# VITELLOGENESIS AND ASPECTS OF ITS PITUITARY REGULATION
# IN TELEOSTS WITH EMPHASIS ON WINTER FLOUNDER
## (Pseudopleuronectes americanus)

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by


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**ABSTRACT**

The ovarian uptake of the homologous serum proteins vitellogenin (VG) and very high density lipoprotein II (VHDL II) (formerly peak A protein) were studied as potential yolk precursors involved in vitellogenesis in winter flounder (Pseudopleuronectes americanus). The major yolk precursor appears to be VG based on the quantity of yolk protein that recognizes the VG and VHDL II antisera by Western blotting. The rate of uptake of VG by the ovary is about three times greater than VHDL II. Internalized VG is processed into a 280,000 relative molecular mass ($M_r$) yolk protein (lipovitellin) that contributes to the major fraction (82%) of ovarian protein. Accumulation of VHDL II occurs in an unprocessed form and contributes to a fraction of ovarian protein representing 12% of the total. Phosvitin and a low $M_r$ phosphoprotein were apparent but in small amounts.

In vitro ovarian incubations done during the prespawning to early vitellogenic phases of the reproductive cycle in winter flounder showed that pituitary extract stimulates estradiol-17$\beta$ ($E_2$) production only during the vitellogenic phase, while induced testosterone (T) production was greatest shortly before spawning. These observations were reflected in the seasonal pattern of serum levels of $E_2$ and T in female winter flounder. To investigate the effect of sockeye salmon carbohydrate-poor (Con A I) and carbohydrate-

rich (Con A II) pituitary protein fractions on $E_2$ production, ovarian follicles with (intact) or without the surface epithelium-thecal cell layer (defolliculated) from rainbow trout (_Oncorhynchus mykiss_) were incubated _in vitro_. It was demonstrated that Con A I is capable of significantly increasing $E_2$ production in defolliculated ovarian follicles while under similar conditions Con A II (containing the maturational gonadotropin) was not. Purified salmonid and pleuronectid growth hormones (GHs) were tested for their ability to increase either $E_2$ and T production during _in vitro_ ovarian incubations in both rainbow trout and winter flounder respectively, but were found to be inactive.

Growth hormones were isolated from the pituitaries of sockeye salmon (_Oncorhynchus nerka_) and American plaice (_Hippoglossoides platessoides_). A bioassay based on the increase of serum triiodothyronine in rainbow trout was developed to follow GH biological activity during pituitary fractionation. The isolation of a pituitary protein from sockeye salmon that was active in the bioassay was confirmed as monomeric GH by an amino-terminal (N-T) amino acid sequence. In plaice GH variants were isolated from two $M_r$ regions within the pituitary, 42,000 and <33,000, that were active in the bioassay and had identical N-T amino acid sequences. The 42,000 $M_r$ form predominates in the plaice pituitary making up 93% of the total.

## ACKNOWLEDGEMENTS

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

Ab, antibody

AcA 54, Ultrogel AcA 54

ALPP, alkali-labile protein phosphorus

$B/B_o$, 50% binding/0% binding

BSA, bovine serum albumin

C-T, carboxyl-terminal

Con A, Concanavalin A Sepharose

Con A I, carbohydrate-poor fraction

Con A II, carbohydrate-rich fraction

cpm, counts per minute

dpm, degradations per minute

$E_2$, estradiol-17$\beta$

G, gravity

GH, growth hormone

GSI, gonadosomatic index

GTH, gonadotropin

$^3$H, tritium

$^{131}$I, $^{131}$iodine

ip, intraperitoneal

iv, intravenous

2-ME, 2-mercaptoethanol

mGTH, maturational-type gonadotropin

$M_r$, relative molecular mass

MSRL, Marine Sciences Research Laboratory

N-T, amino terminal

PAGE, polyacrylamide gel electrophoresis

PE, pituitary extract

Pk A, peak A protein

$R_f$, relative mobility

RIA, radioimmunoassay

S-300, Sephacryl S-300

SDS, sodium dodecyl sulfate

SE, standard error

SSOE, salt soluble ovarian extract

T, testosterone,

$T_3$, triiodothyronine

$T_4$, thyroxine

TCA, trichloroacetic acid

Vo, void volume

VG, vitellogenin

VHDL, very high density lipoprotein

# CHAPTER 1

## GENERAL INTRODUCTION

A major component of oocyte growth in all oviparous vertebrates derives from the accumulation of blood plasma lipoproteins laid down as yolk in the ovary (Wallace 1985). This yolk represents the sole nutritional reserve of the eventual offspring. The process of yolk protein formation and its hormonal regulation can be broadly termed vitellogenesis. The most well studied yolk precursor protein is vitellogenin (VG) (Wallace 1985). Vitellogenin, synthesized in the liver and released into the blood, enters the oocyte via receptor-mediated endocytosis and is proteolytically cleaved to form the yolk proteins lipovitellin, phosvitin and smaller units called phosvettes. In amphibians the majority of yolk formed is attributed to uptake of VG (Wallace et al. 1972). However, in birds serum very low density lipoprotein is actively acquired by the ovary and contributes more to the yolk than VG (Wallace 1985). With respect to fish VG is established as a yolk precursor (Weigand 1982, Ng and Idler 1983, Wallace et al. 1987, Tyler et al. 1988a,b).

There are differences amongst marine teleosts and other oviparous vertebrates with respect to yolk formation and composition. The yolk proteins lipovitellin and phosvitin

are present in a soluble form in most marine teleost
oocytes. This is in contrast to other egg laying vertebrates
where the yolk is insoluble due to its formation into
crystalline structures termed yolk platelets (Wallace et al.
1966, Wallace and Selman 1981, Wallace 1985). Some marine
teleosts may have little or no phosvitin within the yolk
(Jared and Wallace 1968) and teleosts in general have been
regarded as having atypical yolk protein patterns when
compared with other oviparous vertebrates (Wallace 1985).
Finally, in teleosts plasma lipids, carotenoids,
glycoproteins and vitamin-binding proteins also gain access
to the ovary in addition to VG (Weigand 1982, Mommsen and
Walsh 1988). Relative to VG the specific contributions of
these other extraovarian components to teleost yolk protein
are not known.

The pituitary gland regulates reproduction in teleosts
(Idler and Ng 1983). Gonadotropins are the pituitary
hormones that regulate reproductive processes and in female
fish their biological actions can be categorized as
vitellogenic and maturational. Vitellogenic activities are
involved with yolk formation which entails extraovarian
lipoprotein synthesis and its ovarian uptake. Maturational
activities deal principally with the regulation of ovarian
sex steroid hormones before spawning which re-initiate
meiosis within the oocytes and regulate ovulation.

Initially fish were thought to have one gonadotropin

(GTH) which was responsible for both vitellogenic and maturational events. To date there is no consensus as to the number of definitive GTHs that regulate these reproductive processes. However, Idler et al. (1975a,b) proposed the existence of a second pituitary GTH besides the "classical" GTH known in fish and showed using chum salmon (Oncorhynchus keta) pituitaries how it could be separated on Concanavalin A affinity media due to its low carbohydrate content. This led to the recognition of two groups of GTHs in fish termed Con A I (carbohydrate-poor) and Con A II (carbohydrate-rich). Subsequently, Ng and Idler (1979) determined that American plaice (Hippoglossoides platessoides) and winter flounder (Pseudopleuronectes americanus) pituitary Con A I facilitated the uptake of radiolabelled leucine and phosphorus into the developing ovary of winter flounder, while Con A II was primarily involved with maturational activities. Later, a purified protein ($R_f$ 0.7 protein) from chum salmon Con A I was shown to stimulate ovarian uptake of radiolabelled VG in landlocked Atlantic salmon (Salmo salar ouananiche) (Idler and So 1987). The work of Idler and colleagues suggest that Con A I type GTH is vitellogenic while Con A II type GTH controls maturational events, although the Con A II GTH is thought to regulate estrogen synthesis which induces hepatic VG production (Ng and Idler 1983, Mommsen and Walsh 1988). More recently Swanson et al. (1987a,b) and Suzuki et al. (1988a,b) have isolated multiple

glycoprotein GTHs from coho (<u>Oncorhynchus</u> <u>kisutch</u>) and chum salmon respectively, but these preparations have biological activities such as inducing gonadal steroid production that classify them as maturational-type GTHs (mGTH).

Vitellogenin produced by the liver is under the control of ovarian estrogens, principally estradiol-17$\beta$ ($E_2$), in fish (Ng and Idler 1983, Mommsen and Walsh 1988). The site of $E_2$ biosynthesis in the salmonid ovary is the granulosa cell layer within the ovarian follicle (Kagawa et al. 1982). This follows from the "two-cell" model first proposed in mammals by Falck (1959) whereby testosterone (T) synthesized in the outer thecal cell layer is aromatized via the aromatase enzyme in the inner granulosa cell layer to $E_2$. In salmonids ovarian $E_2$ synthesis is controlled by the pituitary and mGTH (i.e. Con A II) is considered the hormonal mediator distinct from vitellogenic GTH (i.e. Con A I) (Idler and Campbell 1980, Ng et al. 1980). A number of experimental studies both <u>in</u> <u>vivo</u> (Billard et al. 1978, Breton et al. 1985) and <u>in</u> <u>vitro</u> (Kagawa et al. 1982, Zohar et al. 1982, Young et al. 1983b, Van Der Kraak and Donaldson 1986, Kanamori et al. 1988) have demonstrated that mGTH can increase ovarian $E_2$ production. In contrast accumulated evidence from studies on natural blood levels of mGTH and $E_2$ in female salmonids during the reproductive cycle do not indicate an interdependence of one with the other (Billard et al. 1978, Scott and Sumpter 1983, Sumpter et al. 1984).

During the vitellogenic period when plasma levels of $E_2$ increase progressively to their highest levels in female rainbow trout (<u>Oncorhynchus mykiss</u>) (Scott and Sumpter 1983, Scott <u>et al</u>. 1980) plasma levels of mGTH remain very low (<0.5 ng/ml) and unchanged (Sumpter and Scott 1989). In the amago salmon (<u>Oncorhynchus rhodurus</u>) mGTH was shown to be inactive in enhancing $E_2$ production by ovarian granulosa cell preparations incubated <u>in vitro</u> with exogenous T as aromatizable substrate (Kagawa <u>et al</u>. 1982, Young <u>et al</u>. 1983a). The inability of mGTH to affect granulosa cell aromatization persists throughout the reproductive cycle of this species (Kanamori <u>et al</u>. 1988). Clearly discrepancies remain concerning the role of mGTH in regulating $E_2$ production in the salmonid ovary.

Another hormone of pituitary origin, growth hormone (GH), has recently been found to influence the production of $E_2$ by the ovary. Studies showed that GH could increase the level of ovarian $E_2$ production, both <u>in vivo</u> and <u>in vitro</u>, in killifish (<u>Fundulus heteroclitus</u>) and by rainbow trout ovarian fragments <u>in vitro</u> (Singh <u>et al</u>. 1988). In goldfish (<u>Carassius auratus</u>) GH appears to potentiate the effect of mGTH in stimulating both $E_2$ and T production by ovarian follicles <u>in vitro</u> while by itself it is inactive (Van Der Kraak <u>et al</u>. 1990). These studies indicate that GH could have effects on the synthesis of ovarian sex steroids. This is particularly interesting since GH is found in the Con A I

pituitary fraction (Komourdjian and Idler 1979, Idler et al.
1989). Previous studies in male teleosts have documented
steroidogenic capabilities of the Con A II fraction (i.e.
containing mGTH) and have also reported some androgen
stimulating capacity in the Con A I fraction (Ng and Idler
1979, Van Der Kraak and Peter 1987). It is possible that
components of the Con A I fraction may be involved in the
synthesis of ovarian sex steroids particularly when mGTH is
unavailable or unable to regulate processes attributable to
the pituitary gland.

   The objectives of this study therefore are to: 1) explore
the uptake and processing of VG and another homologous serum
protein, as potential yolk precursors in the winter
flounder, 2) examine the pituitary regulation and possible
involvement of GH, in ovarian $E_2$ synthesis of winter
flounder and rainbow trout, and 3) isolate the salmonid and
pleuronectid GHs to be used for objective #2.

# CHAPTER 2

## OOCYTE YOLK FORMATION IN THE WINTER FLOUNDER

### INTRODUCTION

Studies have been undertaken to ascertain if serum proteins other than VG become associated with the ovary of winter flounder. Bhat (1986) and So and Idler (1987) have provided evidence that other serum proteins besides VG are incorporated into winter flounder ovaries. All the crude serum protein fractions separated based on molecular mass that are normally present in the sera of adult females were shown to become associated with the ovary to some extent (So and Idler unpublished). Of particular interest is a high relative molecular mass ($M_r$) serum protein (1,170,000) found in both sexes. This protein has been termed peak A protein (Pk A) in reference to it being the first serum protein eluting on Sephacryl S-300 (S-300) gel filtration media. By comparison winter flounder VG has a native $M_r$ of 514,000. Peak A protein in terms of its biochemical characteristics is more like VG than any other serum protein both being glycolipophosphoproteins. However, native and denaturing polyacrylamide gel electrophoresis (PAGE), enzymatic degradation and immunocross-reaction studies indicate that the two proteins are structurally distinct

from one another (Bhat 1986).

From a comparative viewpoint Pk A appears a likely candidate to contribute to ovarian yolk protein along with VG due to their similar biochemical makeup although a number of questions remain. What are the seasonal serum concentrations of these two proteins in reproductively active females? In other female teleosts described to date VG levels are typically elevated during the vitellogenic period (So et al. 1985, Scott and Sumpter 1983). Vitellogenins are usually characterized as very high density lipoproteins (VHDL) (Babin and Vernier 1989, Schjeide and Schjeide 1981) but the lipoprotein density of both winter flounder VG and Pk A are not known. The identification of the eventual end product(s) of Pk A in the developing oocyte would allow a comparison with yolk proteins derived from VG. In summary, the specific contribution by VG and Pk A towards winter flounder yolk both in terms of qualitative and quantitative characteristics is unknown.

To date no other studies on fish have compared the uptake of VG and any other homologous serum protein known to be incorporated by the ovary during the reproductive cycle. The present study sought to compare VG and Pk A in terms of their seasonal serum levels by radioimmunoassay (RIA), plasma lipoprotein class, uptake by the ovary, and qualitative and quantitative relationship to yolk proteins in the winter flounder.

# MATERIALS AND METHODS

## Fish

Mature winter flounder (_Pseudopleuronectes americanus_
Walbaum) were captured throughout the years 1986-1990 by
SCUBA divers using handnets from Conception Bay,
Newfoundland. Fish were brought to the Marine Sciences
Research Laboratory (MSRL) where they were maintained in
tanks (10-15 fish/240 l tank) with ambient seawater and
natural photoperiod. Fish held from June to October were fed
chopped caplin (_Mallotus villosus_) to satiation three times
per week. Since feeding normally ceases in this species
during winter and early spring (Fletcher and King 1978) no
food was provided at this time of year. Experiments reported
herein were conducted from October to January during the
mid-vitellogenic phase of the female reproductive cycle
which is characterized by rapid gonad growth as a result of
yolk formation within the developing oocytes. Female winter
flounder typically weighed 425-750 g with average
gonadosomatic indices (GSI) (ovary weight/body weight-ovary
weight x 100) that varied from 10 to 18. Sampling and
experiments were always conducted approximately 2 weeks
after the fish were obtained.

**Isolation of serum vitellogenin and peak A protein**


To increase the serum VG titre fish were injected intraperitoneally (ip) with $\beta$-estradiol-3-benzoate (Sigma) suspended in peanut oil (10 mg/kg) 1 month before blood collection (So et al. 1985). Serum VG degradation was prevented by injecting all fish via caudal vessels with 2 ml of aprotinin (15-30 trypsin inhibitor units/ml) (Sigma) 15 minutes prior to blood sampling (Wallace and Selman 1982). Blood was sampled by needle and syringe from the caudal vasculature of unanesthetized fish, usually in the morning, and allowed to clot in sterile plastic tubes (# 2006, Falcon) overnight at 4°C. Serum was separated by centrifugation at 13,000x gravity (G) the following day and then used immediately or frozen at -60°C for up to 3 years.

The blood serum proteins of female winter flounder were separated based on their $M_r$ by low pressure gel filtration column chromatography on S-300 (2.6 x 90 cm) (Pharmacia) using VG buffer (pH 8.0) (So et al. 1985) containing 0.4 $\mu$g aprotinin/ml as column eluent. Column chromatography was conducted at 5°C in a cold room at a flow rate of 15 ml/hour using ascending elution. The column was calibrated with thyroglobulin (669,000), ferritin (440,000) and aldolase (158,000) $M_r$ standards supplied by Pharmacia. The optical density at 280 nm of the eluate fractions were monitored in a spectrophotometer (Ultrospec II, LKB) using a 1 cm quartz

cuvette. To isolate VG the peak tubes were concentrated and
re-chromatographed on S-300 to remove any contamination from
adjacent peaks. Both the VG and Pk A fractions were further
purified by affinity chromatography on Concanavalin A
Sepharose (Con A) (Pharmacia) collecting the adsorbed
fraction following elution with α-methyl-D-glucoside (Idler
and Hwang 1978). Only the peak tubes were utilized when
recovering serum proteins. Purified Pk A and VG were
aliquoted, stored at -60°C and never re-frozen afterward.

Serum protein concentration was determined by
trichloroacetic acid (TCA) precipitation followed by the
Lowry method (Lowry et al. 1951) using bovine gamma globulin
(BioRad) as standard.

Native (analytical) PAGE of serum proteins isolated on S-
300 was done using the method of Davis (1964). Briefly, 10
cm gels (5% acrylamide: 0.2% N,N'-methylenebisacrylamide, pH
8.9) were cast in 5 x 125 mm glass tubes and electrophoresed
using a discontinuous buffer system (5 mM tris, 38 mM
glycine, pH 8.3) in a BioRad Model 150 electrophoresis cell.
Electrophoresis was conducted at a constant 2
milliamperes/gel (300 volts) at room temperature with
tapwater cooling until the bromophenol blue tracker dye
reached 1 cm from the bottom of the gel (usually about 3
hours). Following electrophoresis gels were stained with
0.1% Coomassie R-250 and destained by diffusion in 7% acetic
acid.

Denaturing sodium dodecyl sulfate (SDS) PAGE of VG and Pk A were performed on uniform 10% slab gels (33.5% acrylamide: 0.3% N,N'-methylenebisacrylamide, pH 9.1, containing 10% SDS), 1.5 x 120 x 140 mm$^3$, with a 1 cm 4% stacking gel (30% acrylamide: 0.44% N,N'-methylenebisacrylamide, pH 6.8, containing 10% SDS) following Dreyfuss et al. (1984). Samples were mixed with the same volume of sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol) containing 10% 2-mercaptoethanol (2-ME) and placed in boiling water for 5 minutes before application onto the gels. A Hoeffer Model SE 400 was used at room temperature run at constant 100 volts for 16 hours (or until the bromophenol blue tracker dye had run off the gel) with a typical reservoir buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS). Gels were fixed for 1 hour in 50% methanol-10% acetic acid, stained for 2 hours in 0.3% Coomassie Blue R-250 in 50% methanol-15% acetic acid and de-stained by diffusion in 10% methanol-10% acetic acid. Relative molecular masses were estimated from protein standards (LMW and HMW, Pharmacia) run on adjacent lanes of the gels. Densitometric scans (Model 1650, BioRad) were used to calculate the relative mobility ($R_f$) of standards and unknowns, and a semi-logarithmic plot of standard's log $M_r$ vs $R_f$ (Fig. 1) was used to determine the $M_r$ of the unknowns based on their $R_f$.

Protein phosphorus determinations of serum proteins were made using the alkali-labile protein phosphorus (ALPP)
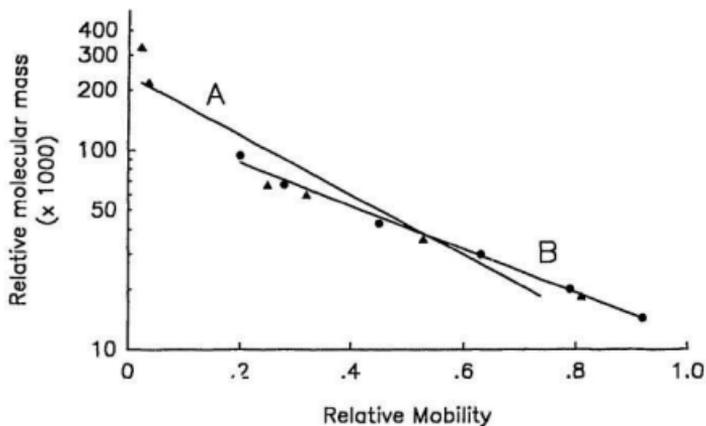
Figure 1: Calibration lines for sodium dodecyl sulfate
polyacrylamide gel electrophoresis, represented by
semi-logarithmic plots of protein standard's log
relative molecular mass versus their relative
mobility. A = HMW Pharmacia, B = LMW Pharmacia.

protocol outlined in Nagler et al. (1987).

The amino acid composition of Pk A and VG were determined by phenyl-thiocyanate derivitization (Pico-Tag, Waters) and quantification by reverse-phase (C-18) high pressure liquid chromatography (Spectra-Physics).

For ultracentrifugation analysis blood was obtained from 15 females and pooled. Plasma was isolated by placing blood in heparinized glass tubes (Vacutainer 4783, Becton-Dickinson), kept at 4°C overnight and centrifuged as above the following day. Five ml aliquots of plasma were used to isolate the various lipoprotein classes based on the same density intervals used to separate human plasma lipoproteins: very low density lipoprotein, d<1.006; low density lipoprotein, d=1.006-1.063; high density lipoprotein, d=1.063-1.21, and VHDL, d>1.21. Preparative ultracentrifugation was conducted in polycarbonate tubes with a Beckman L5-50 (50 Ti rotor) at 140,000x g (10°C) following the method of Ewing et al. (1965). Lipoproteins recovered were frozen at -60°C until used. Protein content was determined by the TCA-Lowry method described above using bovine gamma globulin as standard.

## Radiolabelling of vitellogenin and peak A protein

Vitellogenin and Pk A were radiolabelled with $^{131}$iodine ($^{131}$I) (NEN DuPont) in vitro using Iodogen (Pierce) following

the protocol described by So et al. (1985). This involved
reacting 10 μg of either VG or Pk A (in 25 μl of 0.5 M
phosphate buffer, pH 7.4) in a 6 x 50 mm borosilicate glass
tube with 10 μg of Iodogen and 1.5 mCi of $^{131}$I for 12
minutes. The reaction was terminated by adding 500 μl of 50
mM phosphate buffer (pH 7.4) for a further 12 minutes before
applying the mixture to a disposable Sephadex G-25M column
(PD-10, Pharmacia) to separate labelled protein from free
isotope. The first radioactive fraction eluted containing
the labelled protein was subsequently cleaned-up before use
on a disposable plastic column (0.8 x 18 cm, Kontes) packed
with S-300. The column was previously rinsed with 1 ml 5%
bovine serum albumin (BSA) (Sigma) and only the peak tube
from the first radioactive fraction eluted in each case was
recovered. Iodinated VG and Pk A were used in RIA and for
short term ovarian uptake studies in vivo .

Vitellogenin and Pk A were also radiolabelled with
tritium ($^3$H), for longer term ovarian uptake studies by both
in vivo and in vitro techniques. Labelling of VG in vivo
involved ip injection of females with β-estradiol-3-benzoate
one day prior to ip injection of a [$^3$H] amino acid
hydrolysate (TRK 440, Amersham) carrier-free at 0.5 mCi/kg
fish. After two weeks fish were blood sampled and the
resulting serum applied on S-300 as previously described for
the isolation of VG (pg. 10). The protocol was similar for
$^3$H labelling of Pk A in vivo, except that the β-estradiol-3-

benzoate injections were omitted and fish were blood sampled after four weeks. For both proteins the fraction containing the [$^3$H]VG or [$^3$H]Pk A from a number of chromatographic separations were pooled, concentrated by ultrafiltration (YM10, Amicon) and frozen at -60°C until required.

Proteins were labelled in vitro by modifications to the method of Asher et al. (1983). This method, used for radiolabelling locust VG, is based on chemical modification of lysine residues by reaction with [$^3$H]-succinimidyl propionate. Proteins were ultrafiltered (YM5, Amicon) against 0.1 M borate buffer, pH 8.5. Succinimidyl propionate, N-[propionate-2,3-$^3$H] (56.8 Ci/mmol), was purchased from NEN DuPont. Modifications to Asher et al. (1983) included 1) increasing the amount of protein reacted (38 μg), 2) decreasing the reaction time when it was found that 1 hour at room temperature gave a better recovery than 16 hours at 4°C, 3) stopping the reaction with 250 μl of borate buffer, and 4) applying the reaction mixture directly onto a disposable plastic column (0.8 x 18 cm, Kontes) packed with S-300 at 5°C, previously rinsed with 1 ml 5% BSA and using borate buffer as eluent. Peak tubes of the first radioactive fraction eluted containing either [$^3$H]propionyl-VG or [$^3$H]propionyl-Pk A were lyophilized and reconstituted with 0.9% saline for injection.

### Radioimmunoassays for vitellogenin and peak A protein

Polyclonal antibodies were raised for 6 months in male
New Zealand White rabbits against purified Pk A and VG
following the protocol of Idler and Ng (1979). The Pk A
antibody (Ab) obtained was diluted at 1:600,000 while those
of VG 1:150,000 to give 50% binding of radioiodinated
antigen, respectively. The RIA protocol, using a double-Ab
method, was the same as that described for salmon VG by So
et al. (1985). The log-logit weighted regression lines for
Pk A and VG, respectively, had $r^2$s = 0.993 and 0.995, slopes
of -1.936 and -1.506, intercepts of 0.826 and 1.876 and 50%
binding/0% binding ($B/B_o$) of 2.67 and 17.6 ng. The useful
range of the standard curve for Pk A was 0.13-65.4 ng and
for VG 0.85-219 ng (Fig. 2). Vitellogenin had 0% cross-
reaction in the Pk A RIA and Pk A had 0% cross-reaction in
the VG RIA. Blood samples were collected monthly during
1986-1987 from female winter flounder to provide seasonal
serum profiles for Pk A and VG.

### Salt soluble ovarian extract immunoreactivity with
### vitellogenin and peak A protein antisera

Ovarian follicles are small (<0.5 mm) and difficult to
isolate individually in any number, therefore pieces of
ovary from 2 female winter flounder were dissected free of
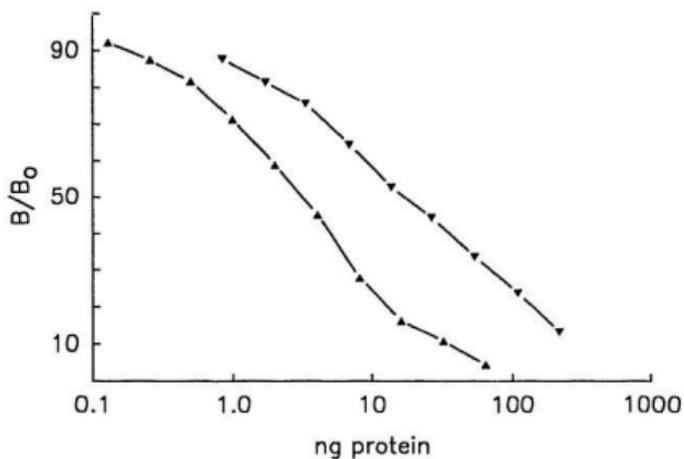
Figure 2: Standard curves developed from radioimmunoassays, represented as %binding/0% binding (B/B$_0$) versus log dose for winter flounder vitellogenin (▼) and peak A protein (▲).

the ovarian wall and utilized to obtain salt soluble ovarian
extracts (SSOE). Teleost yolk proteins are soluble in 0.5 M
NaCl (Wallace et al. 1966, Plack et al. 1971). Portions of
ovary (330 mg) were homogenized (power setting 3, Polytron,
Brinkmann) and extracted with 4 volumes of VG buffer. After
24 hours at 4°C the salt soluble portion of the ovarian
extract was separated from follicle cell and vitelline
envelope debris by centrifugation at 19,000x G for 1 hour.
Salt soluble extracts were then placed in dialytic bags
(6,000-8,000 $M_r$ cut-off, SpectraPor), precipitated by
dialysis against 5 mM $CaCl_2$, and separated by centrifugation
at 19,000x G for 1 hour. The precipitated fraction was re-
dissolved in VG buffer and chromatographed on S-300.
Following chromatography every fifth eluate fraction from
across the profile was measured for immunoreactivity in both
the VG and Pk A RIAs.

## Denaturing electrophoresis of oocyte proteins

Oocyte proteins for denaturing SDS PAGE were obtained
from freshly removed ovaries of 2 female winter flounder.
Follicles (0.2-0.3 mm) were dissected from stromal tissues
with fine forceps under a stereoscopic microscope and
transferred with a glass Pasteur pipette. They were rinsed
twice in modified (158 mM NaCl) FO solution (a physiological
fish oocyte buffer; Wallace and Selman 1978) and collected

each time by gentle centrifugation. Batches of 10 or 20
follicles were placed in 500 $\mu$l polypropylene micro test
tubes (BioRad) with a minimum of FO solution, lyophilized,
and stored desiccated at -20°C until used. Lyophilized
follicles were ground up with a small glass rod, taken up in
sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 12.5%
glycerol, 40 mM dithiothreitol and 0.002% bromophenol blue)
and placed in boiling water for 5 minutes before
centrifuging to remove follicular debris. Total protein
content of oocyte proteins (in sample buffer without
bromophenol blue) was estimated using the Pierce Coomassie
protein assay reagent and BSA standard. Samples were applied
to 5-20% linear gradient slab gels (30% acrylamide:0.8%
N,N'-methylenebisacrylamide, pH 8.8, containing 10% SDS)
(Hames 1981), 1.5 x 120 x 140 mm$^3$, overlaid with a 1 cm 3%
stacking gel (acrylamide ratio as above, pH 6.8, containing
10% SDS). Electrophoresis conditions, staining and de-
staining were identical to the protocol outlined for serum
proteins described earlier (pg. 12). The $M_r$s were estimated
from protein standards (SDS-7 and SDS-6H, Sigma) run on
adjacent lanes of the gels. Densitometric scans were used to
calculate the $R_f$ of standards and unknowns allowing the
lowest $M_r$ standard (14,200) an $R_f$ value of 1.0. Similar to
SDS PAGE analysis for serum proteins a semi-logarithmic plot
of standard's log $M_r$ vs $R_f$ was used to determine the $M_r$ of
the unknowns based on their $R_f$.

# Western blotting and immunoassay analysis of oocyte proteins

Oocyte proteins separated by SDS PAGE above were electrophoretically transferred onto nylon membranes (Zeta-Probe, BioRad) in a Hoeffer Model TE 52 equipped with a heat exchanger. The gel was incubated in 200 ml of transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine) for 10 minutes and then placed in the transfer cassette sandwiched against the nylon membrane between sheets of blotter paper (3MM, Whatman) and foam pads, and clamped together. Transfer conditions were typically 0.6 amperes at 2°C for 10 hours to remove all the protein from the gel.

Following transfer the membranes were analyzed using an enzyme-immunoassay employing a primary Ab (either Pk A or VG) and a secondary alkaline phosphatase conjugated Ab (Protoblot, Promega) for immunodetection and subsequent colour reaction. Modifications to the protocol supplied by Promega included blocking the nylon membrane with 10% BSA in Tris-buffered saline with Tween 20 for 12 hours at 50°C, incubating with the primary Ab in 1% BSA + Tris-buffered saline with Tween 20 for 2 hours and similarly with the secondary Ab for 1 hour.

**Experiments using radioiodinated vitellogenin and peak A protein**

To establish the stability of injected [$^{131}$I]Pk A and [$^{131}$I]VG in the blood up to 48 hours post injection six female winter flounder were anesthetized with tricaine methanesulfonate (125 mg/l; Crescent Research Chemicals, Inc.) and 3 each injected intravenously (iv), via caudal vessels, with either [$^{131}$I]Pk A or [$^{131}$I]VG at 0.5 $\mu$Ci/kg fish. After 48 hours 2 ml of blood was withdrawn from each fish and diluted 4:1 with PMSF solution (a VG stabilizing solution; de Vlaming et al. 1980) on ice. The blood was centrifuged and 1 ml plasma aliquots from fish of each group pooled and chromatographed on S-300 as above. Eluate fractions were monitored for absorbance at 280 nm and specific activity in a gamma scintillation counter (Packard 5650).

To assess short-term (48 hour) ovarian uptake 6 females were anaesthetized and injected, as above, with either radiolabelled [$^{131}$I]Pk A or [$^{131}$I]VG at 10 $\mu$Ci/kg fish. Two fish per group were killed by decapitation at 12, 24, and 48 hours post injection and the ovary dissected free of the ovarian wall. The specific activity in 2.5 g of ovary was determined in a gamma scintillation counter as above.

**Experiments using tritium labelled vitellogenin and peak A protein**

Two experiments were undertaken to examine longer term (2 week) ovarian uptake by both in vivo and in vitro [$^3$H] labelled VG and Pk A. In the first experiment 2 female winter flounder were anaesthetized and injected as above with either 13.6 x 10$^6$ degradations per minute (dpm) [$^3$H]propionyl-VG or 7.4 x 10$^6$ dpm [$^3$H]propionyl-Pk A/kg fish. The fish, killed after 2 weeks were weighed, blood sampled and plasma prepared as above. Ovaries were removed, weighed and placed on ice.

Plasma aliquots (3 ml) from each fish were chromatographed on S-300 with VG buffer, modified by addition of the proteolytic enzyme inhibitors pepstatin A and leupeptin (Sigma) each at 0.4 μg/ml, as column eluent.

Salt soluble ovarian extracts were prepared from 2 g portions of ovary as described previously (pg. 17) but using modified VG buffer (see above) and omitting the dialysis step in order to obtain all the salt soluble ovarian proteins. Aliquots (4 ml) of the SSOE, always used fresh, were chromatographed on S-300 or Ultrogel AcA 54 (AcA 54) (2.6 x 84 cm) (IBF Biotechnics) using modified VG buffer as column eluent. The AcA 54 column was used at a flow rate of 12 ml/hour with upward elution and calibrated using

ovalbumin (43,000) and carbonic anhydrase (29,000) (Sigma),
and ribonuclease A (13,700) $M_r$ standards (Pharmacia).

Eluate fractions were monitored for absorbance at 280 nm.
To determine radiolabel content 1 ml eluate aliquots were
added to 14 ml of scintillation fluid (Ready-Safe, Beckman
or Aquasol-2, NEN DuPont) in glass counting vials and
counted in a Packard Tri-Carb 300C liquid scintillation
counter adjusted for quenching. Plasma aliquots (100 $\mu$l,
n=5) were combined with 0.5 ml 0.5 M tissue solubilizer
(Protosol or Solvable, NEN DuPont), digested at 55°C
overnight and neutralized with 50 $\mu$l acetic acid before
adding scintillation fluid. Similarly, ovarian fragments
(180-200 mg, n=5) dissected free of the ovarian wall were
digested in 1 ml tissue solubilizer and prepared for
scintillation counting as above.

The protein content of eluate fractions was measured by
the TCA-Lowry method using the Pierce BSA standard.

An ALPP determination was made on eluate fractions by the
following procedure. Aliquots (200 $\mu$l) were incubated for 15
minutes at 100°C in 2 M NaOH, neutralized with HCL (Wallace
and Jared 1968) and precipitated with 2.5 volumes of cold
15% TCA for 10 minutes at room temperature. This was
followed by centrifugation at 4,000x G for 10 minutes and
removal of 1 ml of the supernatant which was combined with 1
ml each of the colorimetric reagents ammonium molybdate and
ammonium vanadate. The colour intensity of the complex was

measured spectrophotometrically against a standard curve derived from $KH_2PO_4$ as in Nagler et al. (1987).

The second experiment used 4 females, 2 each injected with either [$^3$H]VG or [$^3$H]Pk A at 9.2 x 10$^5$ and 5.0 x 10$^5$ dpm/kg fish, respectively. Plasma and ovary samples were obtained and prepared for column chromatography or scintillation counting as above. In addition, aliquots of the SSOE (0.5 ml, n=5) and the insoluble pellet fraction (divided into two 0.5 ml aliquots) from the ovarian extraction procedure were also digested and prepared for scintillation counting.

## RESULTS

### Biochemical analyses of vitellogenin and peak A protein

Pooled sera from 5 female winter flounder, taken during the vitellogenic phase of the reproductive cycle, was prepared to provide a representative assessment of the serum protein profile on S-300. This sera was separated into 4 fractions labelled A, B, C, and D (Fig. 3). These fractions had $M_r$s of 1,170,000, 500,000, 270,000, and 150,000, respectively. The serum of an estrogenized female on S-300 demonstrates the massive production of VG, due to $E_2$, which elutes in the region of peak B (Fig. 4). It is noted that estrogen treatment reduces the amounts of other serum
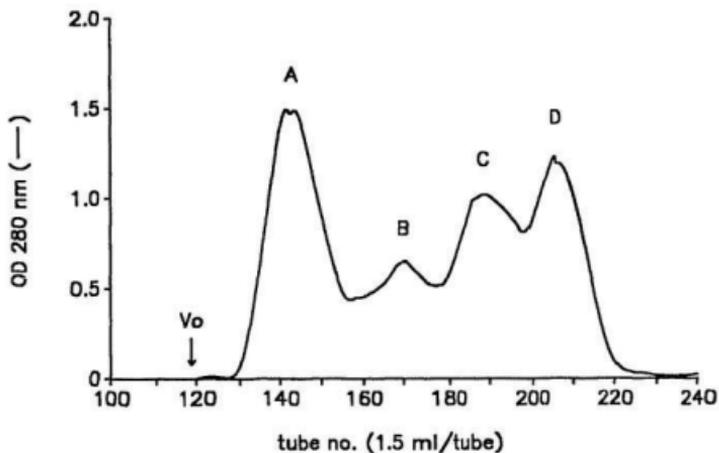
Figure 3: Gel filtration chromatography of 3 ml of female winter flounder sera on Sephacryl S-300 and monitored for absorbance. Void volume (Vo, $1.5 \times 10^6$ $M_r$) indicated with an arrow and peaks labelled A-D.
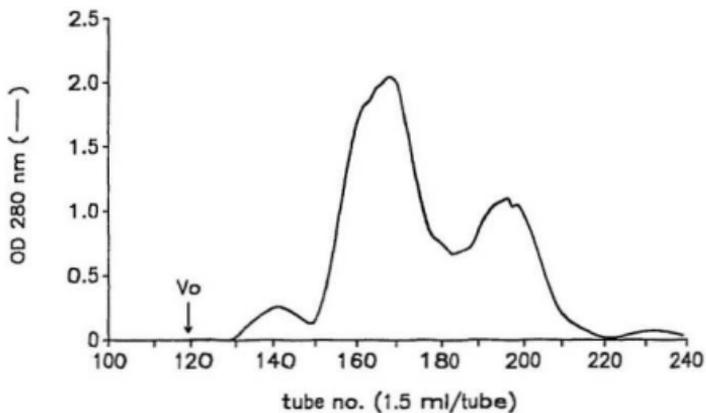
Figure 4: Gel filtration chromatography of 3.5 ml of serum from an estrogenized female winter flounder as in Fig. 3.

proteins particularly Pk A. Native PAGE analysis revealed
that the peak A fraction contained one protein (Pk A) of low
$R_f$, 0.06 (Fig. 5, lane a). Following estrogen treatment two
proteins at previously low levels in the serum (Fig. 5, lane
b) appear as two distinct bands with $R_f$s of 0.30 and 0.35 on
native PAGE and are attributable to VG. On SDS PAGE Pk A is
separated into three subunits, sharp bands at 210,000 and
70,000 $M_r$, and a broad diffuse <14,000 $M_r$ band (Fig. 6, lane
b). Vitellogenin gives a major band at 180,000 $M_r$ plus some
minor components (Fig. 6, lane c). Comparisons of the amino
acid compositions for Pk A and VG show that they are not
greatly different the only amino acids differing by 50% or
more between the two proteins being histidine, isoleucine
and ½ cystine (Table 1). Methionine was not determined in
either case.

## Ultracentrifugation analysis of female winter flounder plasma

Ultracentrifugation of female plasma indicated 4 density
classes of lipoprotein (Table 2). The VHDL fraction
representing 27.5% of the total plasma protein contained
both Pk A and VG. The concentration of Pk A (1470 µg/ml) was
about twice that for VG (737 µg/ml) in the VHDL fraction. A
small amount of VG was also found in the high density
lipoprotein fraction.

Figure 5: Native polyacrylamide gel electrophoresis
analysis of winter flounder peak A protein,
lane a; serum proteins taken from peak B
(Fig. 3), lane b; and winter flounder
vitellogenin, lane c. 25 μg protein applied to
each gel.
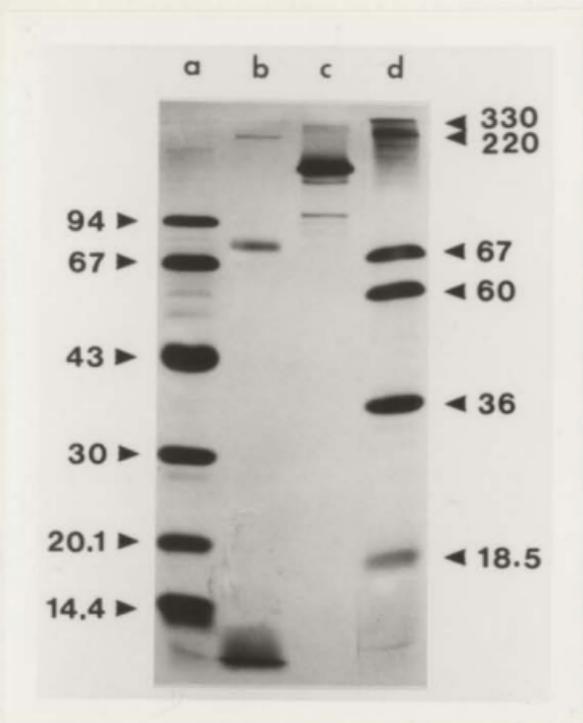
Figure 6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of LMW standards (Pharmacia), lane a; winter flounder peak A protein, lane b; winter flounder vitellogenin, lane c; and HMW standards (Pharmacia), lane d. $M_r$ (x1,000) as calculated in Materials and Methods indicated by arrowheads.

Table 1: Amino acid composition (%) of female winter
flounder vitellogenin and peak A protein.

| Amino Acid | Vitellogenin | Pk A protein |
|---|---|---|
| aspartic acid | 10.5 | 7.3 |
| glutamic acid | 15.0 | 10.8 |
| serine | 8.3 | 9.5 |
| glycine | 8.0 | 7.4 |
| histidine | 0.5 | 2.4 |
| arginine | 7.5 | 4.5 |
| threonine | 6.8 | 10.6 |
| alanine | 4.9 | 4.2 |
| proline | 7.6 | 9.8 |
| tyrosine | 3.7 | 3.1 |
| valine | 5.7 | 7.7 |
| isoleucine | 2.2 | 4.5 |
| leucine | 5.5 | 6.7 |
| phenylalanine | 2.8 | 4.1 |
| lysine | 9.0 | 6.5 |
| ½ cystine | 2.0 | 1.0 |

Table 2: Ultracentrifugation analysis of female winter
flounder plasma.

| Lipoprotein Fractions | Density (g/ml) | Protein (mg/ml) | Vitellogenin ($\mu$g/ml) | Peak A protein ($\mu$g/ml) |
|---|---|---|---|---|
| [1]VLDL | < 1.006 | 0.01 | 0 | 0 |
| LDL | 1.006 - 1.063 | 2.91 | 0 | 0 |
| HDL | 1.063 - 1.21 | 26.6 | 25 | 0 |
| VHDL | > 1.21 | 11.2 | 737 | 1470 |

[1]VLDL = very low density lipoprotein, LDL = low density
lipoprotein, HDL = high density lipoprotein, VHDL = very
high density lipoprotein.

## Radiolabelling of vitellogenin and peak A protein

Both VG and Pk A were successfully radiolabelled with $^{131}I$, $^3H$ and [$^3H$]-succinimidyl propionate. The specific activities on average for [$^{131}I$]VG, [$^3H$]propionyl-VG and[$^3H$]VG were 16.8 and 0.82 $\mu Ci/ug$, and 15 $nCi/mg$ respectively. Similarly, specific activities for [$^{131}I$]Pk A, [$^3H$]propionyl-Pk A and [$^3H$]Pk A were 13.6 and 0.66 $\mu Ci/ug$, and 4 $nCi/mg$ respectively.

## Seasonal serum profiles of vitellogenin and peak A protein

The RIAs developed for Pk A and VG allowed the determination of seasonal serum profiles of these two proteins in female winter flounder (Fig. 7). Both Pk A and VG follow the same general pattern over the annual reproductive cycle with Pk A concentrations usually about twice those of VG. During the summer following the spawning period (May-June) the levels are typically low but increase dramatically by late August and September, drop in October and increase again to reach the highest levels in early December. Levels decline over the winter and into the spring with the onset of spawning.
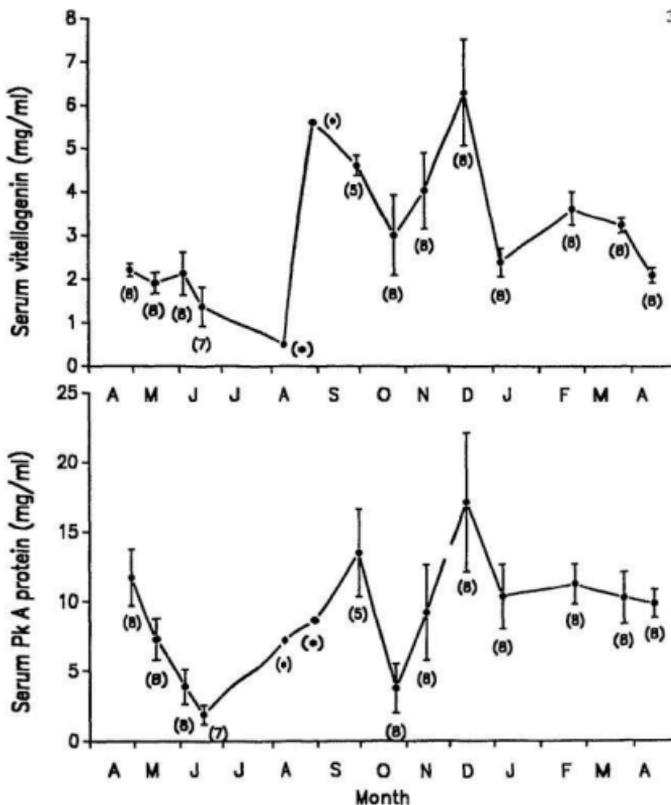
Figure 7:  Seasonal serum profile of vitellogenin (A) and
          peak A protein (B) in female winter flounder
          (1986–87). Each point represents the mean ±
          standard error (SE) (number of fish); * = pooled
          sera from 5 fish.

Stability of radiolabelled vitellogenin and peak A protein
injected in vivo in the blood

The integrity of radiolabelled proteins in the blood,
following iv injection, were determined by the elution
position of fractions containing radiolabel when plasma from
treated fish were chromatographed on S-300. In fish sampled
48 hours after [$^{131}$I]VG injection most of the radioactivity
was found in fractions eluting at the approximate $M_r$ region
of VG (500,000), although there were several obvious peaks
of radioactivity separated on S-300 (Fig. 8). However, in
[$^{131}$I]Pk A injected fish after 48 hours all the radioactivity
measured was found in fractions associated with the first
protein peak on S-300 (Fig. 9). With [$^3$H]propionyl-VG and
[$^3$H]VG injected fish after 2 weeks a single peak of
radioactivity was measured amongst fractions eluting at the
approximate $M_r$ region of VG (500,000) (Figs. 10 and 11). A
single peak of radioactivity was apparent for [$^3$H]propionyl-
Pk A and [$^3$H]Pk A injected fish after two weeks that was
found in fractions eluting with the first protein peak
(Figs. 12 and 13). The clearance from the blood of all
radiolabelled VG preparations is rapid, as reflected by the
amount of radiolabel present in the plasma, in comparison
with similarly radiolabelled Pk A preparations after 48
hours or 2 weeks. The clearance of radiolabelled VGs in all
cases is at least 20 times that of comparable Pk A
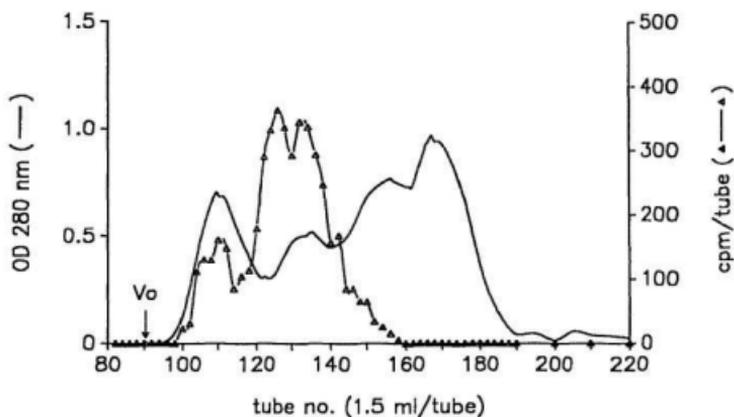
Figure 8: Gel filtration chromatography of 3 ml of female
winter flounder plasma on Sephacryl S-300 from
fish injected 48 hours earlier with
[$^{131}$I]vitellogenin at 0.5 $\mu$Ci/kg and monitored for
absorbance and radioactivity (counts per minute
[cpm]); void volume (Vo, 1.5 x 10$^6$ $M_r$) indicated
with an arrow.
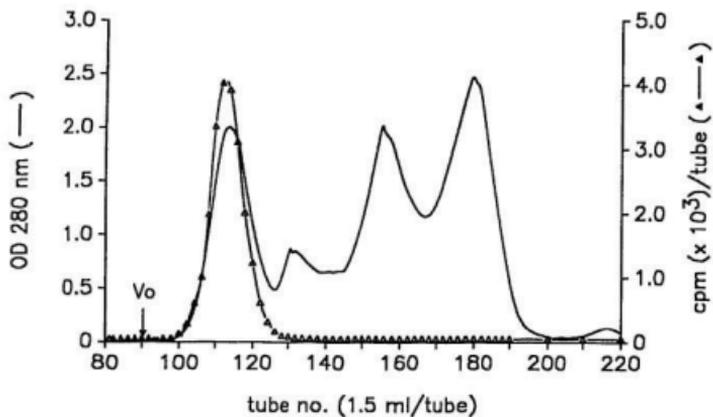
Figure 9:  Gel filtration chromatography of 3 ml of female
           winter flounder plasma from fish injected 48
           hours earlier with [$^{131}$I]peak A protein at 0.5
           μCi/kg, as in Fig. 8.
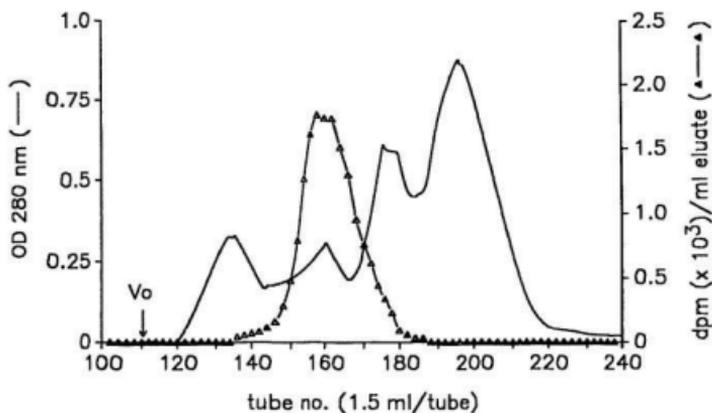
Figure 10: Gel filtration chromatography of 3 ml of female
winter flounder plasma from fish injected 2
weeks earlier with 13.6 x 10^6 degradations per
minute (dpm) [³H]propionyl-vitellogenin, as in
Fig. 8.
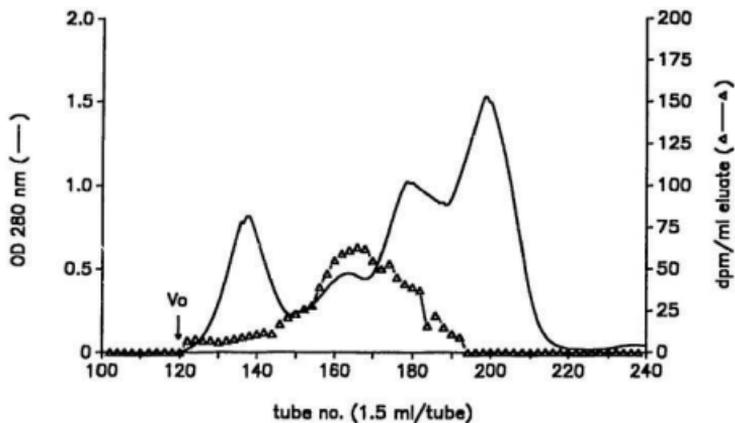
Figure 11: Gel filtration chromatography of 3 ml of female
winter flounder plasma from fish injected 2
weeks earlier with 9.2 x 10$^5$ degradations per
minute (dpm) [$^3$H]vitellogenin, as in Fig. 8.
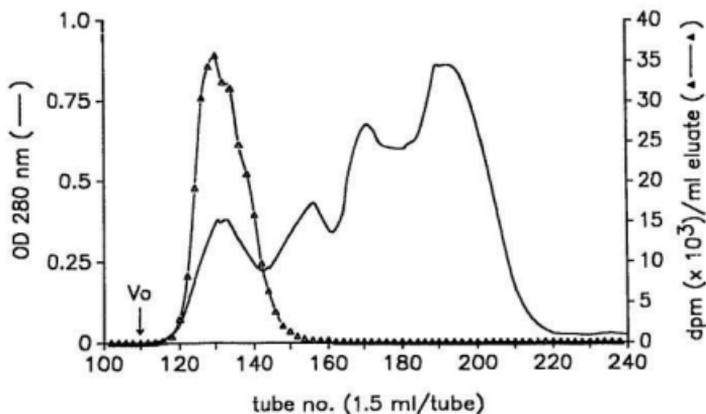
Figure 12: Gel filtration chromatography of 3 ml of female winter flounder plasma from fish injected 2 weeks earlier with 7.4 x 10^6 degradations per minute (dpm) [$^3$H]propionyl-peak A protein, as in Fig. 8.
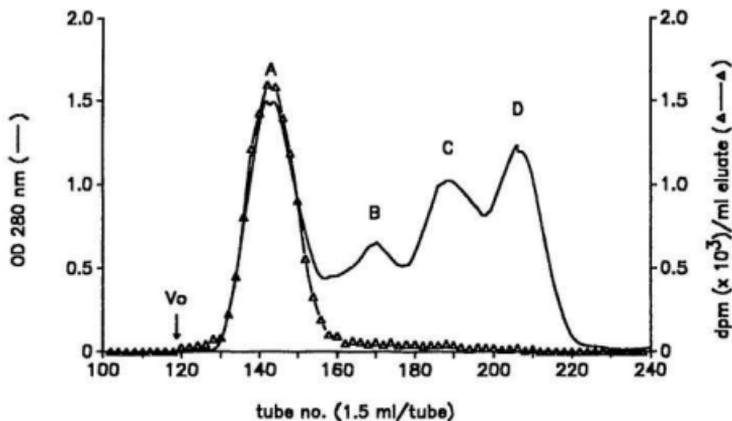
Figure 13:   Gel filtration chromatography of 3 ml of female
             winter flounder plasma from fish injected 2
             weeks earlier with $5.0 \times 10^5$ degradations per
             minute (dpm) [$^3$H]peak A protein, as in Fig. 8.

radiolabels when the different specific activities are taken into account (Figs. 8-13). In fish sampled two weeks after [$^3$H]VG and [$^3$H]Pk A injection the injected radiolabel calculated to be remaining in the plasma was 2 and 52%, respectively, of the total injected.

**Immunoreactivity of the salt soluble ovarian extract with vitellogenin and peak A protein antisera**

Following dialysis SSOE could be separated into two major protein peaks by S-300 chromatography, the first near the void volume (Vo) of the column and the second at 280,000 $M_r$ (Fig. 14). The best immunoreactivity for Pk A occurred on the trailing edge of the first protein peak and to a lesser extent in the second peak. However, for VG the major site of immunoreaction occurred on the second peak and in a concentration dependent fashion relative to the absorbance. There was some minor immunoreactivity also on the first peak and the trailing edge of the second.

**Relationship of oocyte proteins to vitellogenin and peak A protein**

Oocyte proteins from vitellogenic ovarian follicles separated on SDS PAGE produced 5 major subunits; 101,400,
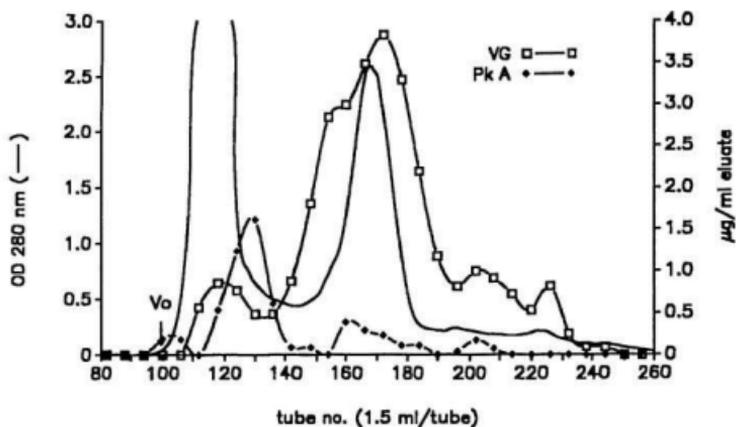
Figure 14: Gel filtration chromatography of 2 ml of ovarian
extract on Sephacryl S-300 and monitored for
absorbance. An aliquot of every sixth eluate
fraction was measured in the peak A protein and
vitellogenin radioimmunoassays as indicated.

94,400, 68,700, 25,500 and 22,500 $M_r$, and numerous minor
components (Fig. 15). Western blots of these proteins
separated by SDS PAGE and immunoassayed using the VG Ab
identified 3 protein subunits corresponding to 101,400,
94,400 and 22,500 $M_r$ (Fig. 16). A similar run utilizing the
VHDL II Ab as primary Ab identified 1 major protein subunit
of 68,700 $M_r$ (Fig. 17).

## Ovarian uptake of radiolabelled vitellogenin and peak A protein

The short term ovarian uptake experiment using
radioiodinated preparations showed that maximal levels of
both [$^{131}$I]PkA and [$^{131}$I]VG were accumulated by the ovary
after 24 hours, with twice as much radioactivity
attributable to VG present in the ovaries as Pk A (Fig. 18)
after 48 hours.

Longer term studies compared ovarian uptake of tritiated
preparations over a two week period. Chromatography of SSOE
on S-300 without prior dialysis revealed three absorbance
peaks (Fig. 19). The first peak elutes immediately after the
Vo, the second has a $M_r$ of 280,000 and the third elutes at
the total volume (10,000 $M_r$) of the column.

When eluate fractions were monitored for radioactivity
two distinct peaks corresponding to the second and third
absorbency peaks were found for [$^3$H]propionyl-VG injected
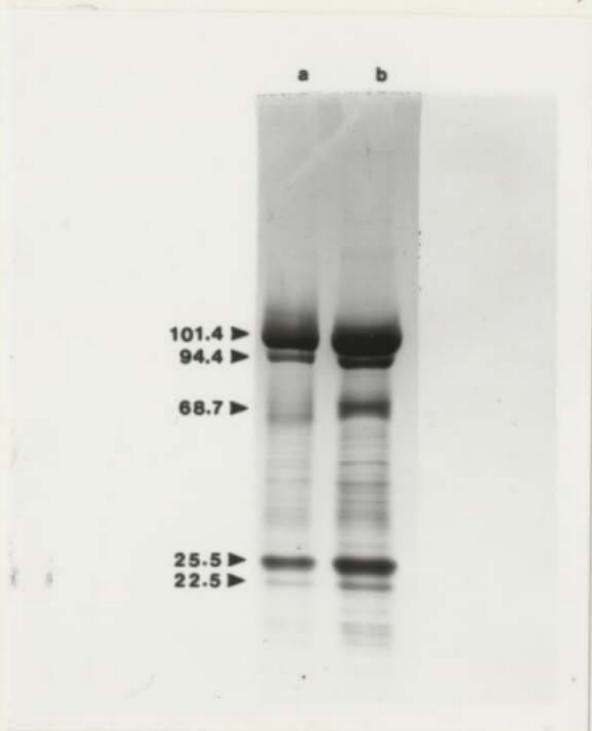
Figure 15: Sodium dodecyl sulfate polyacrylamide gel
electrophoretic separation of winter flounder
oocyte proteins; lane a represents 12 $\mu$g and lane
b 24 $\mu$g protein. $M_r$ (x1,000) as calculated in
Materials and Methods indicated by arrowheads.

Figure 16: Western blot of winter flounder oocyte proteins
1 μg (lane a) and 2 μg (lane b) subjected to
sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS PAGE) and probed with
vitellogenin antibody (1:5,000x in immunoassay).
For comparison 20 μg (lane c) of protein on an
adjacent lane of the SDS PAGE stained with
Coomassie R-250. $M_r$ as in Fig. 15.

Figure 17: Western blot of winter flounder oocyte proteins
48 $\mu$g (lane a) and 72 $\mu$g (lane b) subjected to
sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS PAGE) and probed with peak
A protein antibody (1:500x in immunoassay). For
comparison 20 $\mu$g (lane c) of protein on an
adjacent lane of the SDS PAGE stained with
Coomassie R-250. $M_r$ as in Fig. 15.
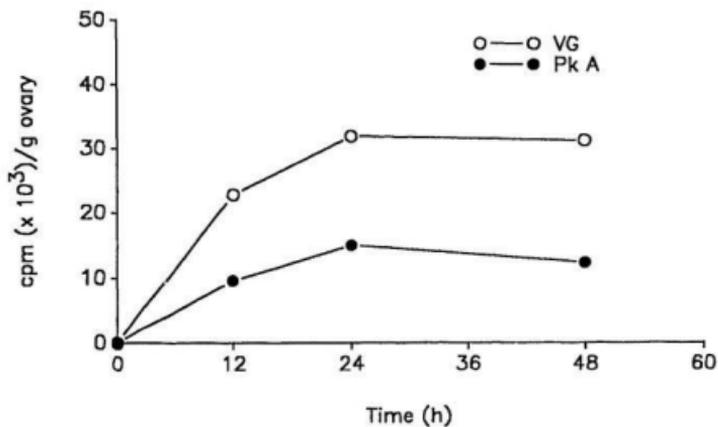
Figure 18: Time course plot of [$^{131}$I]vitellogenin and [$^{131}$I]peak A protein uptake by the ovaries of female winter flounder. Each point represents a mean of 2 fish.
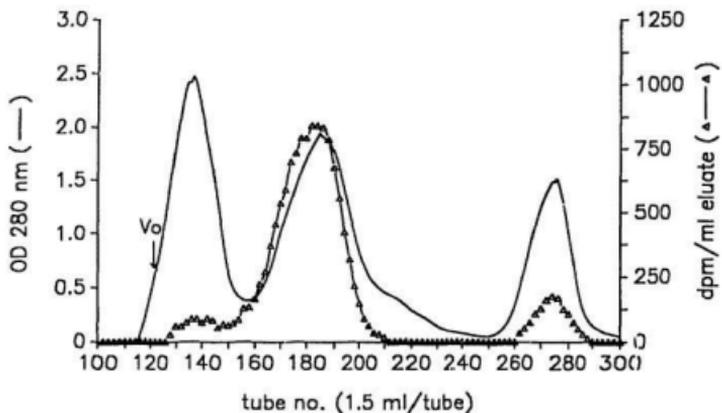
Figure 19: Gel filtration chromatography of 4 ml of salt soluble ovarian extract on Sephacryl S-300 from female winter flounder injected 2 weeks earlier with $13.6 \times 10^6$ degradations per minute (dpm) [$^3$H]propionyl-vitellogenin and monitored for absorbance and radioactivity; void volume (Vo, $1.5 \times 10^6$ $M_r$) indicated with an arrow.

fish although there was also some radioactivity associated
with the first peak (Fig. 19). The most prominent peak of
radioactivity was associated with the second absorbency peak
corresponding to 280,000 $M_r$. In [$^3$H]VG injected fish most of
the radioactivity eluted with the second peak (280,000 $M_r$),
although again some radiolabel appeared near the Vo (Fig.
20). In [$^3$H]propionyl-Pk A injected fish only one small peak
of radioactivity was observed corresponding with the first
absorbency peak near the Vo (Fig. 21). Negligible levels of
radioactivity in chromatographed SSOE from [$^3$H]Pk A injected
fish prevented any assessment of radioactive elution
relative to absorbance.

Two peaks of protein were found after aliquots of eluate
fractions from SSOE chromatographed on S-300 were measured
by the TCA-Lowry method (Fig. 22). The first peak, between
tubes 120 and 150, represents 12% of the total SSOE protein.
It corresponds to the first absorbency peak noted in Figs.
19-21 and the elution position of all radioactivity found in
[$^3$H]propionyl-Pk A injected fish (Fig. 21). The second and
larger peak, between tubes 160 and 210, makes up 82% of
total SSOE protein. This peak appears at the same elution
position as the second absorbency peak of 280,000 $M_r$ noted
above and the elution position of the major radioactive peak
in [$^3$H]propionyl-VG and [$^3$H]VG injected fish (Figs. 19 and
20).

Alkali-labile protein phosphorus measurements on aliquots
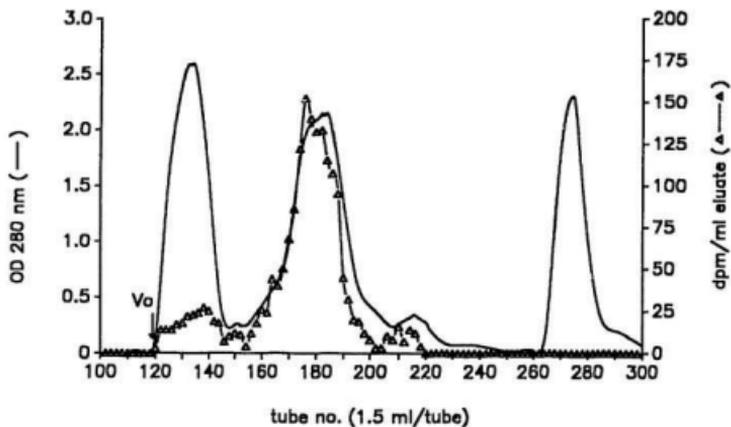
Figure 20: Gel filtration chromatography of 4 ml of salt
soluble ovarian extract from female winter
flounder injected 2 weeks earlier with
$9.2 \times 10^5$ dpm [$^3$H]vitellogenin as in Fig. 19.
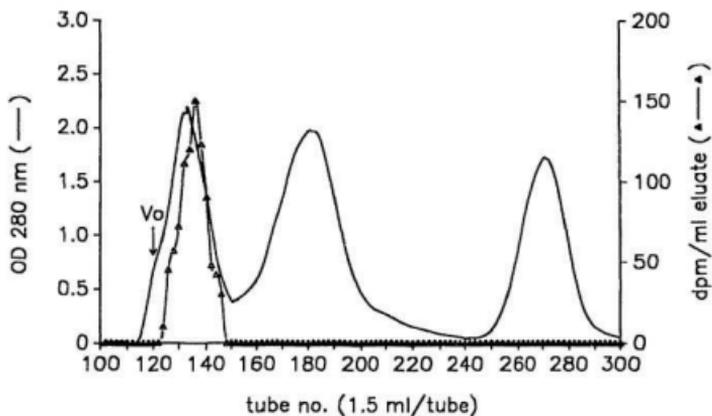
Figure 21: Gel filtration chromatography of 4 ml of salt soluble ovarian extract on S-300 from female winter flounder injected 2 weeks earlier with $7.4 \times 10^6$ dpm [$^3$H]propionyl-peak A protein as in Fig. 19.
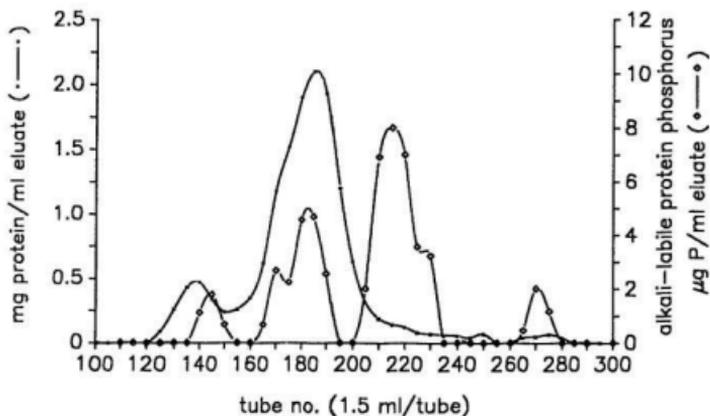
Figure 22: Gel filtration chromatography of salt soluble
ovarian extract as described in Fig. 19, with
every fifth tube monitored for protein and
alkali-labile protein phosphorus.

of selected eluate fractions from SSOE chromatographed on S-300 revealed four main peaks of ALPP (Fig. 22). The first ALPP peak between tubes 135 and 155 appears to relate to the first peak of absorbency and protein, and the radioactivity noted in [$^3$H]propionyl-Pk A injected fish (Fig. 21). The second ALPP peak, spanning tubes 160 to 195, corresponds with the second observed peak of absorbency and protein and the major peak of radioactivity in [$^3$H]propionyl-VG and [$^3$H]VG injected fish (Figs. 19 and 20). However, the third and largest ALPP peak, between tubes 200 and 235, had a $M_r$ of about 80,000 and is associated with little protein and negligible absorbency or radioactivity. The fourth ALPP peak, between tubes 265 and 280, also is not associated with significant amounts of protein but does compare to the third absorbency peak and second smaller radioactive peak at the total volume noted in [$^3$H]propionyl-VG injected fish (Fig. 19). The peak tube from each of the four ALPP peaks noted above had alkali-labile phosphorus contents of 0.5, 0.2, 5.4, and 4.1% respectively.

To achieve more resolution in the low $M_r$ range an ovarian extract from [$^3$H]propionyl-VG injected fish was chromatographed on AcA 54 and monitored for absorbance and radioactivity (Fig. 23), and protein and ALPP (Fig. 24). This revealed four peaks of absorbance the first and largest peak, between tubes 100 and 120, eluting with the Vo (70,000
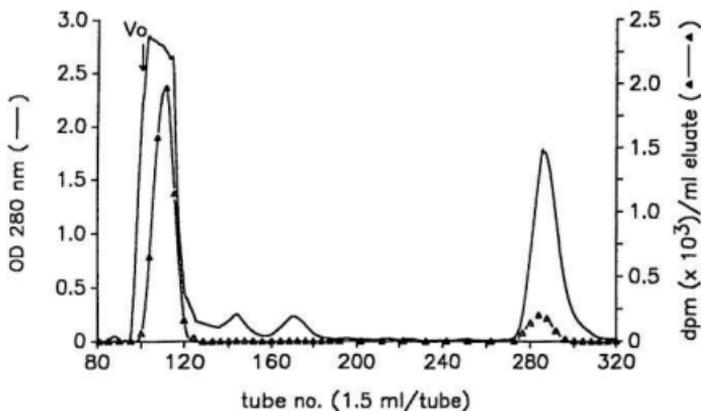
Figure 23: Gel filtration chromatography of 3 ml of salt
soluble ovarian extract on Ultrogel AcA 54 from a
female winter flounder injected 2 weeks earlier
with $13.6 \times 10^6$ degradations (dpm) [$^3$H]propionyl
-vitellogenin and monitored for absorbance and
radioactivity; void volume (Vo, 70,000 $M_r$)
indicated with an arrow.
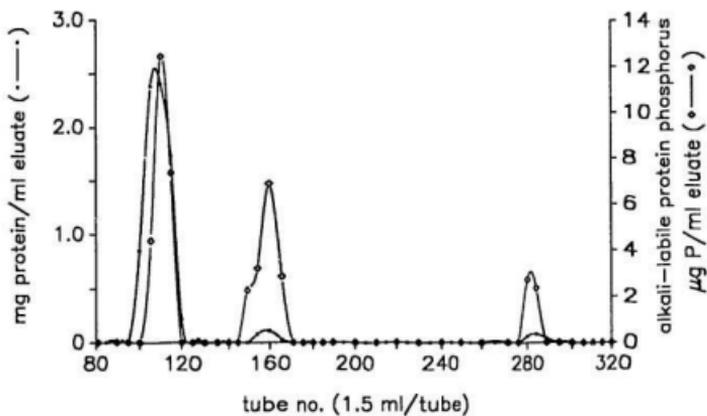
Figure 24: Gel filtration chromatography of salt soluble
ovarian extract as described in Fig. 23 and
monitored for protein and alkali-labile protein
phosphorus.

$M_r$) of the column (Fig. 23). The small second and third peaks were found between tubes 135 to 150, and 160 to 180, respectively. The fourth peak eluted at the total volume (6,000 $M_r$) of the column between tubes 275 and 305. Two peaks of radioactivity were recorded, the larger corresponding to the Vo, while the smaller peak eluted at the total volume. Three peaks of protein and ALPP which corresponded to one another were found (Fig. 24). The major peak of protein and ALPP eluted at the Vo while the smaller second and third peaks had $M_r$s of 40,000 and about 6,000 (total volume) respectively.

A summary of the ovarian uptake of $^3$H labelled VG and Pk A in winter flounder over the two week period is presented in Table 3. Winter flounder ovaries always accumulated more radiolabelled VG, in general, about three times the amount of Pk A. With both proteins there was a trend of greater ovarian uptake of in vivo $^3$H labelled preparations over in vitro $^3$H labelled (i.e. [$^3$H]-succinimidyl propionate) in terms of % uptake relative to the amount injected. The greatest proportion of radiolabel due to VG and Pk A in ovarian extracts were found in the salt soluble fraction (Table 4). However, a greater proportion of Pk A (mean = 25.9%) was found associated with the salt insoluble fraction relative to VG (mean = 8.9%).

Table 3: Comparison of the ovarian uptake of tritiated ($^3$H)
vitellogenin and peak A protein preparations
labelled _in vivo_[a] and _in vitro_[b] in female winter
flounder two weeks after injection.

| Fish No. | Radiolabelled Protein | $^3$H in ovary (% of injected) |
|----------|----------------------|-------------------|
| 1. | [$^3$H]vitellogenin[a] | 73.7 |
| 2. | [$^3$H]vitellogenin[a] | 73.8 |
| 3. | [$^3$H]propionyl-vitellogenin[b] | 52.0 |
| 4. | [$^3$H]peak A protein[a] | 21.7 |
| 5. | [$^3$H]peak A protein[a] | 15.4 |
| 6. | [$^3$H]propionyl-peak A protein[b] | 13.4 |

Table 4: The proportion of injected radioactivity due to [$^3$H]vitellogenin and [$^3$H]peak A protein present in salt soluble and insoluble fractions of ovarian extracts of female winter flounder two weeks after injection.

| Fish No. | Radiolabelled Protein | Tritium in ovarian fraction | |
|---|---|---|---|
| | | Salt soluble | Salt insoluble |
| | | (% of injected) | |
| 1. | [$^3$H]vitellogenin | 91.0 | 8.6 |
| 2. | [$^3$H]vitellogenin | 90.9 | 9.1 |
| 4. | [$^3$H]peak A protein | 75.0 | 25.0 |
| 5. | [$^3$H]peak A protein | 73.2 | 26.8 |

## DISCUSSION

Vitellogenin was identified primarily by the fact that it was the serum protein inducible with $E_2$ and blood levels increased dramatically following injection (Fig. 4). The chromatographic procedures used to isolate Pk A and VG gave essentially pure (>99%) proteins as demonstrated by native PAGE analysis (Fig. 5). The appearance of two bands for winter flounder VG on native PAGE is different from the single bands reported for landlocked Atlantic salmon (So et al. 1985) and rainbow trout (Campbell and Idler 1980) VGs. However, de Vlaming et al. (1980) found three protein bands for goldfish VG on native PAGE that were attributed to a dimer ($R_f$ 0.34) and two monomeric forms ($R_f$ 0.40 and 0.50) of the protein. The dimer and one of the monomeric forms ($R_f$ 0.50) appear about equal in terms of staining intensity (concentration) similar to the relationship between the two bands of winter flounder VG ($R_f$ 0.30 and 0.35) suggesting a dimer-monomer situation. This idea is further supported by SDS PAGE analysis of winter flounder VG which gave one major protein subunit presumably resulting from one parent protein (Fig. 6). The subunit $M_r$ of winter flounder VG (180,000) on SDS PAGE is about midway between the extremes of the range of $M_r$s (127,000-220,000) reported for other teleost VGs (Hara and Hirai 1978, Hori et al. 1979, de Vlaming et al.

1980, Roach and Davies 1980, Selman and Wallace 1983, So et al. 1985, Bradley and Grizzle 1989).

It was noted from the seasonal serum profiles for VG and Pk A in female winter flounder that a trend exists with the concentration of Pk A always about twice VG. However, the molar concentration would be about the same since Pk A has twice the native $M_r$ of VG. A more important point however is the close resemblance of the Pk A profile with that of VG. This is good evidence for a role of Pk A in ovarian development. The serum levels of Pk A in the blood start to increase by early August, before VG (i.e. late August), with the onset of the vitellogenic phase. The blood levels of both Pk A and VG remain high throughout the period (September-January) when the flounder ovary is growing the fastest (Fig. 7). Serum VG levels decrease through the late winter and the approach of the spawning season which is typical of the pattern found in salmonids (Scott and Sumpter 1983, So et al 1985). The significant drop in serum levels of both VG and Pk A in October was unexpected since VG levels in salmonids normally follow a unimodal pattern over the annual reproductive cycle (Scott and Sumpter 1983, So et al. 1985). The bimodal pattern in the winter flounder may be a consequence of rapid serum VG and Pk A ovarian incorporation during September to November, the period when the most rapid ovarian growth occurs based on the increase in GSI (see Chapter 3). Hepatic synthesis may not be great

enough to maintain blood concentrations during this period of rapid oocyte growth which causes a drop in blood levels.

Ultracentrifugation of female winter flounder plasma to isolate the four density classes of lipoprotein (Table 2) showed that most of the protein (65.3%) was found in the high density lipoprotein fraction. A characteristic feature of plasma lipoproteins throughout non mammalian species examined so far is a predominance of protein in the high density lipoprotein fraction (Babin and Vernier 1989, Schjeide and Schjeide 1981). The VHDL fraction, representing 27.5% of the total plasma protein, contained both Pk A and VG. This is the first report of a plasma VHDL involved with the ovary yet different from VG. It is proposed from now on to refer to Pk A as VHDL II recognizing the "vitellogenins", the classical constituents of the VHDL class (Schjeide and Schjeide 1981), as VHDL I. A small amount of VG found in the high density lipoprotein fraction may represent some contamination during removal of this fraction following centrifugation.

Salt soluble ovarian extracts indicated immunoreactivity in both the VHDL II (Pk A) and VG RIAs with the most cross-reaction in terms of quantity toward VG (Fig. 14). From earlier studies in landlocked Atlantic salmon it was recognized, that although VG Ab immunoreactivity exists within SSOE, due to the processing of VG into the yolk proteins lipovitellin and phosvitin it is not possible to

directly quantify amounts of incorporated VG (So et al. 1985). These experiments served to show that some component of the winter flounder ovarian extract was recognized by the VHDL II Ab and that this component did not directly crossreact with portions of ovarian extract recognized by the VG Ab.

Oocyte proteins from vitellogenic ovarian follicles, when separated on SDS PAGE, produced 5 major protein subunits 101,400, 94,400, 68,700, 25,500 and 22,500 $M_r$, and numerous minor components (Fig. 15). In one study on the killifish (Wallace and Selman 1985) 5 major protein subunits were found in mid-vitellogenic ovarian follicles, 3 of which, 103,000, 26,000 and 22,000 $M_r$, have similar $M_r$s to those found in the winter flounder. Unfortunately, many studies have been conducted on egg proteins in which ovulated mature eggs were collected from gravid females but not on oocytes during earlier stages of vitellogenesis. This is very relevant since previous work (Wallace and Begovac 1985, Wallace and Selman 1985, Greeley et al. 1986) has shown the marked changes that occur in teleost oocyte protein patterns with the onset of final maturation. The apparently high level of proteolytic activity shortly before ovulation makes comparisons between eggs and earlier stages of oocyte development difficult.

Western blots of oocyte proteins separated by SDS PAGE and immunoassayed using the VG Ab identified 3 protein

subunits corresponding to 101,400, 94,400 and 22,500 $M_r$ (Fig. 16). The two high $M_r$ subunits 101,400 and 94,400 $M_r$ must result from lipovitellin since native fish phosvitin is usually in the order of 19,000-43,000 $M_r$ (Ng and Idler 1983) and does not stain well with Coomassie Blue R-250 (Wallace and Begovac 1985). An examination by SDS PAGE of the subunits of partially purified goldfish lipovitellin revealed two domains LV1 110,000 and 105,000 $M_r$, and LV2 19,000-25,000 $M_r$ (de Vlaming et al. 1980). In winter flounder the low $M_r$ subunit 22,500 $M_r$ could originate from LV2 similar to goldfish. The run utilizing the VHDL II Ab identified 1 major protein subunit of 68,700 $M_r$ (Fig. 17) which therefore must originate from serum VHDL II. Another study related to serum protein involvement during oogenesis occurred with the red sea bream (Pagrus major) (Hara et al. 1987). An antiserum to crude female red sea bream blood serum was utilized in a Western blot of oocyte derived protein that yielded 4 immunoreactive subunits 105,000, 86,000, 69,000 and 60,000 $M_r$. I suggest that the 105,000 and 69,000 $M_r$ subunits may have their origin as VG and VHDL II, respectively, in this fish.

My results indicate that quantitatively VG gives rise to the majority (3 of 5) of the major proteins contained in the ovarian follicle. From the staining intensity of ovarian follicle proteins on SDS PAGE the VHDL II derived protein appears to exist in smaller quantities than those associated

with VG. The VG Ab used in the immunoassay was very
sensitive to ovarian proteins separated by SDS PAGE. This
was in contrast to the VHDL II Ab where 10 fold lower
primary Ab dilutions and 30 fold larger amounts of protein
had to be applied to gels to achieve a positive signal.
There may be several explanations for these results. Certain
inherent differences in the 2 Abs' ability to react with the
secondary alkaline phosphatase conjugated Ab may be one
explanation. However, there may simply be less VHDL II
immunoreactive protein present.

All radiolabelled preparations with the possible
exception of [$^{131}$I]VG appeared to remain intact in the blood
after various time periods. The appearance of radiolabels
specifically and singularly in the $M_r$ regions from which
they were isolated on S-300 was accepted as the criterion
for this conclusion (Figs. 8-13). An explanation for the
appearance of plasma from [$^{131}$I]VG injected fish on S-300 is
not forthcoming since although radiolabelled fractions
appear to be separable on the column they are not widely
divergent and no low $M_r$ fragments are evidenced. Most of the
radioactivity is in the region from which one would expect
to isolate native VG although there is some radioactivity
associated with the first protein peak. The contrast of
[$^{131}$I]VG with that of tritiated VG preparations prompted use
of the latter in subsequent long term (two week) ovarian
uptake studies.

The short term ovarian uptake experiment (Fig. 18) showed that both VHDL II and VG were accumulated by the ovary after 48 hours similar to other work involving VG in the rainbow trout (Tyler et al. 1988a,b) and African clawed toad (Xenopus laevis) (Wallace and Jared 1969). Since equal amounts of specific activity (per μg) for each radiolabelled preparation were injected and VHDL II contains a similar number of tyrosine residues to VG (Table 1) and VHDL II is approximately twice the $M_r$ of VG; on a per mole basis VG is acquired by the ovary at about four times the rate of VHDL II. In terms of absolute mass VG would be incorporated by the winter flounder oocyte in twice the quantity of VHDL II. These conclusions are based on three assumptions, 1) that both proteins have receptor-mediated uptake on the oocyte surface, 2) that there is equal molar competition of cold VG and VHDL II for their respective radiolabelled counterparts and 3) that all tyrosine residues are radiolabelled on both proteins with $^{131}$I. Support for some of these assumptions can be generated for it is known that VG receptors exist on coho salmon oocytes (Stifani et al. 1990), and chicken (Gallus domesticus) VG and very low density lipoprotein have a common oocyte receptor (Stifani et al. 1989). It can be concluded from the seasonal serum profiles of VHDL II and VG that equal molar competition is certainly conceivable as there are substantial quantities (i.e. mg/ml) of both proteins in the blood when the experiments were conducted.

Longer term ovarian uptake experiments using $^3$H labelled protein preparations essentially corroborated the outcome of the short term studies. Labelled VG was accumulated by the ovary at least three times more than VHDL II over a two week period (Table 3). Calculations of ovarian uptake using $^3$H labelled preparations were presented in terms of % radiolabel accumulated by the ovary relative to the total amount injected. This differs from the short term study using $^{131}$I but compensates for different specific activities of the two proteins and the amounts injected into the fish in each case. The results of both short and long term ovarian uptake studies taken together contrast with earlier studies in African clawed toads where VG uptake by the ovary was 25 times the rate of other blood proteins (Wallace and Jared 1969) and VG accounts for 99% of the yolk proteins (Wallace et al. 1972). This suggests that the winter flounder ovary may not be as selective in terms of which plasma proteins are incorporated.

The ovarian uptake experiments of two week duration permitted an examination of the ovarian processing of $^3$H labelled VG and VHDL II preparations. Both [$^3$H]propionyl-VG and [$^3$H]VG which from the blood eluted intact at approximately 500,000 $M_r$ (Figs. 10 and 11), were processed into smaller 280,000 $M_r$ yolk proteins within the oocyte (Figs. 19 and 20). This 280,000 $M_r$ yolk protein is identified as lipovitellin similar to the findings of Tyler

et al. (1988a) for the processing of [³H]VG into lipovitellin upon uptake by the ovary of rainbow trout in vivo. The $M_r$ (280,000) and alkali-labile phosphorus content (0.2%) of crude lipovitellin reported here are both lower than previous estimations of these values in winter flounder (Ng and Idler 1979) but are in line with data reported for other fish lipovitellins (Ng and Idler 1983). The lipovitellin peak in terms of protein (Fig. 22) represents 82% of the total present in the SSOE. Therefore it is apparent that VG cleaved upon ovarian uptake into lipovitellin contributes to the major source of yolk protein.

By comparison with VG the ovarian uptake pattern of [³H]propionyl-VHDL II indicated that this serum protein was not processed by the oocyte since the single peak of radioactivity in ovarian extracts eluted near the Vo (Fig. 21) where intact plasma VHDL II also elutes (Fig. 12). This is in contrast to the processing of VG in winter flounder (this study) and rainbow trout (Tyler et al. 1988a) or heterologous proteins such as the situation reported for bovine serum albumin (BSA) in rainbow trout (Tyler et al. 1988b). Unfortunately, no radioactivity was measurable in fractions eluted during chromatography of SSOE from [³H]VHDL II injected fish presumably due to the low specific activity. This negates a comparison between the elution pattern of ovarian extracts from [³H]propionyl-VHDL II and

[$^3$H]VHDL II injected fish. Yet [$^3$H]VHDL II is taken up and in terms of the whole ovary is accumulated similar to [$^3$H]propionyl-VHDL II on a % injected basis (Table 3). The elution position of [$^3$H]propionyl-VHDL II from SSOE (Fig. 21) corresponds with the first peak of protein which accounts for 12% of the total (Fig. 22) which may not all be due to incorporated VHDL II. Therefore VHDL II relative to VG makes only a modest contribution to ovarian yolk protein. This does not prevent VHDL II from having other important, but as yet unknown, function(s) within the oocyte.

The existence of winter flounder phosvitin was established from ALPP measurements on SSOE chromatographed on S-300 (Fig. 22) and AcA 54 (Fig. 24). In both cases a major ALPP peak was found that had a high alkali-labile phosphorus to protein content (5.4%) and no absorbance at 280 nm. The $M_r$ of 42,000 over 80,000 determined from AcA 54 rather than S-300 chromatography is considered more accurate since AcA 54 has better resolution in the low $M_r$ range. These characteristics compare favourably with previous information on fish phosvitins (Ng and Idler 1983). There was no radioactivity associated with the ALPP peak representing phosvitin in [$^3$H]propionyl-VG as opposed to [$^3$H]VG injected fish which indicated a small amount of radiolabel. This suggests that [$^3$H]-succinimidyl propionate labelling of VG does not label the phosvitin portion of the VG molecule. There may be several explanations for this,

winter flounder phosvitin may be deficient in lysine the amino acid that reacts with $[^3H]$-succinimidyl propionate or more likely phosvitin makes up a very small component of the winter flounder VG molecule. To substantiate the latter point it is noted that the protein contribution by phosvitin to the SSOE was negligible (Figs. 22 and 24) and even with multiple amino acid labelled $[^3H]VG$ little radiolabel was apparent that could be attributed to phosvitin. Some marine teleosts have been reported to have little or no phosvitin (Jared and Wallace 1968) and it appears that ovarian quantities of phosvitin in the winter flounder are indeed small.

The second peak of radioactivity observed in SSOE from $[^3H]$propionyl-VG injected fish eluted at the total volume on both S-300 (Fig. 19) and AcA 54 (Fig. 23) indicating a $M_r$ as low as 6,000. Conversely, there was no evidence of any radioactivity eluting at the total volume in SSOE from $[^3H]VG$ injected fish (Fig. 20). The radioactivity eluting at 6,000 $M_r$ in $[^3H]$propionyl-VG injected fish was associated with protein unlike the report by Tyler et al. (1988a) of protein-free radioactivity eluting at the column total volume (4,000 $M_r$) when ovarian homogenates from $[^3H]VG$ injected rainbow trout were chromatographed by gel filtration. The 6,000 $M_r$ protein found in this study contained 4.1% alkali-labile phosphorus and may be analogous to small phosphoproteins called phosvettes described in the

African clawed toad (Wiley and Wallace 1981) and the chicken (Wallace and Morgan 1986). It is not clear why this peak of low $M_r$ radioactivity was only found in [$^3$H]propionyl-VG injected fish but it may be due to either the low specific activity of [$^3$H]VG or differential processing of the two radiolabels within the oocyte.

This is the first report of the use of in vitro $^3$H labelled VG and VHDL II in fish as opposed to various in vivo labelled VG preparations which have been used to study VG uptake in fish (Selman and Wallace 1982, 1983, Tyler et al. 1988a,b, this study). The obvious advantages of in vitro [$^3$H]-succinimidyl propionate labelled preparations are that once the protein is obtained in a pure form it can be quickly labelled for use and has much higher specific activity. There was no evidence that $^3$H preparations labelled in vitro degrade in the blood or that the molecular conformation of the protein is altered following labelling such that they are no longer recognized by ovarian membrane receptors. The ovarian incorporation of VG radiolabelled in vivo and in vitro were quite similar. However, the processing that occurs within the oocyte may not be quite so clear due to the appearance of the low $M_r$ radiolabelled peak in [$^3$H]propionyl-VG injected fish as discussed above.

CHAPTER 3

# PITUITARY REGULATION OF VITELLOGENESIS IN WINTER FLOUNDER AND RAINBOW TROUT

## INTRODUCTION

Pituitary involvement in vitellogenesis entails regulating the synthesis of extraovarian yolk precursors and their uptake by the oocyte. The hepatic synthesis of VG in fish is controlled by ovarian $E_2$ (Mommsen and Walsh 1988) and the pituitary is implicated in the regulation of ovarian estrogen synthesis (Fostier et al. 1983). Although mGTH introduced experimentally in salmonids has been shown to increase $E_2$ production under in vivo and in vitro conditions its absence from the blood in rainbow trout during the vitellogenic phase of development (Sumpter and Scott 1989) does raise an important question. Is mGTH responsible for $E_2$ synthesis or are other pituitary factors at play during vitellogenesis? Another factor to be considered is T, a major precursor of $E_2$, and how it's production may be influenced by pituitary hormones?

The seasonal concentrations of $E_2$ and T in serum are known for a number of female teleosts, goldfish (Shreck and Hopwood 1974), plaice (Pleuronectes platessa, Wingfield and Grimm 1977) rainbow trout (Scott et al. 1980), catfish

(<u>Heteropneustes</u> <u>fossilis</u>, Lamba <u>et</u> <u>al</u>. 1983), brown bullhead
(<u>Ictalurus</u> <u>nebulosus</u>, Burke <u>et</u> <u>al</u>. 1984), red seabream
(Ouchi <u>et</u> <u>al</u>. 1988) and sea bass (<u>Dicentrarchus</u> <u>labrax</u> L,
Prat <u>et</u> <u>al</u>. 1990). A greater number of additional studies
have concentrated on $E_2$ and/or T levels during more
restricted parts of the reproductive cycle, particularly
during the maturational and spawning phases (Campbell <u>et</u> <u>al</u>.
1976, Billard <u>et</u> <u>al</u>. 1978, Crim and Idler 1978, Dindo and
MacGregor 1981, MacGregor <u>et</u> <u>al</u>. 1981, van Boheman and
Lambert 1981, Kagawa <u>et</u> <u>al</u>. 1983, Scott <u>et</u> <u>al</u>. 1984,
Truscott <u>et</u> <u>al</u>. 1986, Pankhurst and Conroy 1987, Yeung and
Chan 1987, Norberg <u>et</u> <u>al</u>. 1989). Characteristically both $E_2$
and T are usually low following the spawning phase of the
reproductive cycle and gradually increase over the period of
active ovarian growth. Typically, serum levels of $E_2$ begins
to decrease prior to final maturation while serum levels of
T are usually maintained until ovulation and spawning have
started.

In this chapter the seasonal serum concentrations of $E_2$
and T, and GSIs were investigated in female winter flounder.
In order to understand the influence of the pituitary on
serum levels of sex steroids during this period the ability
of ovarian fragments to produce $E_2$ and T in response to
homologous pituitary extract was examined <u>in</u> <u>vitro</u>.

Some technical limitations are obvious in trying to use
the winter flounder as a model species to study all aspects

of pituitary regulation of oocyte growth. The small size of the winter flounder ovarian follicle (maximum diameter <0.5 mm) precludes their use during in vitro incubation studies requiring considerable numbers of individual follicles or manual manipulations such as dissection of specific follicle cell layers. The relatively large salmonid ovarian follicle (maximum diameter 4-5 mm) does allow some degree of dissection and has been exploited to study sex steroid synthesis by discrete cell layers in vitro (Kagawa et al. 1982, Young et al. 1983a). When the thecal cell layer, the major T synthesizing tissue of salmonid ovarian follicles (Kagawa et al. 1982, Wright and Zhao 1988), is manually removed (= defolliculated follicle) $E_2$ production by granulosa cells can be assessed free of thecal cell influence (Young et al. 1983a). Testosterone is not produced by salmonid granulosa cells in response to mGTH (Kagawa et al. 1982, Wright and Zhao 1988). In this study rainbow trout ovarian follicles, with and without the surface epithelium-thecal layer, were used to examine the pituitary regulation of ovarian $E_2$ synthesis in vitro during the early vitellogenic phase. Two established teleost pituitary protein fractions, Con A I and Con A II, isolated from the closely related sockeye salmon (Oncorhynchus nerka) were used.

There have been two reports in the literature demonstrating the effects of GH on the production of ovarian

sex steroids in fish (Singh et al. 1988, Van Der Kraak et al. 1990). These findings suggested that GH alone or by potentiating the action of mGTH could increase $E_2$ and/or T production in ovarian incubates in vitro. With the knowledge that GH resides in the Con A I fraction (Komourdjian and Idler 1979) the idea arose that GH could be involved in aromatase enhancement. This prompted a study of ovarian $E_2$ and T production in vitro in winter flounder and rainbow trout, testing American plaice and sockeye salmon GH preparations, respectively.

The second aspect of pituitary control during vitellogenesis involves ovarian uptake of blood borne yolk precursors. Although it is known that surgical removal of the pituitary gland (i.e. hypophysectomy) in fish negatively affects ovarian development (Barr 1963), and ovarian uptake of radiolabelled leucine and phosphorus (Campbell and Idler 1976, Ng and Idler 1979), other aspects of pituitary involvement remain to be defined. One of these aspects is the seasonal impact of the pituitary on ovarian uptake of specific serum proteins. A procedure for ensuring complete hypophysectomy in winter flounder by monitoring serum thyroxine ($T_4$) levels after operation was developed and experiments conducted over the vitellogenic period to determine the effect of hypophysectomy on ovarian uptake of radiolabelled VG and VHDL II.

## MATERIALS AND METHODS

**Fish**

Mature winter flounder were obtained and held as
described in Chapter 2. Females caught monthly throughout
the year 1988-89 were killed within 18 hours of arrival at
the MSRL to provide samples for a seasonal study. Blood was
taken, serum prepared, aliquoted and frozen at -60°C as
previously described (pg. 10). Body weight and ovary weight
were recorded for calculation of the GSI for each fish. Fish
used as donors for ovarian incubations in vitro and
collection of pituitary glands were obtained in April 1990
and maintained in the lab as previously described. These
fish were in the prespawning phase of the reproductive
cycle.

Mature female rainbow trout were used from a stock of
winter spawning fish maintained at the MSRL. Fish had been
held in tanks (15 fish/ 240 l tank) supplied with flowing
freshwater at ambient temperature and natural photoperiod
and fed a commercial trout food (Rainbow Trout Brood Stock
Food, #8 Gr., Martin Feed Mills, Elmira, ON) once daily.
Females were selected over a period of approximately 6-8
months prior to spawning for their first (age 4+) or second
time (age 5+) during which their GSIs ranged between 0.5 and
2.5. Once the GSI in female rainbow trout reaches 0.16 and

an oocyte diameter of 0.6 mm is attained vitellogenesis is expected to begin (Sumpter et al. 1984).

Immature (age 1+) rainbow trout were obtained from Rainbow Springs Trout Farm, Thamesford, ON and held similarly to mature females. Fish were fed a commercial trout food (Trout and Salmon Food, #5 Gr., Martin Feed Mills, Elmira, ON) twice daily.

## In vitro incubations using ovarian tissues

Individual female winter flounder were sampled at monthly intervals from April to September encompassing the prespawning to early vitellogenic phase of their reproductive cycle. Fish were killed by decapitation, ovaries removed and placed on ice. Ovarian fragments (40-50 mg) dissected free of the ovarian wall were taken using scissors. The small size of the winter flounder ovarian follicle (<0.5 mm) precludes the isolation of a sufficient quantity of individual follicles. It was noted that ovarian follicles (n=20) from ovarian fragments used in May were classified as Stage 1 (i.e. germinal vesicle central, Ng and Idler 1978a) and June fish were postspawned. Ovarian fragments were placed in 12 x 75 mm borosilicate glass tubes with incubation medium consisting of modified FO solution (pg. 19) supplemented with 2% BSA (Sigma) and the antibiotics streptomycin sulfate and penicillin G (Sigma),

both at 100,000 I.U./l. The addition of BSA dramatically altered the ability of ovarian fragments to synthesize $E_2$ in response to winter flounder pituitary extract (PE). The level of 2% BSA, since it represented a compromise between maximum levels of $E_2$ and T produced, was selected for subsequent incubations from preliminary studies described in Figs. 25 and 26. The final volume of incubation media in assay tubes was 0.5 ml. Tubes were arranged in triplicate for each dose and incubations conducted in the dark with humidified 100% $O_2$ at 8°C in a shaking water bath for 24 hours. Following incubation all media were recovered and used immediately or frozen at -20°C.

Mature female rainbow trout were subdued in a solution of 2-phenoxyethanol (0.4 ml/l) (Sigma) and quickly killed by decapitation, ovaries removed and immediately placed in cold incubation medium. The incubation medium used was trout balanced salt solution pH 7.5 (Jalabert et al. 1973, Jalabert 1976). Intact follicles (1.0-2.5 mm) were dissected from the ovaries using fine forceps (N5, Dumont) and spring scissors (IR-116-11.5 cm, IREX) under a stereomicroscope (Wild M5A) with a water cooled stage. Defolliculated follicles (following the terminology of Schuetz and Lessman 1982) were prepared from intact follicles which had been separated from the ovary the previous day and left in incubation medium at 4°C. A small nick was made in the surface epithelium-thecal layer with the scissors and by
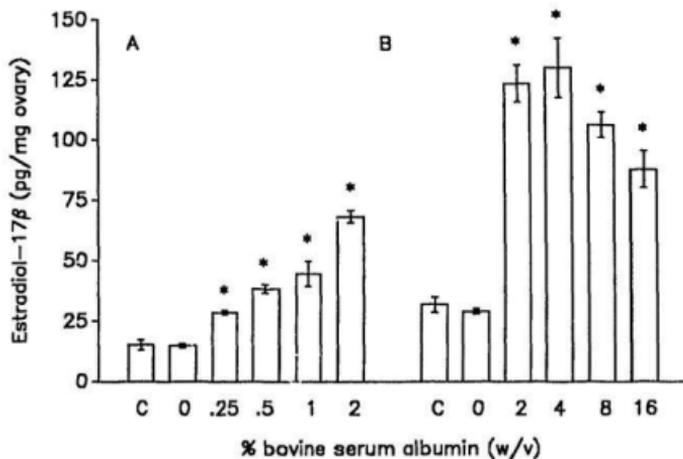
Figure 25: The effect of the addition of bovine serum albumin (BSA) to incubation media on production of estradiol-17$\beta$ _in vitro_ by winter flounder ovarian fragments in response to homologous pituitary extract (PE; 0.5 pituitary equivalents/ml media). A and B represent experiments using different fish. Controls (C) had no BSA or PE added. Each bar represents a mean ± SE; *=significantly different from C at p<0.05.
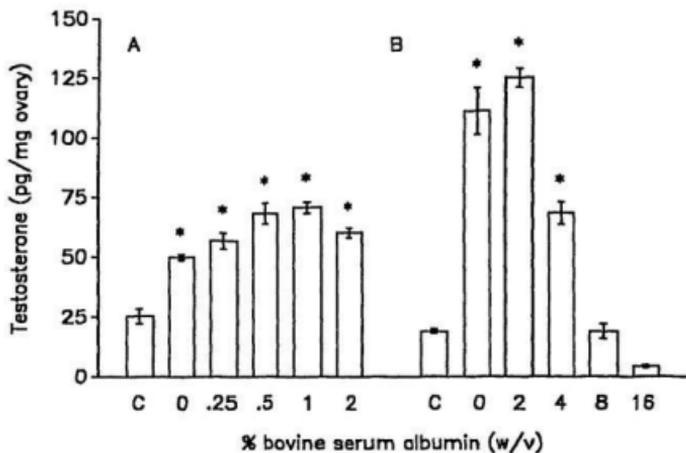
Figure 26: The effect of the addition of bovine serum albumin to incubation media on production of testosterone _in vitro_ by winter flounder ovarian fragments in response to homologous pituitary extract as in Fig. 25.

holding one of the edges and gradually enlarging the opening this layer could be removed. The defolliculated preparation remaining consisted of an intact oocyte surrounded by a relatively undisturbed granulosa (follicular epithelium) cell layer verified by observations using a scanning electron microscope (Hitachi S570).

Groups of 5 follicle preparations (either intact or defolliculated) were placed in 12 x 75 mm borosilicate glass tubes containing 0.5 ml incubation medium. Triplicate tubes were used for each dose. Incubations were conducted identical to those for winter flounder above except at 15°C. Following incubation all media were recovered and either extracted immediately or frozen at -20°C. All follicle preparations in each assay were derived from a single fish and all assays were repeated with a different fish. Results between assays in a given experiment were always reproducible, however control levels and the magnitude of response varied significantly between fish preventing the pooling of assays from each experiment. A representative assay is therefore shown for each experiment.

Ovaries from immature rainbow trout were obtained and used in a manner similar to that for mature females except that ovarian fragments (40-50 mg/tube) utilized for _in_ _vitro_ incubations were pooled from 6 fish and randomly placed in the tubes.

**Hormone preparations**

Winter flounder ovarian fragments were incubated in the
presence of either winter flounder PE, American plaice GH
(isolation described on pg. 124) or T. Pituitary extract was
used since purified winter flounder GTH preparations are not
yet available. The PE was derived from equal numbers of
fresh male and female winter flounder pituitary glands
prepared in April, June and August and used in incubations
for that month and the one following (i.e. April PE used for
April and May incubations). Pituitaries were collected from
both sexes to provide a sufficient quantity for all
incubations. The pituitaries were homogenized in 4 volumes
of incubation media (modified FO solution) which was left at
4°C overnight. The homogenate was centrifuged at 13,000x G
for 5 minutes at room temperature the next day, the
supernatant removed and the pellet extracted again with 2
volumes of incubation media, left for 2 hours and
centrifuged as above to recover the supernatant.
Supernatants were pooled, diluted as required and used
immediately or frozen at -60°C. Testosterone (Steraloids)
dissolved in 100% ethanol (glass distilled) was added to
incubation tubes, warmed at 45°C in a water bath and the
ethanol evaporated under a stream of $N_2$ before adding the
ovarian fragments and incubation media.

For experiments dealing with rainbow trout, pituitary protein fractions were prepared from sockeye salmon since the quantity required was unobtainable from rainbow trout and both species belong to the same genus. Sockeye salmon pituitary glands (80 g) were collected from fish caught commercially in the Strait of Juan de Fuca, BC, Canada, during August. Pituitaries were obtained from mixed sexes since there was no opportunity to sex the fish. The fish were approximately 2 months from spawning and females (n=4) sampled had GSIs of about 4. Pituitaries were kept on dry ice following removal until they could be stored at -60°C. A crude PE was derived by homogenizing pituitaries in 4 volumes of Buffer B (Idler and Hwang 1978) with 3% aprotinin. The PE was stored at 4°C for 24 hours, followed by centrifuging at 14,600x G for 1 hour, and collection of the supernatant. Carbohydrate-poor and Con A II protein fractions were isolated from the supernatant by affinity chromatography on Con A (Idler and Hwang 1978). The Con A I fraction was re-run on Con A to reduce any residual Con A II contamination. Using a chum salmon mGTH RIA (Truscott et al. 1986) only 2.8 ng mGTH/µg Con A I (i.e. 0.28% of the total) was measured after the second pass on Con A. Purified mGTH was subsequently isolated from the Con A II fraction by chromatography on AcA 54, collecting the third protein peak eluted (42,000 $M_r$), followed by anion exchange chromatography on diethylaminoethyl Bio-gel A (BioRad) and

isolation of the protein eluting with 0.2 M ammonium
bicarbonate (Idler et al. 1975a). This mGTH preparation
stimulated cyclic adenosine monophosphate and $E_2$ production
in intact rainbow trout ovarian follicles in vitro, and
germinal vesicle breakdown in vitro in preovulatory ovarian
follicles from landlocked Atlantic salmon. Con A I and Con A
II fractions were placed in dialytic sacs (8,000-10,000 $M_r$
cut-off, SpectraPor) and dialysed against trout balanced
salt solution for 48 hours at 5°C before they were used for
incubations. Protein concentrations of the Con A I and Con A
II fractions were determined by the TCA-Lowry method using
BSA (Pierce) as a standard.

Sockeye salmon GH isolation is described in Chapter 4
(pg. 124). Testosterone was dissolved in 100% ethanol and
diluted in incubation media resulting in a final ethanol
content of less than 0.01%.

## Steroid radioimmunoassays

Antisera specific for $E_2$ and T (Steraloids) were raised
in male New Zealand white rabbits injected with $E_2$ or T (3-
0-carboxy-methyl)-oxime conjugated to BSA as described by
Idler and Ng (1979). The Ab developed for $E_2$ had no cross-
reaction with T even at the high concentrations (10 $\mu g/ml$)
used in some ovarian incubations but significantly cross-
reacted with estrone and estriol. The T Ab had no cross-

reaction with $E_2$, 11 keto-testosterone or $11\beta$-hydroxytestosterone. Tritiated $E_2$ and T purchased from NEN DuPont were cleaned up by paper chromatography prior to their use as both label and recovery tracer. Winter flounder serum aliquots (200 or 500 μl) were extracted twice with diethyl ether, evaporated to dryness under $N_2$ and reconstituted in 0.5 ml 100% ethanol. Extraction efficiency of serum was calculated by recovery of added $[^3H]E_2$ or $[^3H]T$. To overcome the possible interference of either estrone or estriol in determining serum $E_2$ levels the ethanol extracts were further chromatographed on glass columns (6 x 145 mm) of Sephadex LH-20 (Pharmacia) to specifically isolate the $E_2$ fraction (DeJong et al. 1973). Incubation media were extracted using techniques identical to those for serum except that only $[^3H]E_2$ was added as steroid tracer and both $E_2$ and T recoveries estimated from it. Chromatography of media extracts on Sephadex LH-20 prior to RIA was not done since preliminary studies in winter flounder revealed that there was no difference between $E_2$ levels with or without chromatography. In rainbow trout experiments chromatography of media extracts on Sephadex LH-20 to separate $E_2$ fractions from those of estrone and estriol showed that follicular preparations produced predominately $E_2$ (78%). This confirmed that $E_2$ is the major estrogen produced by the rainbow trout ovary similar to conclusions reached by Sire and Dépêche

(1981). All media extracts were assayed direct and values reported are expressed as $E_2$.

For RIA, aliquots of the ethanol extract in duplicate were evaporated under $N_2$ and reconstituted in 200 μl RIA buffer (Simpson and Wright 1977) prepared without sodium azide. Tritiated $E_2$ or T (100 μl) was added, vortexed, and 100 μl of the appropriate antisera ($E_2$ - 1:6,000 dilution or T - 1:30,000 dilution) added, vortexed and left overnight on ice. The following day addition of dextran-coated charcoal (600 μl) followed by centrifugation was used to separate Ab-bound from free tracer (Simpson and Wright 1977). An aliquot (800 μl) added to liquid scintillation fluid (Ready-Safe, Beckman) was then counted in a scintillation counter (Packard Tri-Carb Model 300C). Serially diluted $E_2$ (2-500 pg) or T (15-1000 pg) were similarly carried through the above procedures to provide standard curves. The log-logit weighted regression lines for $E_2$ and T, respectively, had $r^2$s = 0.998 and 0.986, slopes of -2.177 and -2.620, intercepts of 3.610 and 5.924 and $B/B_o$s of 37.3 and 182.19 pg. Ethanol extracts of the unknowns serially diluted between 25 and 175 μl parallelled the standard curves in both cases.

**Double equilibrium dialysis**

The activity of serum $E_2$ binding proteins were determined
at two points during the annual cycle in winter flounder by
double equilibrium dialysis similar to studies on
transcortin by Freeman and Idler (1966). Pooled sera of 5
fish from October (vitellogenic phase) and April (pre-
spawning) were diluted 10 fold with 0.9% saline solution and
0.8 ml pipetted into 4 mm diameter dialysis tubing (12,000-
14,000 $M_r$ cut-off, Spectrapor) which was then knotted at
both ends. Controls consisted of sacs containing 0.8 ml 0.9%
saline only. Sacs (n=3 for each treatment) were placed in 15
ml screw top test tubes containing 12 ml 0.9% saline and
[$^3$H]$E_2$ (76,000 dpm; < 1% of the lowest serum $E_2$
concentration). Tubes were attached to a turntable and
rotated at 50 rpm and an angle of 45° for 48 hours at 4°C.
Aliquots from the dialysis sacs (100 $\mu$l) were added to
liquid scintillation fluid and counted in a scintillation
counter to determine the % bound [$^3$H]$E_2$.

**Experiments on hypophysectomy and ovarian uptake of
vitellogenin and very high density lipoprotein II**

To confirm hypophysectomy in winter flounder a study was
undertaken to examine serum thyroxine ($T_4$) levels before and
after the operation as a criterion for it's success. Adult

winter flounder (males and females) were sham-operated or
hypophysectomized according to the procedure of Campbell and
Idler (1976). Fish were subsequently blood sampled at 2 or
3, 5 and 10 days intervals post operation, serum prepared
and frozen as described earlier (pg. 10). After the last
sampling all the fish were killed, the cranium exposed and
the presence or absence of the pituitary checked to ensure
that fish were completely sham-operated or hypophysectomized
respectively. Serum $T_4$ levels were measured in 100 $\mu$l serum
samples following the RIA procedure of Brown and Eales
(1977). Radiolabelled [$^{125}$I]$T_4$ with a specific activity of
1250 $\mu$Ci/$\mu$g was purchased from NEN DuPont. Standard
solutions (15-1000 pg/100 $\mu$l) were made up from L-$T_4$
(Sigma). The $T_4$ Ab (Calbiochem) was used at 1:5,000 dilution
in the RIA. A typical log-logit weighted regression line for
the standard dose-response curve had an $r^2$ = 0.997, a slope
of -1.922, an intercept of 4.324 and a $B/B_0$ of 182.7 pg. All
serum samples from a given fish were run in the same assay
to minimize interassay variation.

The presence or absence of the pituitary gland was
examined in relation to ovarian uptake of [$^{131}$I]VG and
[$^{131}$I]VHDL II $\underline{in}$ $\underline{vivo}$. Fish were hypophysectomized or sham-
operated and successful hypophysectomy determined as above
by measurement of serum $T_4$ levels 5 days post operation.
After 14 days fish were injected iv with either 1.0 mCi
[$^{131}$I]VG or [$^{131}$I]VHDL II/kg fish and returned to their tanks;

48 hours later fish were killed by decapitation, body and ovary weights taken and 2 gm portions of ovary (dissected free of the ovarian wall) counted in a gamma scintillation counter (Packard 5650).

## Statistical analyses

Analyses involved analysis of variance followed by Tukey's multiple comparisons test (Zar 1984) to determine significant differences between monthly steroid levels, in vitro incubation treatment groups or the ovarian accumulation of [$^{131}$I]VG or [$^{131}$I]VHDL II in hypophysectomized and sham-operated fish.

## RESULTS

### Seasonal gonadosomatic indices and serum levels of estradiol-17β and testosterone in female winter flounder

The ovaries in winter flounder undergo a typical increase in size relative to body weight during the annual reproductive cycle (Fig. 27). The maximum GSIs occur in March and April immediately preceding the spawning period. The spawning period of winter flounder was broadly delineated by the first occurrence of spawned out females
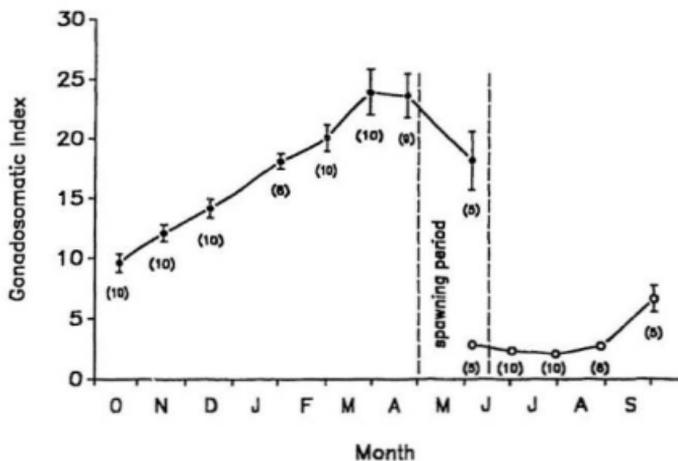
Figure 27: Seasonal gonadosomatic indices for female
winter flounder from Conception Bay,
Newfoundland. Each point represents mean ±
SE; (number of fish). Points without error
bars were too small to be shown; closed
symbols = prespawned fish, open symbols =
postspawned fish.

and the latest evidence of gravid females during repeated samplings from the Conception Bay location.

Serum levels of $E_2$ steadily increase over the annual reproductive cycle to reach their highest levels immediately before the spawning period after which levels dropped precipitously (Fig. 28). A significant ($p < 0.05$) peak in serum $E_2$ was noted in November during the vitellogenic period. The associated serum levels of T also increase progressively with the onset of the spawning season (Fig. 29). Notable is a significant ($p < 0.05$) peak in T approximately one month before spawning, followed by a drop and then a resurgence to the highest annual levels during the mid-spawning period. Serum levels of both $E_2$ and T decreased dramatically after the fish spawned (Figs. 28 and 29) and 500 $\mu$l of serum was required for extraction of sufficient assayable steroid for assay in the RIA. Double equilibrium dialysis revealed similar levels of serum $E_2$ binding protein in April and October, 79 and 78% of the added $[^3H]E_2$ was bound, respectively.

**Ovarian estradiol-17$\beta$ and testosterone responses to pituitary extract _in vitro_ during the prespawning to vitellogenic phase**

Ovarian incubations were conducted _in vitro_ coincident with the prespawning to early vitellogenic phases of the
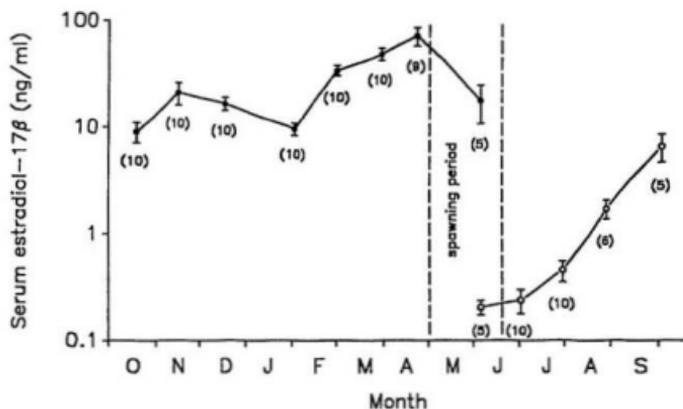
Figure 28: Seasonal serum concentrations of estradiol-17β
(E₂) in female winter flounder from Conception
Bay, Newfoundland. For symbol information see
Fig. 27. Significant differences are discussed in
the text. Note that E₂ levels are presented on a
log scale.
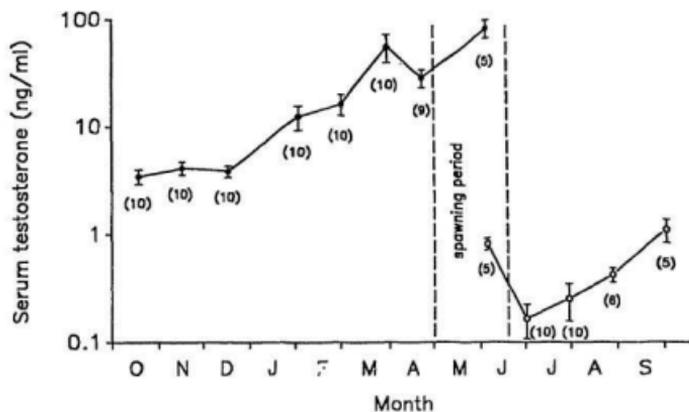
Figure 29: Seasonal serum concentrations of testosterone
(T) in female winter flounder from Conception
Bay, Newfoundland. For symbol information see
Fig. 27. Significant differences are discussed in
the text. Note that T levels are presented on a
log scale.

reproductive cycle when the most dramatic fluctuation in serum $E_2$ and T were observed. During the prespawning (April) and spawning (May) periods $E_2$ production was no different between controls and PE treated groups (Fig. 30). Amongst control groups $E_2$ synthesis was much greater during the prespawning as opposed to the spawning period. In postspawned fish a steady increase in $E_2$ synthesis by ovarian fragments incubated with PE was observed throughout the early vitellogenic phase (July-September). Conversely, T production by ovarian fragments in response to PE increased from the prespawning period to reach maximal levels during the spawning period (May; Fig. 31). Following spawning and throughout the early vitellogenic phase T production _in vitro_ was low.

The addition of increasing amounts of T (10-1,000 ng/ml) to ovarian incubations _in vitro_ during the early vitellogenic phase demonstrated a rapid dose related biosynthesis of $E_2$ compared to the control after 24 hours (Fig. 32).

**Ovarian aromatase regulation in rainbow trout ovarian follicles by sockeye salmon pituitary protein fractions _in vitro_**

Initially, the response by intact follicles of mature rainbow trout to produce $E_2$ in the presence of either
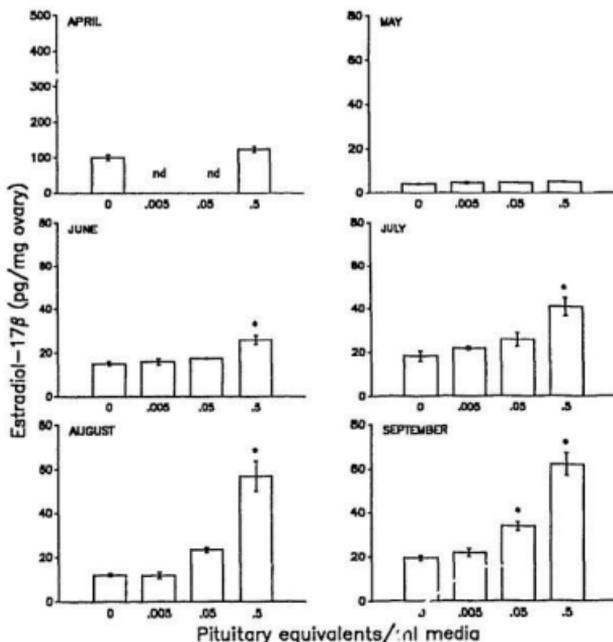
Figure 30: Production of estradiol-17$\beta$ by winter flounder
ovarian fragments *in vitro* in response to
homologous pituitary extract during the
prespawning to early vitellogenic phases of the
reproductive cycle. Each bar represents a mean ±
SE; * = significantly different from
the control (0) at p<0.05; nd = not determined.
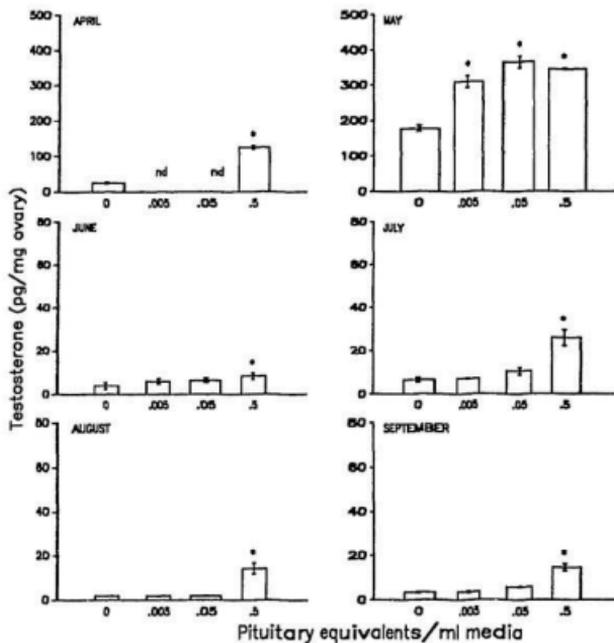
Figure 31: Production of testosterone by winter flounder ovarian fragments *in vitro* in response to homologous pituitary extract as described in the legend of Fig. 30.
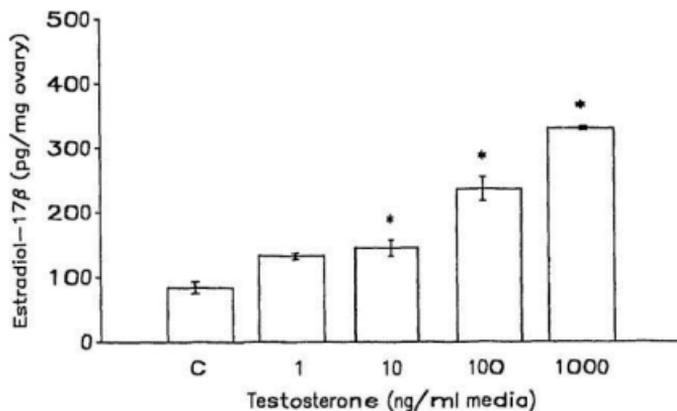
Figure 32: Production of estradiol-17β by winter flounder
ovarian fragments in vitro incubated with
different doses of testosterone during the early
vitellogenic phase (August) of the reproductive
cycle. Bars and * as in Fig. 30.

sockeye salmon Con A I or Con A II was examined. In this experiment the Con A II preparation significantly increased $E_2$ levels above the control levels (Fig. 33). A dose response was observed at Con A II doses of 20 and 200 $\mu g/ml$ and $E_2$ levels rose to 0.8 and 1.22 ng/ml respectively compared to 0.08 ng/ml for the control. Incubations treated with Con A I were not significantly different (p>0.05) from the control level at any of the doses tested.

To establish that elevated levels of T increase $E_2$ production different amounts of T were incubated with intact follicles. Testosterone added to the incubation media dramatically increased the amount of $E_2$ normally produced (Fig. 34). The doses of 10-1,000 ng T/ml produced incremental increases in $E_2$ statistically significant from the control level.

To determine the response by granulosa cells to exogenous T different doses of T were incubated with defolliculated follicles (Fig. 35). All doses of T beyond 0.1 $\mu g/ml$ significantly elevated $E_2$ production. However, beyond the dose of 0.1 $\mu g$ T/ml increasing amounts of T caused no further increase in $E_2$ production and appeared to decrease production. Although not statistically significant high levels of T may inhibit $E_2$ production under these conditions. The level of 1 $\mu g$ T/ml was chosen for future experiments as substrate for aromatase since this level gives maximum $E_2$ production similar to 0.1 $\mu g$ T/ml,
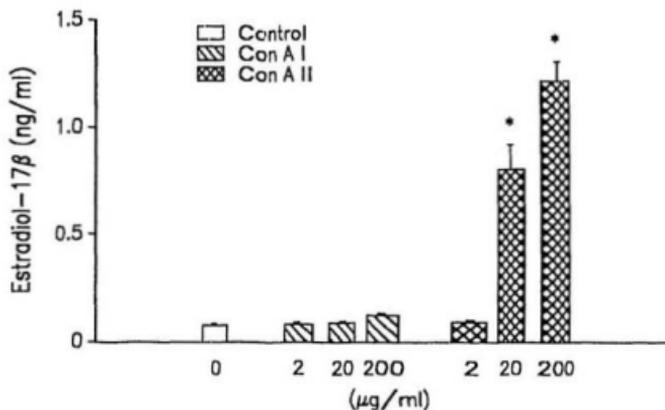
Figure 33: Production of estradiol-17β by isolated intact ovarian follicles from rainbow trout incubated *in vitro* with different concentrations of sockeye salmon pituitary protein carbohydrate -poor (Con A I) or carbohydrate-rich (Con A II) fractions. Each bar represents a mean + SE; * = significantly different from the control at $p < 0.001$.
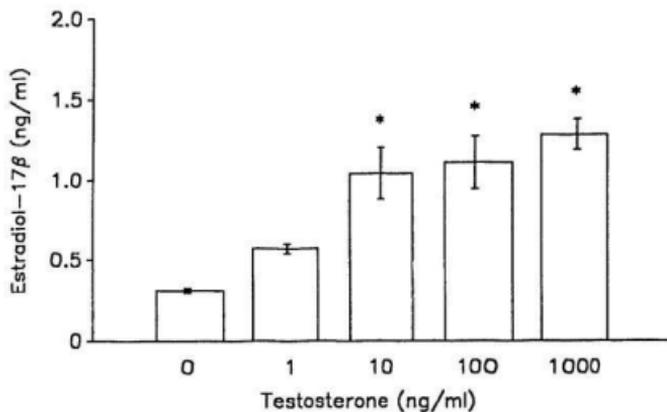
Figure 34: Production of estradiol-17β by isolated intact
ovarian follicles from rainbow trout incubated
in vitro with different concentrations of
testosterone. Bars as in Fig. 33; * =
significantly different from the control at
p<0.005.
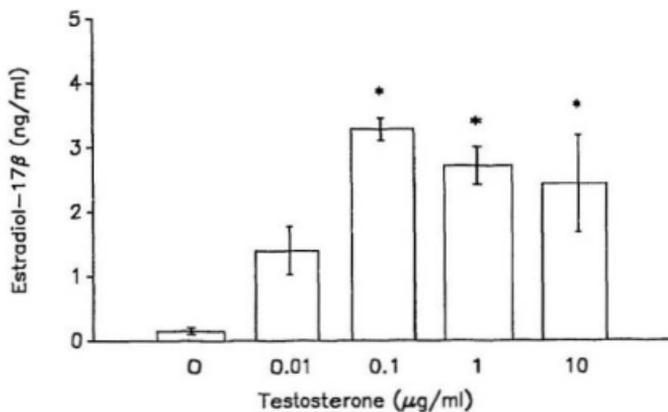
Figure 35: Production of estradiol-17β by isolated defolliculated ovarian follicles from rainbow trout incubated *in vitro* with different concentrations of testosterone. Bars as in Fig. 33; * = significantly different from the control at p<0.025.

yet provides an excess of exogenous T in the medium available for further aromatization.

The fourth experiment tested the effect of Con A I and Con A II on $E_2$ production by defolliculated follicles incubated in the presence of exogenous T at 1 µg T/ml as determined in the previous experiment (Fig. 35). The rationale being that Con A I and/or Con A II might enhance the aromatase activity within granulosa cells resulting in further aromatization of available exogenous T and higher $E_2$ levels. Only the Con A I preparation was able to significantly elevate $E_2$ production (Fig. 36). Although a dose response is suggested only the Con A I level of 200 µg/ml significantly ($p < 0.025$) increased $E_2$ levels to 11.75 ng/ml relative to 2.4 ng/ml for the control. All doses of Con A II resulted in $E_2$ levels no different from the control. Assays conducted later in the reproductive cycle during mid to late vitellogenesis (intact follicle diameter >3 mm) demonstrated a diminished effect of the Con A I fraction in stimulating $E_2$ synthesis under the above conditions.

**The effect of growth hormone on ovarian estradiol-17β and testosterone production _in vitro_ in rainbow trout and winter flounder**

Two series of experiments were conducted to test the
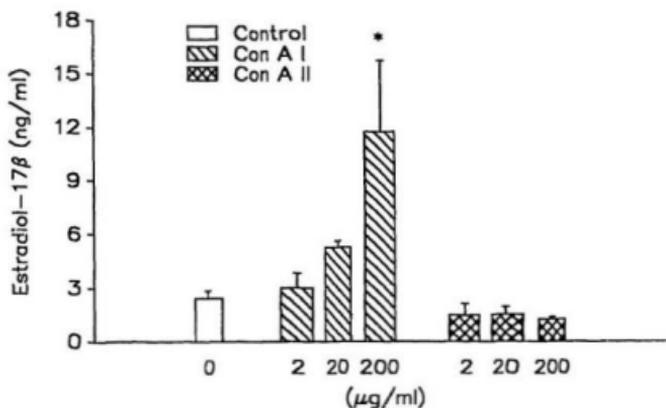
Figure 36: Production of estradiol-17β by isolated
defolliculated ovarian follicles from rainbow
trout incubated in vitro with testosterone
(1 μg/ml), and different concentrations of
sockeye salmon pituitary carbohydrate-poor
(Con A I) or carbohydrate-rich (Con A II)
fractions. Bars as in Fig. 33; * = significantly
different from the control at $p < 0.025$.

effects of GH on ovarian $E_2$ and T production in vitro: 1) using plaice GH on winter flounder and 2) sockeye salmon GH on rainbow trout. In winter flounder 4 assays were done in which both 21,000 and 42,000 $M_r$ forms of plaice GH were tested on ovarian fragments taken from fish during June-August (i.e. early vitellogenic phase). It could not be shown that plaice GH (10-1,000 ng/ml) affected ovarian $E_2$ or T production (p>0.05) although in all cases winter flounder PE (0.25 pituitary equivalent/ml) significantly (p<0.05) increased both steroids above control levels.

Similar experiments testing the effect of sockeye salmon GH (1-1,000 ng/ml) on ovarian fragments and intact ovarian follicles from immature and mature rainbow trout, respectively, had no effect (p>0.05). The use of sockeye salmon mGTH (100 ng/ml) consistently stimulated levels of $E_2$ and T significantly higher than the controls (p<0.05). No differences in ovarian $E_2$ or T production were observed between sockeye salmon mGTH (10 and 100 ng/ml) added alone or the addition of sockeye salmon GH (100 ng/ml) to mGTH (10 and 100 ng/ml) during incubation of intact follicles. Similarly, incubates of ovarian follicles in which sockeye salmon GH (1-1,000 ng/ml) was added along with T (1 μg/ml) were no different from T (1 μg/ml) alone in respect to $E_2$ production.

**Thyroxine status as a criterion for successful**
**hypophysectomy**

Hypophysectomy resulted in a dramatic reduction of serum
$T_4$ levels when compared to sham-operated controls (Figs. 37
and 38). In males $T_4$ levels were significantly lower
($p<0.05$) 3 days after hypophysectomy and by 10 days had
reached the lower limit of detection in the RIA. Thyroxine
levels in sham-operated controls although increasing 3 days
after the operation by 10 days were still within the range
of $T_4$ measurements before operation. Female $T_4$ levels
following hypophysectomy appeared to take longer to decrease
but by 5 days post operation they were significantly lower
($p<0.05$) than controls. The $T_4$ levels continued to decrease
up to 10 days similar to the males. However $T_4$ levels in
female sham-operated controls increased, and by 10 days
after the operation were significantly higher than the level
before operation.

**Effect of hypophysectomy on ovarian uptake of [$^{131}$I]-**
**vitellogenin and [$^{131}$I]very high density lipoprotein II**

Hypophysectomy significantly reduced the ovarian uptake
of both [$^{131}$I]VG (Table 5) and [$^{131}$I]VHDL II (Table 6) in
winter flounder during the early part of the vitellogenic
phase (i.e. GSI = 3). This effect extended in the case of
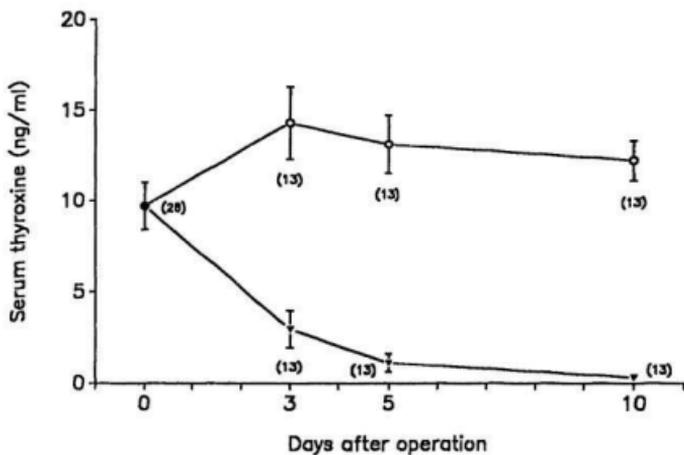
Figure 37: Serum thyroxine levels [mean ± SE (number of
fish)] during November in male winter flounder
sampled at 0,3,5, and 10 days after
hypophysectomy (▼) or sham operation (o). Points
without error bars were too small to be shown.
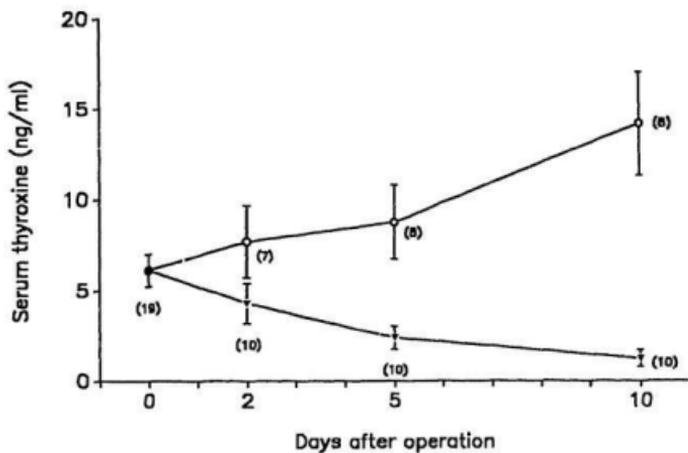
Figure 38: Serum thyroxine levels (data points as in Fig.
37) during January in female winter flounder
sampled at 0,2,5, and 10 days after
hypophysectomy (▾) or sham operation (o).

Table 5: Ovarian uptake of [$^{131}$I]vitellogenin over 48 hours
by hypophysectomized (hypex) and sham-operated
female winter flounder during the mid-vitellogenic
period (September-December).

| Experiment | Treatment<br>(number of fish) | [1]GSI | Ovary count<br>(cpm/g) |
|---|---|---|---|
| 1. | Sham (6) | [2]2.9 ± 0.2 | 3705 ± 293** |
|  | Hypex (7) | 2.4 ± 0.2 | 1683 ± 171 |
| 2. | Sham (8) | 5.4 ± 0.2** | 2575 ± 193** |
|  | Hypex (6) | 3.4 ± 0.4 | 1010 ± 62 |
| 3. | Sham (8) | 15.8 ± 1.3 | 2893 ± 190* |
|  | Hypex (6) | 14.8 ± 1.2 | 2128 ± 137 |
| 4. | Sham (7) | 16.8 ± 1.4 | 5770 ± 702 |
|  | Hypex (8) | 16.9 ± 0.8 | 4194 ± 646 |

[1]gonadosomatic index, [2]data presented as mean ± standard
error; ** p<0.001, * p<0.02.

Table 6: Ovarian uptake of [$^{131}$I]very high density
lipoprotein II over 48 hours by hypophysectomized
(hypex) and sham-operated female winter flounder
during the mid-vitellogenic period (September
-December).

| Experiment | Treatment (number of fish) | [1]GSI | Ovary count (cpm/g) |
|------------|----------------------------|--------|---------------------|
| 1. | Sham (6) | [2]2.9 ± 0.2 | 4906 ± 374* |
|    | Hypex (7) | 2.2 ± 0.1 | 3126 ± 460 |
| 2. | Sham (8) | 4.9 ± 0.2* | 1784 ± 193* |
|    | Hypex (6) | 3.3 ± 0.4 | 1039 ± 130 |
| 3. | Sham (8) | 13.5 ± 0.9 | 814 ± 49 |
|    | Hypex (6) | 14.1 ± 1.0 | 846 ± 61 |

[1]gonadosomatic index, [2]data presented as mean ± standard
error; * $p < 0.02$.

VG until the ovary had attained one half of its eventual GSI (i.e. GSI = 15). However, the ovarian uptake of both [$^{131}$I]VG and [$^{131}$I]VHDL II were not significantly affected by hypophysectomy after GSIs of about 16 and 13 were attained, respectively.

### DISCUSSION

The serum concentrations of $E_2$ and T coincide with the gradual increase in the GSI of female winter flounder over the annual reproductive cycle (Figs. 27-29). This pattern is consistent with data on other female teleosts and the role these sex steroids play in the process of vitellogenesis (Mommsen and Walsh 1988). However, the high serum levels of $E_2$ and T immediately preceding and during the spawning period, respectively, are not as common but have been observed to varying degrees in brown bullhead (Burke et al. 1984), red seabream (Ouchi et al. 1988) and sea bass (Prat et al. 1990). In general, the synthetic capacity of the winter flounder ovary for $E_2$ and T in response to PE in vitro follows the pattern of serum concentrations of these steroids over the prespawning to vitellogenic period (Figs. 30 and 31). There are some exceptions and these are discussed in relation to serum levels below.

The physiological significance of high serum $E_2$ levels during the prespawning period is presently not understood. The level of serum $E_2$ binding protein activity is no different between prespawning and vitellogenic fish ruling out the possibility that most of the $E_2$ reported in pre-spawning fish is protein bound (i.e. biologically inert). The hepatic synthesis of VG is decreasing as reflected by serum VG levels at this time of year (Fig. 7); therefore a

role for $E_2$ in vitellogenesis is not suspected. A mitogenic effect of $E_2$ on the teleost ovary at the time of spawning has been suggested (Fostier et al. 1983), but not explored.

The synthesis of $E_2$ was not stimulated by PE before and during the spawning period (Fig. 30) when serum $E_2$ levels are at their peak (Fig. 28). Only one study similar in approach to the present methodology has been reported for another pleuronectid, plaice, in which ovarian $E_2$ synthesis by homologous PE in vitro was examined (Yaron and Barton 1980). However $E_2$ production was stimulated by PE in a "vitellogenic" female (with an advanced GSI=27), although the proximity to spawning was not reported. The winter flounder ovary has considerable aromatization potential during the prespawning phase judging from the amounts of $E_2$ produced by the control incubations relative to controls during the spawning and vitellogenic phases. Since the PE stimulation of ovarian synthesis of T is very strong during the prespawning phase this is probably the reason for high levels of $E_2$. The substantial aromatase activity during prespawning therefore is probably not linked to PE (i.e. mGTH) which is in agreement with other in vitro studies involving aromatase activity and mGTH in salmonids (Kagawa et al. 1982, Young et al. 1983a, this study below).

The addition of BSA (<2%) to the incubation media had a profound effect by increasing $E_2$ synthesis by ovarian fragments in response to PE (Fig. 25). In fact, BSA was

required along with PE before any significant $E_2$ production
was evidenced unlike T in which BSA addition only slightly
enhanced production. It is known that $E_2$ is sensitive to
atmospheric oxidation and one explanation may be that under
high $O_2$ concentrations during _in vitro_ incubation BSA
protects $E_2$ from oxidation. Excess BSA (>2%) generally
reduced _in vitro_ $E_2$ and T production. At high BSA
concentrations tissue osmolar integrity could be compromised
resulting in lowered sex steroid production.

The highest seasonal levels of T in serum occurred during
the prespawning and spawning periods (Fig. 29) which was
coincident with the best response by ovarian tissues to
synthesize T _in vitro_ (Fig. 31). These results suggest a
role for T during the final maturation-ovulation process in
winter flounder. There is evidence that a shift in enzyme
biosynthetic pathways from $C_{18}$ and $C_{19}$ to predominantly $C_{21}$
steroid production occurs in salmonids as ovaries approach
final oocyte maturation leading to the formation of
progestogens (Scott and Canario 1987). Progestogens are
important for oocyte maturation in salmonids and 17$\alpha$,20$\beta$-
dihydroxy-4-pregnen-3-one is an established maturation
inducing steroid (Jalabert 1976, Goetz 1983). In the
Atlantic croaker (_Micropogonias undulatus_) the maturation
inducing steroid appears to be 17$\alpha$,20$\beta$,21-trihydroxy-4-
pregnen-3-one (Trant _et al_. 1986). In winter flounder the
maturation inducing steroid is not established but other

progestogens along with 17α,20β-dihydroxy-4-pregnen-3-one
are being studied as possibilities in plaice (<u>Pleuronectes</u>
<u>platessa</u>) and dab (<u>Limanda</u> <u>limanda</u>) (Canario and Scott
1990). Certainly, at least in winter flounder, the $C_{19}$
steroid pathway is very active during the prespawning period
including final maturation. The role of T as a maturation
inducing steroid should be examined in winter flounder.

Production of T <u>in</u> <u>vitro</u> is low following spawning and
does not appear to increase with the onset of the
vitellogenic period (Fig. 31) although serum levels increase
steadily (Fig. 29). The most likely explanation is that T
being a precursor of $E_2$ is rapidly converted by aromatase to
$E_2$ during <u>in</u> <u>vitro</u> incubation as was evidenced in Fig. 32.
Normally <u>in</u> <u>vivo</u> there would be an opportunity for T release
into the blood as well as T acting as a precursor for $E_2$ in
the ovary.

Experiments with rainbow trout demonstrated that the
sockeye salmon pituitary Con A I fraction is capable of
enhancing aromatase activity <u>in</u> <u>vitro</u> in the rainbow trout
ovarian follicle during the early vitellogenic phase of the
reproductive cycle (Fig. 36). A proposed mechanism for the
regulation of salmonid $E_2$ synthesis can be generated from
these results and earlier studies (Kagawa <u>et al</u>. 1982, Young
<u>et al</u>. 1983a). Maturational-type GTH induces T synthesis by
the thecal cells, which in turn is aromatized to $E_2$ by
granulosa cells. The aromatase enzyme, present in the

granulosa cells, is likely regulated by a component(s) of the Con A I pituitary fraction. On this basis mGTH (i.e. Con A II) has an indirect affect on $E_2$ synthesis by providing T produced by the thecal cells as aromatizable substrate. Endogenous levels of ovarian aromatase are sufficient to produce elevated levels of $E_2$ as observed in Fig. 33. This explains the action observed in many studies that mGTH in vitro is able to increase $E_2$ synthesis by intact follicles (Kagawa et al. 1982, Zohar et al. 1982, Young et al. 1983a, Van Der Kraak and Donaldson 1986, Kanamori et al. 1988, Swanson et al. 1987a, Suzuki et al. 1988b). Alternatively, the presence or absence of mGTH made no difference to $E_2$ production by follicle preparations containing only granulosa cells incubated with exogenous T in this study and others (Kagawa et al. 1982, Young et al. 1983a). These results provide good evidence that mGTH is not directly involved in $E_2$ synthesis.

The level of ovarian aromatase activity appears to undergo a seasonal cycle that peaks during the mid-vitellogenic phase and decreases toward spawning time in the amago salmon (Young et al. 1983a, Kanamori et al. 1988). It was apparent from control levels in this study that aromatase enzyme is present in the granulosa cells of the ovarian follicle from rainbow trout during the early vitellogenic period (Fig. 35). The mechanism by which Con A I enhances ovarian $E_2$ production is not understood but it

may function to increase the amount of aromatase. A maximum amount of aromatase is probably attained by the mid-point of the vitellogenic phase since it was found that the ability of Con A I to enhance $E_2$ diminished once follicle diameters >3 mm were attained.

Established components that have been isolated from the Con A I pituitary fraction from teleost fish which could possibly have some affect on aromatase activity are GH (Komourdjian and Idler 1979, Wagner and McKeown 1985, Idler et al. 1989, this study below), prolactin (Idler et al. 1978, Song et al. 1988) and the Con A I $R_f$ 0.5 and 0.7 pituitary proteins isolated using preparative PAGE by Idler and So (1987). Growth hormone has been shown to variously increase ovarian $E_2$ synthesis in vitro in a number of fish species (Singh et al. 1988, Van Der Kraak et al. 1990). However, in this study under similar in vitro conditions neither salmonid or pleuronectid GHs were active using rainbow trout and winter flounder ovarian preparations, respectively. A possible explanation could relate to species differences, although rainbow trout were used in this study and another study that demonstrated increased gonadal synthesis of both $E_2$ and T by GH (Singh et al. 1988). Presently it is not known what component of the Con A I fraction enhances ovarian aromatase.

The use of serum $T_4$ levels as a criterion for successful hypophysectomy in winter flounder was particularly

appropriate since removal of the pituitary (the source of thyroid stimulating hormone) quickly lowered $T_4$ blood levels by 80% within 3 days in males and by 73% in 5 days in females (Figs. 37 and 38). Clearly this technique in either sex could be used 5 days after operation to indicate whether the pituitary has been successfully removed. The $T_4$ level in females on Day 0 was lower than that of males reflecting possible differences in sex or season. Eales and Fletcher (1982) have shown that plasma $T_4$ is at its lowest level, 2 ng/ml, during December-February in winter flounder. In addition, the decrease in $T_4$ levels in females after hypophysectomy appeared to take longer than in males. This may also be explained by a difference in sex but more probable is the effect of season. The females were tested in winter when water temperatures were 0°C. This would slow metabolism and reduce the clearance rate of $T_4$. The $T_4$ levels in sham-operated females also exhibited a different pattern over the experimental period than that found in males. Female $T_4$ levels increased from 6.1 ng/ml on Day 0 to reach a highly significant level of 14.1 on Day 10 unlike males where there was no appreciable change. This difference is most likely due to sampling stress which has been reported to elevate $T_4$ in fish (Brown et al. 1978, Eales and Fletcher 1982) and again must be attributed to differences in sex or season since fish were handled identically in both experiments.

The effect of hypophysectomy in female winter flounder on radioiodinated VG and VHDL II uptake by the ovary confirms the role the pituitary gland plays in regulating uptake of blood proteins into the ovary (Tables 5 and 6). Hypophysectomy was more effective during earlier stages of oocyte development with its effect diminished as the ovary developed, but not before the ovary in winter flounder typically ceases to grow (i.e. January-February). This suggests that whatever aspect(s) of the ovarian uptake mechanism that the pituitary influences the ovary eventually attains a state of development that no longer requires pituitary regulation.

It must be mentioned that other unsuccessful approaches were attempted to examine the pituitary control of yolk precursor uptake. The first employed hypophysectomized female winter flounder in which pituitary hormones were replaced by injection with pituitary fractions and given radiolabelled VG to monitor ovarian uptake. Although a similar technique has been used before with crude pituitary fractions (Ng and Idler 1979) the biochemical isolation of sufficient quantities of highly purified pituitary fractions for _in vivo_ experiments made this approach impractical. Also, an effort was made to develop a hormone mediated VG uptake bioassay _in vitro_ using isolated defolliculated rainbow trout ovarian follicles. At present the lack of

hormone specificity and dose response do not constitute a bioassay.

CHAPTER 4

**PITUITARY GROWTH HORMONE ISOLATION**

**INTRODUCTION**

Growth hormones have been isolated and characterized from
the pituitary glands of a number of different teleost
species, tilapia (Sarotherodon mossambica, Farmer et al.
1976b), coho salmon (Wagner and McKeown 1985, Nicoll et al.
1987), chum salmon (Wagner and McKeown 1985, Kawauchi et al.
1986, Idler et al. 1989), Japanese eel (Anguilla japonica,
Kishida et al. 1987), yellowtail (Seriola quinqeradiata,
Kawazoe et al. 1988), bonito (Katsuwonus pelamis, Noso et
al. 1988), tuna (Thunnus albacares, Kariya et al. 1989), and
Atlantic cod (Gadus morhua, Rand-Weaver et al. 1989) besides
other vertebrate groups (Singh et al. 1974a, Farmer et al.
1974, 1976a). One of the unique characteristics to emerge
from these studies is the heterogenous nature of GH. In
mammals GH has been found to exist in forms differing in
molecular weight (Singh et al. 1974a), net charge (Lewis et
al. 1979) and with varying degrees of glycosylation (Sinha
and Lewis 1986, Sinha and Jacobsen 1987).

The existence of GH structural heterogeneity in
vertebrates may arise due to the different biological
activities GH has been involved with (Aramburo et al. 1989).

In fish GH can influence growth (Clarke et al. 1977, Donaldson et al. 1979, Komourdjian and Idler 1979, Wagner and McKeown 1985, Kawauchi et al. 1986, Kawazoe et al. 1988), metabolism (Donaldson et al. 1979, Farbridge and Leatherland 1988), seawater adaptation in anadromous fish (Komourdjian et al. 1976, Clarke et al. 1977, Bolton et al. 1987), sex steroidogenesis (Singh et al. 1988) and antifreeze protein synthesis (Idler et al. 1989). Different GH variants may be selectively released from the pituitary and/or have specific receptors that allow regulation of these complex processes.

Studies involving fish GH have demonstrated molecular forms with different net charge (Farmer et al. 1976b, Wagner and McKeown 1985, Kawauchi et al. 1986, Idler and So 1987, Kishida et al. 1987, Idler et al. 1989) and amino acid structure (Agellon et al. 1988, Sekine et al. 1989). The existence of molecular weight variants differing from the monomeric form (20,000–22,000 $M_r$) have been reported but lack assessment of biological activity (Rand-Weaver et al. 1989) or biochemical characterization (Kawauchi et al. 1986).

One of the difficulties during isolation of GH from fish pituitary glands is a reliable test with which to assay numerous fractions for biological activity. The classical bioassay for GH involves repeated injection of putative preparations while simultaneously measuring length and

weight gain of large groups of juvenile fish (Clarke et al.
1977, Donaldson et al. 1979, Komourdjian and Idler 1979,
Wagner and McKeown 1985). Growth promotion bioassays
although definitive require substantial amounts of test
material, involve extensive fish handling and are time
consuming (3-12 weeks).

Growth hormone from fish and other vertebrate groups can
enhance the peripheral conversion of $T_4$ to triiodothyronine
($T_3$) resulting in elevated plasma $T_3$ levels in fish. This
was demonstrated by de Luze and Leloup (1984) in the eel
(Anguilla anguilla) using tilapia GH to enhance $[^{125}I]T_4$ to
$[^{125}I]T_3$ conversion and increase plasma $T_3$ at 24 and 48 hours
post injection. Subsequently, Farbridge and Leatherland
(1988) showed that chronic ovine GH treatment elevated
plasma $T_3$ in rainbow trout and de Luze et al. (1989)
introduced the enhancement of $[^{125}I]T_4$ to $[^{125}I]T_3$ conversion
in eel as a potential bioassay for GH. Human GH treatment in
rainbow trout increases both hepatic 5'-monodeiodinase
activity and plasma $T_3$ levels in a dose dependent fashion as
early as 8 hours post injection (MacLatchy and Eales 1990).
Although GH effects on the peripheral conversion of $T_4$ to $T_3$
is established in fish the specificity of this action
relative to other fish pituitary proteins is not known.

The purpose of this study was to isolate salmonid and
pleuronectid GH from pituitary glands for studies on ovarian
sex steroid production in rainbow trout and winter flounder.

To facilitate GH isolation a sensitive, specific and rapid assay to monitor biological activity was developed. During a typical isolation from sockeye salmon pituitaries the short-term (24 hour) enhancement of serum $T_3$ in rainbow trout, measured by RIA, was used to monitor isolated fractions for GH activity.

## MATERIALS AND METHODS

### Fish

Immature (1+) rainbow trout were obtained and held as described in Chapter 3 (pg. 77). About 1 month before bioassay 50 similar sized fish (75-100 g) were transferred to identical tanks enclosed with opaque black plastic to minimize disturbance. These assay tanks were supplied with aerated, temperature controlled (10-12°C) freshwater (Neslab, Portsmouth, NH) under a 12 hour light:12 hour dark photoperiod. Under these acclimation conditions fish were fed normally up to 72 hours before injection after which they were starved until blood sampling was completed.

### Pituitary protein fractionation

Sockeye salmon pituitary glands were collected as described in Chapter 3 (pg. 83). Pituitary glands were

obtained from mixed sexes of American plaice caught during
May in the commercial fishery off the northeast coast of
Newfoundland and landed at Hant's Harbour. Pituitaries were
kept on dry ice following removal until they could be stored
at -60°C. Crude PE was derived from homogenizing pituitaries
(sockeye salmon-40 g; plaice-41 g, in 4 volumes of Buffer B
(Idler and Hwang 1978) with 3% aprotinin (Sigma), storing
the homogenate at 4°C for 24 hours, followed by centrifuging
at 14,600x G for 1 hour and collecting the supernatant. The
Con A I and Con A II protein fractions were isolated from
the PE by affinity chromatography on Con A (5 x 25 cm
column) (Pharmacia) (Idler and Hwang 1978). The Con A I
fraction was re-run on Con A to reduce any contamination
from Con A II (Ng and Idler 1978a,b). For sockeye salmon the
Con A I fraction was further subdivided into Con A I >25,000
and Con A I <25,000 $M_r$ fractions by gel filtration
chromatography on AcA 54. Two AcA 54 columns were joined in
series (2[2.6 x 90 cm]) and run at 15 ml/hour using
ascending elution with Buffer F (Ng and Idler 1978a) as
column eluent. Finally, the Con A I <25,000 $M_r$ fraction was
subjected to preparative PAGE (Idler and So 1987) to isolate
all the proteins present similar to chum salmon (Idler et
al. 1989). In the case of plaice the Con A I fraction was
subdivided into three fractions designated peaks A (>160,000
$M_r$), B (45,000-160,000 $M_r$) and C (<45,000 $M_r$) on Ultrogel AcA
44 (2[5 x 82 cm]; IBF Biotechnics) similar to the technique

of Ng and Idler (1978a). The fraction referred to as Con A I peak C (<45,000 $M_r$) was chromatographed on AcA 54 yielding a profile that was pooled to give fractions labelled F I, F II and F III. The F II fraction was re-run on AcA 54 to determine the extent of cross-contamination after pooling. Re-chromatography of the F III fraction was conducted on a single 2.6 x 90 cm AcA 54 column because of small sample size under the same conditions used previously for the double AcA 54 columns. Preparative PAGE was used to isolate all the proteins present in the F II and F III fractions identical to sockeye salmon above. All chromatography steps and preparative PAGE were conducted at 5°C in a cold room. The individual pituitary proteins isolated on preparative PAGE were identified by their $R_f$ on native PAGE as described in Chapter 2 (pg. 11) with the only change being a 7.5% running gel (7.5% acrylamide: 0.2% N,N'—methylene bisacrylamide) and a 2.5% stacking gel (2.5% acrylamide: 0.62% N,N'—methylenebisacrylamide). Denaturing SDS PAGE on a uniform 15% gel followed the method outlined in Chapter 2 (pg. 12) using sample buffer with and without the addition of 2-ME. It was noted that two standard proteins glyceraldehyde-3-phosphate dehydrogenase (36,000 $M_r$) and egg albumin (45,000 $M_r$) do not run as discrete bands under non-reducing conditions and were not used in $M_r$ calculations. Amino acid sequence analysis was performed on a gas—phase amino acid sequenator (Model 470A, Applied Biosystems) at

the Protein Sequencing Facility, Department of Medical
Biochemistry, University of Calgary. Protein concentration
was determined using the Pierce Coomassie protein assay
reagent and BSA standard. All pituitary protein fractions
were diluted in 0.9% saline for injection.

## Development of growth hormone bioassay

In the first experiment a group of 50 rainbow trout were
anaesthetized with 2-phenoxyethanol (0.4 ml/l; Sigma), and 8
were blood sampled to provide a 0 hour control group. Blood
(0.5 ml) was withdrawn by syringe from caudal vessels,
transferred to 1.5 ml polypropylene micro test tubes
(BioRad) and serum prepared as described previously. The
remaining fish were injected ip with sockeye salmon PE (0.5
pituitary equivalents/100 μl/fish) using a glass microliter
syringe (Hamilton, Model 710N). At 2,4,6, and 24 hours after
injection 7-11 fish were removed, anesthetized as above and
blood sampled.

The second series of experiments used 2 or 3 groups of 15
rainbow trout each, 1 as a control (0.9% saline; 100
μl/fish) and 1 or 2 test group(s). The sockeye salmon Con A
I and Con A II fractions were tested at 0.5 pituitary
equivalents/100 μl/fish, and bovine thyroid stimulating
hormone (Sigma) at 15 μg/100 μl/fish. The experimental fish
were subdued before capture by infusing 2-phenoxyethanol

into the tank to give a concentration of 0.04 ml/l after which they could be easily netted and transferred to a stronger concentration of anaesthetic (0.4 ml/l) before ip injection. At this time they were also colour tagged (Floy Tags, Seattle, WA) for identification. Twenty-four hours after injection fish were anaesthetized as above and blood sampled. Fish used in one bioassay were used again after a 3 week period under acclimation conditions.

The third group of experiments evaluated the different individual proteins from the sockeye salmon Con A I <25,000 $M_r$ fraction (10 or 12 $\mu$g/100 $\mu$l/fish) isolated by preparative PAGE. Two groups of 10 rainbow trout were used in each bioassay, a control and test group. Fish were anaesthetized, injected and blood sampled as above.

The fourth experiment tested different doses of purified sockeye salmon GH (3.65, 5.48, 7.30, 8.43 and 10.96 $\mu$g/100 $\mu$l/fish). Two or 3 groups of 15 rainbow trout each were used, a control and 1 or 2 test group(s). Fish were anaesthetized, injected and blood sampled as above.

Serum $T_3$ levels were measured in 200 $\mu$l serum samples by the RIA procedure of Brown and Eales (1977). Radiolabelled ($^{125}$I) $T_3$ with a specific activity of 1110 $\mu$Ci/$\mu$g was purchased from NEN DuPont. In the $T_3$ RIA L–$T_3$ (Sigma) was used as the standard (78–10,000 pg/100 $\mu$l) and the $T_3$ antisera (Calchemical) diluted at 1:40,000. A typical log-logit weighted regression line for the standard dose-

response curve had a $r^2 = 0.99$, a slope of $-1.74$, an intercept of 5.13, and a $B/B_o$ of 881 pg. All fish in a particular bioassay were analyzed in the same RIA.

Both the $R_f$ 0.20 (20-400 ng) and 0.32 proteins (10-200 ng) were assayed in a chum salmon prolactin RIA set up as described in Song et al. (1988).

The preceding bioassay used to monitor biological activity during the isolation of GH from sockeye salmon was applied during the fractionation of plaice pituitaries. Routinely each bioassay consisted of 2 or 3 treatment groups, a control and 1 or 2 test group(s).

## Statistical analyses

Differences between test groups were determined by either a t test (2 groups) or analysis of variance followed by Tukey's multiple comparison test (3 or more groups) to identify groups significantly different from the control (Zar 1984). It was found that the control levels of $T_3$ varied significantly between assays (p<0.001) and in order to compare different pituitary proteins used in separate assays a relative potency (mean $T_3$ level in test group/mean $T_3$ level in control) measure was used (Hubert 1980). To correct for non-constant variance amongst the responses to different doses of sockeye salmon GH a logarithmic transformation (base e) of the serum $T_3$ values were made

before the means and ratio of treated and control groups were calculated. The statistical significance (slope>0; $p<0.05$) of the log-linear dose response regression equation was tested by analysis of variance (Zar 1984).

## RESULTS

### Sockeye salmon growth hormone isolation

In the first experiment sockeye salmon PE was tested for it's ability to affect serum $T_3$ levels over a 24 hour period. A schedule of sampling within the first 24 hours was devised to determine the early pattern of serum $T_3$ response to sockeye salmon PE in rainbow trout. It was evident that serum $T_3$ levels in rainbow trout increase significantly by 6 hours following injection of sockeye salmon PE and are still increasing by 24 hours (Fig. 39). These observations suggested 24 hours would be a practical time interval between injection and blood sampling allowing serum $T_3$ levels to increase substantially. A 24 hour period between injection and blood sampling was adopted for all further experiments.

The second series of assays were designed to determine how specific amongst other pituitary proteins the serum $T_3$ response was. From earlier studies (Komourdjian and Idler 1979, Idler et al. 1989) it was assumed that monomeric GH
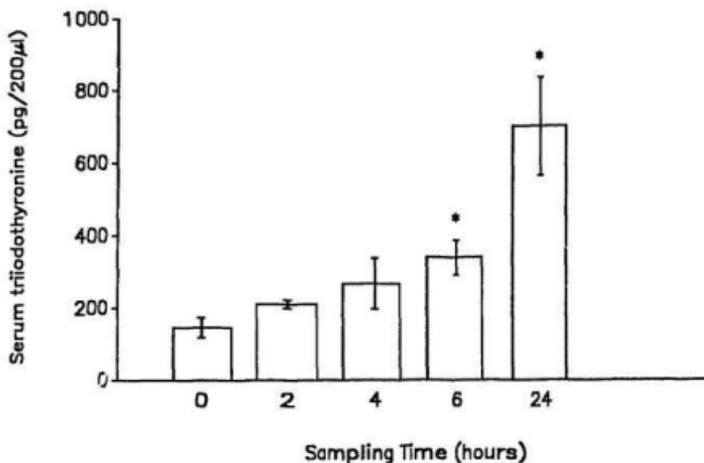
Figure 39: The effect of sockeye salmon crude pituitary extract (0.5 pituitary equivalents/fish) on serum triiodothyronine levels (mean ± SE) at different time intervals over a 24 hour period in rainbow trout. Statistical significance relative to control (0 h), * = p<0.004; the other sampling times were not different.

being a non-glycoprotein would reside in the Con A I <25,000 $M_r$ fraction. It was established that only the Con A I <25,000 $M_r$ fraction relative to the other pituitary protein fractions all tested at 0.5 pituitary equivalents/fish had the ability to significantly elevate serum $T_3$ after 24 hours (Figs. 40 and 41). Bovine thyroid stimulating hormone was inactive in the assay.

Subsequently, all the sockeye salmon Con A I <25,000 $M_r$ pituitary proteins individually isolated on preparative PAGE were tested to determine which protein(s) were bioactive. Only the $R_f$ 0.32 and 0.46 proteins significantly elevated serum $T_3$ at a dose of 10 µg/fish (Table 7). The $R_f$ 0.32 protein was more potent in elevating $T_3$ with a relative potency of 3.27 compared to the $R_f$ 0.46 protein at 1.89. From semi-quantitative native PAGE analysis, based on staining intensity, the $R_f$ 0.32 protein was the most prominent protein in the sockeye salmon Con A I <25,000 $M_r$ fraction. Sodium dodecyl sulfate PAGE analysis of the $R_f$ 0.32 protein resulted in separation of two proteins with $M_r$s of 21,000 and 21,500 (Fig. 42, lane $\underline{A}$). The N-T sequence of the first 25 amino acids of the $R_f$ 0.32 protein are: Ile/Met-Glu-Asn-Gln-Arg-Leu-Phe-Asn-Ile-Ala-Val-Asn/Ser-Arg-Val-Gln-His-Leu-His-Leu-Leu-Ala-Gln-Lys-Met-Phe with two amino acid residues at positions 1 and 12. The $R_f$ 0.32 protein is hereafter referred to as sockeye salmon GH. The effect of sockeye salmon GH on serum $T_3$ elevation in
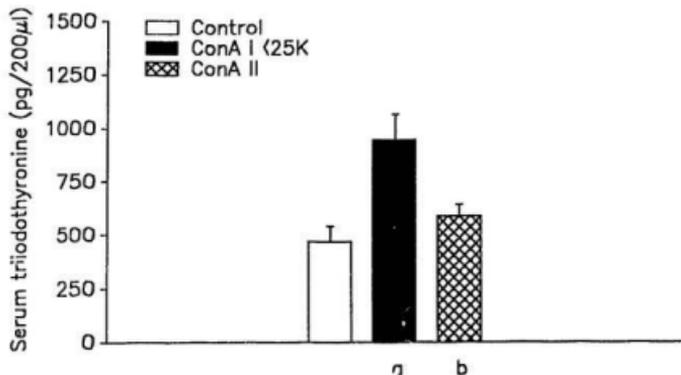
Figure 40: The effect of sockeye salmon carbohydrate-poor
(Con A I) <25,000 $M_r$ and carbohydrate-rich
(Con A II) fractions tested at 0.5 pituitary
equivalents/fish on serum triiodothyronine
levels (mean + SE) in rainbow trout 24 hours post
injection. Statistical significance relative to
the control, a = $p < 0.05$, b = $p > 0.05$.
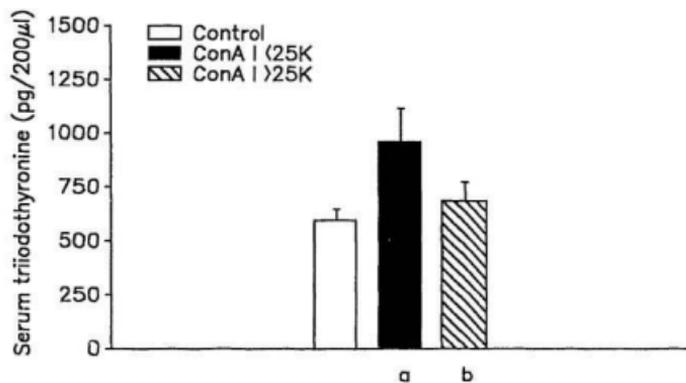
Figure 41: The effect of sockeye salmon carbohydrate-poor
(Con A I) <25,000 $M_r$ and Con A I >25,000 $M_r$
fractions tested as in Fig. 40.

Table 7: The effect of different sockeye salmon carbohydrate
-poor <25,000 $M_r$ pituitary proteins, isolated by
preparative polyacrylamide gel electrophoresis,
on serum triiodothyronine elevation in rainbow
trout 24 hours post injection.

| Relative Mobility ($R_f$) | Dose ($\mu g$/fish) | Relative Potency | Statistical Significance |
|---|---|---|---|
| 0.20 | 10 | 1.33 | ns* |
| 0.32 | 10 | 3.27 | p<0.001 |
| 0.46 | 10 | 1.89 | p<0.02 |
| 0.59 | 10 | 1.46 | ns |
| 0.70 | 10 | 1.00 | ns |
| 0.79 | 12 | 1.03 | ns |
| 0.85 | 12 | 0.93 | ns |

* ns = p>0.05.
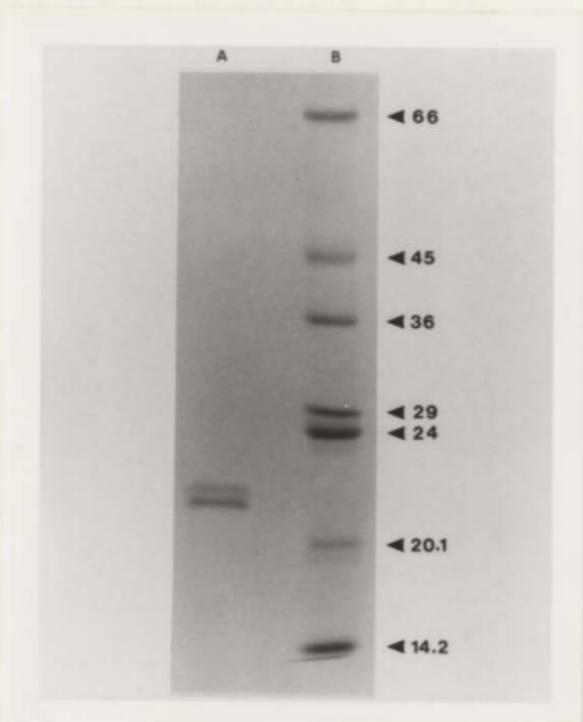
Figure 42: Sodium dodecyl sulfate polyacrylamide gel
electrophoresis of 10 μg of sockeye salmon growth
hormone (lane A) and 15 μg of the SDS-7 (Sigma)
protein standards (lane B). The $M_r$ (x1,000) of
each standard is indicated by an arrowhead.

immature rainbow trout after 24 hours was tested at various concentrations (Fig. 43). All concentrations above 3.65 $\mu$g significantly elevated serum $T_3$ above the controls ($p<0.05$). The lowest dose of 3.65 $\mu$g although having a higher serum $T_3$ level than the control was not significantly different ($p>0.05$). Serum $T_3$ levels represented as log relative potency increased with increasing amounts of sockeye salmon GH and the log-linear dose-response equation is log $Y =$ 0.11X + log0.23 ($p<0.05$) (Fig. 43,inset).

## American plaice growth hormone isolation

It was established using plaice PE that the bioassay based on serum $T_3$ elevation in immature rainbow trout responded indicating its suitability to monitor GH isolation from plaice pituitaries (Fig. 44). All subsequent fractions from plaice pituitaries were tested similarly.

Beginning with the plaice Con A I fractions only the Con A I peak C fraction was active in the bioassay indicated by serum $T_3$ levels significantly higher than the control (Fig. 44). The fractions F I, F II and F III resulting from AcA 54 chromatography of the plaice Con A I peak C fraction (Fig. 45) had a range of $M_r$s which can broadly be designated >43,000, 43,000 and <43,000 respectively; the F II being the major fraction. Re-chromatography of the F II and F III fractions separately on AcA 54 revealed essentially one peak
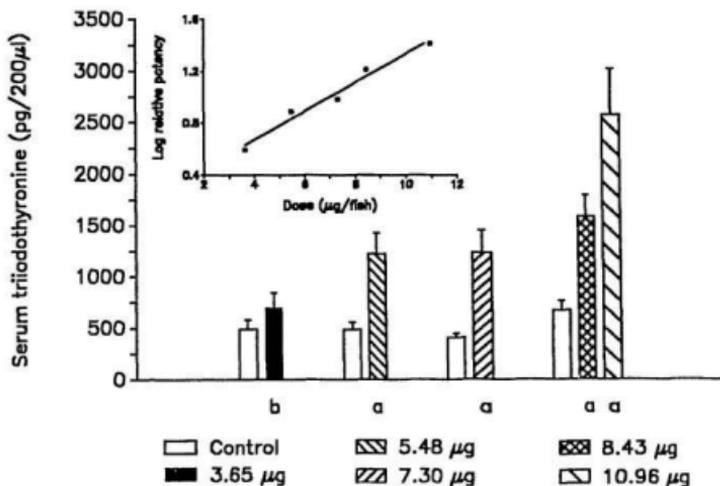
Figure 43: The effect of different doses of sockeye salmon
growth hormone (GH) tested as in Fig. 40.
Statistical significance relative to the adjacent
control, a = p<0.05, b = p>0.05. Inset is the
log-linear dose-response relationship between
different doses of purified sockeye salmon GH and
serum triiodothyronine levels in rainbow trout 24
hours post injection represented as log relative
potency (mean log test group – mean log control
group).
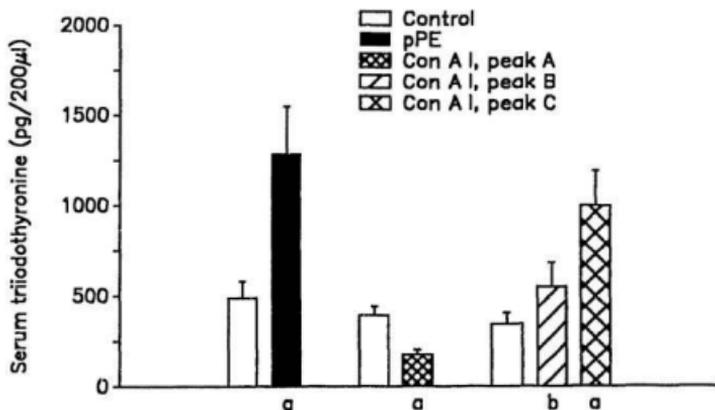
Figure 44: The effect of plaice crude pituitary extract
(pPE; 1 pituitary equivalent/fish) and
carbohydrate-poor (Con A I) peaks A,B and C (1.5
pituitary equivalents/fish) on serum
triiodothyronine levels (mean + SE) in rainbow
trout 24 hours post injection. Statistical
significance relative to the adjacent control,
a = p<0.05; b = p>0.05.
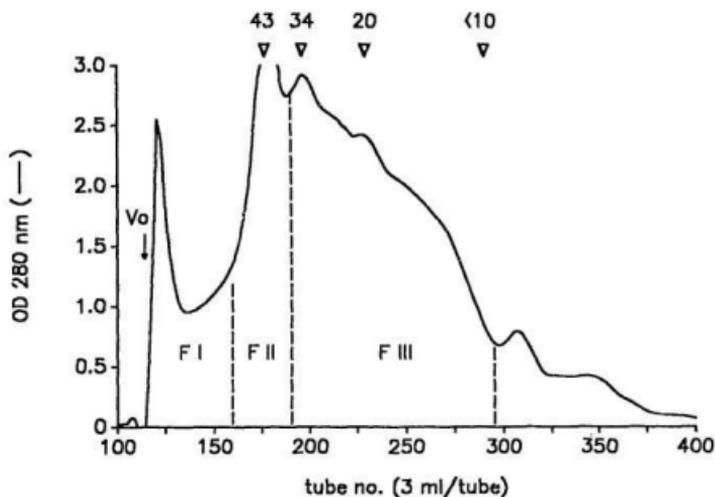
Figure 45: Gel filtration chromatography of plaice
carbohydrate-poor peak C (293 mg) on Ultrogel AcA
54 monitored for absorbance. Dashed lines
demarcate fractions pooled as F I, F II and F
III. Void volume (Vo, 70,000 $M_r$) indicated by
arrow while $M_r$s (x1,000) across the profile are
indicated by arrowheads along the top.

of 42,000 $M_r$ for the F II (Fig. 46A), and three peaks for
the F III, 33,000, 20,000 and 13,500 $M_r$ respectively (Fig.
46B). No appreciable contamination of the F II or F III
fractions with components of the other fraction was evident.
The F I, F II, and F III fractions were tested and both the
F II and F III contained almost equivalent biological
activity, determined from the bioassay (Fig. 47). The F I
fraction was not active.

The F II and F III fractions were subjected to
preparative PAGE analyses and the individual proteins
comprising these two fractions were isolated. In the F III
fraction 6 major proteins with $R_f$s 0.16, 0.22, 0.37, 0.49,
0.65 and 0.77 were found. From this group all tested at 15
$\mu g$/fish only the $R_f$ 0.22 protein was significantly higher
than the control (Fig. 48). The F II fraction contained two
proteins with $R_f$s of 0.22 and 0.28 both of which
demonstrated significant biological activity (Fig. 49). The
$R_f$ 0.28 protein although contaminated with a small amount of
the $R_f$ 0.22 protein (see Fig. 50 below) was considered to be
active in the bioassay (Fig. 49). For comparison the F III
$R_f$ 0.22 protein and the F II $R_f$ 0.22 protein were tested at
15 $\mu g$/fish in the same bioassay, and although the F II
protein appears more potent there is no significant
difference between the two (Fig. 49). Yields of the F II $R_f$s
0.22 and 0.28, and FIII $R_f$ 0.22 proteins were 1,170, 103 and
95 $\mu g$/g wet pituitary respectively.
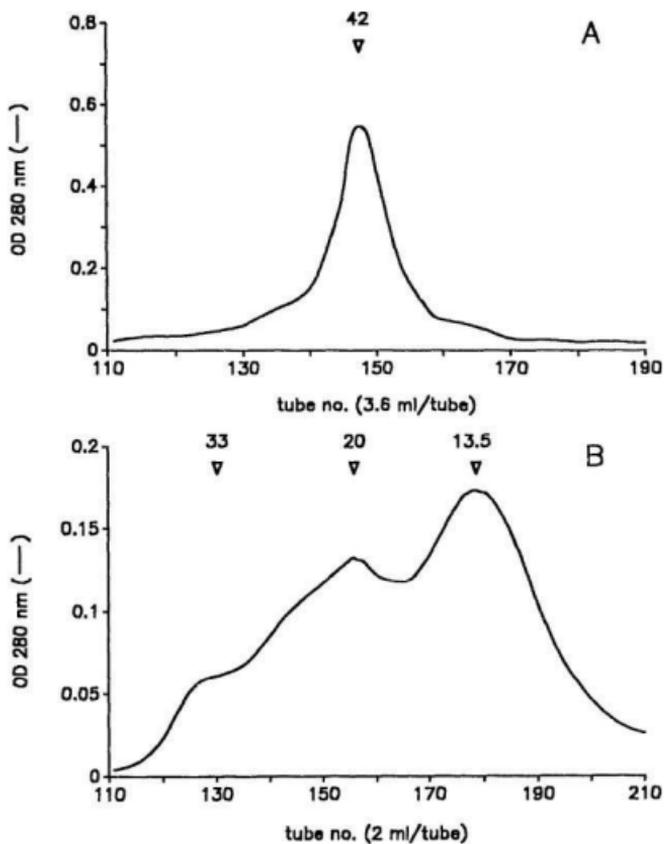
Figure 46: Re-chromatography of aliquots of plaice (A) F II
(52 mg) and (B) F III (19 mg) fractions on
Ultrogel AcA 54 as in Fig. 45.
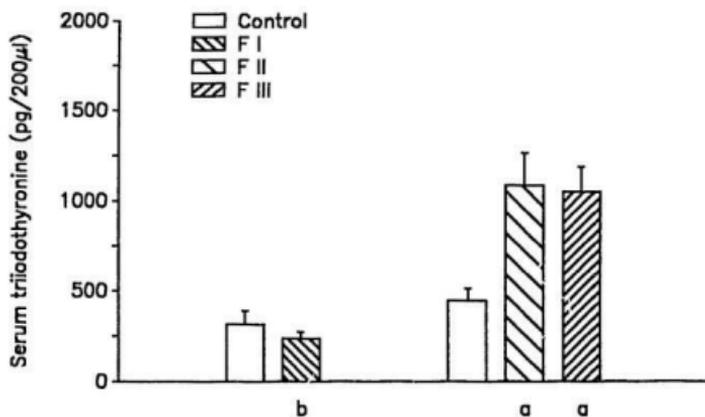
Figure 47: The effect of plaice F I, F II and F III

fractions (3 pituitary equivalents/fish) on serum

triiodothyronine levels in rainbow trout as in
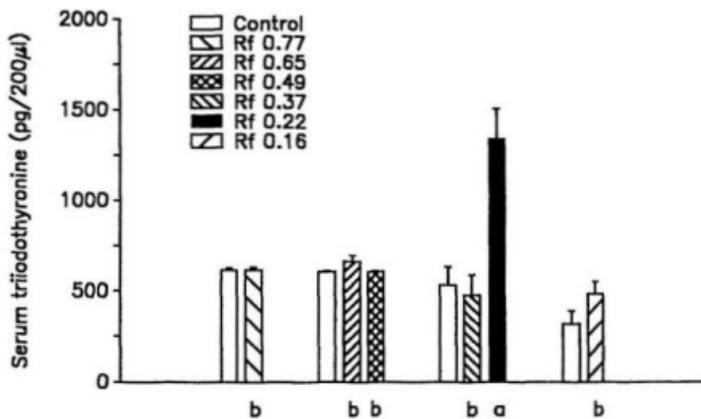
Fig. 44.

Figure 48: The effect of plaice F III fractions (15 μg/fish) on serum triiodothyronine levels in rainbow trout as in Fig. 44.
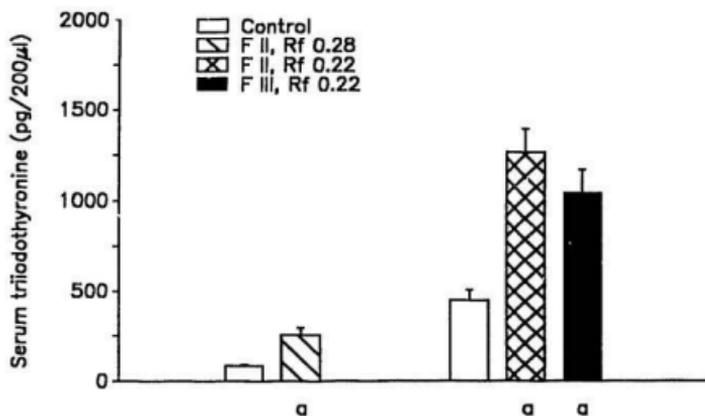
Figure 49: The effect of plaice F II R$_f$s 0.22 and 0.28, and

F III R$_f$ 0.22 proteins (15 µg/fish) on serum

triiodothyronine levels in rainbow trout as in

Fig. 44.
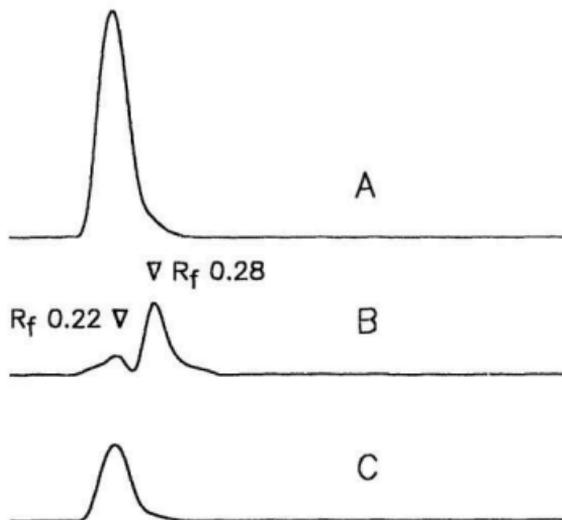
Figure 50: Densitometric scans of plaice F II $R_f$ 0.22 (20 µg;scan A), $R_f$ 0.28 (25 µg;scan B) and F III $R_f$ 0.22 (14 µg;scan C) on native PAGE. Arrowheads indicate the 2 relative mobilities ($R_f$s) observed.

Electrophoretic analyses on native PAGE of both the F II $R_f$ 0.22 and F III $R_f$ 0.22 proteins indicated single homogeneous bands, however the F II $R_f$ 0.28 could not be completely separated free of the F II $R_f$ 0.22 protein (Fig. 50). Sodium dodecyl sulfate PAGE revealed that $M_r$s were dependent on whether the protein samples were reduced (Fig. 51). Under non-reducing conditions (i.e. sample buffer with no 2-ME) $M_r$s were higher for all proteins compared to reduced conditions when 2-ME was included in the sample buffer. The F II $R_f$ 0.22 and 0.28 proteins had identical $M_r$s of 21,000 (Fig. 51, lane $\underline{c}$ and $\underline{D}$) while the F III $R_f$ 0.22 protein was 20,000 (Fig. 51, lane $\underline{B}$) under non-reducing conditions. Following reduction the F II $R_f$ 0.22 and 0.28 proteins yielded similar major bands at 16,000 $M_r$ (Fig. 51, lane $\underline{b}$ and $\underline{c}$) while the F III $R_f$ 0.22 protein gave a single band at 15,000 $M_r$ (Fig. 51, lane $\underline{a}$).

Amino-terminal amino acid sequences were made on the $R_f$ 0.22 proteins from the F II (42,000 $M_r$) and F III (<33,000 $M_r$) fractions representing the major biologically active components of each molecular weight range. Twenty-nine of the first 30 N-T amino acids were derived for the F III $R_f$ 0.22 protein while only 19 of the first 20 were possible from the F II $R_f$ 0.22 protein (Fig. 52). Both proteins had completely identical amino acids at each position although no amino acid could be determined at position 17.
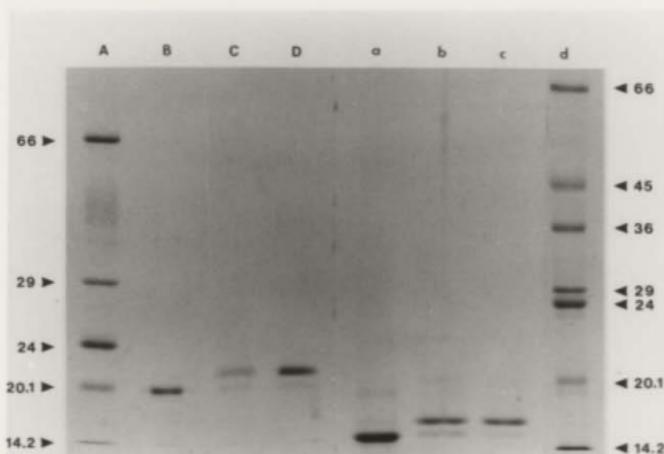
Figure 51: Sodium dodecyl sulfate polyacrylamide gel
electrophoresis under non-reducing (A-D) or
reducing conditions (a-d). In each case 15 μg of
the SDS-7 (Sigma) protein standards (lanes A,d),
7 μg of F III $R_f$ 0.22 (lanes B,a), 8 μg of F II
$R_f$ 0.28 (lanes C,b) and 5 μg of F II $R_f$ 0.22
(lanes D,c) were used. The $M_r$ (x1,000) of each
standard are indicated by arrowheads.

F II : Ser$^1$-Val-Gly-Val-Asp-Glu-Ser-Phe-Arg-Arg$^{10}$-
Asn$^{11}$-Tyr-Glu-Leu-Phe-Ala-X-Phe-Lys-Lys$^{20}$

F III: Ser$^1$-Val-Gly-Val-Asp-Glu-Ser-Phe-Arg-Arg$^{10}$-
Asn$^{11}$-Tyr-Glu-Leu-Phe-Ala-X-Phe-Lys-Lys$^{20}$-
Asp$^{21}$-Met-His-Lys-Val-Glu-Thr-Tyr-Leu-Thr$^{30}$

Figure 52: Amino-terminal amino acid sequences of the plaice
F II and F III relative mobility 0.22 proteins. X
represents an undetermined amino acid.

## DISCUSSION

The use of a bioassay dependent upon increases in serum $T_3$ levels in immature rainbow trout has been successfully employed to monitor biological activity during GH isolation from sockeye salmon pituitary glands. Only proteins derived from the Con A I <25,000 $M_r$ fraction were active in this bioassay, with sockeye salmon GH ($R_f$ 0.32) the most effective protein increasing serum $T_3$ levels more than three-fold compared to the control group after 24 hours (Figs. 39-41). The other bioactive pituitary protein in the Con A I <25,000 $M_r$ fraction, $R_f$ 0.46, was of lower potency and quantity relative to sockeye salmon GH (Table 7). The $R_f$ 0.46 protein is probably another minor form of GH similar to GHs with $R_f$ 0.44-0.45 previously reported in coho (Wagner and McKeown, 1985) and chum salmons (Wagner and McKeown 1985, Wagner et al. 1985, Idler and So 1987, Idler et al. 1989). In all these studies multiple forms of salmon GH have similar but distinct $R_f$s which is consistent with the multiple salmonid GH molecular structures that have been described (Agellon et al. 1988, Sekine et al. 1989).

The Con A I <25,000 $M_r$ protein with the lowest mobility, $R_f$ 0.20, has been identified as sockeye salmon prolactin by its parallel cross reaction in the chum salmon prolactin RIA and is not active in the assay. As well, the Con A II fraction containing mGTH and expected to contain sockeye

salmon thyroid stimulating hormone, and bovine thyroid
stimulating hormone were also inactive in the bioassay.
These results establish the specificity of this bioassay for
identifying salmonid pituitary GH exclusive of other
pituitary hormones.

The sockeye salmon GH isolated in the present study with
$R_f$ 0.32 on native PAGE appears to consist of a mixture of
two forms of GH differing slightly in $M_r$ as evidenced by SDS
PAGE (Fig. 42) and amino acid sequence substitutions at
position 1 and 12 (pg. 131). This sequence was 100%
homologous with comparable N-T sequences for chum salmon GH
(Idler et al. 1986, Kawauchi et al. 1986). It also had
homologies of 95 and 87.5% for chum salmon GH I and II
(Sekine et al. 1989) and 95 and 92.5% for rainbow trout GH I
and II (Agellon et al. 1988) c-DNA amino acid sequences,
respectively. Growth hormone heterogeneity involving Ile/Met
at position 1 and Asn/Ser at position 12 have been
previously reported amongst Oncorhynchus sp. (Agellon et al.
1988, Sekine et al. 1989). It was found that Ile and Ser,
and Met and Asn occur separately in GH I and GH II c-DNA
amino acid sequences respectively.

The sockeye salmon GH produced a dose response in terms
of serum $T_3$ elevation in rainbow trout 24 hours post
injection (Fig. 43). A log-linear increase in serum $T_3$ was
found at doses between 3.65 and 10.96 $\mu$g/fish. In our
bioassay the lowest dose of sockeye salmon GH tested that

produced a statistically significant elevation in serum $T_3$ was 54.8 ng/g fish assuming an average of 100g per fish. This compares well with a study by de Luze et al. (1989) using a teleost GH in which the lowest dose of tilapia GH influencing the $[^{125}]T_3/[^{125}]T_4$ ratio was 45.4 ng/g fish (22.7 ng/g fish x 2 injections). The sensitivity of our bioassay (i.e. 54.8 ng GH/g fish) is well within the typical range of 10-1,000 ng piscine GH/g fish utilized in previous growth promotion bioassays (Donaldson et al. 1979, Komourdjian and Idler 1979, Wagner and McKeown 1985).

This bioassay was sensitive, specific and rapid, and facilitated the screening of numerous fractions originating from a typical pituitary fractionation scheme. It could also be used to compare potencies of purified preparations. With a 24 hour interval between injection and blood sampling a typical bioassay can be completed in 3 days. The $T_3$ RIA system (Brown and Eales 1977) is designed to assay large numbers of serum samples.

Carbohydrate-poor proteins were also isolated from American plaice pituitary glands that exhibit GH biological activity in the GH bioassay previously used to isolate sockeye salmon GH (Fig. 44). Based on gel filtration chromatography these GHs originate from two $M_r$ regions, 42,000 (F II) and <33,000 (F III), in their native state (Figs. 45 and 46). The 42,000 $M_r$ region yielded two forms of GH that differ in terms of quantity and net charge as

evidenced by native PAGE, a major variant with $R_f$ 0.22 and a lesser variant with $R_f$ 0.28. The <33,000 $M_r$ region produced a single GH species with $R_f$ 0.22 identical to the major 42,000 $M_r$ variant (Fig. 50). Upon SDS PAGE without reduction both GH variants from the 42,000 $M_r$ region gave $M_r$s of 21,000 while the GH from the <33,000 $M_r$ region was 20,000 $M_r$ (Fig. 51). These $M_r$s are typical of monomeric vertebrate GHs (Lewis et al. 1980, Farmer et al. 1974, 1976a). It is suggested that proteins making up the 42,000 $M_r$ region are GH dimers since upon SDS PAGE they gave 21,000 $M_r$ peptides. A $M_r$ of 20,000 for the $R_f$ 0.22 peptide from the <33,000 $M_r$ region probably represents a GH monomer in its native state corresponding to the second peak in Fig. 46B.

An unusual N-T amino acid sequence identical for both the 42,000 and 20,000 $M_r$ $R_f$ 0.22 forms was found (Fig. 52). Comparisons with complete GH sequences from other fish species (Agellon et al. 1988, Kawazoe et al. 1988, Momota et al. 1988a,b, Kariya et al. 1989, Koren et al. 1989, Lorens et al. 1989, Sekine et al. 1989) indicated that the plaice N-T sequence obtained in this study did not match. However, there is a 30 amino acid match region near the C-T end of these fish GH sequences that varies between amino acids 130 and 196 depending on the species. Homologies within this match region for the plaice GH sequence are 80% (red sea bream, Momota et al. 1988a, tuna, Kariya et al. 1989), 76.7% (yellowtail, Kawazoe et al. 1988), 73.3% (coho salmon,

Nicoll et al. 1987, rainbow trout GH I and GH II, Agellon et
al. 1988, chum salmon GH I and II, Sekine et al. 1989), 70%
(Atlantic salmon, Salmo salar, Lorens et al. 1989), 60%
(flounder, Paralichthys olivaceus, Momota et al. 1988b) and
50% (carp, Cyprinus carpio, Koren et al. 1989). These
proteins are identified as plaice GHs and represent good
evidence for different GH molecular mass forms in this
teleost.

The N-T amino acid sequences for the 42,000 and 20,000 $M_r$
$R_f$ 0.22 forms of plaice GH obtained in this study are
unusual but can readily be explained by reference to the
mammalian literature. The structure that is consistent with
these findings is the enzymically cleaved variant of human
GH that has been isolated from pituitaries (Lewis et al.
1980). The best homologies of the plaice GHs with other fish
GHs occurs in the region beginning at amino acid 130
analogous to the region of human GH susceptible to enzymic
cleavage (Singh et al. 1974b). The opening up of the N-T
group would create a second sequence if the other N-T amino
acid was not blocked. There was no evidence during
sequencing for other than a single free N-T amino acid
confirming that the only unblocked N-T amino acid is the one
released by enzymic cleavage. It is highly unlikely that the
plaice GH molecule is radically different from other
teleosts with regions commonly found near the C-T end in
other teleost GH sequences now present at the N-T end of

plaice GH. The blockage of the N-T amino group by acetyl has been reported for "fast human GH" (Lewis et al. 1980) and is not uncommon in other peptides with acetyl or formyl groups usually blocking the N-T amino acid (Darbre 1986). The question that needs to be answered is whether enzymic cleavage occurred during homogenization of the pituitaries and isolation as is the case with the cleaved form of human GH (Singh et al. 1974b). It is difficult to believe that proteolysis occurred during isolation since similar procedures employing a proteolytic inhibitor (aprotinin) in all buffers during chromatographic steps (at 5°C in a cold room) have been used previously during isolation of monomeric GH in chum and sockeye salmon (Idler et al. 1989, this study). However, we cannot now exclude the possibility that plaice have pituitary proteinases not found in the less phylogenetically advanced Pacific salmons. It is of interest that enzymically cleaved human GH can have biological activities which are absent from intact human GH or which are greater than the latter (Singh et al. 1974b).

Similar to previous studies on GHs from a number of vertebrate species plaice GH exists in forms that can be separated based on net charge by native PAGE. Plaice 42,000 $M_r$ GH charge variants were found with $R_f$s 0.22 and 0.28 (Fig. 50). These $R_f$s in general are slightly lower than other fish GHs reported to date. Typically $R_f$s of other fish GHs on native PAGE are reported between 0.32 and 0.45

(Wagner and McKeown 1985, Kishida et al. 1987, Idler et al. 1989, this study). It is noted that two GH variants have been found in chicken pituitary extract with $R_f$s of 0.22 and 0.30 (Aramburo et al. 1989).

Differences observed in this study between SDS PAGE analyses of plaice GH, with or without the inclusion of the reducing agent 2-ME, are probably due to the binding of SDS in the presence or absence of disulfide bonds (Fig. 51). Two disulfide bonds are reported for human GH (Singh et al. 1974b) and two proposed in Atlantic cod GH (Rand-Weaver et al. 1989). Disulfide bonds between cysteine amino acids would tend to retain the natural conformation of the protein such that it would migrate on SDS PAGE with a mobility indicative of its real $M_r$. Following reduction of plaice GHs faster mobilities were recorded resulting in lower $M_r$s that must represent the protein in an unfolded state.

One of the lowest homologies found (60%) between the plaice GH sequence and other established fish GH sequences occurred with another flounder species, Paralichthys olivaceus (Momota et al. 1988b). It has been acknowledged by the authors and confirmed in our work that a 14 amino acid deletion is evident beginning at Asn[140]. This deletion region represented in the red sea bream GH sequence (Momota et al. 1988a) between Tyr[140] and Thr[153] includes some of the first 12 amino acids of the plaice GH sequence. If the latter 18 amino acids of the plaice GH sequence are compared to the

flounder GH sequence starting at $Glu^{142}$ 17 out of 18 amino acids correspond (94.4% homology). The undetermined amino acid at position 17 of the plaice GH sequence is probably cysteine.

The vast majority of GH present in plaice pituitaries, 93%, exists in a dimeric form (42,000 $M_r$) of which 86% is present as the $R_f$ 0.22 variant. It is not known whether this is a premature form of GH that is cleaved to the monomer upon release or if in fact the dimer is released. There are reports of high $M_r$ forms of GH apparent in human and rat plasma (Stolar et al. 1984, Stolar and Baumann 1986, Silverlight et al. 1985) and released from the rat pituitary (up to 88,000 $M_r$) during in vitro organ culture (Farrington and Hymer 1990). The plaice GH dimer (42,000 $M_r$, F II) had comparable biological activity to the fraction containing a monomeric GH form (<33,000 $M_r$, F III) in contrast to dimeric human GH which has no biological activity (Lewis et al. 1980). It remains to be investigated why the plaice has such large quantities of GH present in dimeric form within the pituitary unlike for example the sockeye salmon where pituitary GH only appears to exist in monomeric form.

## CHAPTER 5

## GENERAL CONCLUSION

In summary, it appears that VG, a VHDL, is the major serum yolk precursor accumulated by vitellogenic ovaries of winter flounder similar to earlier studies in amphibians and in contrast to birds (Wallace, 1985). This is supported by the quantity of yolk proteins within the oocyte demonstrating immunoreactivity toward VG antisera by Western blotting. Ovarian uptake studies using radiolabelled VG show that lipovitellin is the primary cleavage product of incorporated VG contributing to the major fraction (82%) of salt soluble ovarian protein. By comparison, a serum protein known previously as Pk A and now as VHDL II can be considered to provide a lesser contribution to yolk protein. Very high density lipoprotein II is taken up by the ovary at levels one-third those of VG and does not appear to be processed to any degree. In addition, VHDL II does not accumulate in the ovary to the extent that VG does and contributes to a fraction that represents 12% of salt soluble ovarian protein. Phosvitin and a lower $M_r$ phosphoprotein resembling amphibian and avian phosvettes were apparent but in very small amounts.

Seasonal serum levels of $E_2$ and T were determined in female winter flounder that indicated prespawning maxima and

postspawning minima. Production of ovarian $E_2$ _in vitro_ was only stimulated by homologous PE during the postspawning to early vitellogenic period (June-September). Alternatively, production of ovarian T _in vitro_ in response to PE is high at least one month prior to and increases up to spawning, suggesting a role for T during the final maturation process in winter flounder. High serum $E_2$ levels during the pre-spawning period (April) represent biologically active steroid. Although levels of serum $E_2$ binding protein are substantial (79% of the $E_2$ is bound) they are no different from levels of $E_2$ binding protein during the vitellogenic phase (October).

The role of Con A I and Con A II pituitary protein fractions isolated from sockeye salmon were investigated in relation to $E_2$ production by rainbow trout ovarian follicles _in vitro_. During the early vitellogenic phase of the rainbow trout reproductive cycle, using defolliculated ovarian follicle preparations, it was demonstrated that the Con A I fraction was capable of increasing $E_2$ production in the presence of exogenous T as substrate. Under similar conditions the Con A II fraction (containing the mGTH) was inactive. However the Con A II fraction or T separately increased $E_2$ production by intact ovarian follicles whereas the Con A I fraction did not. A mechanism proposed to explain the regulation of ovarian $E_2$ synthesis involves the Con A I fraction enhancing aromatase activity in granulosa

cells permitting an increased conversion of T to $E_2$.
Although the Con A I fraction enhanced the ovarian synthesis
of $E_2$ it was determined in vitro that this action is not due
to GH, a known component of the Con A I fraction. It could
not be shown that pleuronectid or salmonid GH affected the
production of ovarian $E_2$ or T during in vitro ovarian
incubations from winter flounder or rainbow trout,
respectively.

The measurement of serum $T_4$ in both male and female
winter flounder was established as a useful criterion to
assess the success of hypophysectomy in this fish. A
significant drop in serum $T_4$ levels after 5 days in either
sex indicated that the pituitary was completely removed.
Hypophysectomy in reproductively active females during the
period of major ovarian growth indicated that ovarian uptake
of both radiolabelled VG and VHDL II were decreased to a
greater extent earlier in the vitellogenic phase (October)
rather than later (December). This suggests that the
pituitary gland regulates the uptake of yolk precursors
until a critical level of ovarian development is attained
after which the ovarian uptake of yolk precursors can
continue without a requirement for the pituitary.

To permit the isolation of teleost GH from pituitaries a
bioassay based on the increase of serum $T_3$ in rainbow trout
measured by RIA was developed to follow biological activity
during a typical fractionation protocol. Growth hormone was

isolated from sockeye salmon and American plaice pituitary
glands using established techniques of affinity and gel
filtration chromatography, and preparative PAGE. Amino-
terminal amino acid sequence analysis and subsequent
comparison with established GH sequences from other
<u>Oncorhynchus</u> sp. were used to confirm the isolation of
sockeye salmon GH. In American plaice GHs were isolated with
two different molecular masses from the pituitary, 42,000
and <33,000 $M_r$, in their native state. The 42,000 $M_r$ region
yielded two forms of GH that differ in terms of quantity and
net charge as evidenced by native PAGE, a major variant with
$R_f$ 0.22 and a lesser variant with $R_f$ 0.28. The <33,000 $M_r$
region has a single GH species with $R_f$ 0.22. Upon SDS PAGE
without reduction both GH variants from the 42,000 $M_r$ region
gave $M_r$s of 21,000, while the GH from the <33,000 $M_r$ region
was 20,000 $M_r$, typical of monomeric vertebrate GHs. The
proteins making up the 42,000 $M_r$ region are proposed as GH
dimers since they yield 21,000 $M_r$ peptides. The <33,000 $M_r$
region contains a GH monomer (20,000 $M_r$) in its native
state. An amino-terminal amino acid sequence identical for
both the 42,000 and 20,000 $M_r$ $R_f$ 0.22 forms has good
homologies with other complete fish GH sequences near their
C-T regions (varying between amino acids 130-196). The GH
dimers (42,000 $M_r$) predominate in the plaice pituitary
contributing 93% of the total of which 86% gives rise to the
$R_f$ 0.22 variant.

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