucous secretion.
7. Decreased ion and water absorption from intestine.
8. Diuresis and increased glomerular size.
9. Decreased water and increased ion absorption from bladder.
10. Hypercalcaemia

All of these effects will act to reduce salt loss from the body fluids to the environment, stimulate salt uptake from the environment, reduce water uptake from the environment and enhance urinary water excretion: all functions necessary for the survival of a fish in FW and in direct opposition to osmotic and ionic regulation in a marine environment.

It is worth emphasizing that these functions of prolactin have been elucidated with the use of mammalian prolactins, either by direct injection into the whole animal or by administration to isolated tissues. Only a few studies (Nicoll et al., 1981; Hirano et al., 1985; Prunet and Boeuf, 1985; Prunet et al., 1985;
Hasegawa et al., 1986; Hirano, 1986) have used a homologous teleost hormone or radioimmunoassay in studies of prolactin physiology. This also implies that only a few species of teleosts have been used in investigating the comparative physiology of teleost prolactin: tilapia (Oreochromis mossambicus, Nicoll et al., 1981), rainbow trout (Salmo gairdneri, Prunet and Boeuf, 1985; Prunet et al., 1985; Hasegawa et al., 1986; Hirano, 1986; McKeown et al., 1987), ayu (Plecoglossus altivelis, Hasegawa et al., 1986), Japanese eels (Anguilla japonica, Hasegawa et al., 1986), chum salmon (Oncorhynchus keta, Hasegawa et al., 1986; Hirano et al., 1985) and Atlantic salmon (S. salar, Prunet and Boeuf, 1985). There have been no studies done on homologous prolactin physiology in coho salmon (O. kisutch).

The present study had two objectives. First, to determine the response of plasma prolactin by both post-smolts and smolts of coho salmon upon transfer to FW, as measured using a homologous RIA. This transfer was expected to alter the physiological requirements for prolactin. Secondly, to determine if plasma prolactin of coho salmon smolts was affected by manipulations of environmental calcium levels, an environmental ion known to be involved in water and electrolyte permeability of teleost gills (Loretz and Bern, 1982; Ogasawara and Hirano, 1984) and a plasma ion thought to be partially regulated by prolactin (Hirano, 1986, vide infra). The use of smolt and post-smolt coho salmon in the present study allowed the determination of plasma prolactin changes and osmoregulatory events that occurred upon the transfer of salmon to different salinities in the absence of confounding reproductive phenomena.
Materials and Methods

Experimental Animals

SW-adapted post-smolt coho salmon (249 g mean weight, 63 g SD, n=132) were obtained from SW net pens at Deep Cove, North Vancouver in April, 1986 and were transported to the West Vancouver labs of the Department of Fisheries and Oceans where they were held in four 1100 l tanks in running seawater for one week prior to experimentation.

FW-adapted smolts of coho salmon (15.2 g mean weight, 2.7 SD, n=89) were obtained in July, 1986 from the Pacific Biological Station, Nanaimo, B.C. and were transported to SFU where they were immediately placed into 1100 l SW holding tanks and left for one month to acclimate to SW prior to experimentation. These fish were approximately the size where they would normally smoltify and migrate to SW. They were maintained on a natural photoperiod and fed standard food ad libitum. For logistical reasons, they were maintained in recirculating filtered SW tanks; 90% of the water was replaced twice weekly.

Effects of FW transfer on SW-adapted post-smolts and smolts

Transfer of post-smolts

The transfer experiment of post-smolts was done in the flow through SW tanks described above. Prior to transfer of postsmolts to FW, eight fish were sampled (as below). These fish were the zero hour group. Immediately following the zero time sampling, the seawater supply to the tanks was shut off and all tanks were drained to a minimal quantity of water. In two of the tanks, the sea water supply was turned back on (SW − SW) and in the other two tanks, the fresh water supply was turned on (SW − FW).

Sampling of the fish (as below) from both treatments was done at 0.5, 1, 2, 4, 8, 24, 48 hours and one week post transfer. Eight fish from each treatment (four from each tank) were sampled each time except that only four fish were available in the one week SW − FW group.
At each sampling time, the unanesthetized fish was restrained in a net and blood was collected from the caudal artery with a heparinized syringe. Following blood collection, the fish was decapitated and the brain exposed. Rostral reflection of the brain exposed the pituitary gland in the sella turcica which was then removed using fine forceps. After centrifugation of the blood and removal of the plasma fraction, plasma and pituitary gland samples were immediately frozen on dry ice and stored at −20 C until analysed.

Transfer of smolts

Smolts were transferred from the SW holding tanks to 2 smaller (175 l) experimental tanks containing SW. They were left for one week to recover from the handling prior to salinity transfer. Fish were not fed during this time. After one week, experimental fish were transferred directly into 2 tanks containing FW and control fish were transferred into 2 tanks containing new SW.

At the time of transfer, 8 fish (4 from each tank) were anesthetized in 1:20,000 MS-222 and a blood sample taken from the caudal artery with a heparinized syringe. Eight fish (4 from each tank) from each of the two transfer groups were similarly sampled at 1, 4, 8, 24, 48, and 72 hours post-transfer. Following sampling, blood was centrifuged and the plasma fraction was frozen immediately on dry ice; plasma was stored at −20 C until analyzed.

This experiment was done twice, once on Aug. 24, 1986 and once on Sept. 22, 1986; only in the second trial was plasma sodium determined.

Effects of transfer to calcium enriched FW on SW-adapted smolts

In this experiment, the effects of maintaining the environmental calcium concentration in FW at SW levels upon plasma prolactin, calcium, sodium, and osmotic pressure were determined in SW-adapted smolts. As previously, the control group was a SW to SW transfer. The experimental group was transferred from SW to an extremely hard FW (HFW). This was FW that had sufficient calcium chloride added such that environmental calcium levels approximated SW levels (9.9 mM calcium in the HFW, 9.6 mM calcium in SW).
Fish were transferred from the SW holding tanks to experimental tanks (175 l) and were left (unfed) for one week to recover from the handling prior to salinity transfer. After one week, the experimental fish were transferred directly into 2 tanks containing HFW and control fish were transferred into 2 tanks containing new SW.

At the time of transfer, 8 fish were anesthetized in 1:20,000 MS–222 and a blood sample taken from the caudal artery with a heparinized syringe. Eight fish (4 from each tank) from each of the two transfer groups were similarly sampled at 1, 4, 8, 24, 48, and 72 hours post-transfer. Following sampling, blood was centrifuged and the plasma fraction was frozen immediately on dry ice; plasma was stored at −20 C until analyzed.

**Effects of transfer to calcium–free SW on SW–adapted coho smolts**

In this experiment, the effects of eliminating environmental calcium in SW upon plasma prolactin, calcium, sodium, and osmotic pressure were determined. The formulation of the ASW was from Spotte (1970), based on a commercial recipe (Instant Ocean).

Fish were transferred from the holding tanks to experimental tanks (175 l) containing SW and were left (unfed) for one week to recover from the handling prior to salinity transfer. After one week, the experimental fish were transferred directly into 2 tanks (175 l) containing ASW and control fish were transferred into 2 tanks containing new SW.

At the time of transfer, 8 fish were anesthetized in 1:20,000 MS–222 and a blood sample taken from the caudal artery with a heparinized syringe. Eight fish (4 from each tank) from each group were similarly sampled at 8 and 24 hr post-transfer. Following sampling, blood was centrifuged and the plasma fraction was frozen immediately on dry ice; plasma was stored at −20 C until analyzed.
Assays

Prolactin was measured by radioimmunoassay (Chapter One). Routinely, 20 μl aliquots of plasma were diluted to 100 μl with RIA buffer for use in the RIA. Samples were assayed in duplicate.

For the measurement of the pituitary prolactin content, the glands were homogenized in 1 ml of RIA buffer, on ice, and diluted to 10 ml. Between each homogenization, the homogenizer was rinsed with acid acetone (1:28 v/v conc. HCl:acetone) and RIA buffer. The homogenate was frozen until assayed. Preliminary assays with these homogenates showed that a sample volume of 100 μl of a 1:400 dilution of the homogenate was appropriate for use in the RIA. Samples were assayed in duplicate. As the size of the pituitary would be dependent upon the size of the fish, the value obtained for prolactin content was divided by the weight of the fish and is expressed as ng pituitary prolactin / g body weight.

For analysis of sodium, plasma was diluted 1:1000 with distilled water and analysed by flame emission spectrophotometry. For calcium analysis, plasma was diluted 1:100 with 0.1% lanthanum chloride and analysed by atomic absorption spectrophotometry. Both sodium and calcium analyses were done on a Pye–Unicam SP191 Atomic Absorption Spectrophotometer. Plasma osmotic pressure was measured on a Wescor Vapour Pressure Osmometer Model 5100A.

Statistical analysis was done by analysis of variance and t-tests or the Student–Newman–Keuls multiple range test; differences were considered significant if the p value was less than 0.05.
Results

*Effects of FW transfer on SW-adapted post-smolts and smolts of coho salmon*

The plasma prolactin concentrations, following transfer to FW, of post-smolts is shown in Fig. 26. For the post-smolt salmon, none of the points for the SW to SW group are significantly different from the value at zero time (7.56 ± 0.18 ng/ml, mean ± 1 SEM) (Fig. 26) After one hour post-transfer, the plasma prolactin concentration in the FW-transferred fish showed a steady significant increase until 24 hrs after which the levels remained elevated until the end of the experiment. The values obtained at 2, 4 and 8 hours post-transfer, as a group, are not significantly different from each other; also the values obtained at 24, 48 hours and one week post-transfer, as a group, are not significantly different from each other although the groups were significantly different both from each other and from the controls.

The pituitary prolactin levels of the post-smolts following FW transfer were also measured (Fig. 27a + b). None of the values obtained from the control fish were significantly different from the level seen in the zero time group although there was a consistently lower level after 1/2 hour. In the experimental fish, only the decreases in pituitary content seen at 1/2 and 2 hours post-transfer were significant. The variability of the pituitary prolactin levels was much greater than that of the plasma levels.

The plasma calcium and sodium concentrations of the post-smolts are shown in Fig. 28 and 29, respectively. Transfer to FW induced a hypercalcaemia, evident at 2 hours post-transfer (Fig. 29) in the post-smolts. At this time, plasma prolactin in the FW-transferred fish was significantly elevated from the control fish. The hypercalcaemia was still evident after 1 week in FW, although the levels at 4, 8 and 24 hours post-transfer are not significantly different from control values.

In contrast to the plasma calcium responses, transfer to FW did not greatly affect the plasma sodium concentration of the post-smolts (Fig. 29). The control fish, however, quickly became hypernatremic, peaking at two hours post-transfer. Plasma sodium levels in the control fish were stable after 8 hours post-transfer for the duration of the experiment. Post-smolts transferred into both FW and SW had
Figure 26: Plasma prolactin response of post-smolts transferred to FW
The FW transferred fish had significantly higher plasma PRL than the controls at all times following one hour post-transfer. (p < 0.05, mean ± 1 SEM). X axis represents time post-transfer. "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
Plasma Prolactin (ng./ml.)

Time (hours)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

0 24 48 1 Week

SW to FW

SW to SW
Figure 27: Pituitary gland prolactin response of post-smolts transferred to FW.

a: Only at 1/2 hour and 2 hours post-transfer did the FW-transferred post-smolts show a significantly different pituitary gland PRL content from the controls (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW. For clarity, the results of this experiment have been split into two graphs.

b: Second part of the previous graph. No significant differences were detected between the two groups (p < 0.05, mean ± 1 SEM).
Co-Pituitary Prolactin (ng./g. body weight)

Time (hours)

1 week

- SW to FM
- SW to SW
Figure 28: Plasma calcium response in the post-smolts transferred to FW.

Plasma calcium concentrations in the FW-transferred fish were significantly different from the controls at 48 hr and one week post-transfer (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW. "SW to FW" indicates the experimental fish transferred to FW.
Plasma Calcium (mM)

Time (hours)

0 1 4 8 24 48 2 Week

SW to FW

2.75 2.50 2.25
Figure 29: Plasma sodium response in the post-smolt transferred to FW.

Plasma sodium concentrations in the FW-transferred fish were significantly different from the controls at 1, 2, 4, 8 and 24 hours post-transfer (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
identical plasma sodium concentrations at 48 hours and 1 week following transfer.

The much smaller smolts showed a different response to FW transfer than did the post-smolts (Fig. 30a + b). The smolts did not show the expected increase of plasma prolactin concentration after transfer to FW; rather they exhibited more stable PRL levels than did the SW-transferred control fish.

The plasma calcium response to FW transfer in the SW-adapted smolts (Fig. 31 + 32) differed from the post-smolt fish in that no difference was observed between the SW-SW transferred controls and the FW-transferred smolt coho in either of the two experiments, except that at 72 hours post-transfer in the second experiment where the plasma calcium concentration was significantly depressed in the FW-transferred smolts.

The FW-transferred smolts also differed from the post-smolts in the response of plasma sodium to FW transfer (Fig. 33). These fish were unable to maintain their plasma sodium at control levels: at times after 8 hours post-transfer, the FW-transferred fish had mean sodium concentrations 79% of controls. The plasma osmotic pressure, changes in which will indicate fluid shifts, was determined in the coho salmon smolts (Fig. 34 and 35). Little difference was seen between the FW-transferred fish and the controls, indicating that over the course of the experiment a large influx of water from the environment into the fish did not occur. The slight (mean of 91.8% of control values) but consistent depression of plasma osmotic pressure that occurred at times after 24 hours post-transfer to FW (Fig. 35) seen in the second experiment was not of sufficient magnitude to account for the larger depression of plasma sodium seen in these same fish (Fig. 33). Plasma osmotic pressure was not determined in the post-smolt coho salmon.

**Effects of transfer to calcium enriched-FW on SW-adapted smolts**

In this experiment, the smolts were subjected to a calcium challenge by transfer to a FW environment that had the same calcium concentration as SW (HFW; 9.9 mM calcium).

The smolts that were transferred to this environment showed a different response from the smolts that were transferred to normal FW. The plasma prolactin concentration (Fig. 36) had risen significantly
Figure 30: Plasma prolactin response of smolts transferred to FW.

a: Only at 8 and 24 hours post-transfer were the FW-transferred smolts significantly different from the controls (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.

b: Second transfer of smolts to FW. Only at 4, 8 and 48 hours post-transfer were the FW-transferred smolts significantly different from the controls (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
Plasma Prolactin (ng./ml.)

Time (hours)

0 1 4 8 24 48 72
Plasma Prolactin (ng/ml)
Figure 31: Plasma calcium response of smolts transferred to FW.

No significant differences between the two groups were detected (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW. "SW to FW" indicates the experimental fish transferred to FW.
Figure 32: Plasma calcium response of second transferral of smolts to FW.

Only at 72 hours post-transfer were the FW transferred fish significantly different from the controls (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
Figure 33: Plasma sodium response of smolts transferred to FW.

FW-transferred smolts has significantly lower plasma sodium than controls at all times following 4 hours post-transfer (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
Plasma Sodium (mM)

Time (hours)

0  1  4  8  24  48  72

100 110 120 130 140 150 160 170

SW to FW

SW to SW
Figure 34: Plasma osmotic pressure response of smolts transferred to FW.

No significant differences were detected between the two groups. "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
Figure 35: Plasma osmotic pressure of second transfer of smolts to FW.

Plasma osmotic pressure was significantly different in the FW-transferred smolts, compared to controls, at 8, 24, 48 and 72 hours post-transfer (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to FW" indicates the experimental fish transferred to FW.
Figure 36: Plasma PRL response of smolts transferred to calcium enriched FW.

HFW refers to the FW medium containing 9.9 mM calcium. At 8, 24, and 48 hours post-transfer the plasma prolactin in the HFW transferred fish was significantly above control values (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to HFW" indicates the experimental fish transferred to calcium enriched FW.
above the control levels by 8 hr following transfer and remained elevated until 48 hr post-transfer. By 72 hr post-transfer, plasma prolactin concentrations had fallen to control values.

The response of plasma calcium also was different in this experiment (Fig. 37). The HFW-transferred fish quickly showed a significant hypercalcaemia, maximal at 24 hr post-transfer. This hypercalcaemia occurred even though haemodilution, as evidenced by the depression in plasma osmotic pressure (Fig. 38) at this time, was also occurring. Plasma calcium concentrations were restored to control levels by 48 hr post-transfer.

Plasma sodium (Fig. 39) however did not show a different response to high calcium FW than to normal FW (Fig. 33), which, at times following 8 hr post-transfer, were significantly depressed. This depression may be accounted for by haemodilution, as shown by the depression in plasma osmotic pressure (Fig. 38) seen in these fish.

**Effects of transfer to calcium-free SW on SW-adapted smolts**

When SW-adapted smolts were transferred to a calcium-free artificial SW (ASW), somewhat different results were obtained. Plasma prolactin (Fig. 40) was elevated above control levels at 8 hr post-transfer but was significantly below control levels at 24 hr post-transfer. Plasma calcium concentrations were significantly depressed (Fig. 41) at both 8 and 24 hr post-transfer while plasma sodium concentrations (Fig. 42) were stable at both times although well below the control levels. Plasma osmotic pressure (Fig. 43) showed a minor but significant haemodilution at 24 hr post-transfer, similar to SW-fish transferred to FW at the same time (Fig. 35). This haemodilution could not account for either the hypocalcaemia or the lowered prolactin levels in these fish by a simple dilution of the plasma.
Figure 37: Plasma calcium response of smolts transferred to calcium enriched FW.

At 24 hours post-transfer, the HFW transferred fish showed a significant hypercalcaemia (p < 0.05, mean ± 1 SEM), "SW to SW" indicates the control fish transferred to SW, "SW to HFW" indicates the experimental fish transferred to calcium enriched FW.
Figure 38: Plasma osmotic pressure response of smolts transferred to calcium enriched FW.

Plasma osmotic pressure was significantly different in the HFW–transferred smolts, compared to controls, at 1, 24, 48 and 72 hours post-transfer (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW, "SW to HFW" indicates the experimental fish transferred to calcium enriched FW.
Figure 39: Plasma sodium response of smolts transferred to calcium enriched FW.

At times following 8 hours post-transfer, the smolts transferred to HFW had significantly lower plasma sodium than the controls (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW. "SW to HFW" indicates the experimental fish transferred to calcium enriched FW.
Figure 40: Plasma PRL response of smolts transferred to calcium–free artificial SW.

At 8 and 24 hours post-transfer, the ASW transferred fish has plasma PRL levels significantly different from controls (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW, "SW to ASW" indicates the experimental fish transferred to calcium–free artificial SW.
Plasma Prolactin (ng/ml)
Figure 41: Plasma calcium response of smolts transferred to calcium-free artificial SW.

Significantly lower plasma calcium concentrations were measured in the ASW transferred smolts at 8 and 24 hours post-transfer (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW. "SW to ASW" indicates the experimental fish transferred to calcium-free artificial SW.
Figure 42: Plasma sodium response of smolts transferred to calcium-free artificial SW.

Plasma sodium concentrations in the ASW transferred fish, compared to controls, were significantly different at 8 and 24 hours post-transfer (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW, "SW to ASW" indicates the experimental fish transferred to calcium-free artificial SW.
Figure 43: Plasma osmotic pressure response of smolts transferred to calcium–free artificial SW.

At 24 hours post transfer, the ASW transferred fish had plasma osmotic pressures significantly different from the controls (p < 0.05, mean ± 1 SEM). "SW to SW" indicates the control fish transferred to SW. "SW to ASW" indicates the experimental fish transferred to calcium–free artificial SW.
Plasma Osmotic Pressure (mOsm/l)

Time (hours)

24 8 4 0

Plasma Osmotic Pressure (mOsm/l)

SW to SW

SW - ASW

778
Discussion

Although coho salmon would normally make a SW to FW transition only during their spawning migration as reproductively competent adults, a time when the FW adapting functions of prolactin are most likely to be needed, there are several reasons why the present study did not use such animals.

Aside from the facts that they are large, requiring extensive experimental facilities, do not withstand experimental manipulation well and are commercially valuable in their own right, the main reason why spawning adults are unsuitable concerns the endocrine reproductive events that are occurring in such animals at this time in their life cycle. Being semelparous animals, Pacific salmon turn a large part of their physiology towards reproduction for the production of gametes. The high production of gonadotropins by the pituitary gland is bound to confuse studies on basic prolactin physiology; e.g., it is known that the reproductive steroid 17β-oestradiol has profound effects on prolactin secretion from the pituitary gland. The large amounts of vitellin in the plasma of reproductively maturing female salmon will, due to its calcium-binding properties, make interpretations of plasma calcium determinations difficult (Watts et al., 1975). Also, such animals are difficult to obtain in sufficient numbers; juveniles being much more readily available.

The SW-adapted coho salmon used in these experiments, upon transfer to FW, were subjected to greatly decreased environmental concentrations of sodium and calcium and an increased environmental water concentration. There is a large body of evidence that suggests a role for prolactin in teleost FW osmoregulation (see Clarke and Bern, 1980; Hirano, 1986) acting to maintain plasma ion (sodium and calcium) concentrations at levels much higher than those present in the environment. Without prolactin, or the mechanisms under its control, many FW fish show extremely reduced plasma ion concentrations. The FW transfer would be expected to provide a stimulus for the pituitary release of prolactin, to ameliorate adverse changes in plasma ions and water balance.
The post-smolt coho used in these experiments had spent 2 years in SW prior to experimentation. Upon transfer to FW, they showed a prolonged increase in plasma PRL, peaking at 48 hr post-transfer. This response is qualitatively similar to that reported for large sedentary rainbow trout *(Salmo gairdneri*, Prunet *et al.*, 1985) which showed a peak in plasma PRL 15 days after transfer from SW to FW, and mature female chum salmon *(Hirano et al.*, 1985) which showed a peak after 1 day following transfer to FW. Mature male chum salmon did not show a PRL response to transfer to FW *(Hirano et al.*, 1985). The three salmonid species examined to date do, however, show quantitative differences. The PRL elevation in rainbow trout was greater (1400%) than in female chum salmon (800%) *(Hirano et al.*, 1985, Prunet *et al.*, 1985) or coho salmon (173%, present study). Also, in rainbow trout, this elevation was much more prolonged; PRL was still greatly elevated 20 days post-transfer in rainbow trout while chum salmon plasma levels had returned to near control values after one week *(Hirano et al.*, 1985, Prunet *et al.*, 1985). In coho, PRL titres were still elevated after one week post-transfer. These quantitative differences may be related to the extent of anadromy of which each species is capable: Rounsefell *(1958)* described the extent of anadromy in salmonids and found that chum salmon showed a greater anadromous capability than coho salmon whose capability was greater than rainbow trout. The differences between the two salmon species may be related to their reproductive condition, as prolactin is known to have reproductive effects *(Hirano, 1986, Clarke and Bern, 1980)*. The female chum salmon were mature while the post-smolt coho salmon immature. Although the comparable experiment has not been done in tilapia, PRL levels are much higher in FW-adapted than SW-adapted tilapia and animals in intermediate salinities had intermediate plasma PRL levels *(Nicoll et al.*, 1981).

The pituitary gland responses are similar in rainbow trout *(Prunet et al.*, 1985) and coho salmon (the present study), although more pronounced in rainbow trout: pituitary PRL levels decreased in both species following transfer. However, in coho salmon the SW controls showed the same response as did the FW transferred fish up to one week post-transfer. In rainbow trout, the FW-transferred fish showed depressed levels of pituitary gland prolactin up to 20 days post-transfer while the SW control fish showed higher levels after one week *(Prunet et al.*, 1985). Neither study demonstrated an activation of lactotropes
as reflected by pituitary gland prolactin content. In tilapia, pituitary PRL levels were related to the salinity of adaptation: FW animals showed much higher levels than did SW animals (Nicoll et al., 1981). Thus, at least in tilapia, a decrease in salinity leads to higher plasma and pituitary gland PRL levels and, likely, a higher metabolic turnover rate (Brewer and McKeown, 1980)

Thus the post-smolt coho salmon responded similarly to other salmonid species, showing a rapid and prolonged increase in plasma prolactin titre to FW exposure. The pituitary gland response was not so "clear-cut"; only at 2 hours post-transfer was the pituitary prolactin content significantly lower than the control level. As the pituitary gland is the site of storage and release of prolactin into the circulatory system, it would be expected that an increased plasma PRL concentration initially would result from the release of stored hormone from the pituitary gland, thus lowering the pituitary gland content of PRL. As Brewer and McKeown (1980) showed that the metabolic half-life of iodinated ovine PRL was much shorter in FW than in SW coho salmon, over a longer term exposure to FW, the pituitary gland production of PRL would be expected to be stimulated and increased plasma levels of PRL would then reflect newly synthetized material.

Both the post-smolt coho salmon in the present study and the rainbow trout in Prunet et al. (1985) showed decreases in the pituitary gland PRL content in the SW control fish and in neither study did the plasma PRL concentration change to reflect a release of stored hormone from the pituitary gland. This could be explained if the metabolic turnover rate of plasma PRL was increased. This, however, is unlikely. SW-SW transfer does not induce synthesis of new PRL (Brewer and McKeown, 1980) and lactotropes are inactivated in SW (Wendelaar Bonga et al., 1985; Clarke and Bern, 1980). Some fish (e.g., Gasterosteus aculeatus, the three-spined stickleback) have extensively atrophied RPD lobes in SW, so much so that they have been referred to as being "physiologically hypophysectomized" (Lam, 1972). Prunet et al. (1985) tentatively suggested that these effects in the control fish might be due to short-term starvation. However in their experiments, they sampled their fish once daily and thus their first indication of reduced pituitary gland prolactin content in the SW control fish was detected 24 hours post-transfer. In the present study, SW controls showed reduced pituitary gland PRL content after one hour post-transfer, eliminating
short-term starvation as an explanation. As Brewer and McKeown (1980) showed that protein incorporation of tritiated leucine by lactotropes of the coho salmon was unaffected by a similar transfer (but was immediately stimulated by FW transfer) it is unlikely that a depression in the synthesis of new prolactin occurred which otherwise might have explained the observed reduction in pituitary gland PRL content. Perhaps one effect of handling stress in SW-adapted salmonids may be to enhance intracellular lysosomal activity upon the stored prolactin without affecting synthesis. Degradation of intrapituitary gland prolactin is known to be associated with starvation (Nagy et al., 1978) and with removal of secretory stimuli (Chertow, 1981; Dannies and Rudnick, 1980; Farquhar, 1969) in rats and with removal of hypothalamic control in salamanders (Mazur and Holtzman, 1969). It has been suggested that release from the cell or intracellular degradation may be alternate fates for secretory granules and that the state of the secretory cell determines which alternative occurs (Smith and Farquhar, 1966, in Mazur and Holtzman, 1969).

The rapid elevation of the PRL titre seen in the FW-transferred post-smolts corresponded with a quick adjustment to the osmotic and ionic regulatory burden implied by this treatment. Plasma levels of sodium were unaffected by this treatment, which presents a polarity reversal from a hypertonic to a hypotonic environment, and plasma calcium levels were consistantly elevated above the SW–SW transferred controls at times after 2 h post-transfer. Although osmotic pressure of the plasma was not measured in this experiment, it is known that salmon and trout show a decrease in plasma osmotic pressure upon transfer to FW (Brewer and McKeown, 1980, Prunet et al., 1985). Prunet et al. (1985) found a concomitant depression of plasma chloride with the decreased plasma osmotic pressure in rainbow trout. Any haemodilution certainly would not account for the stable sodium and elevated calcium plasma levels seen. The hypercalcaemia is especially notable in view of the probable haemodilution and the fact that the environmental calcium was reduced by a factor of 192; administration of exogenous salmon prolactin caused hypercalcaemia in FW coho smolts (vide infra).

A different response to FW-transfer occurred in the much smaller coho salmon smolts. Plasma PRL in these fish did not change while the levels in SW control fish were elevated. This is somewhat similar to the pattern reported for coho smolts by Brewer and McKeown (1980). They reported a 68% drop in
immunoreactive plasma PRL upon transfer to FW, while plasma PRL in SW–SW controls was unaffected, as measured using a heterologous RIA. As pituitary PRL levels showed a concomitant decrease with plasma levels, Brewer and McKeown (1980) attributed an increased clearance of plasma PRL to be associated with the FW transfer.

The plasma ion response to FW transfer is also different in the smaller fish. Plasma calcium was significantly depressed after 48 hr (Fig. 31) and 72 hr (Fig. 32). The large depression in plasma sodium seen in the present study was not found by Brewer and McKeown (1980); their fish showed very minor ionic disturbances. This difference is probably due to differences in the method of transfer. Brewer and McKeown (1980) employed a gradual transfer of the smolts to FW and found that this procedure produced the least ionic perturbations; when they abruptly transferred larger fish more pronounced ionic disturbances occurred. In present study fish were abruptly transferred from SW to FW.

The effects seen in the coho smolts cannot be entirely accounted for by changes in plasma osmotic pressure. A decrease in plasma osmotic pressure will result from haemodilution by an increased net influx of water from the FW environment or efflux of plasma ions to the environment. Changes in plasma osmotic pressure were seen in only one (Fig. 35) of the two experiments where SW-adapted coho smolts were transferred to FW. These fish showed an 8% decrease in plasma osmotic pressure from 24 hours post-transfer till the end of the experiment. Thus the sodium depression seen in these fish represents a net loss as the haemodilution does not account for the sodium depression by simple dilution of the plasma; nor can the effects on plasma calcium be explained by such a mechanism. However, as it is likely that plasma PRL clearance was increased (Brewer and McKeown, 1980), pituitary prolactin secretion might have been increased to maintain the stable plasma prolactin levels seen in the face of the haemodilution.

Thus the pattern of adaptation to FW in salmonids may be a size-dependent (or stage-dependent) event. Larger salmon and trout (above 100 gms) (Hirano et al., 1985, Prunet et al., 1985, Hirano, 1986) showing increased plasma concentrations of prolactin and minimal ionic disturbances while salmonids under this size show depressed (Brewer and McKeown, 1980) or unchanged plasma prolactin titres with
more pronounced ionic disturbances (present study). This is known to be the case in the reverse situation: SW adaptation in salmonids is a size-dependent phenomenon (Parry, 1958), even in salmonids that do not undergo the parr-smolt transformation (Fargher, 1977, Folmer and Dickoff, 1980). It is possible that the hormonal differences may relate to the clearance kinetics of PRL: smaller fish may show a shorter turnover time or faster metabolic clearance rate than do larger fish.

The abrupt exposure to a decreased environmental calcium concentration upon FW transfer may also lead to a size differential response as environmental calcium is known to reduce the osmotic permeability of fish gills (Clarke and Bern, 1980; Ogasawara and Hirano, 1984). SW fish would thus have a low gill osmotic permeability as SW calcium levels are approximately 10 mM. Upon transfer to FW, where environmental calcium concentrations are much reduced (to the micromolar range), the lack of external calcium should result in an immediately increased osmotic water influx (Ogasawara and Hirano, 1984). Mammalian prolactin is known to increase active uptake of sodium from the environment and to decrease the passive loss of sodium from the gill (Loretz and Bern, 1982). Mammalian prolactin also reduces the branchial osmotic water permeability in the absence of environmental calcium (Ogasawara and Hirano, 1984) thus lowering the rate of water influx. As smaller fish would have a greater surface to volume ratio, they would experience a greater osmo-ionic regulatory burden. Thus the difference between the larger post-smolt fish and the smaller smolts on being transferred to FW may reflect a greater need for prolactin by the smaller fish. Plasma prolactin levels in the smolts may be lower because of an increased utilization of prolactin to counter the greater problems in maintaining homeostasis in these fish.

When the SW-adapted smolts were transferred to the calcium-enriched FW (HFW), the water and sodium concentration gradients should be the same as those encountered by the FW-transferred smolts and the response of plasma sodium and osmotic pressure was similar to both treatments. However, the HFW-transferred fish are in a hypercalcic environment, similar to SW, and these fish showed a transient hypercalcaemia. Plasma prolactin was elevated; qualitatively similar to the larger post-smolt fish. A similar elevation of plasma prolactin (in 100 g rainbow trout) upon transfer to HFW was also reported by Hirano (1986); prolactin was also elevated in FW-transferred trout to a greater degree than in the
HFW-transferred fish.

One possible explanation for the elevation in plasma prolactin seen in the HFW-transferred fish compared to the FW-transferred fish may be that, due to the lowered hypoosmoregulatory burden in the HFW fish (in that these fish do not face the same hypocalcaemic challenge as do the FW-transferred fish) the metabolic clearance rate of plasma prolactin may be lowered. An elevation of plasma prolactin would be expected if the pituitary prolactin secretion was identical in the two groups and if the metabolic clearance rate was lower in one treatment group. In the FW-transferred smolts, regulation of plasma calcium and sodium concentrations above environmental concentrations and regulation of plasma water content below the environmental level is necessary, while in the HFW-transferred fish only plasma sodium and water need to be so regulated as the hypocalcaemic challenge is removed. Thus it would be expected that the FW-transferred fish would have a greater requirement for prolactin than would the HFW-transferred fish.

As Hirano (1986) stated, "the difficulty in the interpretation ... is partly because it is imprecise to relate changes in the circulating level of a hormone with changes in its rate of release into circulation and ultimately with its level at the target sites." Prolactin is both hypernatremic and hypercalcaemic in Oncorhynchus (vide infra) and sensitive to reduced osmolality in the environment (Hirano, 1986); the response seen will be an integrated response to alterations in all these variables.

The converse treatment to HFW-transferral was to expose the SW-adapted coho salmon smolts to calcium-free artificial SW (ASW). In this hypocalcic environment, the necessity for hyper-regulation of plasma sodium and water is removed (although hypo-regulation is required) while presenting a calcium challenge to the fish.

This resulted in hypocalcaemia and depression of the plasma osmotic pressure; plasma sodium was stable in this experiment. Plasma PRL showed an initial rise at 8 h post-transfer and a fall by 24 h post-transfer. This hypocalcaemia, but not the haemodilution, was likely the stimulus for the elevated PRL level seen at 8 h as intact coho salmon smolts showed elevated PRL levels in response to experimentally induced hypocalcaemia (vide infra). That a reduction in plasma osmotic pressure may not
lead to higher levels of plasma PRL was shown by Wendelaar Bonga et al. (1985) in tilapia where a reduction of plasma osmotic pressure was accompanied by a reduction of PRL synthesis in vivo, although hypo-osmotic medium stimulated both synthesis and release in vitro (Grau et al., 1986). The depression of plasma PRL at 24h post-transfer is somewhat puzzling as the fish were still hypocalcaemic at this time.

Coho salmon smolts show a variant pattern to this treatment than do immature rainbow trout (100 g fish). Hirano (1986) transferred SW-adapted trout to calcium-free SW for times up to one week; at no time after transfer did the plasma prolactin levels in the ASW fish differ from the SW controls.

Thus, in summary, transfer of SW-adapted coho salmon to FW induced a response by plasma PRL that differed between the post-smolts and the smolts. Post-smolt coho salmon showed a pronounced and prolonged increase in plasma PRL associated with a plasma hypercalcaemia, while smaller coho salmon smolts showed little change in their plasma PRL as well as depression in plasma calcium, sodium and osmotic pressure. Transfer of coho salmon smolts to calcium enriched FW induced an increase in plasma PRL, a brief hypercalcaemia and decreases in both plasma sodium and osmotic pressure. Transfer of coho salmon smolts to acalcic SW caused a brief, rapid elevation of plasma PRL followed by a depression of this hormone as well as a hypocalcaemia and a slight depression in plasma osmotic pressure. It is concluded that PRL is involved in the FW osmoregulation of coho salmon as it is in other teleost species but that the differences seen between the post-smolts and the smolts of coho salmon indicate that both the osmoregulatory problems encountered and the pattern of FW adaptation are different in these two size or age classes. Pituitary production of PRL may be inadequate in the short term adaptation to FW by coho salmon smolts.

There was no clear correspondence between environmental calcium and plasma prolactin in the coho salmon smolts subjected to manipulations of environmental calcium. Transfer to low calcium FW did not induce elevations in the concentration of plasma prolactin, although it is possible that the metabolic turnover rate was affected. Transfer to high calcium FW did result in higher titres of plasma prolactin, likely due to depressions in both plasma sodium and osmotic pressure. Transfer to a calcium-free SW resulted in only a brief elevation in plasma prolactin although this transfer did result in hypocalcaemia. It is
clear that the adaptation to a varying environment is a complex phenomenon in smolts of coho salmon and that plasma prolactin in these animals may be responding to reductions in environmental osmotic pressure or some other unknown factor rather than to environmental calcium *per se*. 
CHAPTER III
EFFECTS OF EXPERIMENTALLY INDUCED ALTERATIONS OF PLASMA CALCIUM AND PROLACTIN IN COHO SALMON

Introduction

Calcium is an extremely important electrolyte in vertebrates and precise homeostatic regulation of extracellular calcium levels by endocrine mechanisms is characteristic (Dacke, 1979; Taylor, 1985). In teleost fish, however, the mechanisms of Ca\(^{2+}\) regulation are unclear. Parathyroid hormone (PTH), the major hypercalcemic hormone in tetrapods appears to be absent from fish (Feinblatt, 1982, Taylor, 1985) and PTH has little or no effect in fish (Feinblatt, 1982). Calcitonin (CT), the major tetrapod hypocalcemic factor, is present in fish in the ultimobranchial glands, and in the blood, often in large amounts (Dacke, 1979; Bjornsson et al., 1987), but has not been demonstrated to have a convincing effect on plasma Ca\(^{2+}\) regulation in fish (Dacke, 1979, Feinblatt, 1982) or respond to states of hyper- or hypo-calcaemia (Bjornsson et al., 1987) although CT has been reported to inhibit calcium influx across salmon gills (Milhaud et al., 1980). Vitamin D\(_3\) is abundant in fish but as Taylor (1985) stated, "the role of vitamin D\(_3\) in fish remains enigmatic".

Removal of the corpuscles of Stannius, small organs associated with the kidneys in holosteans and teleosts, results in hypercalcaemia (Pang et al., 1973a, Wendelaar Bonga and Greuven, 1978). Teleocalcin (or hypocalcin), a hormone from the corpuscles of Stannius, has recently been purified from sockeye salmon (Oncorhynchus nerka, Wagner et al., 1986) and is hypocalcemic in that it inhibits branchial uptake of Ca\(^{2+}\) in juvenile rainbow trout (Salmo gairdneri, Wagner et al., 1986).

The pituitary gland of teleosts has been implicated in calcium regulation since 1956 when Fontaine found that hypophysectomy of FW eels causes hypocalcaemia. That the pituitary factor involved is prolactin has been suspected since Pang (1973) found that hypophysectomized killifish (Fundulus heteroclitus) adapted to calcium-deficient seawater showed hypocalcaemia and tetanic seizures while levels
of other electrolytes were unaffected, effects that were corrected by administration of pituitary homogenates or ovine prolactin (Pang et al., 1973b; Pang, 1981). Other studies have shown that mammalian prolactin is hypercalcaemic in a variety of teleost species (Flik et al., 1984; Flik et al., 1986; Hasegawa et al., 1986).

Although the hypernatremic effects of teleost prolactin are well established (Farmer et al., 1977; Grau et al., 1984; Specker et al., 1985; Hasegawa et al., 1986), a hypercalcaemic effect is not established. Only one report (Hasegawa et al., 1986) has shown that prolactin purified from chum salmon (Oncorhynchus keta) is hypercalcaemic in Japanese eel (Anguilla japonica); this hormone was not hypernatremic in chum salmon fry (Hasegawa et al., 1986). Thus the objectives of this study were to determine the hypercalcaemic actions of prolactin purified from Oncorhynchus in Oncorhynchus, and the effects of raising or lowering plasma calcium on plasma prolactin.
Materials and Methods

Fresh water-adapted juvenile coho salmon (*Oncorhynchus kisutch*; 15 gm mean weight) were obtained in July 1986 from the Pacific Biological Station, Nanaimo, B.C. They were placed into 1100-l SW holding tanks and left for four months to acclimate to SW prior to experimentation. These fish were approximately the size where they would normally smoltify and migrate to SW. They were maintained on a simulated natural photoperiod and fed standard food *ad libitum*. For logistical reasons, they were maintained in recirculating, filtered SW tanks; 90% of the water was replaced twice weekly.

These fish were from the same stock as those used in the previous experiments (Chapter Two). At the time of the present experiments their mean weight was 48.2 gm (SD=16.2, n=64).

Experiment One: Injection of EGTA

In this experiment, the effect of lowering plasma calcium on plasma prolactin was determined. Four groups of 8 fish (SW-adapted) were injected with either distilled water (controls) or one of three doses of the calcium chelator (or buffer) EGTA (ethylene glycol bis-(β–aminoethyl ether) tetraacetic acid, Sigma, free acid). EGTA was chosen over EDTA as it is much more selective for calcium than for magnesium. EGTA doses employed were 1.06, 2.13, and 3.16 µmol/10 gm body weight. These doses were chosen to attempt to reduce the extracellular fluid (250 ml/kg (Milligan and Wood, 1986), less the cellular volume) calcium concentration in a 10 gm fish by 0.5, 1.0 and 1.5 mM. Post hoc calculations, using a computer programme which calculates pCa values of calcium buffers, indicated that the computed calcium reduction by the injection of the indicated EGTA doses closely approximated the desired calcium reduction (Tibbets, pers. comm.)

All fish were injected intraperitoneally with 10 µl/gm body weight of their treatment and returned to SW. Blood samples were taken 8 hours after injection from the caudal vessels of anesthetized fish (1:10,000 MS 222). Following sampling, blood was centrifuged, and the plasma fraction was frozen immediately on dry ice and then stored frozen at -20°C until analyzed.


**Experiment Two: Injection of Calcium**

In this experiment, the effect of a chronic elevation of plasma calcium on plasma prolactin was tested, by the injection of either a low (4.35 \( \mu \text{mol}/10 \text{ gm body weight} \)) or a high dose (8.75 \( \mu \text{mol}/10 \text{ gm body weight} \)) of calcium chloride into SW-adapted coho smolts. Two control groups were employed. One was injected with mannitol (131 mM) as an osmotic control, the second was injected with choline chloride (17.5 \( \mu \text{mol}/10 \text{ gm body weight} \)) as an ionic control.

Each group of eight fish was injected intraperitoneally once daily (10 \( \mu l/gm \text{ body weight} \)) for three days and was sampled (as previously) six hours after the last injection. The calcium doses were chosen in an attempt to double and triple normal extracellular fluid calcium levels.

**Experiment Three: Injection of Prolactin into FW-adapted coho smolts**

In this experiment, the SW-adapted coho smolts were transferred back to FW for 3 weeks prior to experimentation. In an effort to conserve valuable hormone, fish were selected on the basis of size (13–20 gm). The control group of 12 fish was injected with the saline vehicle (100mM NaCl, 10 \( \mu l/gm \text{ body weight} \)) and the experimental group of 10 fish was injected with 0.5 \( \mu g \) chum salmon prolactin per gm body weight (10 \( \mu l/gm \)). Fish were injected intraperitoneally once daily for three days and blood samples were taken (as previously) 8 hours after the last injection.

**Assays**

Prolactin was measured by radioimmunoassay (see Chapter one). Routinely, 20 \( \mu l \) aliquots of plasma were diluted to 100 \( \mu l \) with RIA buffer for use in the RIA. Samples were assayed in duplicate.

For analysis of sodium, plasma was diluted 1:1000 with distilled water and analysed by flame emission spectrophotometry. For calcium analysis, plasma was diluted 1:100 with 0.1% lanthanum chloride and analysed by atomic absorption spectrophotometry. Both sodium and calcium analyses were done on a Pye–Unicam SP191 Atomic Absorption Spectrophotometer.
Statistical analysis was done by t-tests or Dunnett's test (Steel and Torrie, 1980): differences were considered significant if the p value was less than 0.05.
Results

Experiment One: Injection of EGTA

The doses of EGTA employed were calculated to reduce the extracellular fluid calcium concentration by 0.5, 1.0 and 1.5 mM. The response of plasma prolactin, eight hours following a single injection of such doses, is shown in Fig. 44. Neither the low nor the intermediate doses of EGTA caused a response significantly different from controls, while the highest dose of EGTA resulted in a significant (p<0.05) elevation of plasma prolactin. Plasma sodium was unaffected by EGTA treatment (Table 3). The plasma calcium concentrations measured (Table 3) seemingly do not support the contention that injection of EGTA lowered the plasma calcium concentration (i.e., increased plasma pCa). However, two facts must be taken into consideration. Firstly, these measurements are measurements of total plasma calcium and reflect all chemical forms of calcium (including EGTA-bound calcium). Secondly, these values were taken eight hours after injection of EGTA during which time the fish will be responding to the experimental manipulation of its plasma pCa, not necessarily by prolactin-mediated mechanisms.

Experiment Two: Injection of Calcium

The two doses of calcium chloride employed in this experiment were calculated to cause an elevation of the extracellular fluid calcium levels by 2.5 or 5.0 mM. The plasma prolactin response of SW-adapted coho smolts to 3 daily injections of either a low or high dose of calcium is shown in Fig. 45.

Neither of the two control groups nor the low dose of calcium significantly affected plasma prolactin while a significant depression (p<0.05) of plasma prolactin was seen in the high dose group. Plasma calcium and sodium determinations (Table 4) showed, firstly that plasma sodium was not significantly altered by the treatments and, secondly, that six hours after the last injection, the plasma calcium concentration was approximately at normal concentrations.
The indicated doses represent μmoles EGTA injected per 10 gm body weight. The distilled water control was an osmotic control. Only the group injected with the highest dose of EGTA had plasma prolactin levels significantly different from controls (p < 0.05, mean ± 1 SEM).
Control

1.06 μmol EGTA

2.13 μmol EGTA

3.18 μmol EGTA

Plasma Prolactin (ng/ml)
Figure 45: Plasma PRL response of SW Coho Salmon injected with calcium.

The doses of calcium represent μmoles calcium injected per 10 gm body weight. The osmotic control was 131 mM mannitol which had the same osmotic pressure as the low calcium dose. The ionic control was a choline chloride solution which had the same chloride concentration and a higher osmotic pressure than the high calcium dose. Only the group injected with the high dose of calcium had plasma prolactin levels significantly different from either control group (p < 0.05, mean ± 1 SEM).
Osmotic Control

Ionic Control

4.38 μmol Ca / 10 gm

8.75 μmol Ca / 10 gm

Plasma Prolactin (ng/ml)
### Table III
*Plasma Calcium and Sodium of EGTA–Injected Coho Salmon*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma Calcium Mean ± 1 SEM</th>
<th>Plasma Sodium Mean ± 1 SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.89 ± 0.2</td>
<td>148.1 ± 2.57</td>
</tr>
<tr>
<td>Low EGTA</td>
<td>3.88 ± 0.15</td>
<td>149.5 ± 1.20</td>
</tr>
<tr>
<td>Mid EGTA</td>
<td>3.90 ± 0.31</td>
<td>157.0 ± 6.17</td>
</tr>
<tr>
<td>High EGTA</td>
<td>4.18 ± 0.12</td>
<td>151.9 ± 1.97</td>
</tr>
</tbody>
</table>

Note: Plasma calcium concentrations are total plasma calcium, as measured by Atomic Absorption Spectrophotometry. Thus they represent both the relevant ionized calcium and the biologically irrelevant calcium bound to EGTA.
Table IV

*Plasma Calcium and Sodium in Calcium–Injected Coho Salmon*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma Calcium Mean ± 1 SEM</th>
<th>Plasma Sodium Mean ± 1 SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>2.72 ± 0.08</td>
<td>158.8 ± 3.59</td>
</tr>
<tr>
<td>Choline</td>
<td>2.84 ± 0.12</td>
<td>171.4 ± 4.88</td>
</tr>
<tr>
<td>Low Calcium</td>
<td>2.88 ± 0.12</td>
<td>169.7 ± 3.73</td>
</tr>
<tr>
<td>High Calcium</td>
<td>3.02 ± 0.11</td>
<td>160.6 ± 3.56</td>
</tr>
</tbody>
</table>
That the prolactin response seen in the high dose calcium group was not due to either perturbations of plasma osmotic pressure or plasma chloride levels (that may have been caused by the type of compounds injected) is shown in that neither the control treatments induced a response.

**Experiment Three: Injection of Prolactin**

The response of FW-adapted coho to 3 daily injections of 0.5 μg/gm body weight salmon prolactin is shown in Figs. 46 and 47. This treatment resulted in significant hypercalcaemia and hypernatremia (p < 0.05) compared to the vehicle injected controls.
Figure 46: Plasma calcium response of FW Coho Salmon injected with sPRL.

The prolactin treated fish had a significantly higher plasma calcium concentration than the controls ($p < 0.05$, mean ± 1 SEM).
Control

0.5 μg Prl / gm

Plasma Calcium (mM)
Figure 47: Plasma sodium response of FW Coho Salmon injected with sPRL.

The prolactin treated fish had a significantly higher plasma sodium concentration than the controls (p < 0.05, mean ± 1 SEM).
Control
0.5 μg Prl / gm

Plasma Sodium (mM)
Discussion

These experiments provide evidence that salmon PRL (sPRL) acts as a hypercalcaemic hormone in *Oncorhynchus*. Lowering of plasma ionized calcium by the injection of the calcium chelator EGTA, resulted in an elevation of plasma PRL. Chronically raising plasma calcium by injection of calcium chloride resulted in lowering of plasma PRL. Injection of sPRL resulted in both elevated calcium and sodium in the plasma of juvenile *Oncorhynchus kisutch*.

The hypernatremic action of mammalian PRL in some euryhaline teleosts has long been known, since the demonstration by Pickford and Burden in 1959 that removal of the pituitary gland from the mummichog, *Fundulus heteroclitus*, abolished FW survival and resulted in depressed plasma sodium levels, effects which were corrected by administration of mammalian PRL. Many other fish (e.g., *Poecilia*, *Oreochromis* and *Gasterosteus*) have also been shown to die in FW following hypophysectomy unless treated with mammalian PRL, and the specific action of mammalian PRL in maintaining plasma sodium levels in FW has been demonstrated many times (for review see Clarke and Bern, 1980). This sodium-conserving action is the basis of bioassays for fish PRL in teleosts (Clarke, 1973; Grau et al., 1984). In brown trout (*Salmo trutta*), a salmonid, PRL has also been shown to be a requirement for FW survival (Oduleye, 1976).

Chum salmon PRL has been shown to have hypernatremic effects in killifish, ayu (*Plecoglossus altivelis*), juvenile rainbow trout (*Salmo gairdneri*), and Japanese eels (*Anguilla japonica*) (Hasegawa et al., 1986). There is a paucity of studies of the actions of PRL in *Oncorhynchus*. Hasegawa et al. (1986) investigated the ionic effects of chum salmon PRL injected into SW-adapted chum salmon fry (*Oncorhynchus keta*), both in SW and after transfer to FW. Neither chum salmon PRL nor ovine PRL (at doses from 1 ng to 1 µg/gm body weight) had any effects on plasma sodium levels in these fish; they did not report any calcaemic effects of these hormones.
The lack of an ionic response to administration of prolactin (Hasegawa et al., 1986) and the positive response obtained in the present study may be due to methodological differences. They sampled their fish 24 hours following a single injection while I injected salmon prolactin once daily for 3 days and sampled 8 hours following the final injection. Bern et al. (1981) reported that prolactin-sensitive organs from a variety of species did not show a short term response to prolactin and concluded that a longer term exposure to prolactin, for a priming action, may be a prerequisite to obtaining rapid effects. Hasegawa et al. (1986) themselves found that the hypernatremia and hypercalcaemia seen in Japanese eels after injection of either ovine or chum salmon prolactin was maximal 24 hours after the last of 3 daily treatments.

The hypercalcaemic action of mammalian prolactin was shown by Pang et al. (1973b) when the hypocalcaemia in hypophysectomized killifish adapted to calcium deficient-seawater was corrected by injection of carp (Cyprinus carpio) pituitary homogenates and that injections of ovine PRL caused a log dose dependent hypercalcaemia (Pang et al., 1978) in hypophysectomized killifish adapted to calcium-deficient SW (Pang et al.; 1978, Pang, 1981) or 1.2% NaCl, but was without effect in hypophysectomized killifish adapted to FW, calcium-enriched FW or 0.6% NaCl (Pang, 1981). Ovine PRL was hypercalcaemic in intact killifish adapted to SW or calcium-deficient SW (Pang, 1981).

Ovine PRL has also been shown to stimulate net uptake of Ca++ from FW by tilapia (Oreochromis mossambicus, Flik et al., 1986) and, in American eels (Anguilla rostrata), to stimulate a high affinity branchial epithelial Ca++-ATPase (Flik et al., 1984) which may be the calcium pump. Ovine PRL is also hypercalcaemic in tilapia (Flik et al., 1986), American eels (Flik et al., 1984) and Japanese eels (Hasegawa et al., 1986). Chum salmon prolactin is also hypercalcaemic in Japanese eels (Hasegawa et al., 1986).

The present study is the first report of direct hypercalcaemic and hypernatremic effects of chum salmon PRL in Oncorhynchus and provides supporting evidence for the hypothesis that PRL is an ionoregulatory hormone in salmon.
SUMMARY

Prolactin was purified from pituitary glands of chum salmon by chromatographic techniques. The molecular weight was estimated to be 25,000 daltons and its purity was established by the detection of a single band in acid-urea PAGE, SDS PAGE, 2–dimensional PAGE and Western blots. The amino acid residue analysis was similar to, and the sequence of the first ten amino acids identical to, reported values. Only one N-terminal amino acid was detected.

The biological activity of the purified hormone was established in the hypophysectomized mummichog bioassay. Immunocytochemical staining of salmon pituitary glands showed a specific reaction only with lactotropes; such staining could be blocked by the pure hormone. A sensitive and specific radioimmunoassay was developed and validated for use in measuring salmon prolactin.

Transfer of SW–adapted juvenile coho salmon to FW induced a size–dependent response of plasma prolactin with post–smolts showing a pronounced and prolonged increase in plasma prolactin, an associated hypercalcaemia and little disturbance of plasma sodium. Smaller smolts, upon transfer to FW, showed little change in plasma prolactin but had depressed levels of plasma calcium, and sodium, and decreased osmotic pressure.

The role of environmental calcium in prolactin physiology was investigated by the transfer of SW–adapted smolts to high calcium FW and to calcium–free SW. There was no clear correspondence between environmental calcium levels and plasma prolactin; transfer to high calcium FW resulted in higher levels of prolactin, a brief hypercalcaemia and decreases in plasma sodium and osmotic pressure; transfer to calcium–free SW caused a brief rapid elevation then depression of plasma prolactin levels with lowered plasma sodium and osmotic pressure levels.

A hypercalcaemic role for salmon prolactin was established by manipulations of plasma calcium and injection of pure hormone. Lowering of plasma calcium caused an elevation of plasma prolactin, raising plasma calcium caused a depression of plasma prolactin and raising plasma prolactin elevated both plasma
calcium and sodium.
REFERENCES

Ball, J.N. 1969. Prolactin (fish prolactin or paralactin) and growth hormone. In: Fish Physiology Vol 2. (W.S.Hoar and D.J. Randall (eds)). Academic Press, NY


APPENDIX A

The following is a copy of the amino acid sequencing results as obtained from the Tri-Partite Microsequencing Centre, University of Victoria.
<table>
<thead>
<tr>
<th>cycle no</th>
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