VITELLOGENESIS IN THE TELEOST *BRACHYDANIO RERIO* (ZEBRA FISH)

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For Mary, Augustine, Carol and Glen

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to this thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Herman A. Fernandes.

June 1994

ABSTRACT

During oogenesis in teleosts, the volume of the oocyte will typically increase several hundred fold mainly due to the accumulation of yolk proteins within the ooplasm. This process is termed as vitellogenesis. In the teleost, as in other oviparous vertebrates, these yolk proteins are derived from a major precursor protein vitellogenin (Vg) produced in the liver in response to estrogen stimulation and released into the blood where it is transported to the gonad, and sequestered into the growing oocytes.

This study concerns vitellogenesis in the zebra fish (*Brachydanio rerio*). The major estrogen inducible protein in the zebra fish liver has been purified to homogeneity by FPLC using anion exchange chromatography (Mono-Q Pharmacia) with purification being monitored by SDS-PAGE electrophoresis. The purified protein was found to have an estimated molecular weight of 325 kilo Daltons (kD) in its native state and an amino acid composition which closely paralleled that found for Vg from other teleost species. As with Vg purified from other teleost species, on treatment with sodium dodecyl sulfate it dissociated to form several peptide species including two major peptides of about 190 and 160 kD molecular weight.

It was also found that, in accord with studies on other species, the synthesis of the putative Vg was enhanced in the liver of female zebra fish and induced in male fish by estradiol-17β (E_2) stimulation. Repeated dosages of E_2 (4μg/gm body weight) to female zebra fish resulted in increased content of native protein such that the putative purified Vg obtained after chromatography constituted 68%, 40% and 46% of the total protein of the serum, liver and gonad respectively.

Hepatocytes in primary monolayer cultures synthesised Vg in response to E_2 stimulation as assessed by SDS-PAGE. The synthesis was dose dependent, with the most

effective dose of E_2 being 10^{-4} M and the least effective was 10^{-8} M. The synthesised protein was secreted into the medium after a lag period of 3 h.

Through the use of polyclonal antiserum prepared to the native protein and Western blotting techniques, Vg was identified in medium collected from stimulated hepatocyte cultures derived from E_2 stimulated male and female fish. Using immunohistochemical techniques, antigen was detected in the liver and gonads from E_2 stimulated fish with other tissues being immunonegative. The polyclonal antiserum was utilised to develop an enzyme linked immunoabsorbent assay (ELISA) to measure the levels of Vg in the serum of E_2 stimulated male and female zebra fish. In the males serum Vg levels of 0.65 mg/mg of the total serum protein were found after the final E_2 stimulation, compared with 0.14 mg/mg in the serum of mature females increasing to 0.84 mg/mg after the final E_2 stimulation. The antiserum was also found to be species specific and showed no cross reaction in the ELISA with serum obtained from vitellogenic chickens a lizard (gecko) and another teleost species, the medaka.

In the other study several steroids were tested for their capacity to induce gonadal maturation in the zebra fish, of the steroids tested 17α-hydroxy-20β-dihydro-4-pregnene-3-one (17α,20β-P)proved the most potent. Ovaries obtained from fish with a gonadosomatic index (GSI) higher then 16% showed an increase in the percentage of oocytes undergoing final maturation *in vitro* in the presence of 17α,20β-P. Oocyte size was recognised as an important factor for final maturation and oocytes with less than 600μm in diameter failed to mature whether or not the steroid was present.

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ABBREVIATIONS

µCi	micro Curie
µg	micro gram
µl	micro litre
µm	micro meter
%	percent
α	alpha
Ab	antibody (antiserum)
ACTH	adrenocortico trophic hormone
Ag	antigen
β	beta
b.w	body weight
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
DAB	diaminobenzidine
DEAE	dietylaminoethyl
DNA	deoxyribonucleic acid
dpm	disintegration per minute
DTT	dithiotheritol
E_2	estradiol-17β
EDTA	Etylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	fetal calf serum
Fig	figure
FPLC	Fast Protein Liquid Chromatography
FSH	follicle stimulating hormone
GAR-AP	alkaline phosphate conjugated goat anti-rabbit immunoglobulin

GAR-HRP	Horse radish peroxidase conjugated anti-rabbit immunoglobulin
gm(s)	gram(s)
GtH	gonadotrophin
h(rs)	hour(s)
HBBS	Hanks balanced salt solution
ip	intraperitoneal
IU	International Units
kD	kilo Dalton
kg	kilogram
L	litre
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
M	Molar
mA	milli Amperes
MEM	Minimum Essential Medium Eagles
mg(s)	milli gram(s)
min(s)	minute(s)
ml(s)	milli litre(s)
mm	milli meter
mM	milli Molar
mOsm	milli Osmolarity
MQ H_2O	Milli Q reagent grade water
mRNA	messenger ribonucleic acid
mU	milli Unit
N_2	liquid Nitrogen
NBT	Nitroblue tetrazolium
NC	nitrocellulose
ng	nano gram
nm	nano meter
NRS	normal rabbit serum

^{o}C	degree Celsius
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PG	prostaglandin
pH	percentage of Hydrogen
PMSF	phenyl methyl sulfonyl floride
PMSG	pregnant mare serum gonadotrophin
PNPP	p-nitrophenyl phosphate
PRL	prolactin
RIA(s)	radio immunoassay
RNA	ribonucleic acid
SDS	sodium dodecyle sulfate
SG-G100	salmon gonadotrophin
Tris-HCl	Trizma Base
TSH	thyroid stimulating hormone
V	Volts
v/v	volume/volume
Vg	vitellogenin
w/v	weight/volume

Figure 1.1. Male and female *Brachydanio rerio* (zebra fish). The zebra fish *Brachydanio rerio* (Hamilton-Buchanan) is a tropical fresh water Cypriniform representative of the family cyprinidae. The adult fish reaches an average length of 4.5 cms, has a slim compressed shape and is a vigorous swimmer. Immature males and females are difficult to distinguish due to indistinct secondary sexual characteristics, however as shown in the figure, in the adult the body contour of the gravid female is different from males which are generally slimmer, with larger anal fins and bearing yellowish tinge on the fins.

CHAPTER 1

1. LITERATURE REVIEW

1.1. INTRODUCTION

In oviparous animals, the eggs provide for the embryo in two ways. They contain developmental instructions to direct the initial phases of embryogenesis after fertilisation and provide a source of nutritive substances in the yolk to support the embryo until it can obtain its own food.

In most oviparous species successful growth and development of the oocytes is finally dependent on vitellogenesis, the process whereby vitellogenin, the major precursor of the protein in yolk is secreted by the liver, and carried to and sequestered within the eggs. This vitally important process is under hormonal control in all oviparous vertebrates, with avian, amphibian, reptilian, and fish species showing many features in common.

The similarity in the regulation of vitellogenesis between species has facilitated the study of the process both *in vivo* and *vitro* and there is now a broad understanding of factors which regulate vitellogenin gene expression, post translational modification of products, secretion and transport, uptake and proteolytic cleavage within the oocyte to form the major proteins of egg yolk namely lipovitellin and phosvitin.

This thesis concerns a study of vitellogenesis in the zebra fish (*Brachydanio rerio*) a species which is now being increasingly recognised for its potential as a major model organism for analysing vertebrate development (Strahle and Ingam 1992).

1.2. OVARIAN YOLK PROTEINS

1.2.1. Purification

In teleosts various attempts have been made by investigators to isolate the yolk proteins from the whole ovaries or eggs. Generally the procedures are based on extracting entire ovaries with a concentrated inorganic salt solution, for example 1.2 M $MgSO_4$ as used in studies of the ovarian yolk proteins of the herring (Barman et al. 1964), or 0.5 M NaCl employed for studies in the trout (Ando 1965; Wallace et al. 1966; Campbell and Idler 1980), Pacific salmon (Markert and Vanstone 1968), Atlantic salmon (Idler et al. 1979) and cod (Plack et al. 1971). The extracted lipovitellin-phosvitin complex from which the yolk proteins are derived, can then be simply dissociated with $(NH_4)_2SO_4$, and further purified by chromatography or gel filtration (Campbell and Idler 1980).

1.2.2. Characteristics

The lipovitellin component of the complex purified from yolk in the above manner was found to be a glycolipophosphoprotein which typically contains about 25% lipid and 0.007% alkali-labile phosphorus (Campbell and Idler 1980). The phosvitin component, by contrast, is typically phosphate rich, with an alkali-labile phosphate content of 15.8%, but lacking lipid and carbohydrate (Campbell and Idler 1980). In salmonids (Mano and Yoshida 1969; Campbell and Idler 1980) and ling (Mano and Lipmann 1966), the high phosphate content of phosvitin is due to its enrichment with serine, which accounts for 42% of the amino acid content. The highly phosphorylated toad and chicken phosvitins are also serine enriched (Redshaw and Follett 1971; Christmann et al. 1977).

In comparison to other vertebrates, teleost yolk proteins are more soluble in water due to their lower level of phosphorylation (Jared and Wallace 1968). Some

workers have reported teleost phosvitin as having lower molecular weight than that of other vertebrates (Schmidt et al. 1965; Wallace et al. 1966; Markert and Vanstone 1971) and that it is sometimes absent from ovarian yolk (Jared and Wallace 1968). However the reported molecular weights of lipovitellin and phosvitin vary significantly. In the trout, for example, the molecular weight for lipovitellin is reported as 300 kD and phosvitin 43 kD (Campbell and Idler 1980) or 19 kD (Mano and Yoshida 1969). In the goldfish lipovitellin was found to dissociate into two subunits of 105-110 and 19-25 kD (de Vlaming et al. 1980) whereas similar extracts obtained from the ovaries of the winter flounder yielded two major components with molecular weights of 500 and 30 kD when fractionated by gel filtration, subsequently identified as lipovitellin and phosvitin, respectively (Ng and Idler 1979).

1.3. VITELLOGENESIS

1.3.1. Mechanism of Vitellogenesis

There are two general mechanism of vitellogenesis: The first, which is common in invertebrates entails the egg producing its own yolk by a process called autosynthetic vitellogenesis, (Boyer 1972.; Huebner and Anderson 1976). The second, which is typical of the more advanced insects and the vertebrates, is one where the egg obtains its yolk from an extraovarian source and is called heterosynthetic vitellogenesis

Based on pioneering studies in the goldfish, Bailey (1957) hypothesised that in heterosynthetic vitellogenesis, there was a yolk precursor protein, vitellogenin, which was synthesised by the liver under the stimulation of ovarian estrogen, which was secreted and transported in the blood plasma to the ovary where it was taken up by the oocytes. This is now recognised as the general mechanism found in wide range of species including the amphibian *Xenopus laevis* (Follett and Redshaw 1974), the

chicken *Gallus domesticus* (Chan et al. 1979), numerous teleosts (Ng and Idler 1983; Mommsen and Walsh 1988) and other nonmammalian vertebrates (Wallace 1978).

1.3.2. The Site of Vitellogenin Synthesis.

In the chicken, yolk protein precursors were first identified in the serum of laying hens from the tendency of the sreum to form a precipitate when diluted with water (McIndoe 1959). An important observation and one which was consistent with the Bailey (1957) hypothesis was that hepatectomy prevented the build up these proteinaceous material named, serumvitellin, in the serum, clearly implicating the liver as a potential site of its synthesis (Ranney and Chaikoff 1951). This was subsequently confirmed by *in vitro* radio isotope tracer studies where it was shown that tissue slices from the liver of laying hens synthesised and released labelled serumvitellin into the medium (Heald and McLachlan 1965). Data obtained in similar studies with *Xenopus laevis* also pointed to the liver as the principal site of yolk protein synthesis in amphibians (Munday et al. 1968; Wallace and Jared 1969).

There is now a considerable body of evidence to indicate that the mechanism of vitellogenesis in the fish is essentially similar to that found in the fowl and amphibian. In the zebra fish for example (Korfsmeier 1966), radioisotope tracer studies have shown that injected labelled amino acids are immediately incorporated into hepatic proteins which subsequently appear in the ovary and analytical studies have shown that the major lipophosphoprotein present in the ovary of zebra fish can also be found in both serum and liver extracts (Heesen and Engels 1973). In the cod, yolk proteins was found in the liver of sexually immature young after estrogen treatment indicating an hepatic source and in radioisotope tracer experiments it has been found that including estrogen in liver tissues incubates *in vitro* promoted the incorporation of L-[^{14}C]-leucine into presumptive yolk proteins in both immature and male cod (Plack and Fraser 1970; 1971). Similarly in the cat fish, radio labelled phosphate has been shown to be rapidly incorporated into hepatic phosphoproteins, with the peak of

incorporation occurring at about 12 hours postinjection, followed by the appearance of labelled vitellogenin in the circulation (Sundararaj and Nath 1981) .

1.3.3. Hormonal Control of Vitellogenesis.

1.3.3.1. PITUITARY REGULATION

In vertebrates vitellogenesis like many other reproductive functions is regulated by gonadal steroids which in turn are controlled by pituitary gonadotrophins, produced as part of the normal functioning of hypothalamo-hypophysio-ovarian axis. However the first studies of the endocrine control of vitellogenesis were made in insects (cockroaches), where vitellogenesis was shown to be controlled by juvenile hormone secreted from the corpora allata (Engelmann 1979; see review by Postlethwait and Giorgi 1985).

In vertebrates the first convincing evidence indicting gonadotrophins in the control of vitellogenesis, came from a study on *Xenopus laevis,* where it was shown that injections of human chorionic gonadotrophin (hCG) both induced vitellogenin synthesis and caused ovulation within 24 hours (Wallace and Jared 1968; Wallace and Dumont 1968). A concurrent study showed hCG treatment to be ineffective in inducing vitellogenin synthesis if the toads were first ovariectomised (Nicholls et al. 1968) clearly identifying a role for ovarian secretions. Speculation that the active ovarian principle was estrogen was subsequently confirmed by the finding that estradiol-17β alone could increase vitellogenesis in the female *Xenopus*, and that the response was not deminished by hypophysectomy (Follett and Redshaw 1968). These experiments clearly indicate that the pituitary hormones were not directly involved in vitellogenin synthesis but act indirectly through increasing steroidogenesis (Redshaw and Nicholls 1971).

In teleosts, vitellogenesis ceases following ablation of the pituitary, as demonstrated, for example, in the plaice *Pleuronectes platessa* (Barr 1963), and

catfish *Heteropneustes fossilis* (Sundararaj and Goswami 1968). Pituitary ablation is also accompanied by a decrease in the gonadosomatic index (GSI), reflecting the lack of ovarian growth due to the interruption in the processes concerned with the build up of yolk within the ovary (Campbell and Idler 1976). Subsequently there have been a series of investigations in which piscine as well as mammalian pituitary and placental hormones have been administered to hypophysectomized and ovariectomised catfish and the effects on vitellogenesis studied (Nath and Sundararaj 1981). In the hypophysectomised catfish, for example, administration of partially purified salmon gonadotrophin (SG-G100) resulted in the expected increase in the ovarian weight which was accompanied by an elevation in the serum levels of vitellogenin. Further, when catfish were ovariectomised during the spawning season, there was a gradual drop in serum vitellogenin, which was offset by administration of estradiol-17β but not SG-G100 (Nath and Sundararaj 1981), consistent with pituitary being involved in the regulation of the synthesis of vitellogenesis indirectly through gonadal estrogen stimulation. These studies taken together provide compelling evidence to show that, with the exception of adrenocorticotrophic hormone (ACTH), most pituitary hormones including luteinising hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL) and thyroid stimulating hormone (TSH) have the capacity to induce vitellogenin synthesis, but without stimulating deposition. Significantly, for the experimentalist (see later), the readily available placental gonadotrophins, hCG and pregnant mare serum gonadotrophin (PMSG) show similar biological actions in fish.

1.3.3.2. STEROIDS

In oviparous vertebrates, the evidence identifying estrogen as the paramount stimulus to the liver to produce yolk precursor proteins is compelling. Estrogen injections into chickens, for example, result in the appearance of yolk proteins in the circulation of both immature pullets and cockerels(Ranney and Chaikoff 1951; Mok et al. 1961; Heald and McLachlan 1964), and in fishes and reptiles act to cause a

discernible rise in the blood protein, lipid, calcium and phosphate (Bailey 1957; Urist and Schjeide 1961). The action is specific for estrogens with testosterone, progesterone and cortisol being ineffective (Redshaw et al. 1969). Of the naturally occurring estrogens, estradiol-17β, was shown to be the more potent than estrone and estriol (Redshaw et al. 1969).

Androgens can be active in some species, example, the gold fish (Hori et al. 1979) but as shown in subsequent more detailed studies, only estrone and estradiol caused the typical elevation in the plasma lipid and phosphate seen in normal vitellogenesis (Wiegand and Peter 1980).

Studies with other fish species provide further confirmatory evidence that estradiol is primararily responsible for the regulation of synthesis of vitellogenic proteins. For example, estrogen has been shown to elevate the plasma content of calcium and vitellogenin in rainbow trout (Elliot et al. 1979; Whitehead et al. 1980) and increase the total serum protein and calcium content in *Tilapia aurea* (Yaron et al. 1977) and in the goldfish (Hori et al. 1979) cause an increase in the calcium, lipids and protein bound phosphate levels, which closely correlated with the changes which occur naturally during ovarian maturation (Yaron et al. 1977). Strong correlation has also been shown between plasma estradiol-17β levels and the gonadosomatic index in both the white-spotted char (*Salvelinus leucomaemis*) and goldfish (Kagawa et al. 1981; 1984 and Wallace 1985).

In the more recent molecular studies of vitellogenesis, all report an increase in the synthesis and secretion of vitellogenin in fish, in response to estradiol. This is particularly evident in studies with medaka (*Oryzias latipes*) (Hamazaki et al. 1987), rainbow trout hepatocytes cultures (Vaillant et al. 1988), and *Oreochromis aureus* (Ding et al. 1989; Lim et al. 1991).

The hypothesis proposed by Bailey (1957) now appears to hold for all types of nonmammalian vertebrates, from hagfish (Yu et al. 1981), sharks (Craik 1978), amphibian and reptiles (Carnevali et al. 1991) and other fish (Wiegand 1982), turtles (Ho et al. 1981) and birds (Bregink et al. 1974).

1.4. VITELLOGENIN

The term vitellogenin was initially proposed by Pan et al. (1969) to describe the female specific, bloodborne yolk precursors found in insects. Since then similar yolk precursor proteins have been described in several vertebrate phyla, vitellogenin has been generally adopted to describe the yolk precursor proteins present in the blood of all oviparous vertebrate species. In most groups of oviparous vertebrates, including teleost fishes, amphibians and reptiles (Urist and Schjeide 1961), vitellogenin exits in the blood as a single protein, whereas in birds it circulates as two separate proteins, phosvitin and lipovitellin (Gruber 1972; Redshaw and Follett 1972) which can cause some confusion in nomenclature. However in general most authors identify the blood borne yolk precursor as vitellogenin and the yolk protein components found in the gonads as phosvitin, lipovitellin or livetins (Ng and Idler 1983). In this review the term vitellogenin is reserved for the blood borne yolk precursors synthesised by the liver.

In the fish, as in other oviparous species, vitellogenesis results in the appearance of lipophosphoprotein(s) in plasma with biochemical and immunological characteristics similar to the egg yolk proteins (Campbell and Idler 1980; Emmersen and Petersen 1976; and Heesen and Engels 1973). As vitellogenin was first only detectable in females it was characterised as being female-specific plasma protein, but it is now recognised to occur in gonads and serum of males (Ding et al. 1989; Goodwin et al. 1992), and can be induced in both the immature and mature male by estradiol (Campbell and Idler 1979; de Vlaming et al 1980; Ding et al 1989).

Interestingly, the molecular nature of fish vitellogenin is highly variable, with the different fish species showing more variability in parameters such as molecular weight, degree of phosphorylation, degree of lipidation, or subunit composition than their amphibian or avian counterparts (see review, Mommsen and Walsh 1988). Even within the same species, there is wide variation in the molecular weight of

vitellogenin reported by the different authors, which cannot always be accounted for by the different methodologies used in the isolation, or differences in the amount of proteolytic breakdown.

1.4.1. Isolation

As previously indicated, yolk proteins were first obtained in a crude form by diluting the serum of laying hens with water (McIndoe 1959). Since then techniques have been refined with the aim of purifying vitellogenin from hen serum, including physical (ultracentrifugal separation) (Redshaw and Follett 1971) and chemical (dimethylformamide precipitation) procedures (Ansari et al. 1971). These same techniques when applied to amphibians also yield vitellogenin, but it requirs a very high titre of vitellogenin in the serum to reduce contamination with other serum proteins. Other more successful approaches towards the isolation of vitellogenin from plasma of *Xenopus, (*as reviewed by Wiley et al. 1979) include selective precipitation by a chelating agent such as EDTA (Ethylenediaminetetraaceticacid) together with the divalent cation Mg^{+2}, followed by DEAE-cellulose (Diethylaminoethyl-cellulose) chromatography.

However, in fish, specifically the goldfish, attempts to isolate vitellogenin using EDTA and Mg^{+2} precipitation alone proved to be ineffective (de Vlaming et al. 1980), but this approach proved useful when combined with chromatography, and this procedure has been used successfully to isolate vitellogenin in a variety of species, most notably atlantic salmon (Idler et al. 1979), rainbow trout (Campbell and Idler 1980), medaka (Hamazaki et al. 1987).

1.4.2. Quantification of Vitellogenin

Initial estimates of the plasma vitellogenin content in teleosts were obtained simply by comparing differences in serum protein content between males and females

(Ho and Vanstone 1961), or measuring the alkali-labile phosphorous content of serum (Emmersen and Petersen 1976; Nath and Sundararaj 1981). This proved useful in some species but could not be used in those species where protein phosphorus was almost or completely absent. Estimate of serum vitellogenin levels have also been obtained using densitometric scanning of polyacrylamide gels following electrophoresis in *Salmo gairdneri* and goldfish (van Boheman et al. 1981; Hori et al. 1979), or by quantitative immunoelectrophoresis as in a study of pike (*Esox lucius*) vitellogenin (Goedmaker and Verbroom 1974) or by indirect semiquantitative immunodiffusion as in a study of vitellogenesis in the maturing cod (Plack et al. 1971). A single-radial immunodiffusion method was also used as a means of providing quantitative estimates of serum vitellogenin in maturing rainbow trout (Gothe et al. 1990) where it was shown that there was a correlation between egg maturation and serum vitellogenin. However in the most recent studies the most common approach has been to use an immunoassay procedure employing antibodies raised to the specific vitellogenin purified from the target species.

An immunological relationships between serum proteins and ovarian extracts was first noticed in studies utilising antibodies raised against yolk proteins (Plack et al. 1971). The development of the first immunoassays for circulating vitellogenin based on these crude antibodies followed (Hara and Hirai 1978). Radioimmunoassays for vitellogenin have since been developed for the salmonids including Atlantic salmon (Idler et al. 1979), and rainbow trout (Campbell and Idler 1980; Sumpter 1981), which has proven useful as a means of distinguishing differences in the vitellogenin content of the serum of male and female fish, and identifying the spawning from nonspawning females. The radioimmunoassays developed have been shown to have high sensitivity and specificity and to be generally reliable but, difficulties have been experienced in the labelling of vitellogenin and stability of the radio labelled protein. The recent development of ELISA procedures has largely overcome these limitations and a range of species specific assays have already been

developed with a sensitivity as high as that reported for the radioimmunoassay (10 ng/ml) (Carnevali et al. 1991; Kwon et al. 1990).

1.4.3. Biochemical Characterisation of Vitellogenin

In all oviparous vertebrates studied to date, vitellogenin undergoes postranslational modification including lipidation, glycosylation, and phosphorylation prior to being secreted into the blood. All these processes occur on the membranes of endoplasmic reticulum and are already being initiated while the polypeptide chain is forming (Tata and Smith 1979). Rather limited information is available for fish with respect to the mechanism, sequential events or locale of these transformations.

In fish as in amphibians, vitellogenin is known to be a rich source of phosphate, due to the serine moieties which constitute a significant part of the vitellogenin structure. In fish, however, the protein phosphate content is only about 50% of its avian and amphibian counterparts, possibly reflecting a lower serine content (Mommsen and Walsh 1988). In ovarian yolk the major source of protein phosphorous is phosvitin which is generally found in a low molecular form (Wallace et al. 1966; Markert and Vanstone 1971; de Vlaming et al. 1980). The amount of phosphorous varies between species and there are also reports of phosvitin being almost or completely absent from yolk preparations (Jared and Wallace 1968; Craik 1982).

In addition to phosphate, teleost vitellogenin also avidly binds ions such as calcium, magnesium, or iron (Hara and Hirai 1978; Hara et al. 1980) and thus provide an important source of minerals for the growing oocytes. In fact, the capacity of vitellogenin to act as a chelating substance is the basis of the use of EDTA to isolate vitellogenin from other plasma proteins (Wiley et al. 1979; Ng and Idler 1983).

In contrast to the relatively low phosphate content of fish vitellogenin, the lipid content is twice that found in other vertebrate groups. Typically the lipid content of fish vitellogenin ranges around 20% by weight as determined in goldfish (Hori et al.

1979), rainbow trout (Weigand and Idler 1982), sea trout (Norberg and Haux 1985) and an elasmobranch, the dogfish (Craik 1978). The bulk of this lipid material, forms the polar lipovitellin moiety of yolk (Hori et al. 1979).

Estimates of the molecular weight of vitellogenin molecule have been made by using either gel electrophoresis or chromatographic procedures. Their reliability is however uncertain due to the different method used, and the amount of proteolytic breakdown. Molecular weights of 460 kD, 390 kD and 600 kD have been claimed for the vitellogenin of *Xenopus laevis* (Wallace 1970; Redshaw and Follett 1971; Ansari et al. 1971) and 235 kD in the chicken (Christmann et al. 1977). In the teleosts, a range of molecular weights has been reported: 330 and 280 kD for goldfish (Hori et al. 1979; de Vlaming et al. 1980), 390 kD for Coho salmon (Markert and Vanstone 1971), 400 kD for cod (Plack et al. 1971), 470 kD for trout (Campbell and Idler 1980), and 550 kD for the flounder (Emmersen and Petersen 1976) and catfish (Nath and Sundararaj 1981). Most fish vitellogenins have been shown to exist as dimers in their native state with subunit molecular mass ranging from 140-220 kD, with the notable exception of the Japanese eel which exists as tetramer having a 350 kD molecular weight in its native state and a subunit molecular mass of 85 kD (Hara et al. 1980).

1.5. HEPATIC EVENTS AND CHANGES RELATED TO VITELLOGENESIS

Liver typically contains highly specific estrogen receptors, which on binding ligand can interact with the DNA to modulate the expression of specific genes (Miesfeld et al. 1984) including the vitellogenin gene. The presence of the estrogen receptors in fish liver has been verified for a number of different species, including the Atlantic salmon (Lazier et al. 1985;), pacific hagfish (Turner et al. 1981) and brown trout (Pottinger 1986). Compared with other vitellogenic vertebrates, the

teleost liver appears to a very rich source of highly specific estrogen receptors, making it an ideal model system to study the induction of receptors and to analyse the mechanism of hormone-receptor and receptor-chromatin interaction in lower vertebrates.

With a time delay of few hours following the binding of the estrogen/receptor complex to the nuclear DNA, a variety of changes are initiated in the liver cells consistent with a substantially increased capacity for protein synthesis and secretion. In naturally vitellogenic fish a much higher rate of hepatic protein synthesis is observed than in non vitellogenic fish (Yu et al 1980; Emmersen and Korsgaard 1983), a difference that can be further enhanced by estrogen administration.

Several ultrastructural differences are observed between liver cells from immature and vitellogenic fish. For example in the red grouper (*Epinephelus akaara*), the vitellogenic liver is characterised by expanded nuclear envelope cisternae, swollen mitochondria and a much enhanced endoplasmic reticulum, Golgi apparatus and secretory vesicles (Ng et al. 1984). In trout, during endogenous vitellogenesis, hepatocytes contain moderately developed rough endoplasmic reticulum, small Golgi bodies containing no electron-dense material, and an increased cytoplasmic glycogen and lipid (van Boheman et al. 1981). There is no difference in the ultrastructure of the liver in either sexes during this stage. During exogenous vitellogenesis the rough endoplasmic reticulum is strongly developed, the Golgi bodies are much enlarged with electron-dense inclusions, the mitochondria possess a densely packed and concentrically arranged membrane configuration, whereas the cytoplasm is depleted of glycogen granules and lipid droplets and the nucleus and nucleolus present an hypotrophied appearance (Ng and Idler 1983). All these ultrastructural changes are consistent with the endoplasmic reticulum being actively involved in protein synthesis, the Golgi with the packaging of secretion of these proteins, and the mitochondria providing the energy demanded by this process. Hepatocytes of naive fish treated with estradiol showed similar, but not entirely identical ultrastructural changes.

Several studies indicate that vitellogenesis is accompanied by an increase in relative liver size (van Bohemen et al. 1982 a,b; Dasmahapatra et al. 1982; Ng et al. 1984). In the red grouper, this appears to be due to the rise in cell lipid and water content rather than cell proliferation. In the atlantic salmon (*Salmo salar*), and flounder, estradiol administration has been shown to result in an increase in liver protein, total RNA, and total nuclear count (Korsgaard et al. 1986; Korsgaard and Petersen 1976) accompanied by a massive increase in rough endoplasmic reticulum (Selman and Wallace 1983); the amount of newly synthesised RNA in the hepatic tissue reflecting the increase seen in the amount of ribosomal RNA.

1.6. OVARY RELATED EVENTS

1.6.1. Steroid Production by Ovarian Follicles During Vitellogenesis

Under natural environmental conditions, ovarian activity including ovarian development, vitellogenesis, maturation and ovulation, is responsive to environmental factors, through the mediation of the hypothalamo-hypophysio-ovarian axis..

Oocyte growth and meiotic maturation in teleosts, like those in other nonmammalian vertebrates, are regulated by gonadotrophins. However as discussed previously, the action of gonadotrophin on yolk is mediated by ovarian production of steroid hormones, particularly estradiol-17β (see review Wallace and Selman 1981)

In vitro studies have confirmed that cells of the ovarian follicular epithelium are responsible for the gonadotrophin induced production of estradiol (Kagawa et al. 1984; Sakai et al. 1987; Sundararaj et al 1982). However a series of investigations, have also shown that under the influence of gonadotrophin (Salmon gonadotrophin, PMSG, and hCG) the same ovarian follicles produce both estradiol-17β and 17α-hydroxy,20β-dihydro-4-pregnene-3-one (17α,20β-P) dependent on the developmental stage (Sakai et al. 1987). In amago salmon (*Oncorhynchus rhodurus*) a two cell-type

model for the production of follicular estrogen, has been proposed which identifies the vascularised thecal layer to be the site of the biosynthesis of androgens which are transported to the granulosa layer and aromatised to 17β-estradiol (Kagawa et al., 1982)

During the reproductive cycle there is a slow increase in estradiol production by the ovary which is reflected in the increase in vitellogenesis. Interestingly, aromatase activity is also found in the brain tissue where it is thought to be involved with behavioural modification (Callard et al. 1981; Lambart and van Oordt 1982). It is also of intrest to note that in comparison to other vertebrates the level of aromatase activity in the teleost brain is exceptionally high.

1.6.2. Passage of Macromolecular Materials Through the Follicular Epithelium

The microscopic structure of the vitellogenic teleost egg has been described in detail (see review Guraya 1978). Once the oogonia give rise to an oocyte within the ovary, they become invested by a layer of follicle cells and connective tissue, thus forming a follicle. The organisation of the growing follicles differs between species to some extent. In general an acellular investment forms immediately adjacent to the oocyte and is penetrated by microvilli from the oocyte and also by processes from an overlying layer of follicle (granulosa) cells. This cellular layer is in turn invested by a basal lamina and vascularised collagenous matrix. The matrix layer, termed the theca is where the specialised steroid-secreting cells are found.

1.6.3. Uptake of Vitellogenin by Growing Oocyte

The ovarian matrix is is contained by a squamous and largely impermeable ovarian epithelium (Dumont and Brummett 1978) and injection of labelled vitellogenin or macromolecular tracers into a variety of oviparous animals has demonstrated that such materials freely penetrate throughout the follicle primarily by

an intercellular route. Circulating materials appear to leave the perifollicular capillaries both by intracellular (pinocytotic) transport across endothelial cells and by passage between adjacent endothelial cells as observed in lizard (Neaves 1972), *Xenopus* (Brummett and Dumont 1977) and the sheepshead minnow (Selman and Wallace 1982a). Substances can then pass from the theca through a basal lamina and penetrate intercellular channels of the follicular epithelium and the pore canals of the vitellin envelope. Thus, the bloodborne proteins have ready access to the growing oocyte during vitellogenesis. A full discussion of this process is presented in the review by Selman and Wallace (1982b).

In female of oviparous vertebrates, circulating vitellogenin is rapidly and specifically cleared from the bloodstream by the growing oocyte. In *Xenopus*, it has been estimated that about 12% of the vitellogenin circulating in the blood is taken up by the gonad per day (Wallace and Jared 1968). In the absence of ovary, as in the estrogen primed males, the vitellogenin continues to accumulate in the circulatory system until it is finally removed by the liver and degraded along with other proteins.

The oocytes take up vitellogenin selectively relative to both plasma proteins normally present in the blood stream (Wallace et al. 1970) and heterologous macromolecules (Wallace and Jared 1976) indicating a receptor mediated process. Studies on *Xenopus* and chicken have shown that the oocyte receptor proteins display low non-specific binding, are saturable, and specifically recognise and bind the phosvitin region of vitellogenin molecule, and that phosphorylation is crucial to the process of receptor recognition and vitellogenin uptake (Opresko et al. 1981; Yusko et al. 1981). This is of special interest as, in the fish species so far studied, the phosphate content of vitellogenin is only about half that of the other vertebrates, raising questions concerning the involvement of phosphate groups in vitellogenin uptake in fish and encouraging further comparative studies which could lead to interesting insights into recognition and receptor mechanism in general.

Detailed studies into the mechanism of vitellogenin recognition and the selectivity of its uptake in fish oocytes was first investigated in rainbow trout

(Campbell and Jalabert 1979). These studies showed that the developing oocytes took up labelled vitellogenin *in vitro* at a rate 10% less than *Xenopus* with no evidence of selective uptake over serum albumin as seen in *Xenopus* under comparable experimental conditions.

The question of selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout (*Salmo gairdneri*), was further investigated by Tyler et al. (1988). Based on their *in vivo* studies of the rates of uptake of radiolabelled vitellogenin and bovine serum albumin (BSA) they concluded that vitellogenin was sequestered into vitellogenic oocytes by receptor mediated endocytosis, whereas BSA, and other extraneous proteins, were taken up adventitiously. They estimated a rate of uptake of vitellogenin to be 60 times that of labelled BSA, with all the vitellogenin that was taken up remaining within the oocyte as yolk protein, whereas greater then half of the BSA entering the oocytes was subsequently released. The subsequent *in vitro* studies on the dynamics of protein sequestration into vitellogenic follicles confirmed that the vitellogenin uptake was both selective and far exceeded that of labelled BSA (Tyler et al. 1990a). This uptake appears to be regulated by the outer epithelial cell layer and was affected by the amount of vitellogenin in the culture medium which induced greater rates of uptake in a dose dependent manner (Tyler et al. 1990b). Uptake of vitellogenin was also found to be temperature dependent, with higher temperature inducing higher levels of incorporation. Size and stage of the follicle had an impact on the incorporation of vitellogenin. Compared with vitellogenic follicles the rate of sequestration was reduced in pre-vitellogenic follicles and those approaching ovulation. Furthermore, vitellogenic follicles showed increases in both the total amount of vitellogenin sequestered and the rate of uptake as the size of the follicle increased. This led Tyler et al. (1991a) to speculate that the increase in size of the vitellogenic follicles resulted in an increase in the number of vitellogenin receptors per unit surface area, thus regulating the rate of uptake, and ensuring that the ability to sequester vitellogenin was appropriate to the developmental stage.

With respect to the hormonal regulation of vitellogenin uptake by the fish oocyte, less information is available, although estrogen does not seem to be involved. There is some evidence that gonadotrophin may stimulate uptake (Ng and Idler 1983), although this aspect is less well documented. In the study conducted by Tyler et al. (1991b) utilising the gonadotrophin GtH I and GtH II species isolated from chum salmon showed that, GtH I increased the uptake of labelled vitellogenin into the oocytes by more than two-fold, effectively doubling the rate of growth *in vivo*, whereas GtH II showed a rate of vitellogenin sequestration similar to controls. Similarly *in vitro* study showed that GtH I stimulated the rate of vitellogenin uptake in a dose dependent manner and GtH II had no effect on the sequestration of vitellogenin in the isolated oocytes. This was the first evidence that GtH I has a primary function in stimulating vitellogenin uptake, and strongly support the contention that at least two functionally distinct GtHs occur in fish (Tyler et al., 1991b).

1.7. VITELLOGENESIS :*IN VITRO* STUDIES

1.7.1. Estrogen Responsive Liver Cultures

Evidence reviewed above clearly indicates that the liver is the site of synthesis and secretion of vitellogenin. This process is amplified in mature females but can be initiated in the males by estrogen, factors which have made vitellogenin synthesis a favourite investigative model for the mechanism of steroid regulation of eukaryotic gene expression. The first *in vitro* studies of the process were initiated when Rudack and Wallace (1968) cultured *Xenopus laevis* liver slices with estrogen. This system has since been widely used as a model system in the study of estrogen regulation of vitellogenin gene transcription. Purified hepatocytes have since been prepared from avian (Mullininix et al. 1976), other amphibian species (Ryffel et al. 1978; Skipper et

al. 1977; Tata et al. 1976) and fish (Plack and Fraser 1971, Bradley and Grizzle 1989).

1.7.2. Hepatocytes Primary Monolayer Cultures

Methods have been developed for isolation and primary culture of hepatocytes from various mammalian species (Miyazaki et al. 1981; Jauregui et al. 1986; Seglen 1973), and these cells have proved invaluable for studying the mechanism of differentiation (Guguen and Guillouzo 1983), hormonal regulation (Laishes and Williams 1976) and pharmacotoxicity (Guzelian et al. 1977). Until now, very few reports have dealt with fish hepatocyte cultures, partly due to the difficulty of getting firm cell attachment of fish liver cells to culture vessels and problems with maintaining cell viability for prolonged periods.

Although various studies have been performed on freshly isolated fish hepatocytes (Bouche et al. 1979; Hayashi and Ooshiro 1978; Saez et al. 1982), their ability to maintain specific gene expression in primary culture remains to be demonstrated. There is only one report of a procedure in the rainbow trout in which high yields of hepatocytes were obtained from liver which showed good viability and formed primary monolayers which retain a capacity to respond to estrogen by de novo synthesis of vitellogenin (Maitre et al. 1986). These have been used in preliminary studies of the kinetics of vitellogenin and its mRNA (messenger ribonucleic acid) (Vaillant et al. 1988).

1.8. OOCYTE MATURATION AND OVULATION

1.8.1. Induced Final Maturation and Ovulation

Although the primary use of induced-spawning techniques is often directed towards those species that fail to reproduce in captivity, there is an increasing realisation that the technique can be used to advantage to alter or synchronise the spawning time of species that do undergo sexual maturation and produce gametes in captivity. Hormone treatments for example can be used to obtain viable gametes outside the normal spawning season thereby lengthening the period available for rearing prior to stocking, transplanting, or release (Donaldson et al. 1981 a,b), and to improve production efficiency, through minimising gamete losses attributable to prespawning mortality (Hunter et al. 1978 and 1981). They can also be used for research to produce ova with known ovulation time for experimental studies such as induced gynogenesis, triploidy (Donaldson and Hunter 1982; Reftstie et al 1982) or transgenesis (Houdebine and Chourrout 1991)

1.8.1.1. Levels Of Intervention

The hypothalamic-pituitary-ovarian axis controlling reproduction allows for intervention at several levels to promote or interfere with the process of maturation and ovulation. Hypophysation, for example, is used widely to control the final maturation stages and ovulation through judicious injection with homologous or heterologous pituitary extracts, typically partially purified piscine gonadotrophin and/or hCG either alone or in conjunction with fish pituitary extracts.(Rowland 1983; Donaldson and Hunter 1983).

Due to the restricted availability of purified gonadotropins in the 1970s alternative approaches aimed at stimulating the production and /or release of

gonadotrophin in the pituitary gland (e.g., antiestrogens and gonadotrophin releasing hormones) have also been developed.

1.8.1.2. Use of specific compounds

(a) Antiestrogens

Gonadotrophin secretion in teleosts, as in other vertebrates, is in part regulated by negative feedback of gonadal steroid. This was demonstrated by showing that estradiol inhibited the compensatory ovarian growth induced by unilateral ovariectomy in catfish, and caused atresia of matured oocytes in catfish (Goswami and Sundararaj 1968). Activation of gonadotrophs was also shown to be reversed by treatment with estradiol in sockey salmon (van Overbeek and Mcbride 1971) and this awareness of the negative feedback control of gonadotrophin secretion by estrogen led to a series of spawning trials of antiestrogens. Clomiphene for example which is widely used as a fertility agent in the human with both anti and pro estrogen activity, was used to induce ovulation in goldfish (Pandey and Hoar 1972), and *Heteropneustes fossilis* (Singh and Singh 1976). The rise in plasma gonadotrophin which preceded ovulation in the latter species is consistent with an action of clomiphene at the pituitary level (Pandey et al. 1973). By contrast, in the carp clomiphene administration resulted in an increase in the proportion of ova having germinal vesicles (GV) in the peripheral position, but failed to induce ovulation (Bieniarz et al. 1979) . This limited research whilst suggestive is insufficient to predict of how antiestrogens would act after final maturation and ovulation.

(b) Gonadotrophin Releasing Hormone

A study of the effect of synthetic natural luteinising hormone releasing hormone (LHRH) on ovulation in a teleost was conducted by Hirose and Ishida (1974). In the

mature female ayu (*Plecoglossus altivelis*) there was a dose dependent increase in ovulation in reponse to a single ip (intraperitoneal) injection of LHRH (viz. 50, 100, or 200 μg LHRH/fish caused a 40%, 50%, and 80% increase in the ovulation rates respectively). LHRH injected ip (10 mg/kg) or intracranially (2 mg/kg) in the goldfish resulted in 75% and 100% ovulation respectively after 4-7 days of treatment (Lam et al. 1975). However ovulation was not observed in all the teleosts following LHRH treatment, Sokolowska et al. (1978) for example, found that daily intrahypophyseal injection of 1 mg/kg caused GV migration and GV breakdown (GVBD) but no ovulations.

The LHRH analogue, des Gly10-(D-Ala^6) luteinising hormone releasing hormone ethylamide (LHRHa D-Ala^6) which has a biological activity 35-50 times that of the native LHRH in mammals (Coy et al. 1974; Fujino et al. 1974). It has also been used in fish alone or in conjunction with gonadotrophin. Van Der Kraak et al. (1983) for example, reported that a single ip injection of LHRHa D-Ala^6 promotes oocyte maturation and ovulation in coho salmon, whereas native LHRH was ineffective, also the analogue was found to cause a more prolonged increase in plasma gonadotrophin levels than LHRH. Combined injection of LHRHa D-Ala^6 (0.02 and 0.2 mg/kg) and chinook salmon gonadotrophin (SG-G100) (0.1 mg/kg) were more effective than a single injection of SG-G100 or LHRHa D-Ala^6 in inducing ovulation in coho salmon (Van Der Kraak et al. 1984). Positive results were obtained in inducing ovulation with LHRHa D-Ala^6 in rainbow shark (*Labeo reythrurus*) and redtail black shark (*Labeo bicolor*) (Shireman and Gildea 1989).

(c) Gonadotropins

Gonadotropins of the piscine and mammalian origin have been used in teleost for induction of final maturation and ovulation. Hypophysation using piscine pituitary extracts is effective in inducing ovulation, but its effectiveness has been shown to be improved if used in conjunction with mammalian gonadotropins (see review

Donaldson and Hunter 1983). Carp pituitary extracts (CPE), for example, used in combination with hCG has proven very effective as a means of inducing spawning in different teleost species (Rowland 1983, Shireman and Gildea 1989). Generally the use of partially purified and purified teleost gonadotrophin is limited by availability, the exception being the generally good availability of a partially purified gonadotrophin from pacific salmon, *Onchorynchus* species, SG-G100 (Donaldson et al. 1972) which is in common use to induce ovulation both in salmonids and non salmonids (Donaldson and Hunter 1983).

The availability, uniformity, and effectiveness of mammalian gonadotrophin is an obvious attraction over fish gonadotrophin. hCG (human Chorionic Gonadotrophin) has been used effectively on the widest range of species either alone, as in goldfish (Stacey et al. 1979), grey mullet (Kuo et al. 1973), rabbit fish (Ayson 1991) or together with fish pituitary extracts (Donaldson and Hunter 1983). PMSG has been used successfully alone in only limited number of species including *Hetropnuestes fossilis* (Sundararaj and Goswami 1966) and gulf croaker (Haydock 1971), and was found ineffective when tested alone in clarius , or the cichlid, *Tilapia nilotica* (Babiker and Ibrahim 1979) whereas hCG alone was effective in both these species under similar conditions.

(d) Steroids

The progesterone (P) derivatives 17α-hydroxyprogesterone (17α-P) and 17α,20β-P were detected in significant quantities in the plasma of maturing sockeye salmon, *Oncorhynchus nerka*, by Idler et al. (1960) and Schmidt and Idler (1962) and later shown to be effective inducers of final maturation in teleost oocytes (Jalabert 1976). These steroids whilst competent in inducing the final maturation of the oocytes do not induce ovulation, implying the need for additional factors. Priming the fish with gonadotrophin before treatment with progestins increases the rate of ovulation as shown in the studies with trout (Jalabert et al. 1978), where

ovulation was induced by a treatment consisting of a primer of 0.25 mg/kg SG-G100 followed two days later with 2 mg/kg of 17α,20β-P.

However, subsequent studies have shown that ovulation will only occur when the endogenous gonadotrophin concentration is sufficient, or when a minimum amount of exogenous gonadotrophin is supplied. Thus the stage of maturity of the fish and the developmental stage of the oocytes need to be considered as a prerequisite for the successful induction of ovulation.

(e) Prostaglandins

The role of prostaglandins has been reviewed by Stacey and Goetz (1983). It is clear from both *in vitro* and *in vivo* studies that prostaglandins are involved in follicular rupture (Jalabert and Szollosi 1975; Kagawa and Nagahma 1981) and PGF2α was found to be the most effective. *In vivo* studies in cyprinids have shown that indomethacin blocks prostaglandin synthesis and inhibits ovulation, and that the inhibition can be overridden by administration of exogenous prostaglandin (Stacey and Pandey 1975; Kapur 1979). There are very few reports on the use of prostaglandin for *in vivo* studies, but from the *in vitro* data (see review Goetz 1983), prostaglandins have great potential in inducing ovulation after a final maturation induction.

1.8.2. *In Vitro* Maturation and Ovulation

The development of the culture techniques for the mammalian oocytes has facilitated investigations of the hormonal regulation of final maturation and ovulation in the lower vertebrates. As a result, knowledge of these events in the teleost fish is accumulating with much of the information gained from the *in vitro* studies which have already been applied to the *in vivo* investigation or been correlated with naturally occurring reproductive events (see review by Goetz 1983).

1.8.2.1. Final Maturation

(a) Characteristics

As reviewed by Wallace and Selman (1981), during the final stage in the maturation of the teleost oocyte, there is a period of rapid growth during which the oocytes enlarge, follicular layers develop and yolk accumulates (vitellogenic phase). Following this phase, or in some species during this phase, the nucleus or germinal vesicle (GV) migrates from the central to the peripheral position. The time and rate at which the GV migrates to a peripheral position varies between species, and following migration the GV disperses; this is termed germinal vesicle breakdown (GVBD). The area in which this occurs may remain rather inconspicuous as in some salmonids, and cyprinids (Stoeckel and Neves 1992) or become quite distinct as in yellow perch (Goetz and Bergman 1978). In most species studied, the oocyte becomes translucent after or during GVBD as observed in yellow perch (Goetz and Bergman 1978), zebra fish (Van Ree et al. 1977), and catfish (Goswami and Sundararaj 1971).

(b) Steroids

As previously discussed certain steroids when applied *in vivo* were capable of inducing final maturation and ovulation in teleosts. To exclude other ovarian factors and examine more precisely the effect of steroids, i*n vitro* systems are now being employed. Goetz (1983) has listed the various species, incubation conditions and efficiency of the gonadotropins, pituitary preparations and steroids so far tested. The composite evaluation of the effect of steroid on final maturation in oocytes of several species, has shown the progestagens to be the most effective as regulators of the final maturation of oocytes, followed by 11-deoxycorticosteroids, androgens with estrogen being the least effective.

A variety of naturally occurring and synthetic progestagens have been demonstrated to be effective in a number of fish species, with 17α,20β-P proving to be the most potent in a number of species (see review by Scott and Canario 1987), also more recently in blue gourami (Degani and Boker 1992), winter flounder (Truscott et al. 1992), and amago salmon (Young et al 1983).

(c) Gonadotropins or Pituitary Preparations

As with steroids, many studies have been undertaken comparing the *in vitro* effects of various pituitary and gonadotrophin preparations. Many different heterologous gonadotropins or pituitary preparations have been shown to induce final maturation in oocytes *in vitro*, but generally piscine preparations are the most effective, but are importantly highly specific (Goetz 1983). There is however considerable variability between species in response, for instance in catfish neither luteinising hormone (LH) nor salmon gonadotrophin could induce greater than 15% of the oocytes to mature over a wide range of concentrations (Goswami and Sundararaj 1971; Goswami et al. 1974). Homologous preparations were also ineffective in these species as were other mammalian pituitary hormones (Goswami and Sundararaj 1971). Similarly, in yellow perch, mammalian gonadotropins and homologous or heterologous piscine pituitary preparations failed to induce significant maturation *in vitro* (Goetz and Bergman 1978). Whereas, Van Ree et al. (1977) were successful in inducing final maturation in zebra fish oocytes using homologous pituitary extracts.

The varying ineffectiveness of gonadotrophin preparations in causing maturation of oocytes *in vitro* of some species could result from a variety of technical as well as biological sources, such as, incubation condition, developmental stage of the oocytes, impermeability of the gonadotrophin, presence or absence of follicular tissue, length of incubation or the concentrations of the hormones.

1.8.2.2. Ovulation

Ovulation in fish is defined as the actual expulsion of the oocyte from the follicle due to active contraction of the follicle. Maturation *in vitro* is seldom followed by spontaneous ovulation, even when oocytes are cultured after initiation of maturation *in vivo*. On the other hand, if ovulation has already started *in vivo* those follicles that are recovered intact can ovulate *in vitro* (Jalabert 1976). Infact follicles recovered from yellow perch ovaries in this manner can be induced to ovulate *in vitro* following 17α,20β-P stimulation, a response which is blocked by indomethacin, and restored by prostaglandins (PG) (Goetz 1983). Ovulation appears to be dependent on the extrafollicular ovarian tissues. When this is removed only 5% of the follicles ovulated following 17α,20β-P stimulation compared with 94% in intact follicles. Prostaglandins PGF and PGE levels were significantly elevated at the time of ovulation in intact follicles incubated in 17α,20β-P, however, there was no significant increase in PGF or PGE in incubates containing individual ovarian components indicates a synergistic action between tissues (Goetz 1983). Prostaglandins have also been reported to stimulate *in vitro* ovulation in rainbow trout and pike (Jalabert 1976).

1.9. AIMS OF THIS STUDY

The present study was aimed at further characterising the process of vitellogenesis in *Brachydanio rerio* as part of an expanding body of further studies on the molecular embryology of this species.

Two hypothesis were formulated to structure the studies namely.

(1) In *Brachydanio rerio* a yolk protein precursor molecule is synthesised and secreted by the liver as a specific response to estrogen and released into the blood for transport to the oocytes, where it is proteolytically cleaved into yolk proteins.

(2) In *Brachydanio rerio* the gonadosomatic index and size of the oocytes play an important role in determining whether or not the oocytes can be maturated successfully in an *in vitro* system.

CHAPTER 2

2. ISOLATION, PURIFICATION AND CHARACTERISATION OF VITELLOGENIN

2.1. INTRODUCTION

The most conspicuous feature of oviparous vertebrate eggs is their large size, which they achieve within a relatively short period of time. The size increase is due to the accumulation of yolk consequent with the synthesis of vitellogenin (Vg) in the liver. As previously discussed (see literature review) a general framework for the hormonal control of vitellogenesis was first proposed by Bailey (1957), and over the years has been tested and modified accordingly.

The natural occurrence of Vg has been reported in a range of mature females of oviparous species; in insects (Pan et al. 1969), chickens (Follet and Redshaw 1974), amphibians (Wallace and Bergink 1974) and fish (Volataire et al. 1984) and its synthesis and secretion in response to estrogen studied in all type of nonmammalian vertebrates, from hagfish (Yu et al. 1981), sharks (Craik 1978) and other fish (Wiegand 1982), turtles (Ho et al. 1981) birds (Bergink et al. 1974) and amphibians (Follet and Redshaw 1974). Vg can also be induced in males and juvenile females by administration of estrogen with estradiol-17β being the most potent(Campbell and Idler 1979; de Vlaming et al. 1980; Ding et al. 1989).

Various techniques have been utilised to purify vitellogenin from the vertebrates studied, these include physical (ultracentrifugal seperation) (Redshaw and Follett 1971) and chemical (dimethylformamide precipitation) procedures (Anasari et al. 1971). These techniques require a very high titre of vitellogenin in the serum. Selective precipitation by a chealating agent such as EDTA together with the divalent cation Mg^{+2}, followed by chromatographic procedure has been successfully employed by many investigators (Wiley et al. 1979; de Vlaming et al. 1980; Campbell and Idler 1980).

As reviewed, biochemical investigations of Vg in some teleost fish (Hara and Hirai 1978; Hori et al. 1979; So et al. 1985), indicate that it is a complex high molecular weight protein containing variable amounts of lipids, carbohydrates and phosphates (Campbell and Idler 1980; Mano and Yoshida 1969). Interestingly, the fish display a higher variability in the different parameters, than their amphibian and avian counterparts.

The aim of the present study was to isolate and purify Vg from zebra fish (*Brachydanio rerio*). This has been successfully achieved using aqueous extraction and anion exchange chromatography from serum, liver and gonadal homogenates after estradiol-17β stimulation from both males and females.

2.2. MATERIALS AND METHODS

2.2.1. MAINTENANCE OF FISH

2.2.1.1. Fish

Brachydanio rerio (Zebra danio) is a teleost fish species which belongs to the Cyprinid family. It is a hardy tropical fresh water fish of south-east Asia. At maturity it reaches a length of 2-3 cms.

Adult zebra fish were obtained from commercial suppliers, and maintained in aquarium glass tanks with both gravel filter and aeration through air pumps and a water filtration system (Ehime, Germany). Fish were always acclimatised to the tank water before introducing them in the stock tanks.

2.2.1.2. Prerequisites for Holding Fish

Water for holding the fish was obtained from the purified water system used in the building, and was dechlorinated either by using dechlorination drops (Genesis, Aquarium products, Australia) or by ageing for several days before use. At least a third of the tank water holding the fish was changed once a week. The gravel substrate was cleaned by siphoning, to remove the faecal material and the food debris that had settled at the bottom. The pH of the water was maintained between 7.2-7.6.

Tank temperature was regulated by using thermostat heaters (220 W, Automatic heaters, Rena, France), the fish stocks were maintained around $20^{o}C \pm 1^{o}C$ and the experimental fish were maintained at $26^{o}C \pm 1^{o}C$ continuously till the end of the experiments. All fish, were kept in an artificial photoperiod of 14 hour (h) light-12 h

dark cycle (14L-12D). Fish stocks were fed twice daily with fish flakes (Tropical food flakes, Wardleys, Australia), the experimental fish were fed twice with flakes and once with frozen brine shrimps.

2.2.2. ESTRADIOL STIMULATION

Adult zebra fish were selected from the stock tank and isolated into 2.5-5 litre (L) containers depending on the density of fish and were fed daily with fish flakes only. The containers were either kept at 26^{o}C with continuous aeration using air lines assisted by an air pump, and water changes were done everyday.

For the induction of Vg, each fish was injected intraperitonealy (ip) with 4 μ g/gm body-weight (b.w) estradiol-17β (E_2), 1,3,5(10)-Estratriene-3,17β-diol (Steraloids, N.Y.). A stock solution of E_2 was prepared in ethanol (10 mg/ml) and an aliquot of the stock was mixed with vegetable oil (1:9; E_2 stock:vegetable oil) with a final concentration of 1 μg/μl for administration. E_2 was administered intraperitonealy (ip) in the maximum volume of 4 μl/fish using micro syringe (Hamilton, Nevada). Control fish were injected with oil alone. All fish were anaesthetised prior to handling in Benzocaine (12 mg/L). Fish were injected with sequential E_2 dose on days 0, 3, and 6, before tissue sampling.

2.2.3. TISSUE COLLECTION AND SAMPLING

Tissues were collected 24 hours (h) after the final administration of E_2 unless mentioned otherwise. Fish were anaesthetised and blood was collected from the caudal vein into Eppendorf tubes containing a solubilisation buffer made up of 50 mM Tris-HCl pH 8.0 [Tris (hydroxymethyl) amino methane. Trizma Base, Sigma], containing 0.15 M NaCl (Sodium Chloride, Fison scientific, England) and 0.1 mM PMSF (phenyl methyl sulfonyl floride). The tubes were vortexed and stored on ice. Fish were sacrificed and serum was collected from the caudal vein directly into the

solubilisation buffer, liver and gonads were collected and immediately rinsed in fish Ringers solution then transferred to the solubilisation buffer on ice. Solid tissues were homogenised and all the samples were allowed to solubilise overnight at 4°C. Samples were then centrifuged at 4°C, and the supernatant was collected. An aliquot of the supernatant was used for protein estimation (Biorad) with BSA (Bovine Serum Albumin, Sigma) as reference standard and the remaining supernatant was either used immediately or stored at –70°C.

2.2.4. PURIFICATION OF VITELLOGENIN BY ANION EXCHANGE CHROMATOGRAPHY

The supernatant collected was loaded directly onto the Mono-Q anion exchange column (HR 5/5 prepacked column, Pharmacia) on the FPLC (Fast Protein Liquid Chromatography) System (Pharmacia). All solutions used were filtered through 0.22 µm filters and degassed before use. The column was equilibrated with three volumes of 50 mM Tris-HCl, pH 8.0 (Buffer A), or until a steady base line had been obtained. A maximum of 10 ml of the sample was loaded onto the column, followed by washing with 15 ml of Buffer A. Both bound and unbound proteins were then eluted with buffer A admixed with 1 M NaCl to create a linear gradient from 0 to 0.05 M NaCl in a total volume of 20 ml. Following the gradient run, the column was washed with 10 ml of 1 M NaCl in Buffer A to ensure that no other proteins remained on the column, after elution of all the protein off the column a further 10 ml of Buffer A was allowed to pass through the column to ensure the absorbance reading had reached the base line profile.

The flow rate throughout the chromatographic procedure was maintained at 1.0 ml/min, and fractions of 1.0 ml were collected using a fraction collector (Pharmacia). The absorbance was measured at 280 nm and all the chromatographic procedures were performed at room temperature. Fractions relating to the peak in abundance were pooled and concentrated using an Amicon concentrator (cut off at 30,000

Daltons) at 4°C and an aliquot was used for protein estimation, the remaining fraction was used immediately or stored at –70°C.

2.2.5. ISOTOPE INCORPORATION STUDY

To investigate protein synthesis *in vivo*, a separate batch of E_2 stimulated and control fish were injected with 10 μCi of ^{3}H-leucine, L-[3,4,5-^{3}H(N) (Du Pont, NEN Products, Boston) 2 h after the final injection of E_2 and the tissues were collected after 6 h. Following extraction and chromatography as described above, the distribution of the radioactive tracer in the column eluates was determined by scintillation counter. (Beckman LS 6000 LL).

2.2.6. ELECTROPHORESIS

2.2.6.1. Polyacrylamide Gel Electrophoresis (PAGE)

All PAGE techniques were carried out using Tris-Glycine system on a vertical slab gels (Hames and Rickwood 1988). Molecular weight determination was performed on 10% PAGE under denaturing condition in the presence of lauryl sulfate (Sodium dodecyl sulfate, SDS) (Sigma) and β-mercaptoethanol (BDH Chemicals. England), using molecular weight markers myosin 200 kilo Dalton (kD), phosphorylase b 97 kD, BSA 69 kD, ovalbumin 46 kD, carbonic anhydrase 31 kD, trypsin inhibitor 21 kD and lysozyme 14 kD (Amersham, Australia). All denaturing, discontinuous gel casting and electrophoresis was performed using Biorad gel apparatus. The purity of the protein and the molecular weight in its native state was assessed by electrophoresis on 5% nondenaturing, discontinuous linear PAGE utilising a small gel apparatus (Hoeffer's) and the molecular weight makers, thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), albumin (69 kD) (Pharmacia).

2.2.6.2. *Sample Preparation, Loading and Electrophoresis*

All extracts loaded onto the gel contained equal amount of protein. For SDS-PAGE analysis all the samples including molecular weight markers were mixed with sample buffer (2% SDS w/v, 5% β-mercaptoethanol v/v, 10% glycerol v/v, 0.002% Bromophenol blue v/v), and were boiled for 5-15 mins and allowed to cool down to room temperature before loading onto the gel, the 10% SDS-PAGE gels were electrophoresed at 200 V (Volts) constant voltage, until the dye front reached the bottom of the gel.

For native PAGE, samples were mixed with sample buffer without SDS and B-mercaptoethanol before loading, and 5% native gels were electrophoresed at 30 mA (milli Amperes) constant current. Electrophoresis run was terminated when the tracking dye approach near the bottom of the gel.

2.2.6.3. *Staining of Electrophoresed Gels*

After electrophoresis gels were immediately transferred to trays for staining with 0.2% Coomassie Brilliant Blue R-250 (Sigma) in 50% Methanol (Ajax Chemicals, Australia), and 5% Acetic acid (BDH Chemicals, England) in water purified in a Milli-Q Reagent Grade Water System (MQ H_2O, Millipore, Bedford, MA) and were left over night. Gels were destained by rinsing them twice with 50% Methanol and then transferred to the destaining solution made up of 20% methanol and 5% acetic acid in MQ H_2O, with regular changes of the destaining solution. Shaking was maintained throughout the staining and destaining procedure. Destained gels were then photographed and stored after drying in the gel drier.

2.2.7. AMINO ACID ANALYSIS

The purified Vg was lyophilised, then hydrolysed in 6.0 N HCl and 0.1% β-mercaptoethanol for 1 hour at 150°C. Amino acid analysis was performed on a Beckman 6300 Amino Acid Analyser.

2.3. RESULTS

2.3.1. CHROMATOGRAPHY OF VITELLOGENIN

The Mono-Q column chromatographic elution profile of the pooled E_2 stimulated male zebra fish serum (Fig 2.1), liver (Fig 2.2) and gonadal homogenate (Fig 2.3) showed a single peak eluting at a chloride ion concentration of 0.35 M. This peak was absent in extracts obtained from unstimulated male and female but was present in the samples from E_2 stimulated female fish serum (Fig 2.4) and liver homogenates (Fig 2.5C).

By contrast, the elution profile of extracts of the E_2 stimulated female gonad showed two peaks (Fig 2.5D), one peak which eluted at a chloride ion concentration of 0.35 M, similar to the major peak of serum and liver extract, together with an additional peak eluting at a chloride ion concentration of 0.25 M. The chromatographic profile of homogenates prepared from the gonads of untreated female fish showed only a single peak eluting at chloride ion concentration of 0.25 M and with no evidence of a peak at 0.35 M (Fig 2.5B).

Fractions associated with the peak at 0.35 M chloride ion concentration were pooled and concentrated and after protein estimation the samples were analysed on denaturing and nondenaturing PAGE.

2.3.2. ISOTOPE INCORPORATION

In isotope incorporation studies, a major peak of radioactivity for the ^{3}H-leucine incorporation experiment corresponded to the peak eluted at 0.35 M chloride ion concentration in the E_2 stimulated liver and gonad of female fish (Fig 2.5), whereas the incorporation of isotope into this peak in the unstimulated extracts was negligible. Since leucine, a nonpolar amino acid is relatively abundant in fish Vg (de Vlaming et al. 1980; Hara and Hiria 1978) and it is known that Vg will incorporate radioactive leucine during synthesis. The absorbance peak at 0.35 M NaCl was tentatively identified as zebra fish Vg.

2.3.3. ANALYSIS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

2.3.3.1. Effect of Estradiol Induction

Analysis of the crude extracts of E_2 stimulated and unstimulated liver, serum and gonad of both male and female zebra fish on 10% SDS-PAGE revealed two major protein bands of about 160 kilo Daltons (kD) (band I) and 190 kD (band II) molecular weight (fig 2.6). Both these bands were totally absent in the unstimulated males, with unstimulated mature female showing faint bands of similar molecular weight.

2.3.3.2. Effect of Sequential Estradiol Dose

To further examine the response to E_2, crude extracts were prepared from individual zebra fish 24 hours after being subjected to sequential E_2 doses. Examination of the extracts by electrophoresis on 10% SDS-PAGE provided evidence of synthesis and secretion of both protein bands (I and II) increasing in both males and females with successive injections (Fig 2.7). In the female, a single injection of 4

μg/gm body-weight caused a significant increase in the circulating Vg concentration level, but in the male, increase in synthesis was only clearly evident after the second injection.

2.3.3.3. *Analysis of Purified Putative Vg*

For determination of native molecular weight, samples were loaded on to 5% discontinuous nondissociating PAGE. When stained with Coomassie blue a single protein band of molecular weight 325 kD was obtained (Fig 2.8).

SDS-PAGE analysis of the purified protein revealed two major protein bands of 160 kD and 190 kD molecular weight (Fig 2.9), similar to those obtained from the crude extracts of E_2 stimulated fish.

2.3.4. AMINO ACID ANALYSIS

Amino acid analysis for zebra fish putative Vg is provided in Table 2.1. It exhibits close parallelism with the composition for gold fish and rainbow trout Vg, with the exception of serine which is higher than that reported for goldfish and rainbow trout.

2.3.5. MEASUREMENT OF VITELLOGENIN

Putative Vg obtained from Mono-Q column chromatography was measured as a percentage of total protein by protein estimation using BSA as standard reference. The purified protein from the serum, liver and gonads was calculated to be 68%, 40%, and 46% of the total protein respectively.

2.4. CONCLUSION AND DISCUSSION

As has been previously stated, oviparous vertebrate Vg is characterised as being an unique high molecular weight glycolipophosphoprotein (Wallace 1978; Tata 1978), synthesised by liver under the stimulation of estrogen (Aida et al. 1973; Hara and Hirai 1978; de Vlaming et al. 1980).

The studies described within this chapter describe the isolation and characterisation of Vg from the zebra fish. Several criteria support this conclusion, firstly the protein component is female specific, yet inducible in the males by estrogen treatment, secondly it is present in all tissues involved in natural vitellogenesis, thirdly its chromatographic and electrophoretic characteristics are similar to those reported for vitellogenin from other vertebrates (Wallace et al. 1980) and finally, amino acid analysis of the purified product indicates a remarkable similarity in composition to Vg purified from other species (Table 2.1).

Vg has been purified using variety of techniques; DEAE-cellulose chromatography (Wiely et al 1979; Hamazaki et al. 1987), selective chemical precipitation (Wiely et al 1979) and ultracentrifugal separation (Redshaw and Follet 1971), usually used in combination. The simple procedure developed in this chapter proved very reliable and yielded a relatively clean Vg preparation as verified by native PAGE (Fig 2.6). As with Vg in other teleosts (de Vlaming et al 1980; Hara and Hirai 1978), zebra fish Vg has a high content of leucine, and this has been used to advantage in the isotope incorporation studies showing that the putative Vg to be synthesised and secreted in response to E_2. The putative zebra fish Vg also exhibits significantly higher level of serine then in other teleosts suggesting that it may be more like amphibian and chicken Vg in phosphate content (Follett and Redshaw 1974).

As reviewed previously a range of molecular weights has been reported for teleost Vg ranging from: 320 and 280 kD for goldfish (Hori et al 1979; de Vlaming et al. 1980), 390 kD for Coho salmon (Markert and Vanstone 1971), 400 kD for Cod

(Plack et al. 1971) and 470 kD for trout (Campbell and Idler 1980). Our estimates of molecular weight of native zebra danio fish Vg was 325 kD by electrophoresis, and 300 kD by amino acid analysis (where cystine and tryptophane were not determined), which is within the range reported for other teleost species (Hori at al 1979; de Vlaming et al. 1980).

The synthesis of Vg in the liver can be greatly enhanced by estrogen treatment regardless of sex, and in the present experiments, steroid treatment resulted in an increase above the preexisting levels of Vg in mature female fish, and detectable production in the males. The most intriguing finding was the detection and isolation of Vg from the gonads of E_2 stimulated male zebra fish. The presence of Vg in the gonads of untreated male *Oreochromis aureus* has been reported by Ding et al. 1989, though in zebra fish untreated males, Vg was totally absent in the gonads. Vg production was also greatly amplified if more than one injection of E_2 was administered, confirming previous observations in other species (Ding et al. 1989; Bergink et al. 1974; Valotaire et al. 1984). Data presented here indicate that the Vg levels determined by protein estimation, represent approximately 68% of the plasma protein after chromatographic procedure consistent with Clemens et al. (1975) claim that 90% of the total increase in serum protein following E_2 stimulation could be attributed to Vg.

Figure 2.1. Chromatographic profile of male serum. Anion exchange chromatography of serum pooled from estradiol stimulated and unstimulated males with a linear NaCl gradient (0.0 M to 0.5 M) on Mono Q column. A single peak eluted at a salt concentration of 0.35 M in the serum of E_2 treated as opposed to the untreated males. The absorbance was monitored at 280 nm.

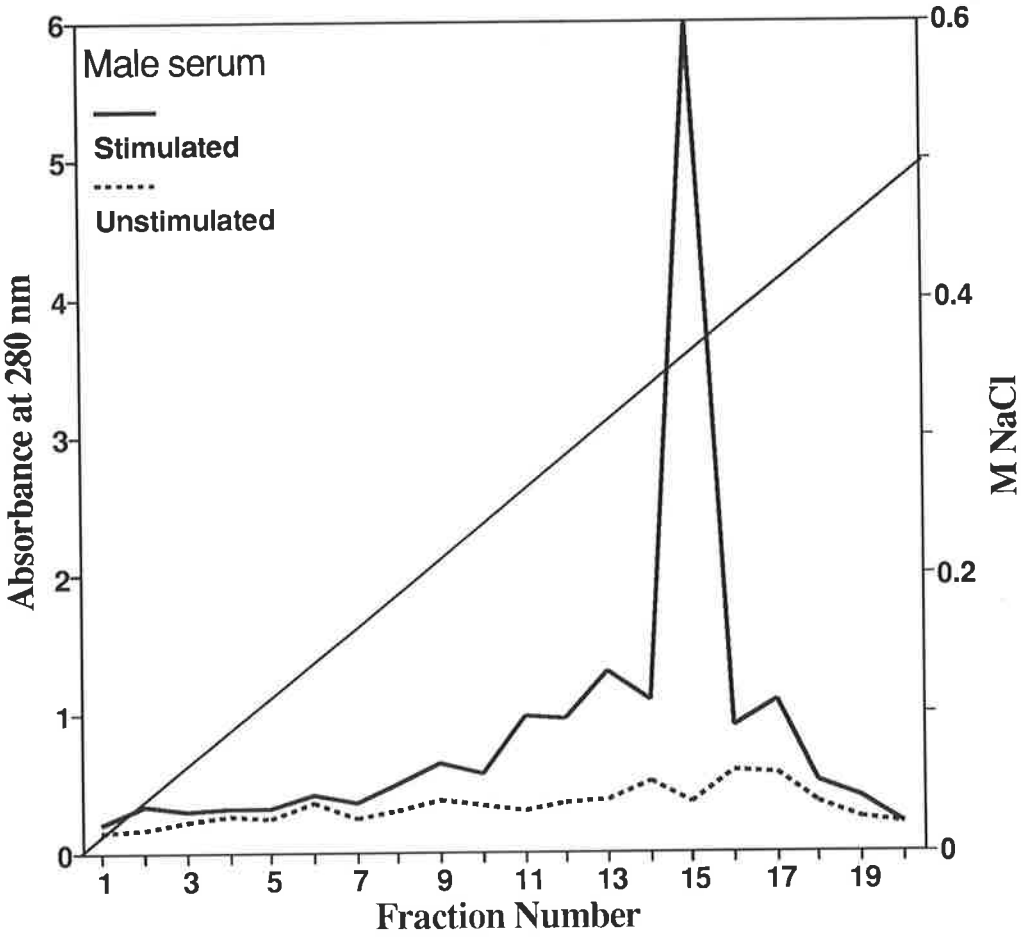

Male serum
Stimulated
Unstimulated
Absorbance at 280 nm
M NaCl
Fraction Number
0
1
2
3
4
5
6
0
0.2
0.4
0.6
1
3
5
7
9
11
13
15
17
19

Figure 2.2. Chromatographic profile of male liver extracts. Anion exchange chromatography of the liver extracts from E_2 stimulated and unstimulated males with a linear NaCl (0.0 M to 0.5 M) gradient on Mono Q column. A single peak was eluted in the E_2 treated males at a salt concentration of 0.35 M this peak which was absent in extracts from the untreated males. The absorbance of the fractions was monitored at 280 nm.

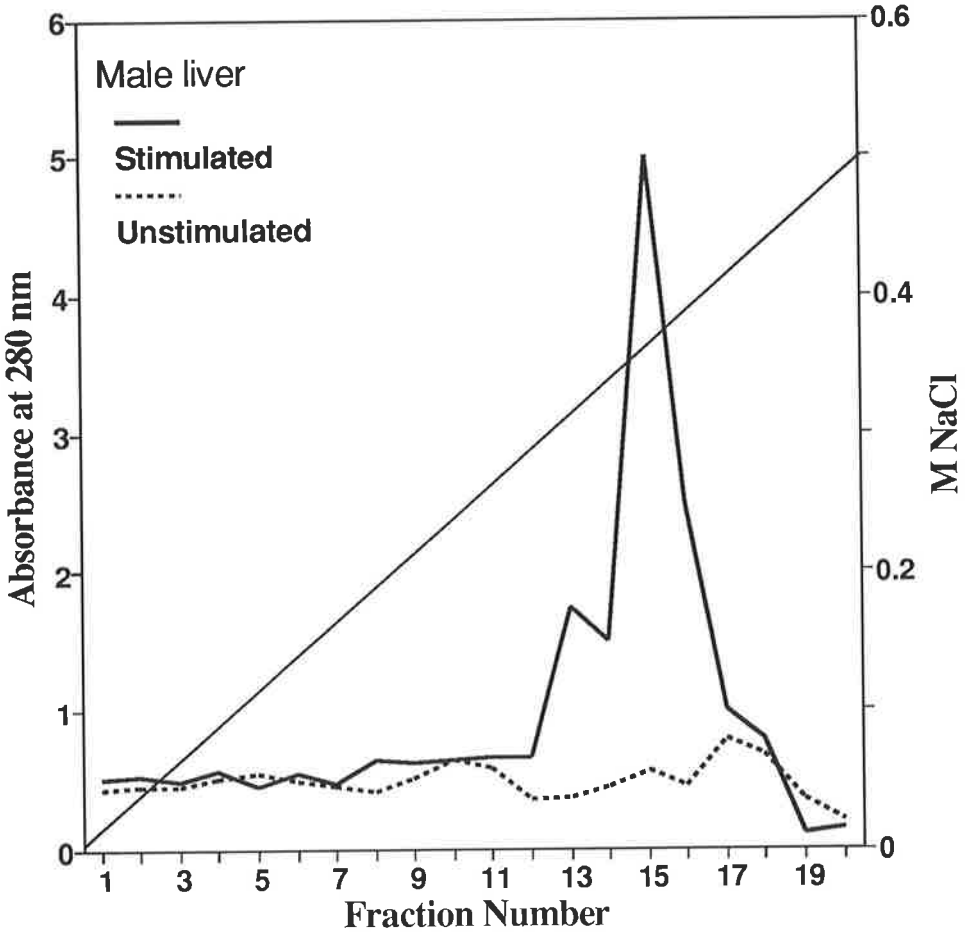

Male liver
Stimulated
Unstimulated
Absorbance at 280 nm
M NaCl
Fraction Number
0
1
2
3
4
5
6
0
0.2
0.4
0.6
1
3
5
7
9
11
13
15
17
19

Figure 2.3. Chromatography of gonadal extracts from males. Anion exchange chromatography of gonadal extracts from E_2 stimulated and unstimulated males with a linear NaCl (0.0 M to 0.5 M) gradient on Mono Q column. A single peak was eluted in the extract of E_2 treated males at a salt concentration of 0.35 M. The absorbance of the fractions was monitored at 280 nm.

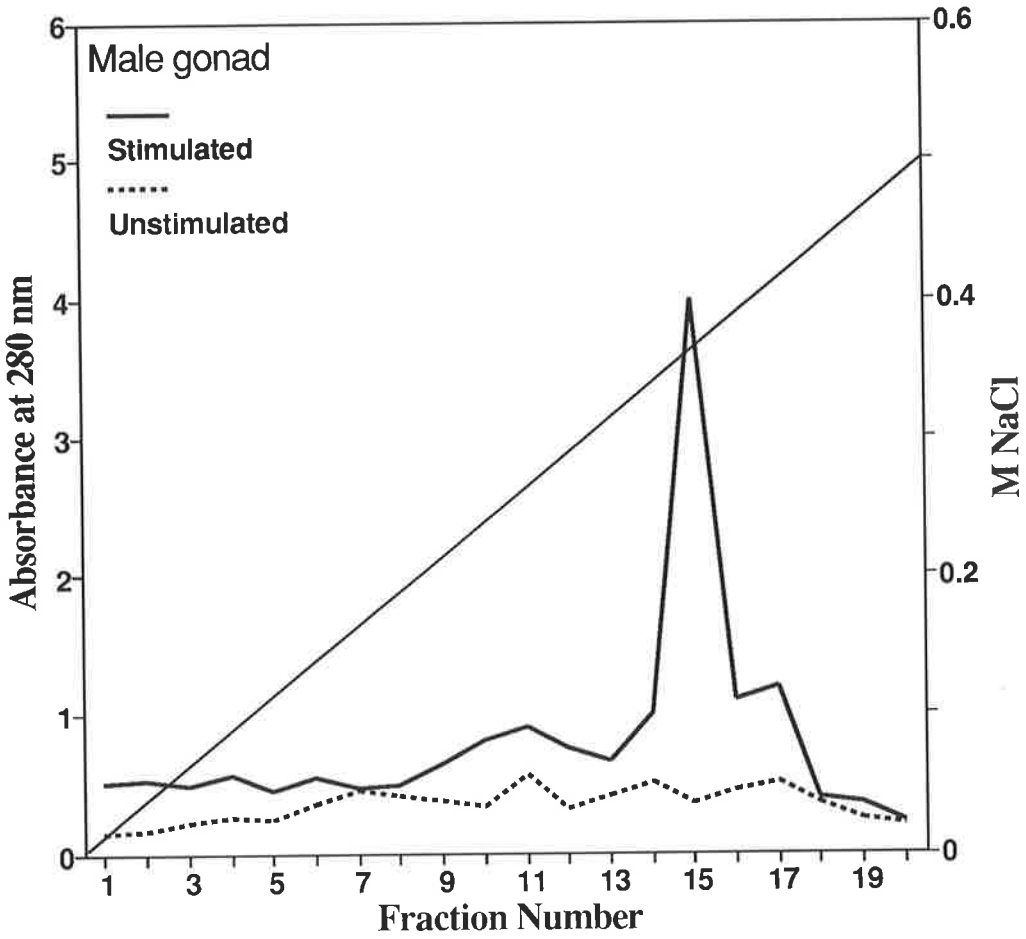

Male gonad
Stimulated
Unstimulated
Absorbance at 280 nm
M NaCl
Fraction Number
0
1
2
3
4
5
6
0
0.2
0.4
0.6
1
3
5
7
9
11
13
15
17
19

Figure 2.4. Chromatographic profile of female serum. Anion exchange chromatography of serum pooled from E_2 stimulated and unstimulated females with a linear NaCl gradient on Mono Q column. A single peak eluted in the plasma of E_2 treated females at a salt concentration of 0.35 M. The absorbance of the fractions was monitored at 280 nm.

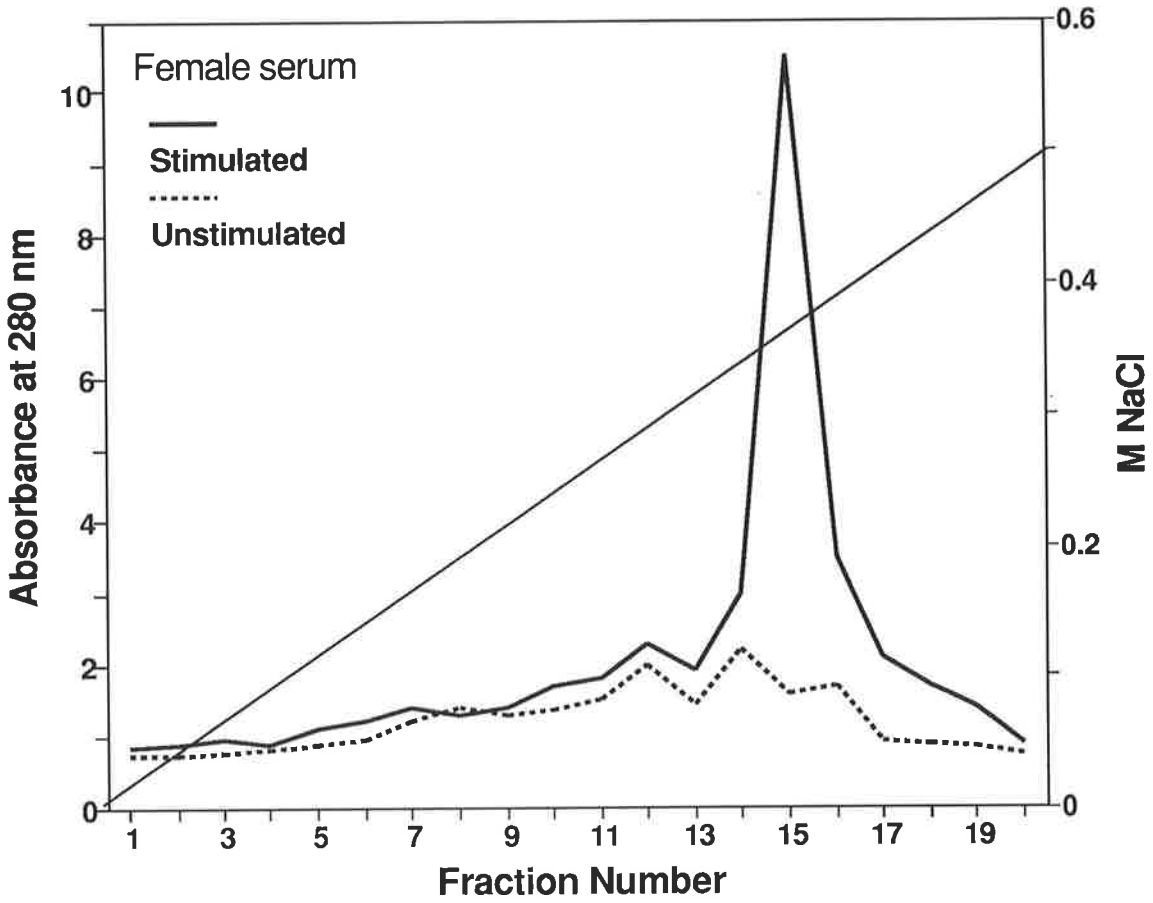

Female serum
Stimulated
Unstimulated
Absorbance at 280 nm
M NaCl
Fraction Number
0
2
4
6
8
10
0
0.2
0.4
0.6
1
3
5
7
9
11
13
15
17
19

Figure 2.5. Chromatography of female liver and gonadal extracts. Anion exchange chromatography of extracts from unstimulated liver (A) and gonad (B) and E_2 stimulated liver (C) and gonad (D) , with a linear NaCl (0.0 M to 0.5 M) gradient on a Mono Q column. Fish were injected ip with 10 μCi of ^{3}H-leucine 2 h after the final E_2 injection (see text for details) and the tissues were collected after 6 h. The incorporation of the isotope in the fractions was measured in dpm (broken line) and the absorbance was monitored at 280 nm (solid line).

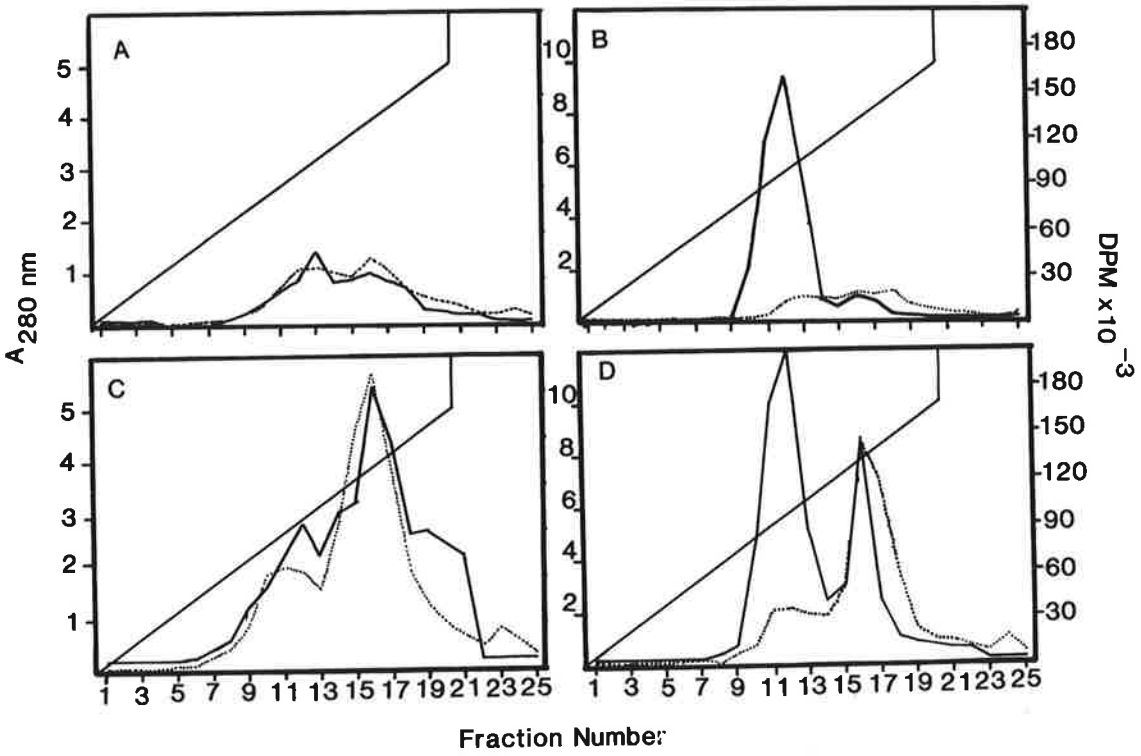

A
B
C
D
$A_{280\ nm}$
DPM x10^{-3}
Fraction Number

Figure 2.6. SDS-PAGE analysis of E_2 stimulated and unstimulated zebra fish tissues. 10% PAGE electrophoresis of tissue homogenates obtained from E_2 stimulated and unstimulated zebra fish tissue homogenates. Lane (1) molecular weight markers (200 kD, 97 kD, 69 kD, 46 kD, and 30 kD). (2) unstimulated and (3) stimulated male liver, (4) unstimulated and (5) stimulated male gonad, (6) unstimulated and (7) stimulated male serum, (8) unstimulated and (9) stimulated female gonads, (10) unstimulated and (11) stimulated female liver, (12) unstimulated and (13) stimulated female serum. Band I and II are the two protein bands of 160 kD and 190 kD induced by estradiol. The gel was stained with Coomassie blue.

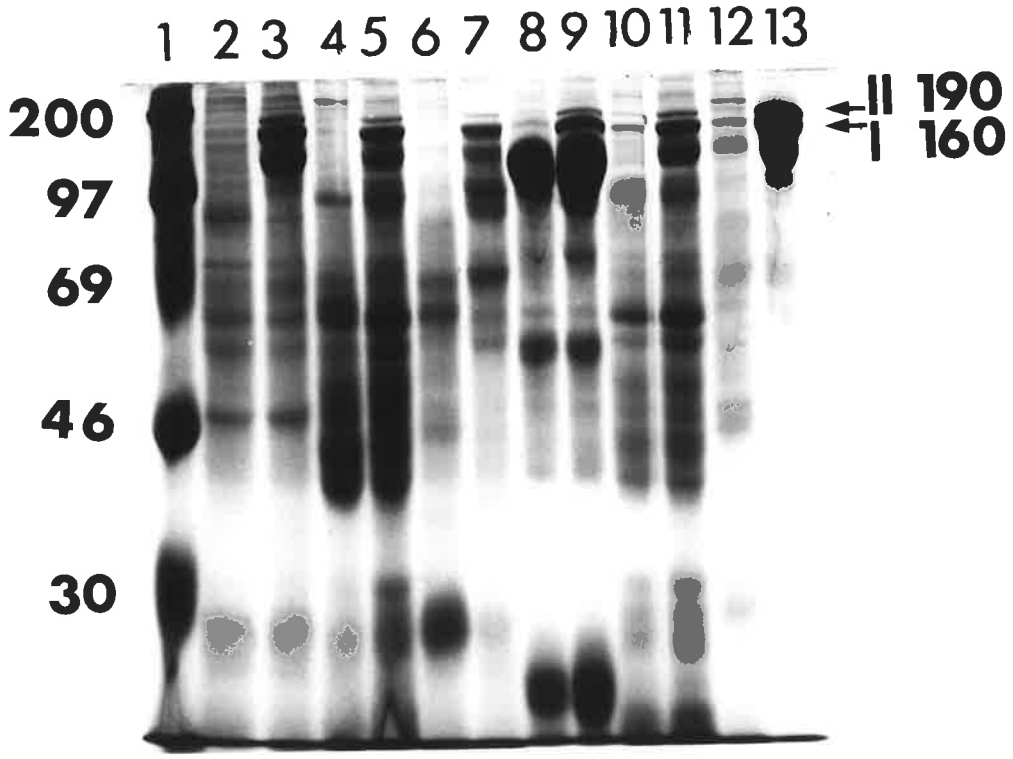
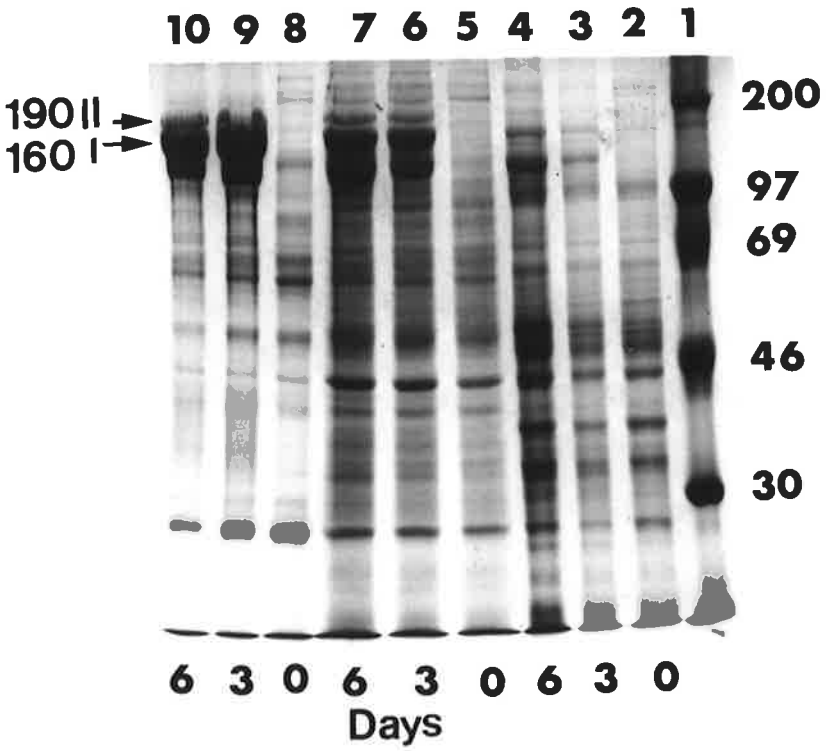

1 2 3 4 5 6 7 8 9 10 11 12 13
200
97
69
46
30
II 190
I 160

Figure 2.7. Effect of sequential E_2 induction on synthesis and secretion of 160 kD (band I) and 190 kD (band II) polypeptides in the zebra fish. 10% SDS-PAGE analysis of serum, liver and gonad homogenates collected 24 h after E_2 injection on days 0, 3, and 6. Lane (1) molecular weight standards (200 kD, 97 kD, 69 kD, 46 kD, and 30 kD). **Top**:- Male zebra fish gonad (2.,3., and 4.), liver (5.,6., and 7.) and serum (8.,9., and 10). **Bottom**:-Female zebra fish liver (2.,3., and 4.), gonad (5.,6., and 7.), and serum (8.,9., and 10.). Gels were stained with Coomassie blue.


10 9 8 7 6 5 4 3 2 1
190 II
160 I
200
97
69
46
30
6 3 0 6 3 0 6 3 0
Days

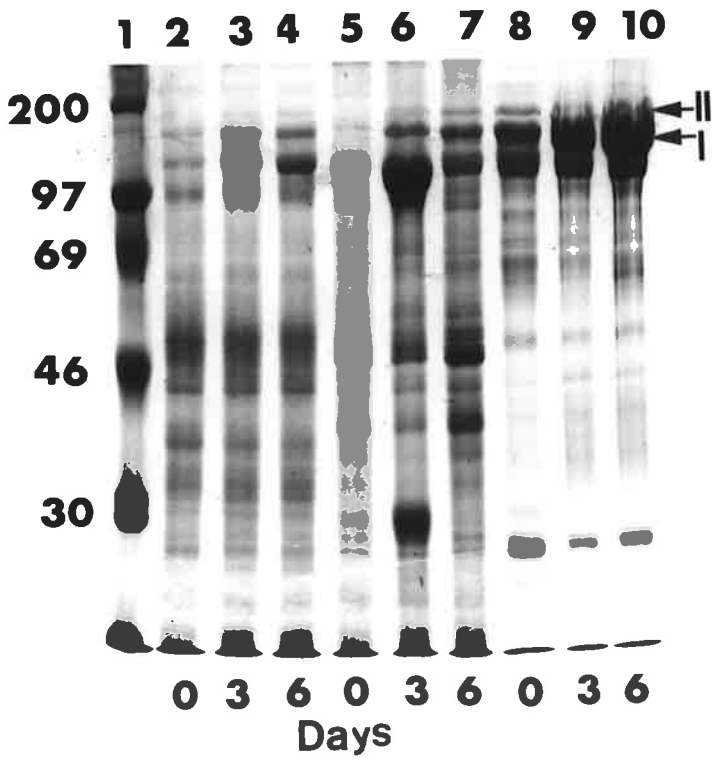

1 2 3 4 5 6 7 8 9 10
200
97
69
46
30
II
I
0 3 6 0 3 6 0 3 6
Days

Figure 2.8. Native gel electrophoresis of purified Vg. Purified protein obtained following Mono Q chromatography was analysed on 5% nondenaturing, nondissociating PAGE and stained with Coomassie blue. Lane (1) molecular weight standards (669 kD, 440 kD, 232 kD, 140 kD and 69 kD) and (2) Purified Vg indicating a molecular weight of approximately 325 kD.

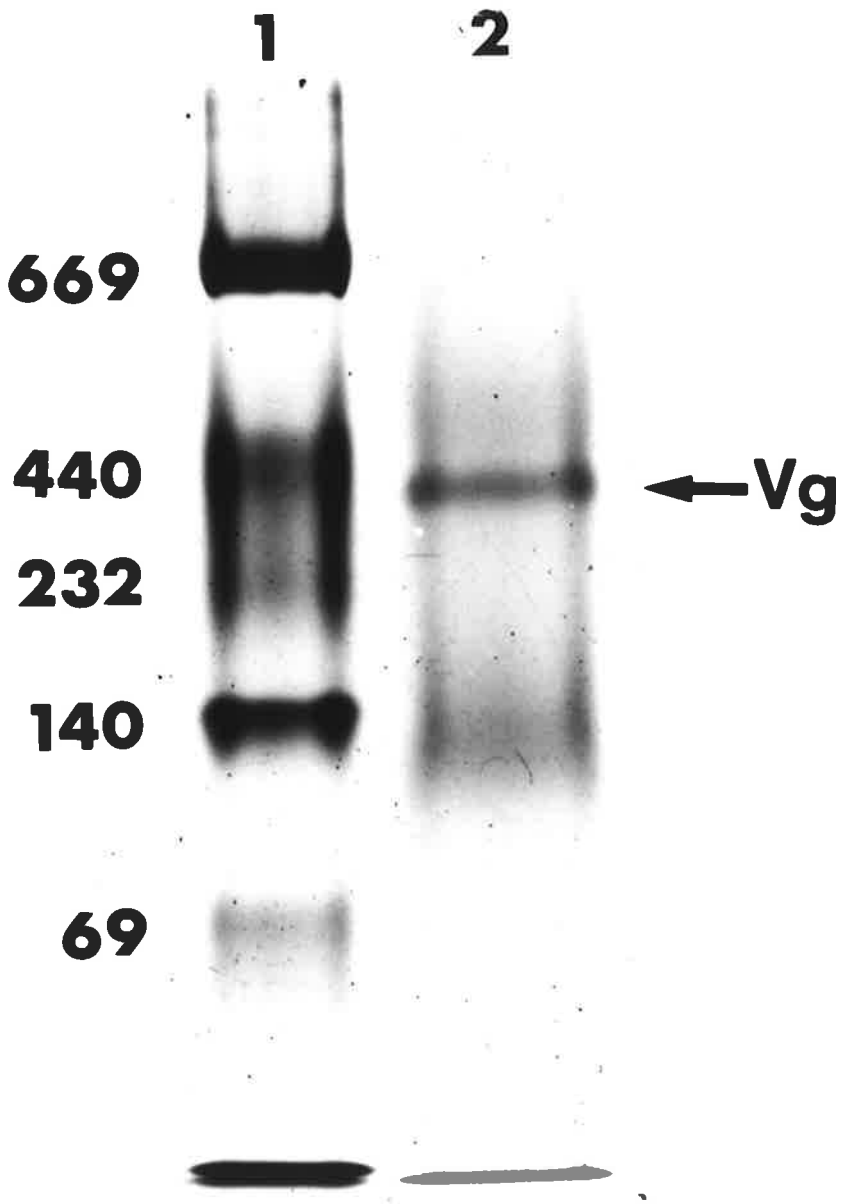

1
2
669
440
232
140
69
Vg

Figure 2.9. SDS-PAGE analysis of purified Vg. Purified Vg obtained following Mono Q chromatography from the, liver (2), gonad (3) and serum (4) homogenates under denaturing conditions of 10% SDS-PAGE resolved to give the two bands of 160 kD and 190 kD probably Vg subunits similar to those identified in the crude extracts of the E_2 treated fish, along with few unidentified epitopes.

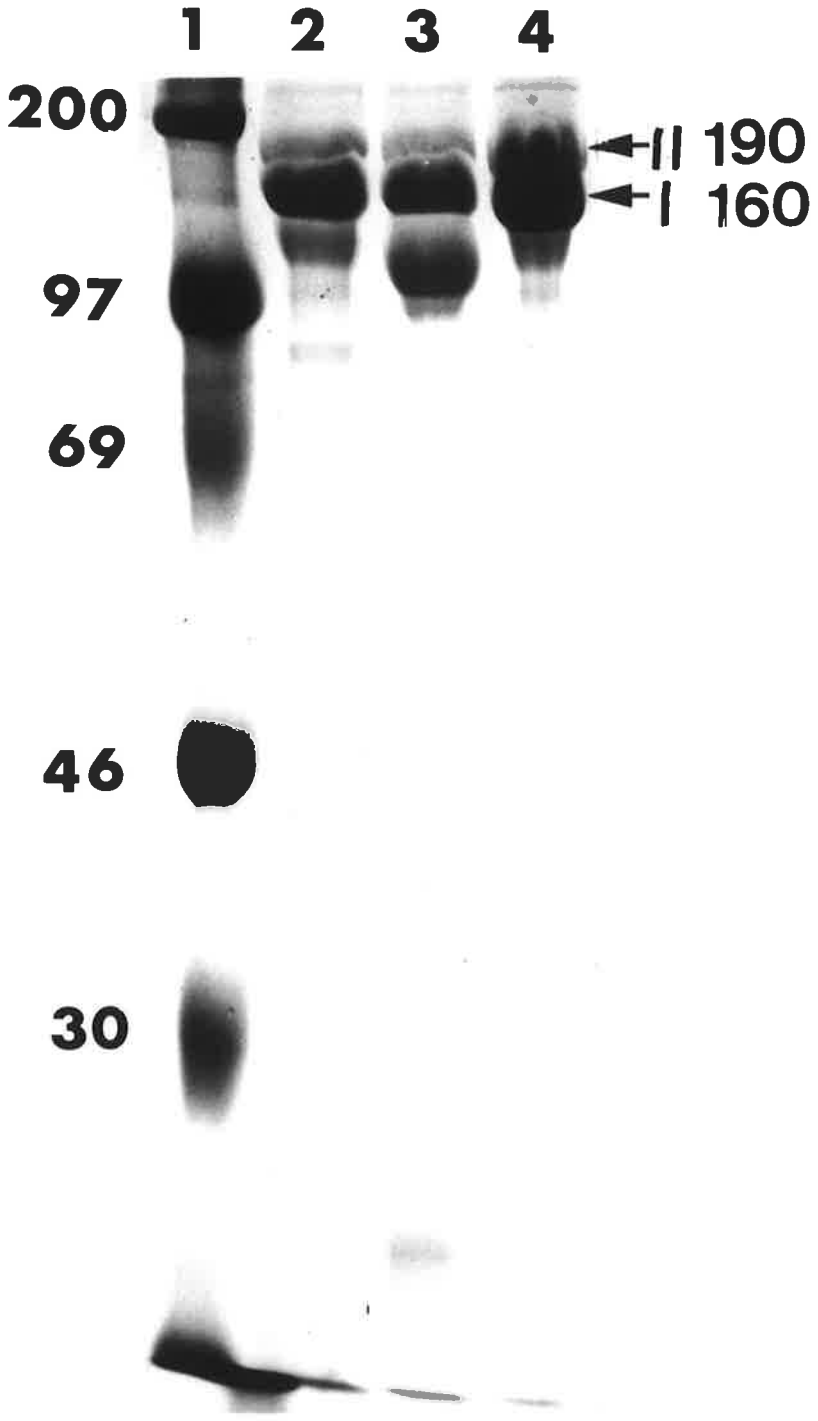

1
2
3
4
200
97
69
46
30
II 190
I 160

TABLE 2.1

AMINO ACID COMPOSITION OF VITELLOGENIN FROM THE ZEBRA FISH, GOLDFISH AND RAINBOW TROUT.

	Mol% of total amino acid		
Amino acids	**Zebra fish**	**Goldfish[a]**	**Rainbow trout[b]**
Asp	7.7	6.5	8.5
Thr	5.6	5.5	5.0
Ser	9.0	6.9	7.5
Glu	10.6	11.9	11.5
Pro	6.1	5.5	5.2
Gly	3.8	4.6	4.2
Ala	12.2	12.8	11.7
Val	7.6	6.9	7.1
Met	2.4	2.0	2.6
Ile	5.9	6.6	5.5
Leu	9.0	10.8	9.5
Tyr	3.2	2.6	3.0
Phe	3.5	2.9	4.0
His	2.0	2.3	2.1
Lys	6.3	7.0	7.1
Arg	5.3	4.9	4.5
Cys	ND[c]		
Tryp	ND		

a. Hara and Hirai 1978.

b. de Vlaming et al 1989.

c. not determined

CHAPTER 3

3. PRIMARY MONOLAYER CULTURES OF HEPATOCYTES AND SYNTHESIS OF VITELLOGENIN

3.1. INTRODUCTION

Hormonal induction of Vg is well established as a model for studying diverse aspects of the regulation of gene expression (Tata and Smith 1979). Most studies involve whole animals but they present some limitations due to the complex dynamics of tissue distribution and metabolism of the hormone, leading to uncertainty about the rate and level of hormone delivery to the target cells. These variables can be controlled much more easily in tissue culture.

In this chapter, experiments were undertaken to investigate whether Vg synthesis could be demonstrated in primary monolayer cultures of zebra fish hepatocytes and whether Vg synthesis would remain responsive to estrogen thus providing a model for further study of hormonal stimulation of Vg. Previous studies in this area were based on studies on whole liver or liver slices (Plack and Fraser 1971; Wallace and Bergink 1974).

Until now very few reports have dealt with the culture of hepatocytes from fish due to the difficulty of providing the appropriate culture conditions to maintain both the viability and function of hepatocytes for prolonged periods.

Although various studies have been conducted with isolated fish hepatocytes (Bouche et al 1979; French et al. 1981; Walton and Cowey 1979), their ability to continue to regulate the expression of specific genes when maintained in primary monolayer culture remains to be demonstrated.

3.2. MATERIALS AND METHOD

3.2.1. PREPARATION OF FISH FOR OBTAINING TISSUE

Zebra fish were maintained as described previously (2.2.1), and the fish chosen to supply for tissue culture were isolated in 5 L containers. Feeding was stopped for at least two days, and regular water changes were done to remove the faecal material. Just before obtaining the tissue, fish were acclimatised and washed with at least two changes of MQ H_2O. Prior to tissue collection, the working area was cleaned with disinfectant and all the necessary sterile conditions were maintained. Fish were anaesthetised and by using cotton swabs the entire fish was sterilised in 70% alcohol, and passed through two changes of fish Ringers solution.

3.2.2. ISOLATION OF HEPATOCYTES AND PREPARATION OF PRIMARY CULTURE

3.2.2.1. Collection Of Tissue

An incision was made on the lateral side from the gill operculum to the gonopore, thin liver lobes were carefully detached off the alimentary canal under stereo microscope (Olympus), without damaging the gut or the gall bladder, with the help of fine sterile forceps. Liver tissue was immediately transferred to either fish Ringer solution or phosphate buffered saline (PBS) containing 1000 IU (International

Units) of each, Penicillin G, potassium (Sigma) and Streptomycin sulfate (Sigma) maintained at pH 7.4 on ice. Tissues were washed twice in cold saline (PBS) before being processed further. All solutions and instruments used were sterilised by autoclaving or filtering through 0.22 μm filters (Millex-Gs;Millipore, Bedford, MA).

3.2.2.2. Preparation Of Cell Suspension And Cell Harvesting

Autoclaved pasture pipettes were used to transfer the tissue to fresh PBS in a petridish and vigorous pipetting was used to break the tissue into fragments which were collected and rinsed in cold PBS. The tissue was then finely minced using scalpel blades until the largest pieces were at most 2 mm in size. Tissue pieces were usually washed several times to remove debris and blood cells, before disaggregation by trypsin (Sigma) at a final concentration of 0.25% prepared in Hanks balanced salt solution, (HBSS, Sigma). Digestion was carried out at room temperature for at least 15 mins with intermittent shaking. Heavy particles were allowed to settle, and the digestion solution containing the cell suspension was collected into 5 ml tubes. To inhibit further action of trypsin, equal amounts of freshly prepared culture medium Minimum Essential Medium Eagle (MEM, Sigma) supplemented with 10% Fetal calf serum (FCS) (Commonwealth Serum Laboratories, Australia) 2 mM glutamine (Sigma) and antibiotics (as mentioned above) (MEM-FCS medium) were added. Harvested cell suspensions were then centrifuged, and the cell pellets obtained were resuspended in MEM-FCS by pipetting. The process of trypsinisation and harvesting was continued by adding fresh trypsin to the tissue pieces until most were digested and disaggregated into cell suspension.

3.2.2.3. Lysis Of Erythrocytes

Harvested cells were pooled in 50 ml rocket tubes, and the cell pellet was obtained by centrifugation. The supernatant was discarded and the cells resuspended

in 5 ml of 0.2% NaCl by pipetting and the suspension allowed to stand for 20-30 seconds. Then, 5 ml of 1.6% NaCl was added rapidly and mixed and, after 5 seconds the salt solution was further diluted with 25 ml of MEM-FCS medium, gently vortexed and centrifuged. The supernatant was discarded and the cells were resuspended in the culture medium and centrifuged at low speed to remove cell debris, this was repeated at least three times. Cells were checked for erythrocytes and counted.

3.2.2.4. Cell Count And Viability

Following erythrolysis, an aliquot of the cell suspension was added to Eppendorf tubes containing 0.4% Tryphan Blue stain (Sigma) to obtain the appropriate dilution factor, and mixed thoroughly and allowed to stand for 5-15 mins. Tryphan blue cell suspension was transferred to both the chambers of the Neubauer haemocytometer (Assistent, Germany) to determine the total cell number and to assess the viability of the cells harvested by tryphan blue dye exclusion method.

3.2.2.5. Coating Of Culture Plates

All sterile plastic culture plates were coated with 1% gelatin (Sigma). the Gelatin solution was prepared in MQ H_2O and filtered through 0.22 μm filters. The culture surface was coated with minimum volume of gelatin, by transferring the solution and ensuring that it spread uniformly over the entire surface. The coated culture plates were allowed to dry overnight, and were rinsed with culture medium before inoculation.

3.2.2.6. Seeding Density For Primary Monolayer Cultures

Following harvesting and cell count, the cell suspension was adequately diluted in the MEM-FCS medium. Coated culture plates were inoculated with 1 to 2 x 10^6

cells/ml. Cultures were routinely examined for confluency and health with the aid of a phase contrast microscope (Olympus)

3.2.3. CULTURE CONDITION

Minimum Essential Medium Eagle (MEM), with Earl's salts and non-essential amino acid but without L-glutamine, phenol red and sodium carbonate, was obtained in powdered form from Sigma. The Medium was prepared in MQ H_2O and osmolarity was adjusted to between 280 and 290 mOsm/kg with water prior to addition of serum, antibiotics and phenol red (0.011 g/L). All the glassware and solutions were sterilised by autoclaving or filtration through 0.22 µm Millipore filter. Medium with and without phenol red was stored as stock solution at 4°C. An aliquot of the stock was used for preparation of the culture medium containing 2 mM L-glutamine, with or without 10% FCS, and 1000 IU of penicillin and streptomycin were added mixed and filtered through 0.22µm filters before use.

Culture plates were incubated in Autoclavable Anaerobic jars which were sealed and fitted with 0.22 µm filters for aeration. Humidity was maintained by placing a sterile plastic petridish with MQ H_2O. at the base of the jars during the culture period. Jars were placed into a water bath used as an incubator maintained at 25°C.

3.2.4. *IN VITRO* VITELLOGENIN INDUCTION

3.2.4.1 Estradiol Stimulation

Hepatocyte monolayer cell cultures were prepared from liver obtained from both male and female fish on 35 mm petridishes as described above. Cells were cultured in MEM-FCS medium for 48 hours (h) and then in serum free MEM in presence of E_2 and 10 mU/ml of insulin (Commonwealth Serum Laboratories, Melbourne, Australia).

E_2 was prepared in propylene glycol and added to the cultures at final concentration of 10^{-4} M, 10^{-6} M, and 10^{-8} M . Control cultures received propylene glycol only.

Every 24 h after E_2 stimulation, medium was collected and fresh medium containing the desired concentration of E_2 was added to the cultures and incubated. Spent medium was collected and centrifuged to remove any debris, the supernatant was dialysed for an hour in MQ H_2O at 4°C, and 0.1 mM PMSF was added as a proteolytic enzyme. An aliquot was used for protein estimation (Biorad) with BSA as reference standard and the remaining supernatant was either used immediately or stored at -70°C.

3.2.4.2 ^{3}H-Leucine Incorporation

Cells were allowed to attach for 48 h in MEM-FCS medium. At the start of labelling the old medium was removed, the cultures were rinsed with two changes of PBS and fresh medium and then incubated in freshly prepared serum free medium with 10^{-4} M E_2 supplemented with 10 mU/ml insulin and 5.0 µCi/ml of ^{3}H-leucine. The labelling medium was collected after appropriate time intervals, centrifuged and supernatant was collected and saved for measurment of secreted protein after extraction using TCA precipitation (Hames and Rickwood 1981), an aliquot was used for protein estimation. The cultures were rinsed at least three times with isotonic saline containing 1 mM leucine and the cells were dissolved in 0.2 N Sodium hydroxide (NaOH) (Stanchfield and Yager 1978). One hundred microlitres of the solution were spotted onto Whatman 3 mm glass fibre paper and processed according to the procedure of Mans and Novelli 1961. Scintillation fluid (Ready safe, Beckman Instruments Inc., Fullerton, CA), was added to vials containing individual paper discs and radioactivity was measured as disintegrations per minute (dpm) in a liquid scintillation counter (Beckman LS 6000 LL).

3.2.5. SDS-PAGE ELECTROPHORESIS

For PAGE technique refer to section 2.2.5, a different staining technique was utilised as mentioned below.

3.2.5.1. Silver Staining SDS-PAGE

After electrophoresis, gels were carefully placed in glass trays and prefixed overnight in mixer of methanol (50% v/v) and acetic acid (7% v/v) in distilled water and soaked for 1 h, and then rinsed with several changes of distilled water. The gels were then placed in the solution containing 5 ug/ml of dithiotheritol (DTT, Sigma) and incubated for 1 h. The DTT solution was decanted and without further rinsing, 0.1% silver nitrate ($AgNO_3$, Sigma) (w/v) solution was added and incubation continued for an hour. Gels were then rinsed once rapidly with small amount of distilled water and then twice rapidly with a small amount of developer (500 µl of 37% formaldehyde in 1 L distilled water and 3% sodium carbonate w/v). They were then soaked in developer until the desired level of staining was obtained, the developer was then poured off and acetic acid (5% v/v) was added. All the incubation procedures were carried out with continuous shaking. Gels were photographed after staining.

3.3. RESULTS

3.3.1. ISOLATION AND VIABILITY

Approximately 1×10^8 cells were obtained from each liver weighing approximately 1 gm to 1.5 gm. They were harvested as clumps of round cells with

refringent plasma membranes as seen by phase contrast microscopy, and were easily dispersed by vigorous pipetting. Cell viability of the suspension was about 92% (Table 3.1) as determined by the tryphan blue exclusion test. Repeated low speed centrifugation of the cell suspension facilitated the removal of the damaged cells and most of the pelleted cells were viable hepatocytes.

3.3.2. MORPHOLOGY OF ISOLATED AND CULTURED HEPATOCYTES

The isolated cells when observed immediately after isolation by phase contrast inverted microscope, appeared spherical with refringent plasma membrane (Fig 3.1A). Detailed examination revealed that cell preparations largely comprised a homogeneous population of parenchymal cells interspersed with a few non-parenchymal cells identified by their dark pigmentation (Stanchfield and Yager 1978). Although the final preparation of zebra fish liver cells appeared remarkably homogeneous, the functional identity of all isolates is unknown and the term hepatocytes was retained as general description of the cell preparation.

Over 90% of hepatocytes isolated from the zebra fish attached to the substrate in serum free medium (Table 3.1). They aggregated and adhered tightly to the culture dishes in about six to eight hours after plating and progressively assumed a flattened appearance with a trabecular colony formation occurring within 24 h. After 48-72 h in culture, cells displayed a polygonal characteristic (Fig 3.1B) and had formed a nearly confluent monolayer.

3.3.3. CELL ATTACHMENT WITH TIME IN CULTURE

Table 3.2 shows the change in cell number over 7 days in culture. There was essentially no loss over at least 3 day period in the serum free medium. This was followed by approximately 50% decline in the number of attached cells by day seven, which was offset by including 10 mU/ml of insulin in the medium.

3.3.4. EFFECT OF SUBSTRATE ON HEPATOCYTE CULTURE

Initially hepatocytes were isolated and cultured on to plastic substrate in MEM-FCS medium. These cells remained in suspension for at least two days before they attached to the substrate, but majority of the hepatocytes remained in suspension. Medium renewal resulted in the loss of the loosely attached cells, with those surviving for a few days in suspension soon forming large aggregates of dying cells. Coating the culture dish with 1% gelatin was found to increase the attachment efficiency and survival, and promoted maintenance of primary monolayer cultures.

3.3.5. KINETICS OF ^{3}H-LEUCINE INCORPORATION

Isotope tracer studies using ^{3}H-leucine incorporation were carried out to provide a measure of the cells ability to synthesise protein. Fig 3.2 illustrates the incorporation of leucine into intracellular and secreted protein. In the continuous presence of the isotope and E_2 the rate of incorporation of tracer into the tissue was found to be linear throughout the culture period, whereas the appearance of radioactivity in the medium remained parallel to the intracellular incorporation for the first two hours, then increased rapidly with time . This 3 h lag period, prior to secretion presumably is the time required for the processing of secreted proteins by the zebra fish hepatocytes.

3.3.6. EFFECT OF INSULIN ON PROTEIN SECRETION IN CULTURE

In mammals, insulin has been shown to affect the functional activity of hepatocytes in culture (Hopgood et al. 1980; Tanaka et al. 1978). In zebra fish, during the culture period of hepatocytes in serum free medium, the total protein in the spent medium was approximately 70 µg/ml for the first three days, but the secretion

decreased with time in culture. In contrast, cells cultured in the medium supplemented with 10 mU/ml insulin maintained a higher secretion rate over controls for the first six days of culture. Cultures without added hormone showed a decline in total protein by approximately 35% (Table 3.2)

3.3.7. EFFECT OF ESTROGEN ON HEPATOCYTES IN CULTURE

Hepatocyte cultures were exposed to increasing amount of E_2 (10^{-8} M to 10^{-4} M) from day 1 to day 7 and the secreted protein was analysed by electrophoresis. It was found that E_2 had stimulated the synthesis of the putative Vg (160 kD band)over controls in a dose related manner.

Vg was the first detected in the medium collected on day 1 from cultures stimulated with 10^{-4} M E_2, and its concentration increased significantly till day 6. A similar response was observed with 10^{-6} M E_2, but 10^{-8} M was less effective. The PAGE analysis of secreted protein from day 1 to day 7, in response to different doses of estradiol along with the controls is illustrated in Figs. 3.3; 3.4; and 3.5.

SDS-PAGE analysis of medium collected (day 1 to day 7) from the controls (Fig 3.6) and E_2 stimulated hepatocyte cultures (Fig 3.7; 3.8; 3.9) clearly indicate that, Vg is secreted in response to estradiol stimulation. After hormone addition, detectable levels of Vg appear in the medium, and on succeeding days the concentration of Vg increases until it becomes the major protein synthesised and secreted by the hepatocytes.

3.4. CONCLUSION AND DISCUSSION

The experiments reported within this chapter indicate that primary cultures of zebra fish hepatocytes can be obtained in high yield. The isolated cells attach efficiently to 1% gelatin coated culture dishes and subsequently form monolayers. Functionally

these monolayer cultures can be maintained in serum free medium for over one week with minimum cell loss and throughout this period retained their competence to respond to estrogen by *de novo* synthesis of vitellogenin, thus offering a useful model for the study of the regulation of gene expression *in vitro*.

Procedures for isolation of hepatocytes for monolayer cultures used routinely for rat hepatocytes have been successfully adapted to trout (Maitre et al 1986) and amphibian (Stanchfield and Yager 1978) liver cells. This procedure requires preliminary *in situ* perfusion of the liver with collagenase to cause enzymatic disruption, an approach which, for technical reasons, is unsuited to the smaller zebra fish. The method reported in this chapter based on enzymatic disruption of isolated livers followed by erythrolysis which consistently resulted in high yields of a fairly homogenous population of adult zebra fish hepatocytes. These cultures were maintained for as long as 10 days and were capable of retaining their differentiated state in a completely defined medium.

In contrast to experience with trout (Maitre et al. 1986), zebra fish hepatocyte culture was improved by use of coated culture vessels. The prompt attachment of the zebra fish hepatocytes maintained the cell numbers fairly constant, whereas in trout (Maitre et al. 1986) attachment occurred more slowly and remained relatively weak, the thus increasing the number of viable cells lost during medium renewal.

In addition to use of coated plates, cell viability was also improved through use of growth factors. Insulin is widely employed to improve the maintenance of vital functions of cells seeded in either serum free or serum supplemented tissue culture medium, and is known to affect the functional capacity of the hepatocytes (Hopgood et al. 1980; Tanaka et al. 1978). Both these approaches proved effective in this study with the zebra fish hepatocytes which attached efficiently, and survived and maintained their functional activity in serum free medium when supplemented with insulin and allowed to attach to gelatin coated vessels.

As demonstrated, the monolayer cultures of zebra fish hepatocytes are able to respond to estradiol stimulation by synthesising higher amounts of Vg. This

observation leads us to conclude that the hepatocytes sustain the ability to keep high functional capacity for several days by active synthesis of a specific protein after a prolonged period in culture, under hormonal stimulation. This also suggest that the expression of specific genes can be maintained in cultured zebra fish hepatocytes.

TABLE 3.1. Hepatocytes isolation and culture inoculation data.

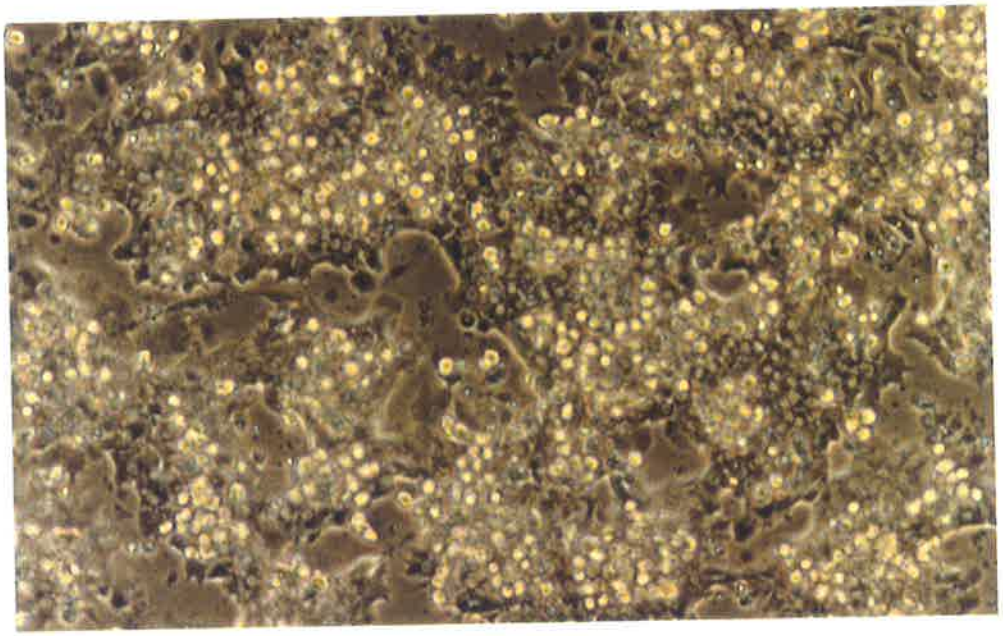
Cell yield	$1x10^8$ cells/gm
Viability[b]	92% ± 1.7 (6)[a]
Attachment efficiency	90% ± 6[c] (6)

[a] Results derived from the number of separate experiments.
[b] Viability judged as the ability to exclude the tryphan blue dye.
[c] Data expressed as the mean ± S.D.

TABLE 3.2. Effect of insulin on cell attachment and protein secretion with time in serum free medium. Hepatocytes were cultured in the presence (+) or absence (-) of insulin 10 mU/ml supplemented medium from the time of inoculation. Every 24 h the medium was collected and an aliquot was used for protein estimation. The cells were harvested by trypsinisation and counted using a haemocytometer.

Day in culture	Number of cells $\times10^6$		µgm protein/ml medium	
	–Insulin	+Insulin	–Insulin	+Insulin
1	2.1	2.02	73	76
2	1.98	1.96	71	71
3	1.90	1.93	68	75
4	1.57	1.89	42	80
5	1.42	1.88	37	72
6	1.30	1.85	29	70
7	1.14	1.83	25	68

Figure 3.1. Phase contrast micrograph of zebra fish hepatocytes. Top: Suspension of freshly isolated cells immediately after inoculating the cultures. Cells appeared spherical with refringent membrane. **Centre:** Colonies of hepatocytes showing flattened and polygonal in shape after 48 h. **Bottom:** Confluent monolayer of zebra fish hepatocytes after 5 days. Cultures were inoculated at a density of 1×10^6 cells/ml medium and cultured at 26°C in the MEM-FCS medium on a gelatin coated culture dishes.



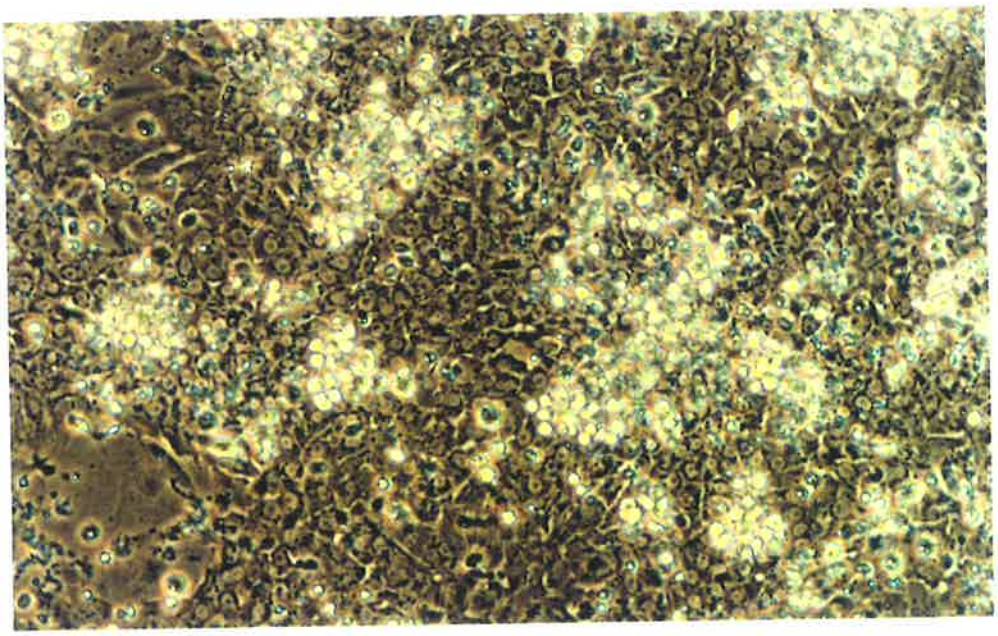


Figure 3.2. Incorporation of ^{3}H-leucine as a measure of cells ability to synthesise protein. Cultures were established at 1.5 x 10^{6} cells/ml, at time zero the cultures were rinsed with fresh medium then incubated with complete serum free medium containing 5.0 µCi/ml ^{3}H-leucine and 10^{-4} M E_2. The incorporation of the isotope tracer into intracellular and extracellular (secreted) material was determined as outlined in the method.

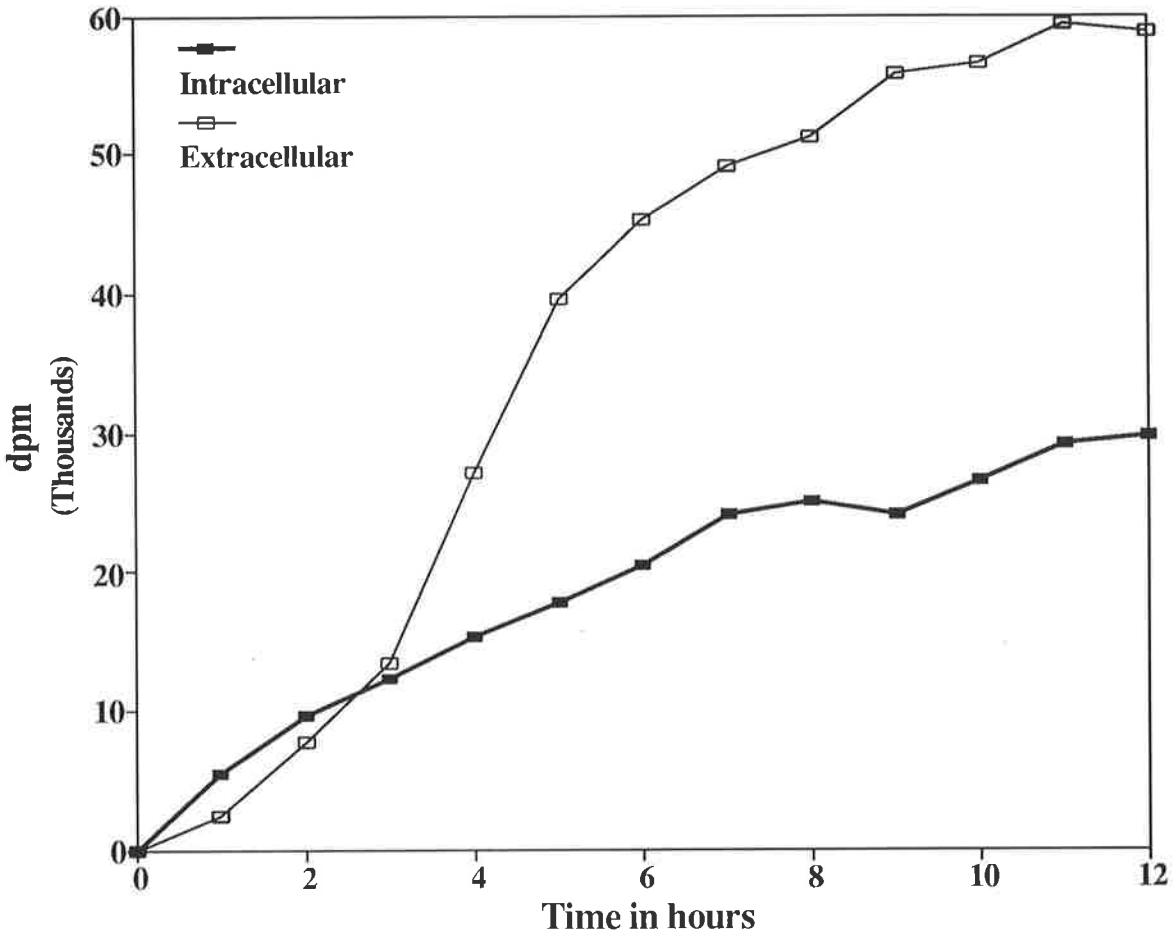

Intracellular
Extracellular
dpm
(Thousands)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Time in hours

Fig 3.3. SDS-PAGE analysis of the protein secreted by hepatocyte cultures in response to different dose of E_2 (day 1 and day 2). Hepatocyte monolayer cultures were established in gelatin coated dishes at a density of $2x10^6$ cells/ml. Cultures were rinsed and serum free medium containing E_2 at 10^{-8}, 10^{-6}, and 10^{-4} M concentration was added on days 0 and 1, in the control cultures E_2 was replaced with propylene glycol. After 24 hrs the medium was changed and the spent medium was analysed on a 10% SDS-PAGE as mentioned in the methods. M represents the standard molecular weights and the figures on the right indicate the molecular weights in kD. C represents the control lane and 160 kD band is indicated by an arrowhead.

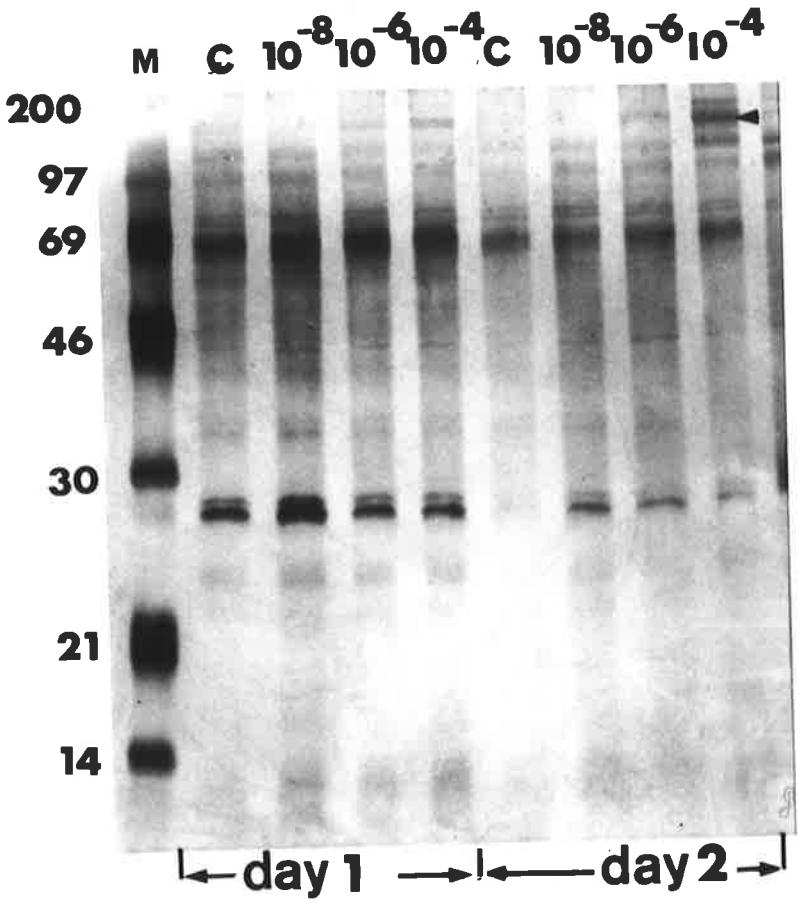

M
C
10-8
10-6
10-4
C
10-8
10-6
10-4
200
97
69
46
30
21
14
day 1
day 2

Fig 3.4. SDS-PAGE analysis of the protein produced by hepatocyte cultures in response to different dose of E_2 (day 3 and day 4). Culture conditions and E_2 stimulation procedures were the same as cited in fig 3.3 except the medium was changed on day 3 and day 4 (day 2 and day 3 of hormone treatment respectively). M represents the molecular wights from 200 kD to 14 kD and the E_2 stimulated protein (160 kD) is shown by an arrowhead.

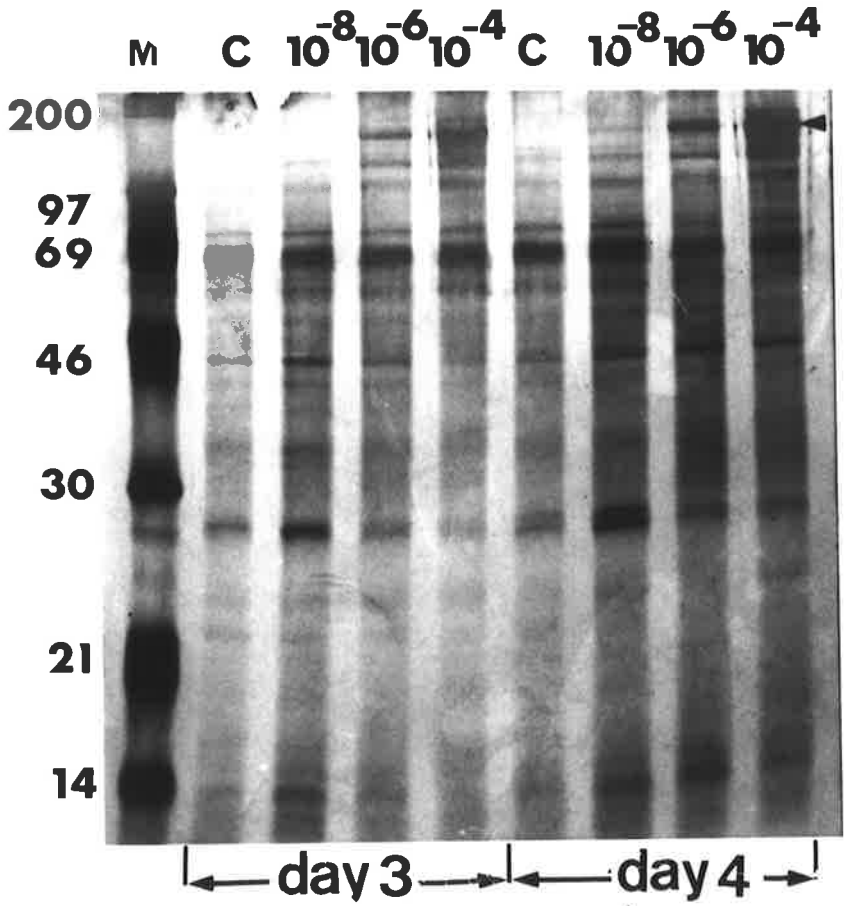

M
C
10^{-8} 10^{-6} 10^{-4}
C
10^{-8} 10^{-6} 10^{-4}
200
97
69
46
30
21
14
day 3
day 4

Fig 3.5. SDS-PAGE analysis of the protein produced by hepatocyte cultures in response to different dose of E_2 (day 5, day 6 and day 7). Culture conditions and E_2 stimulation procedures were same as in fig 3.3 except medium analysed was changed on day 5, day 6 and day 7 (day 4, day 5 and day 6 of hormone treatment respectively). M represents the molecular wights from 200 kD to 14 kD and the E_2 stimulated protein (160 kD) is shown using an arrowhead.

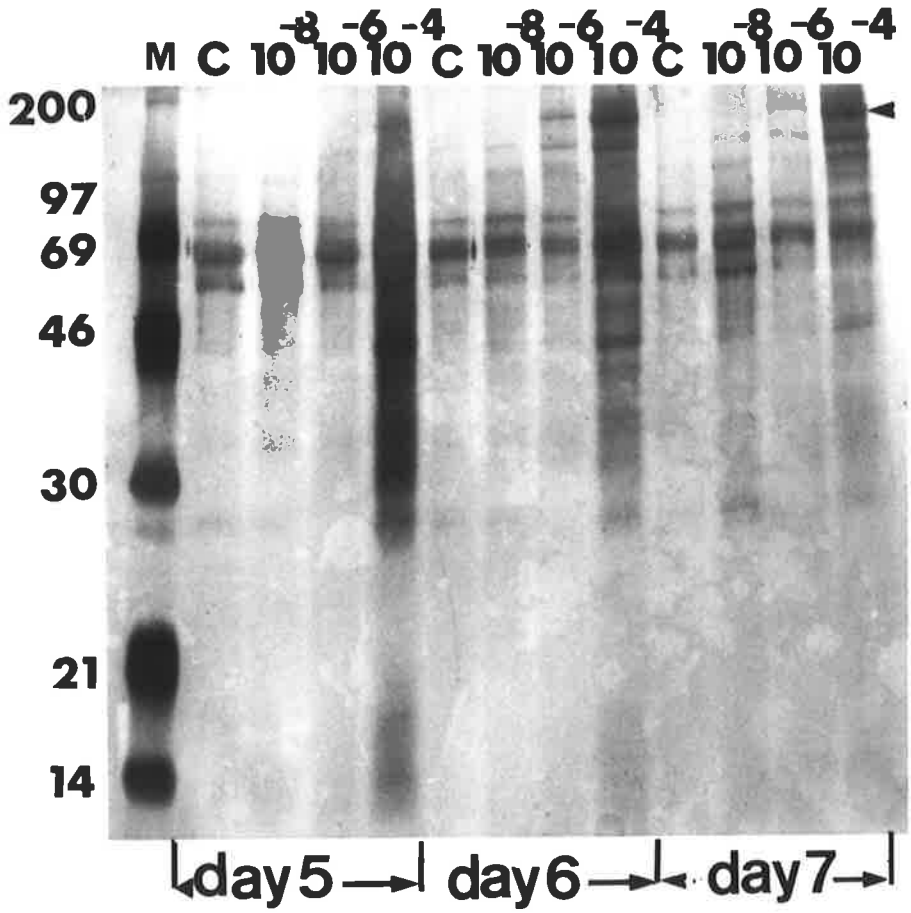

M C 10-8 10-6 10-4 C 10-8 10-6 10-4 C 10-8 10-6 10-4
200
97
69
46
30
21
14
day 5
day 6
day 7

Figure 3.6. Electrophoretic analysis of protein secreted by unstimulated hepatocyte cultures. Hepatocyte monolayer cultures were established in gelatin coated dishes at a density of 2×10^6 cells/ml. Cultures received propylene glycol only in the serum free medium as opposed to the stimulated cultures. Medium was changed every 24 hrs, the spent medium collected over 7 day period was analysed on a 10% SDS-PAGE. M represents the standard molecular weights and the figures on the left indicate the molecular weights (200 kD to 14 kD)

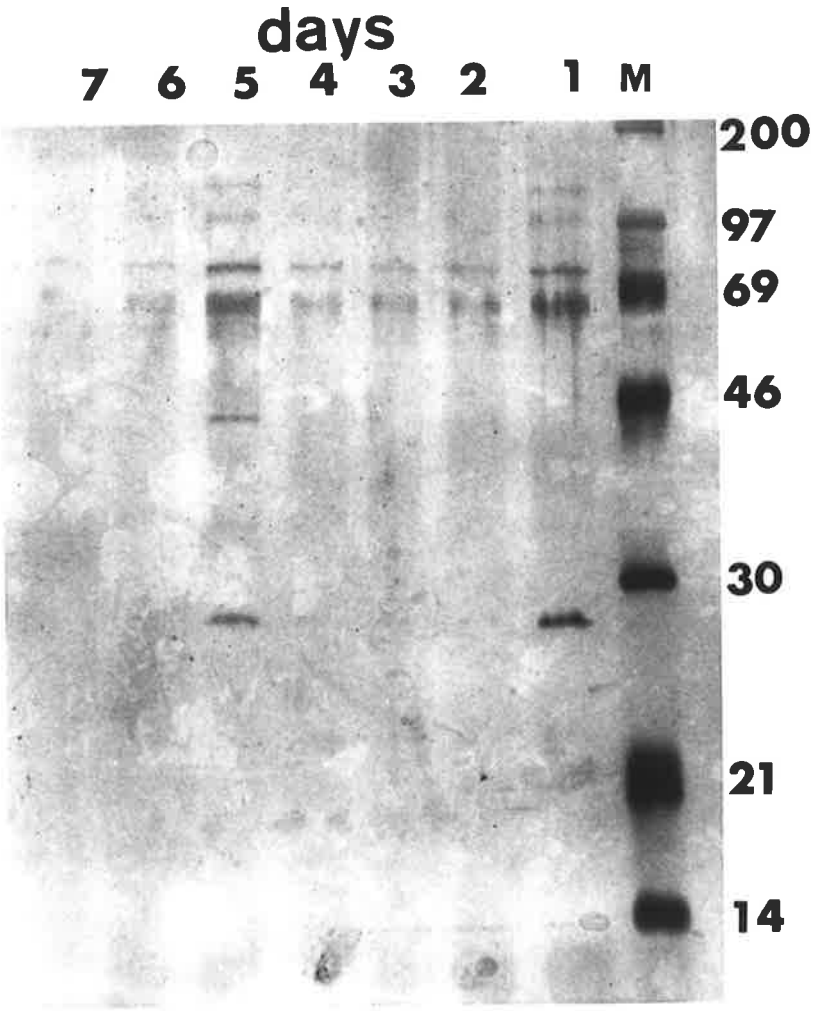

days
7
6
5
4
3
2
1
M
200
97
69
46
30
21
14

Figure 3.7. Electrophoretic analysis of protein secreted by hepatocyte cultures in response to 10^{-8} M E_2 dose. Hepatocyte monolayer cultures were established in gelatin coated dishes at a density of 2×10^6 cells/ml. At the start of the induction the cultures were rinsed and serum free medium containing 10^{-8} M E_2 was added and maintained at this concentration through the culture period. Medium was changed every 24 hrs and the spent medium obtained was analysed on a 10% SDS-PAGE as mentioned in the methods. M represents the standard molecular weights and the figures on the right indicate the molecular weights (200 kD to 14 kD). 160 kD polypeptide is shown by an arrowhead.

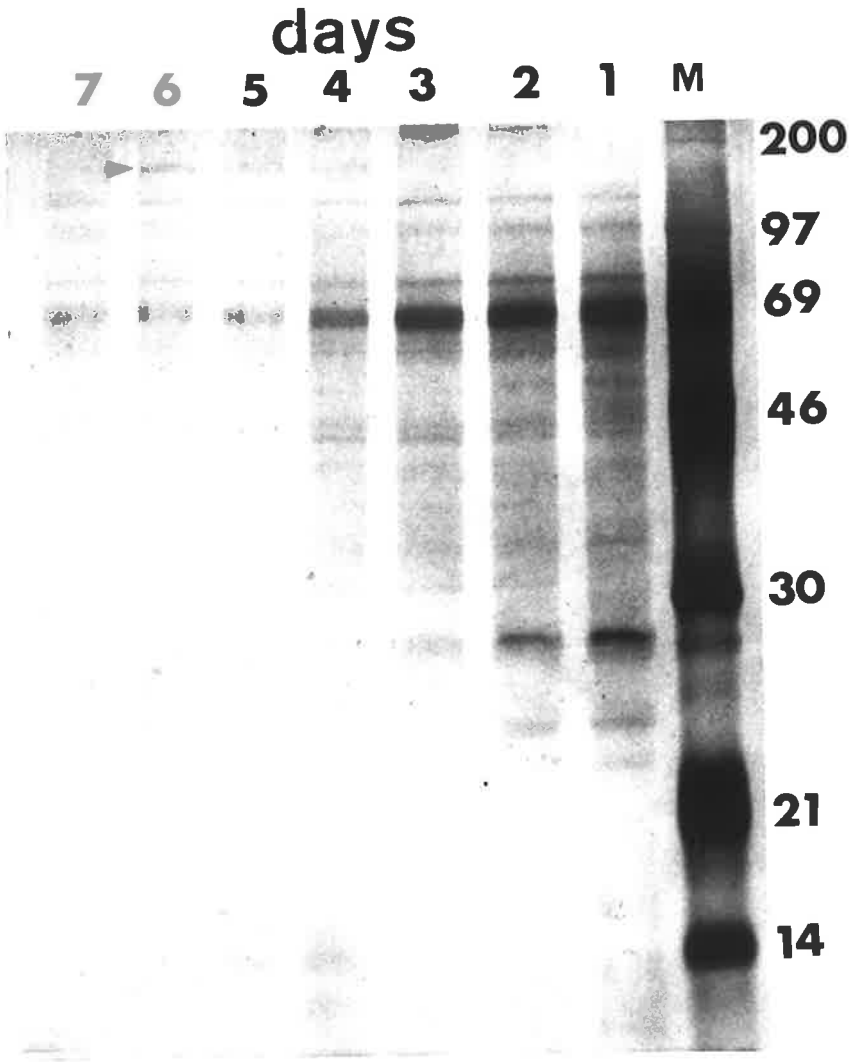

days
7
6
5
4
3
2
1
M
200
97
69
46
30
21
14

Figure 3.8. Electrophoretic analysis of protein secreted by hepatocyte cultures in response to 10^{-6} M E_2 dose. Hepatocyte monolayer cultures were established in gelatin coated dishes at a density of 2×10^6 cells/ml. At the start of the induction the cultures were rinsed and serum free medium containing 10^{-6} M E_2 was added and maintained at this concentration through the culture period. Medium was changed every 24 hrs and the spent medium obtained was analysed on a 10% SDS-PAGE as mentioned in the methods. M represents the standard molecular weights and the figures on the right indicate the molecular weights (200 kD to 14 kD). 160 kD polypeptide is shown by using an arrowhead.

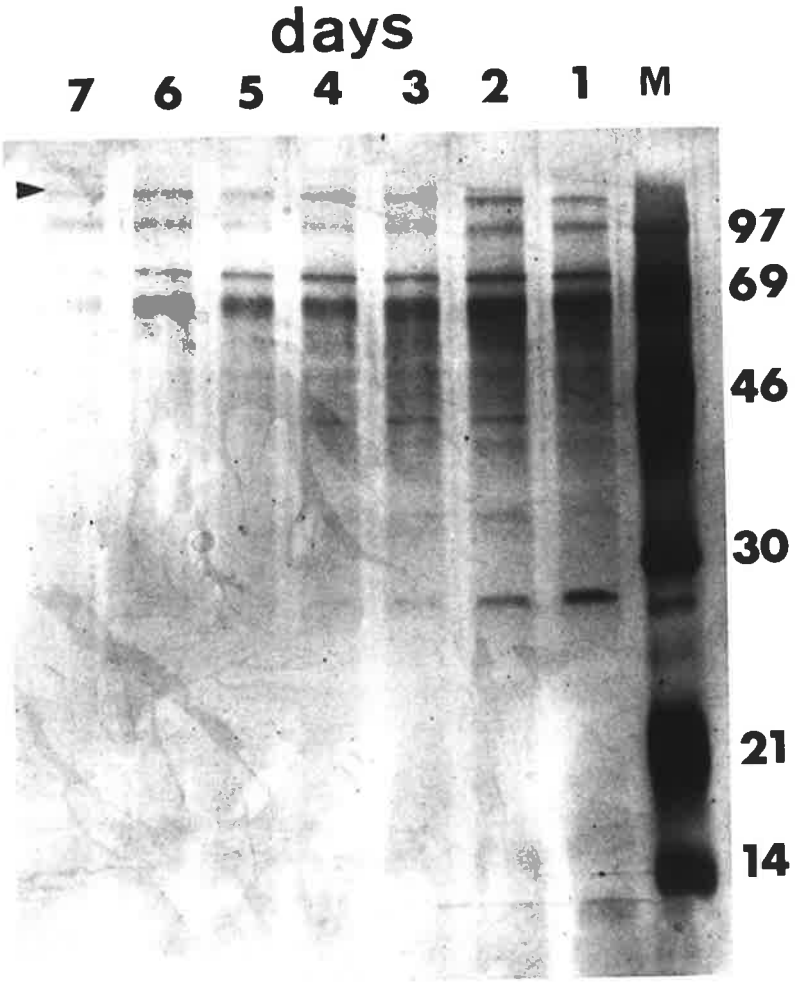
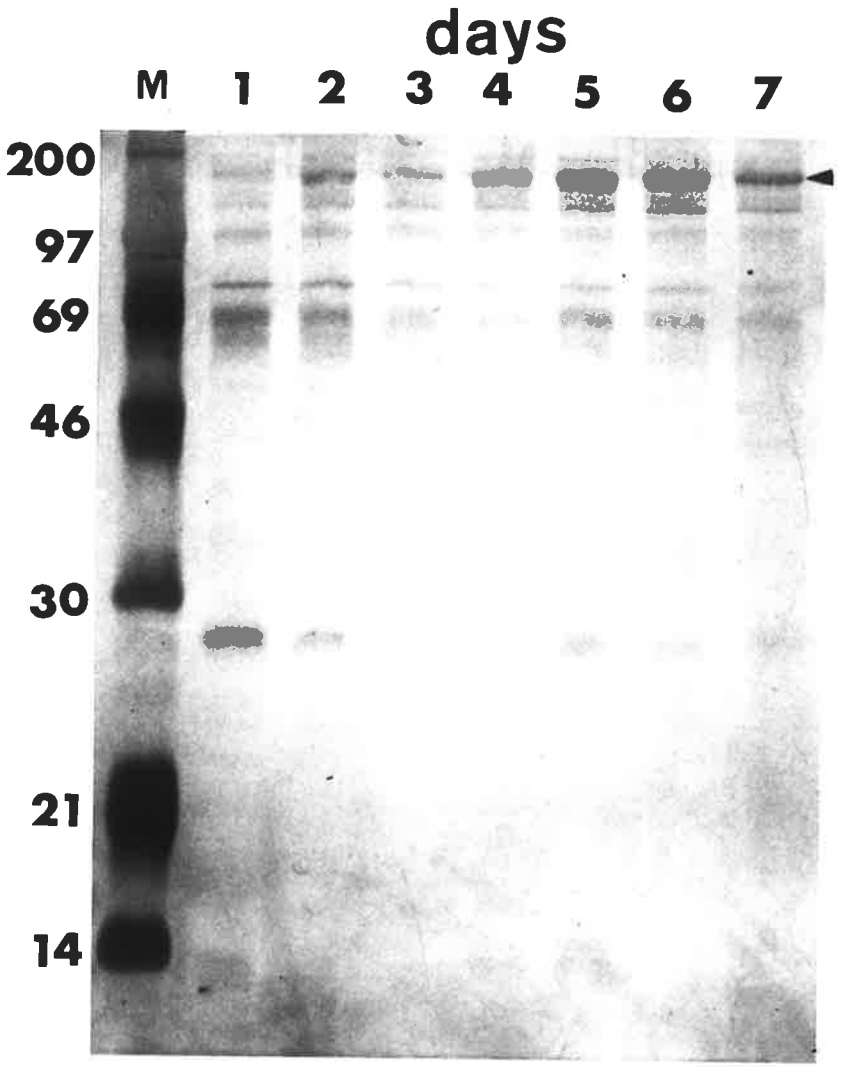

days
7
6
5
4
3
2
1
M
97
69
46
30
21
14

Figure 3.9. Electrophoretic analysis of protein secreted by hepatocyte cultures in response to 10^{-4} M E_2 dose. Hepatocyte monolayer cultures were established in gelatin coated dishes at a density of 2×10^6 cells/ml. At the start of the induction the cultures were rinsed and serum free medium containing 10^{-4} M E_2 was added and maintained at this concentration through the culture period. Medium was changed every 24 hrs and the spent medium obtained was analysed on a 10% SDS-PAGE as mentioned in the methods. M represents the standard molecular weights and the figures on the right indicate the molecular weights (200 kD to 14 kD). 160 kD polypeptide is indicated by an arrowhead.


days
M
1
2
3
4
5
6
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200
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69
46
30
21
14

CHAPTER 4

4. IMMUNOLOGICAL LOCALISATION AND IDENTIFICATIONOF VITELLOGENIN AND RELATED YOLK PROTEINS

4.1. INTRODUCTION

In birds and amphibian Vg incorporated by developing oocytes is enzymatically cleaved into the yolk proteins lipovitellin and phosvitin (Wallace and Bergink 1974; Christmann et al. 1977). In spite of much research on fish Vg, little is known about direct relationship between the serum Vg and the related yolk proteins. Hara and Hirai (1978) provided immunological evidence in rainbow trout, to indicate the cleavage of Vg occured within the oocytes to yield egg yolk proteins, including lipovitellin, and comparative study of Vg and yolk proteins in few fresh water and marine teleosts by Covens et al., (1983) further confirmed that this proteolytic cleavage can occur in the oocytes without destroying all the antigenic determinants.

Vg has been measured in fish serum in a variety of ways including using indirect methods such as the determination of calcium or alkali-labile protein phosphorous (Emmersen and Petersen 1976; Nath and Sundararaj 1981) or more directly by immunological methods such as immunodiffusion (Plack et al. 1971; Hara and Hirai 1978; Gothe et al. 1990) or immunoelectrophoresis (Crim and Idler 1978; Maitre et al. 1985). The later approaches culminated in the development of radioimmunoassays which allow precise and sensitive quantification of Vg in the rainbow trout (Idler et

al. 1979; Campbell and Idler 1980) and more recently enzyme linked immunosorbent assay (ELISA) using polyclonal antibodies has been developed to allow quantification of Vg in the whitespotted charr (Kwon et al. 1990) and common carp (Carnevelli et al. 1991;). There is a recent report of the use of monoclonal antibody for measurement of Vg in channel catfish (Goodwin et al. 1992). Immunologically evidence has indicated that Vg is species specific with studies in salmonids (Campbell and Idler 1980) and cichlids (Ding et al. 1989), showing no cross reactivity even between closely related species.

In this chapter experiments are described which were undertaken to develop quantitative assays to allow further explanation of the process of vitellogenesis and to specifically identify the fate of vitellogenin within the oocytes.

4.2. MATERIALS AND METHOD

4.2.1. ANTIBODY PRODUCTION

Vg, was purified from liver as described (2.2.4), was used as an immunogen to raise antibodies in New Zealand White rabbits. Two rabbits were primed subcutaneously with 1 mg of purified immunogen emulsified with an equal volume of complete Freund's adjuvant (Sigma). Boosters containing 0.5-1 mg of antigen, emulsified in incomplete Freund's adjuvant (Sigma) were given every four weeks. Blood was collected from the marginal ear vein seven days after the booster, and allowed to clot at room temperature, stirred gently and left overnight at 4^{o}C. Antiserum was collected by centrifugation and stored in aliquots at –20^{o}C. The immunisation was terminated after desired titre had been obtained.

4.2.2. ASSAY FOR ANTIBODY ACTIVITY

The antiserum collected was tested by a double immunodiffusion. 1% Agar gel (Difco, USA) containing 0.02% sodium azide (Ajax) was prepared in 50 mM Tris-HCl (pH 8.2) and the mixture was poured into a petridish and allowed to set. Wells (3 mm or 5 mm in diameter) were then cut in the agar using cork-borer or Pasteur pipette. Serially diluted antiserum was placed in a succession of peripheral wells and the antigen at a concentration of 1 mg/ml was placed in the central well. The plates were covered and incubation was carried out in humidified chamber for at least 48 h. In the second set of immunodiffusion the test samples obtained were placed in the peripheral wells and the antiserum (1:100 dilution) was placed in the central well and the reaction was allowed to proceed as mentioned above.

4.2.3. PROTEIN TRANSFER AND ANALYSIS BY WESTERN BLOTTING

Protein samples were electrophoresed on a 10% SDS-PAGE (see 2.2.6). After electrophoresis the gels were soaked in the transfer buffer (20% Methanol, 20 mM Tris-HCl and 50 mM Glycine) for 30 minutes (min). Nitrocellulose (NC) membrane was placed on to the gel and then sandwiched between Whatman filters with a final outer covering of gauge pads, ensuring that all components were wet. The assembly was placed in the blotting caster of Trans-Blot apparatus (Biorad) containing the transfer buffer. Transfer of the protein onto the NC membrane was performed for 14 h at 20 mA or for 2 h at 200 mA.

The NC membrane was removed and non-specific protein binding sites were blocked by incubation it for 1 h in 1% BSA prepared in buffer containing 100 mM Tris-HCl pH 7.5, 1 M NaCl and 0.1% (v/v) Tween-20 (Polyoxyetelene Sorbiton Monolaurate, Sigma) (Buffer 1). The NC membrane was then washed 2 times for 5

min and once for 15 min in Buffer 1 and incubated in anti-Vg antiserum (1:5000 in Buffer 1) for 14 h at room temperature. The NC membrane was rewashed and incubated in alkaline phosphatase conjugate-goat anti rabbit immunoglobulin (GAR-AP, Sigma) (1:1000 in Buffer 1) for 2 h, and washed 2 times for 5 min and once for at least 30 min and twice for 5 min in Buffer 2 (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). Stocks of substrate solutions were prepared by dissolving 75 mg of NBT in 1 ml of 70% Dimethylformamide (Ajax) and 50 mg of BCIP in 1 ml Dimethylformamide. Staining substrate contained 100 µl of each Nitroblue tetrazolium (NBT) (Sigma) and 5-Bromo-4-Chloro-3-indolyl phosphate (BCIP) (Sigma) in 25 ml of Buffer 2. To develop colour, the membranes were incubated in the staining substrate in semidarkness till the desired colour intensity was acquired and the reaction was stopped by replacing the substrate with a stopping solution (1 mM EDTA). Shaking was maintained throughout all the incubation procedures.

4.2.4. IMMUNOHISTOCHEMISTRY

Antiserum prepared against the purified Vg was used for the localisation study of the antigen in the fish tissues. Various tissues, liver, ovary, brain, and gut from E_2 stimulated and unstimulated fish were placed in OCT compound (Tissue-Tek, Miles Inc., Elkhar, IN) and frozen by immersion in liquid N_2 cooled isopentane (BDH Chemicals). Sections (5-6 µm thick) were cut on a Bright (Huntingdon, UK) model OTF cryostat and air dried, then fixed in 96% ethanol (BDH) at 4°C for 10 min. Sections were washed 3 times in PBS and incubated in 1% BSA to block the non-specific protein sites for 1 min. Sections were then incubated in the antiserum (1:100 in PBS) for 14-18 h at 4°C, washed 3 times in PBS and incubated in goat anti rabbit-Horse radish peroxidase conjugate antibody (GAR-HRP) (Dakopatts, Copenhagen) (1:50 in PBS containing 1% BSA and 10% normal sheep serum) for 2 h at 4°C, and washed again 3 times in PBS. All incubations were carried out in humidified

chambers. Reactivity was visualised by incubating slides in diaminobenzidine (DAB, Sigma) (5 mg/ml in 0.05 M Tris-HCl pH 7.2) plus 0.02% hydrogen peroxide for 10 min at room temperature. Tissues were counterstained in Gill's haemotoxylin (Sigma), dehydrated in 2 changes of absolute ethanol, cleared in 2 changes of Safsolvent (Ajax Chemicals, Auburn, NSW), mounted in Depex (BDH) and viewed and photographed using an Olympus BH-2 light microscope and 400 ASA colour film (Fugi, Japan).

Some sections of the tissues were stained in Gill's haemotoxylin only, dehydrated in ethanol, cleared in Safsolvent prior to mounting in Depex.

4.2.4.1. Elimination of Endogenous Peroxidase Activity

All tissue sections that showed endogenous peroxidase activity in the preliminary experiments when treated in the absence of primary antibody, were preincubated in absolute ethanol containing 0.3% H_2O_2 for 30 min at room temperature.

4.2.5. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

4.2.5.1. General Methods.

ELISA was developed using the rabbit antisera with purified serum Vg as competing ligand. The assay was performed by using an indirect method employing polystyrene 96 wells flat bottomed microtitration plates (NUNC, Denmark).

PBS (pH 7.4) was used as a coating buffer, antigen standards of appropriate concentration were prepared by diluting a known amount of purified Vg (1 to 100 µg/ml), and the test samples were diluted serially in the coating buffer. 100 µl was utilised to coat the wells and incubated overnight at 4°C in an humidified chamber. Plates were washed with three changes of PBS containing 0.1% Tween-20 (PBS-Tween), soaked for at least 3 min in PBS-Tween, dried and the remaining sites were blocked with 100 µl of 1% BSA in PBS and incubated at room temperature for 1 h.

Plates were washed as mentioned before in PBS-Tween. Antiserum of desired dilution was prepared in the coating buffer and 100 µl was added to each well, plates were incubated for 2 h at room temperature and washed three times in PBS-Tween. GAR-AP (1:1000) in PBS, was dispensed in each well (100 µl) and incubated for 2 h at room temperature and then washed three times in PBS-Tween. The p-nitrophenyl phosphate (PNPP) substrate solution prepared by dissolving 1 mg/ml of PNPP (Sigma) in 9.7% v/v diethylamine (Sigma) containing 0.02% sodium azide (Ajax Chemicals) adjusted to pH 9.8 with 1 N HCl, and 200 µl added to each well and the reaction was allowed to proceed at room temperature. The reaction was terminated by adding 50 µl of 3 M Sodium hydroxide (Ajax Chemicals). Absorbance was read at 405 nm in the micro ELISA reader (Biotek Instruments, USA).

4.2.5.2. Measurement Of Enzyme Activity Of Alkaline Phosphatase Conjugate

GAR-AP was diluted to 1:1000 in PBS, 20 µl of the diluted conjugate was added to 3 ml of freshly prepared PNPP substrate solution in a glass tube, and incubated at room temperature covered in foil. Aliquots of 200 µl were removed serially every 5 min and transferred to the wells of the ELISA plates, immediately 50 µl of 3 M sodium hydroxide was added as stopping solution. Plates were kept covered between sample application, and when the series was complete the reaction was read at 405 nm in the micro ELISA reader.

4.3. RESULTS

4.3.1. IMMUNODIFFUSION

The results of double immunodiffusion analysis with 1% agarose gel are shown in Fig 4.1. A continuous precipitin line appeared between the antiserum and the purified Vg and the extracts of liver and gonad of the E_2 stimulated male and female zebra fish. A similar precipitin line was also present when antiserum was reacted to the gonadal extract of matured females, but was absent in male liver and gonad, and unstimulated female liver extracts.

4.3.2. WESTERN BLOT ANALYSIS

4.3.2.1. Immunological Identification of Estradiol Induced Polypeptides

Immunopositive polypeptides were detected with the antiserum in all the extracts of E_2 injected fishes, these polypeptides were found to be immunologically related to the major vitellogenin subunits (160 kD and 190 kD) identified in Fig 4.2. In the unstimulated extracts of males, no antigenic determinants were present, whereas in all the extracts of unstimulated matured female, as expected, vitellogenic subunits and the denatured epitopes were positively identified. In the E_2 treated females, the concentration of these vitellogenic polypeptides was much higher compared to the control females. An additional polypeptied of molecular weight around 20 kD, was detected in the ovarian extract, which was speculated as being a product of proteolytic cleavage of vitellogenin after the uptake in the oocytes.

4.3.2.2 *Polypeptide Pattern of Native Vitellogenin*

Vg purified from the serum, liver and gonad was subjected to 10% SDS-PAGE followed by immunoblotting on NC membrane, and probing with the antiserum. At least three major polypeptides were identified (Fig 4.3). These major protein bands along with their breakdown products were also observed in the crude extracts from E_2 treated fish.

4.3.2.3. *Identification of Vitellogenic Polypeptides Produced by Estradiol Stimulated Hepatocytes.*

Western blot analysis of the medium collected from the control, and E_2 stimulated hepatocyte cultures is illustrated in Fig 4.4. The vitellogenin subunit of 160 kD was detected as the major immunopositive polypeptide in the treated cultures. Cultures treated with 10^{-4} M E_2 were found to be most effective on day 5.

4.3.3. IMMUNOHISTOCHEMICAL LOCALISATION OF VITELLOGENIN

i. Localisation in Ovarian Tissue

Vg related proteins were identified in sections of ovarian tissue by immunohistochemistry, using an indirect immunoperoxidase peroxidase technique. Ovaries of sexually matured and E_2 treated females both showed an immunological reaction to the antiserum. The E_2 treated ovarian sections (Fig 4.5 bottom) taken 24 h after treatment showed a high immunoreactivity in the intercellular spaces. Post vitellogenic oocytes containing yolk spheres arranged along the periphery showed a low immunoreactivity in the cytoplasm, but high immunoreaction was observed in

and around the follicular layers of this oocytes. The cytoplasm of the vitellogenic oocytes were stained positive to the anti-Vg antiserum. The immunoreactivity was also observed in the germinal epithelium, and connective tissues of the ovary. Control ovarian sections from sexually matured or E_2 treated females were treated with DAB and H_2O_2 for endogenous peroxidase activity before staining with Gill's haemotoxylin, dehydrating, clearing and mounting (Fig 4.5 top).

ii. Localisation in Liver Tissue

Liver sections from E_2 stimulated male (fig 4.7 bottom) and female fish (Fig 4.6 bottom) revealed antigenic substance localised throughout the section. This immunopositive localisation response was lacking in the liver section from unstimulated male (Fig 4.7 top) and female (Fig 4.6 top) fish. This indicates that vitellogenin is localised in the liver tissue of E_2 stimulated fish but not in the controls.

iii. Brain and Gut of fish

Brain and gut were collected from E_2 stimulated fish. Sections of brain processed for immunoreactivity of antigens, resulted negative for immunohistochemical staining (Fig 4.8). Sections were also treated with DAB and H_2O_2 for endogenous peroxidase activity before staining with Gill's haemotoxylin.

Immunohistochemical study for sections of gut (Fig 4.9) elicited similar results as obtained for brain tissue.

4.3.4. DEVELOPMENT OF ELISA FOR VITELLOGENIN

4.3.4.1. Concentration of Antigen for Coating the Microtiter Plates

An appropriate concentration of antigen was incubated with 100 µl of coating buffer containing 1 µg/ml, 2.5 µg/ml, and 5 µg/ml, of purified Vg for 16 h at 4°C. As shown in Fig 4.10 an acceptable calibration curve was obtained for ligand concentrations ranging from 1 µg/ml to 5 µg/ml. Antigen concentration of 5 µg/ml gave high absorbance values and was selected as the standard coating concentration for all assays. Also at this antigen concentration the antiserum gave a good titre and constituted a working dilution of 1:32000 (OD 1.5). BSA at 10 µg/ml gave a very low background.

4.3.4.2. Standardising the Antibody Working Dilution

The reference ligand (Vg 5 µg/ml) was prepared by dissolving a known amount of purified serum Vg estimated by using BSA as reference (Biorad protein estimation), wells were coated in triplicates with 100 µl of antigen diluted serially with PBS-BSA. After incubation and blocking, antiserum dilution were tested as shown in fig 4.11.

At all the antibody dilutions, BSA (10 µg/ml) coated wells gave a minimum OD readings. The calibration curve representing antibody dilution of 1: 10000 gave high absorbance and very low background in comparison to the other two dilution. This antiserum dilution was utilised for remaining assays.

4.3.4.3. Verification of Antigen Specificity

Duplicate wells were coated with, serial dilution of antigen (serum Vg 5 µg/ml) as positive control, BSA (100 µg/ml) as one control, antigen coated wells with

conjugate alone (replacing the antiserum step with PBS-Tween), and as a third control, antigen with normal rabbit serum (NRS) instead of the antiserum and unstimulated male serum was used as a negative control.

This pilot assay indicated that the BSA, antigen plus conjugate and antigen plus NRS wells gave very low absorbance values in comparison to the standard antigen (Fig 4.12), and there was no cross reaction with the male serum protein. This experimental setup verifies a high specificity for Vg.

4.3.4.4. Species Specificity.

Zebra fish anti-Vg antiserum was utilised against the serum collected from estradiol treated zebra fish, Vg obtained from the teleost *Oryzia latipes*, serum from matured egg laying marbled gecko (*Christinus marmoratus*), and an egg laying chicken. As shown in Fig. 4.13, there was no cross reaction observed between the anti-Vg antiserum from zebra fish and the material obtained from other species.

4.3.4.5. Measurement of Vg

Vg purified from the serum of zebra fish was utilised as an antigen at a concentration of 5µg/ml serially diluted across the plate. The Vg content in the serum of both male and female zebra fish after the first, second, and third injection of E_2 was measured relative to the total serum protein assessed by protein estimation procedure using BSA as the reference standard. Vg in male zebra fish was undetectable after the first E_2 injection but the subsequent injection the Vg content was found to be 0.5 mg/mg of total serum protein, which increased to 0.65 mg/mg of total serum protein after the third and final E_2 stimulation. Control male serum tested negative for the presence of Vg.

In females, the first injection of E_2 in females increased the concentration of Vg in the serum to 0.28 mg/mg which was two folds than the amount measured in the

control fish serum (0.14 mg/mg of total serum protein), second dose of E_2 elevated the Vg content to 0.70 mg/mg, and the final injection increased the serum Vg to 0.84 mg/mg of total serum protein (Table 4.1).

ELISA performed for the serum collected from males and females after the first, second and third injection is represented in Fig 4.14 and 4.15 respectively.

TABLE 4.1. Levels of Vg measured (mg/mg of total serum protein) in the male and female zebra fish using ELISA. Fish were treated with E_2 (4 µgm/gm) on days 0, 3, and 6. 24 hrs after the treatment the serum was collected (days 1, 4, and 7) and quantified using ELISA. Data analysed using Chi-square analysis.

	Males	females
control	0.00	0.14^c
first E_2 (day 0)	0.00	0.28^d
second E_2 (day 3)	0.48^a	0.70^e
third E_2 (day 6)	0.65^b	0.87^f

a,b $p<0.02$; c,f $p<0.001$; c,e $p<0.001$
c,d $p<0.005$; d,e $p<0.001$

4.4. CONCLUSION AND DISCUSSION

These studies indicates that rabbit antiserum prepared against the Vg purified from the liver could be effectively employed to detect the antigen and antigenic determinants in the zebra fish.

Preliminary conclusions drawn from the immunodiffusion examination indicated the presence of the antigen Vg in the liver and gonadal extracts of estradiol stimulated fish, with the single precipitation line in ovarian extracts implying that the antiserum also recognised a Vg like antigen in ovarian tissues.

The Western blotting data indicated that the purified Vg was composed of at least three species of polypeptide (Fig 4.3). As vitellogenin undergoes proteolytic

cleavage after uptake by the oocytes (Clemens 1974; Wallace 1978), the identification of vitellogenic polypeptides in the ovarian extracts by electrophoresis is complicated. In the present studies, two immunoreactive peptides was identified in the ovarian extracts of the unstimulated matured female which had molecular weight of approximately 130 kD and 20 kD (Fig 4.2) but the major Vg subunits (160 kD and 190 kD) were absent.

In experiments designed to demonstrate the localisation of the antigen Vg in tissues, the tissue sections were initially checked for false positive endogenous peroxidase staining and chemically processed before proceeding with immuno staining. Immunopositive staining was detected in both the liver and ovary of estradiol stimulated fish, and in the ovary of mature unstimulated female zebra fish. Brain and gut were also assessed for the presence of antigen, but no immunopositive stain was evident. Localisation of the estradiol stimulated protein in the liver and gonad is consistent with the hypothesis proposed by Bailey (1957).

In the teleost ovary, the transportation of exogenous macromolecules that takes place through the follicular epithelium and into the oocytes has been demonstrated using horse radish peroxidase as a probe (Selman and Wallace 1982 a, b; 1983). Vitellogenin incorporated into the oocytes is then thought to be processed into the yolk material which accumulates within membrane bound yolk bodies (Selman and Wallace 1983; 1986). In sticklebacks it was observed that the immunopositive staining is not the same for all oocytes of the ovary and that in some oocytes the immunopositive reaction localised to granules in the peripheral cytoplasm, whereas other oocytes showed no reaction in the cytoplasm of the oocytes, but sometimes showed immunopositive staining near the theca cell layer (Covens et al. 1987). Similarly, in the ovary of zebra fish it was observed that most of the oocytes showed positive immunoreactivity in the follicular layers, cytoplasm and the yolk spheres, and in addition the connective tissue surrounding the oocyte which was not noted in the other studies reported.

An RIA for the measurement of rainbow trout Vg has been developed by Idler et al. (1979) . However, this technique has not found widespread applications because of the difficulty in isotopic labelling of Vg and instability of the labelled Vg. This problem was partially overcome by using plasma Vg as standard and an egg yolk protein lipovitellin as tracer (Campbell and Idler 1980) and milder iodogen based labelling procedure (Sumpter 1985; So et al. 1985; Copeland et al. 1986). Nevertheless, the problem of instability of the labelled Vg still remains to be solved.

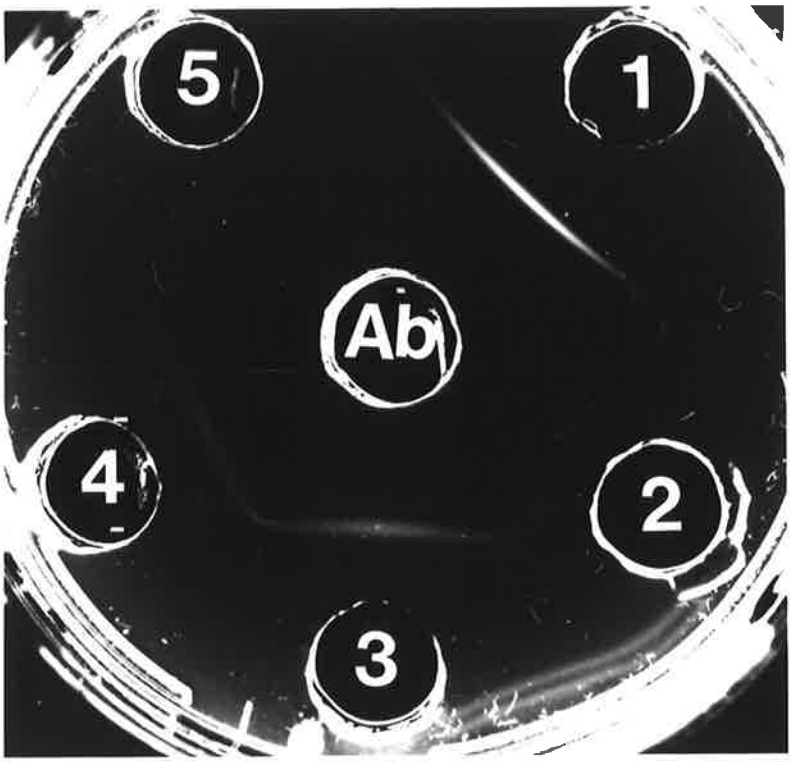
Recently ELISA has been developed for Vg in a few teleost species. Various approaches had been tried including a competitive binding ELISA (Nenuz Rodriguez et al. 1989), non-competitive sandwich method (Kwon et al. 1990) and indirect method (Carnevali and Belvedere 1991). In the present study an ELISA was developed for zebra fish Vg utilising an indirect method employing polystyrene microtitre plates as the solid phase.

To optimize the assay conditions, various developmental steps were performed, as described by Catty and Raykundalia (1989). It was found that sensitising the plates with antigen at 5 µg/ml was quite adequate as all the negative controls gave a very low background (Fig 4.12). Also antigen in the presence of conjugate alone showed no cross-reactivity, indicating that the antigen coating concentration used leaves no spare sites for conjugate to adsorb. The standard curve generated using the antigen Vg verifies the sensitivity of the antiserum to zebra fish Vg with the assay showed no evidence of cross reactivity to sera from vitellogenic fowl and gecko, or the teleost medaka, indicating a high degree of species specificity.

The sensitivity of the assay described in this study allowed the detection of Vg as low as 50 ng/ml, although it is not as sensitive as the RIA developed for rainbow trout (10 ng/ml) (Campbell and Idler 1980) and Atlantic salmon (less than 10 ng/ml) (So et al. 1985) and ELISA for whitespotted charr (l0 ng/ml), this technique is sufficient to quantify serum Vg during the later stages of exogenous vitellogenesis. The ELISA was shown to be useful for quantifying Vg in the serum of both male and female zebra fish, and determine Vg in extracts from the liver and gonads of the estradiol

stimulated fish. Although Vg was not detectable in the plasma of males and immature females, synthesis was induced by treating these animals with estradiol. An amplification effect of successive estradiol treatments was found similar to that previously reported in an amphibian (Tata and Smith 1979), tilapia (Ding et al. 1989) and whitespotted charr (Kwon et al. 1990).

Figure 4.1. Double immunodiffusion analysis of vitellogenin like immunoreactivity in liver and gonadal extracts of zebra fish. 1% Agar gel containing 0.02% sodium azide was prepared in 50 mM Tris-HCl (pH 8.2). Mixer was poured into petridish and allowed to set, wells were cut in the agar using Pasture pipette. Antiserum (Ab) was placed in the central wells at 1:100 dilution, while the peripheral wells contained: **Top**;(1) purified Vg, (2) control female liver, (3) E_2 stimulated female liver, (4) E_2 stimulated male liver and (5) control male liver. **Bottom**; (1) purified Vg, (2) control male gonad (3) female gonad, (4) E_2 stimulated male gonad and (5) female gonad.


5
1
Ab
4
2
3

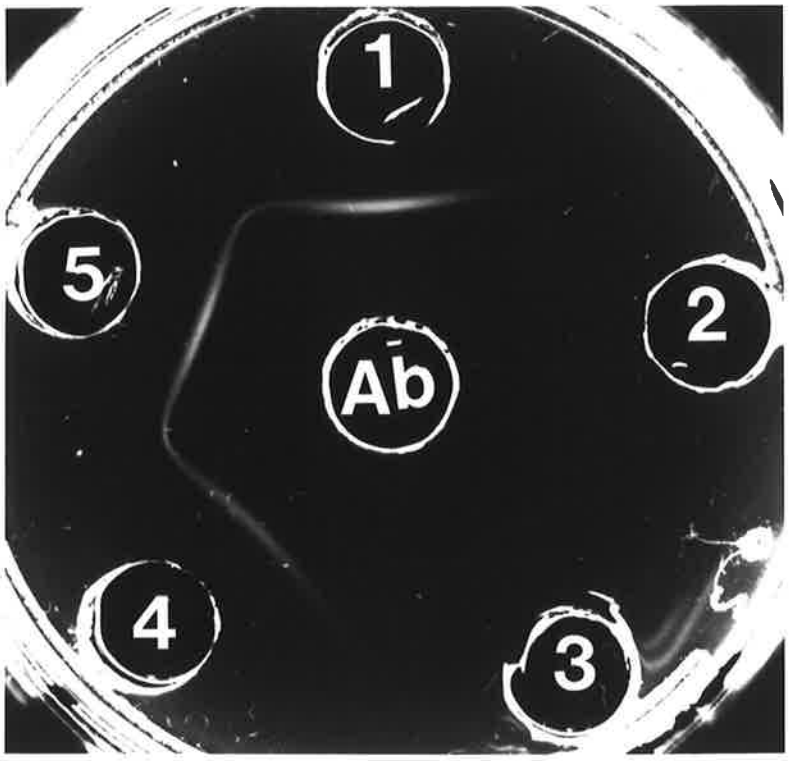

1
5
2
Ab
4
3

Figure 4.2. Western blot analysis of male and female tissues from E2 treated and untreated zebra fish. Protein samples obtained were electrophoresed on a 10% SDS-PAGE, then transferred onto NC membrane and probed with the anti-Vg antiserum (1:5000 dilution). GAR-AP was used as a secondary antibody at 1:1000 dilution. After developing in the presence of BCIP and NBT the immunopositive polypeptides were detected. **Male tissues:** lane (1) untreated and (2) treated liver; (3) untreated and (4) treated gonad; (5) untreated and (6) treated serum. **Female tissues:** (7) untreated and (8) treated gonad; (9) untreated and (10) treated liver (11) untreated serum and (12) treated serum. Bands I and II are the major proteins in the treated fish detected as the immunopositive vitellogenin subunits along with few breakdown products. A single band of approximately 20 kD was detected in the ovarian extracts is presumed to be phosvitin.

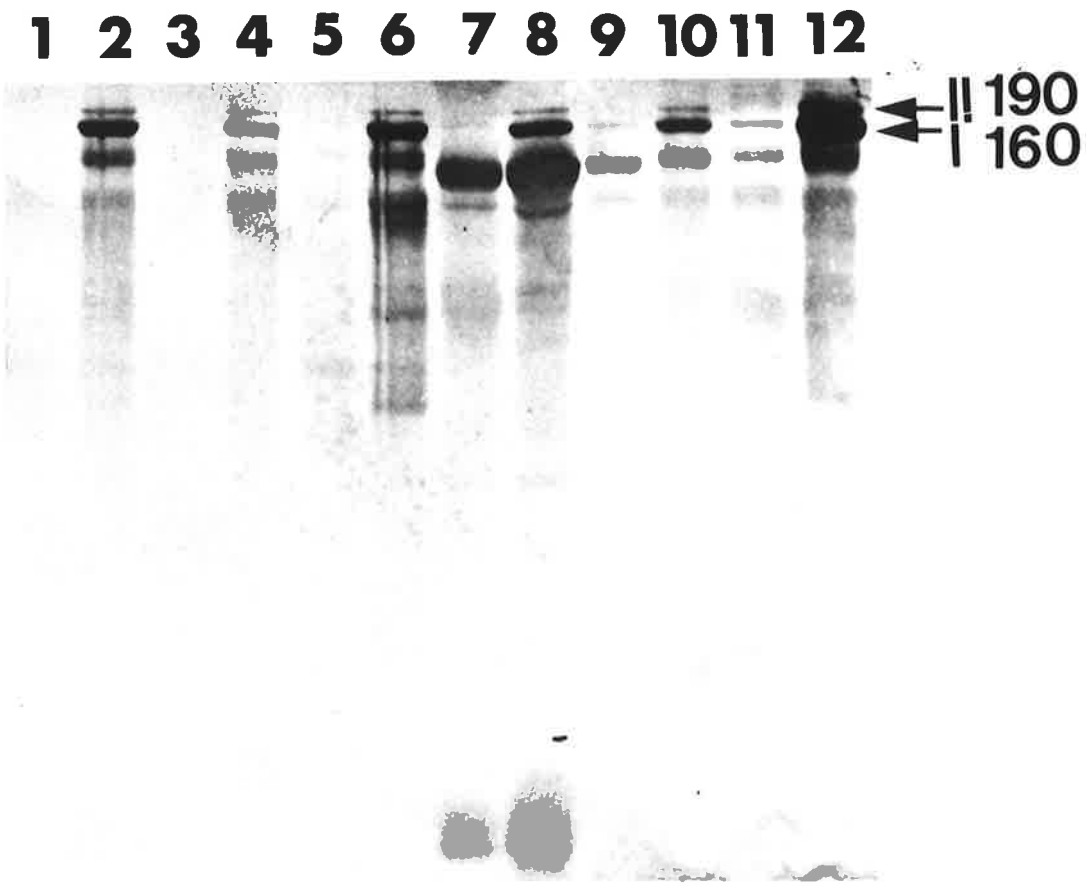

1 2 3 4 5 6 7 8 9 10 11 12
II 190
I 160

Figure 4.3. Western blotting pattern of purified Vg. Vg purified by FPLC from the liver (1), gonad (2),and serum (3) of zebra fish were electrophoresed on 10% SDS-PAGE and transferred onto nitrocellulose membrane and probed using anti-Vg antiserum as the primary antibody (1:5000 dilution) and GAR-AP as the secondary antibody (1:1000 dilution). Polypeptide pattern were detected after developing in the presence of BCIP and NBT. Vg subunits of approximately 190 kD and 160 kD along with a 130 kD molecular weight polypeptide were identified.

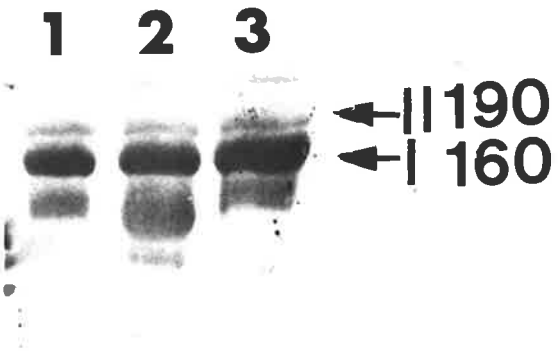

1
2
3
II 190
I 160

Figure 4.4. Western blot analysis of the protein secreted by the hepatocyte cultures in response to E_2 stimulation. Hepatocyte monolayer cultures were established in gelatin coated dishes. Separate cultures were treated with 10^{-8} M, 10^{-6} M and 10^{-4} M concentration of E_2 in the serum free medium and propylene glycol was substituted in the control cultures. Medium was changed every 24 hrs and the spent medium collected on day 4 and day 5 (days when maximum response was observed, see 3.3.7 and fig. 3.9) was electrophoresed on 10% SDS-PAGE and then transferred onto NC membrane. Lanes: (1) control (2) day 4 and (3) day 5, treated with 10^{-8} M E_2. (4) day 4 and (5) day 5, treated with 10^{-4} M E_2. (6) day 4 (7) day 5, treated with 10^{-6} M E_2. 160 kD was detected as the major immunopositive polypeptide in all treatments except the control medium.

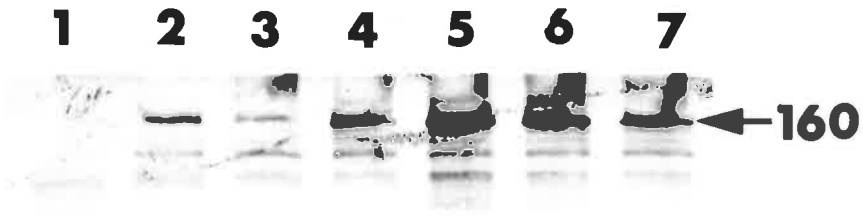
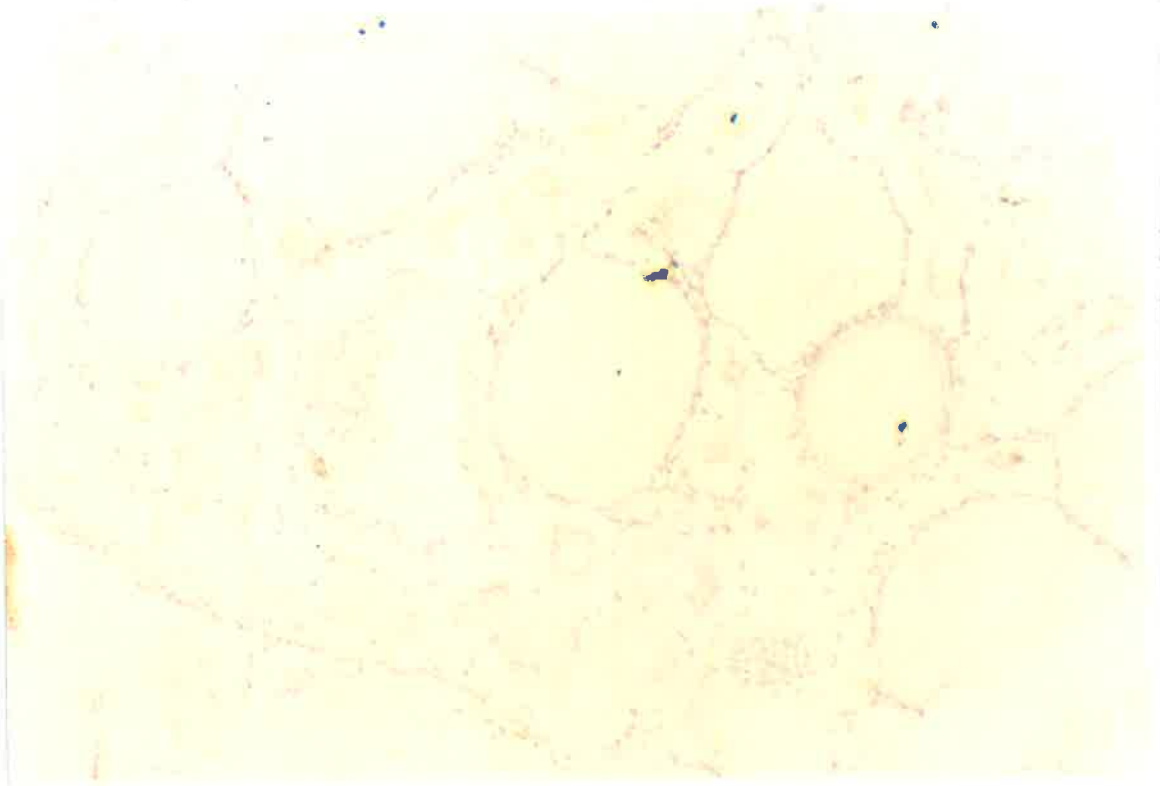

1
2
3
4
5
6
7
160

Figure 4.5. Immunohistochemical localisation of Vg in the ovarian tissue. **Top:** Section of the ovary from matured females was stained in Gill's haemotoxylin. **Bottom:** Sections of the ovary from the matured females were incubated in the antiserum (1:100 in PBS), after washing GAR-HRP was directed towards the rabbit immunoglobulin (1:50 in PBS). Immunopositive reactivity was visualised using DAB and hydrogen peroxide. Tissues were counter stained with Gill's haemotoxylin, dehydrated in ethanol, cleared in Safsolvent, mounted in Depex and viewed and photographed using an Olympus BH-2 light microscope. Vg was localised in the connective tissue surrounding the oocytes, in the ooplasm of majority of the oocytes and the follicular layers of the oocytes.



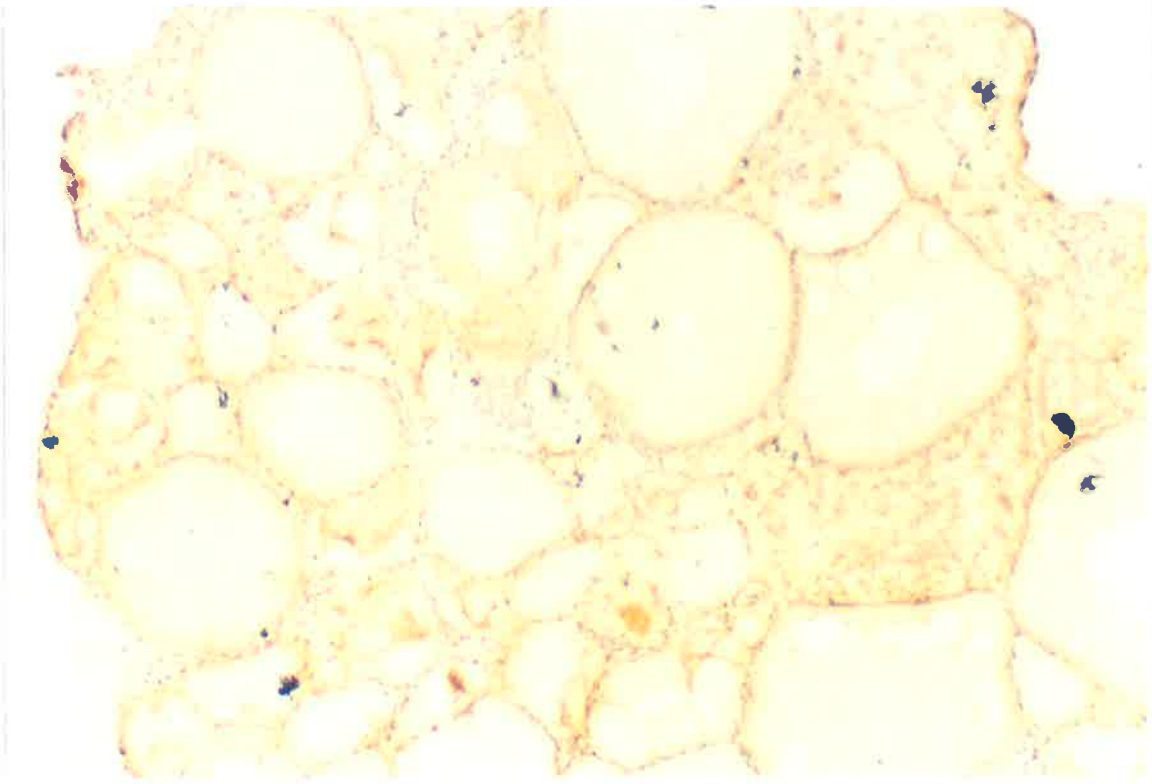


Figure 4.6. Immunohistochemical localisation of Vg in the female liver tissue sections: Liver sections from the unstimulated female (**top**) failed to show any immunopositive staining as opposed to the E_2 stimulated liver (**bottom**) which showed antigenic substance localised throughout the section after incubating in the antiserum (1:100 in PBS) and after washing GAR-HRP was directed towards the rabbit immunoglobulin (1:50 in PBS). Immunopositive reactivity was visualised using DAB and hydrogen peroxide. Tissues were counter stained with Gill's haemotoxylin, dehydrated in ethanol, cleared in Safsolvent, mounted in Depex and viewed and photographed using an Olympus BH-2 light microscope.

Figure 4.7. Immunohistochemical localisation of Vg in the male liver tissue sections: Liver sections from the unstimulated male (**top**) failed to show any immunopositive staining as opposed to the E_2 stimulated liver (**bottom**) which showed antigenic substance localised throughout the section after incubating in the antiserum (1:100 in PBS) and after washing GAR-HRP was directed towards the rabbit immunoglobulin (1:50 in PBS). Immunopositive reactivity was visualised using DAB and hydrogen peroxide. Tissues were counter stained with Gill's haemotoxylin, dehydrated in ethanol, cleared in Safsolvent, mounted in Depex and viewed and photographed using an Olympus BH-2 light microscope.

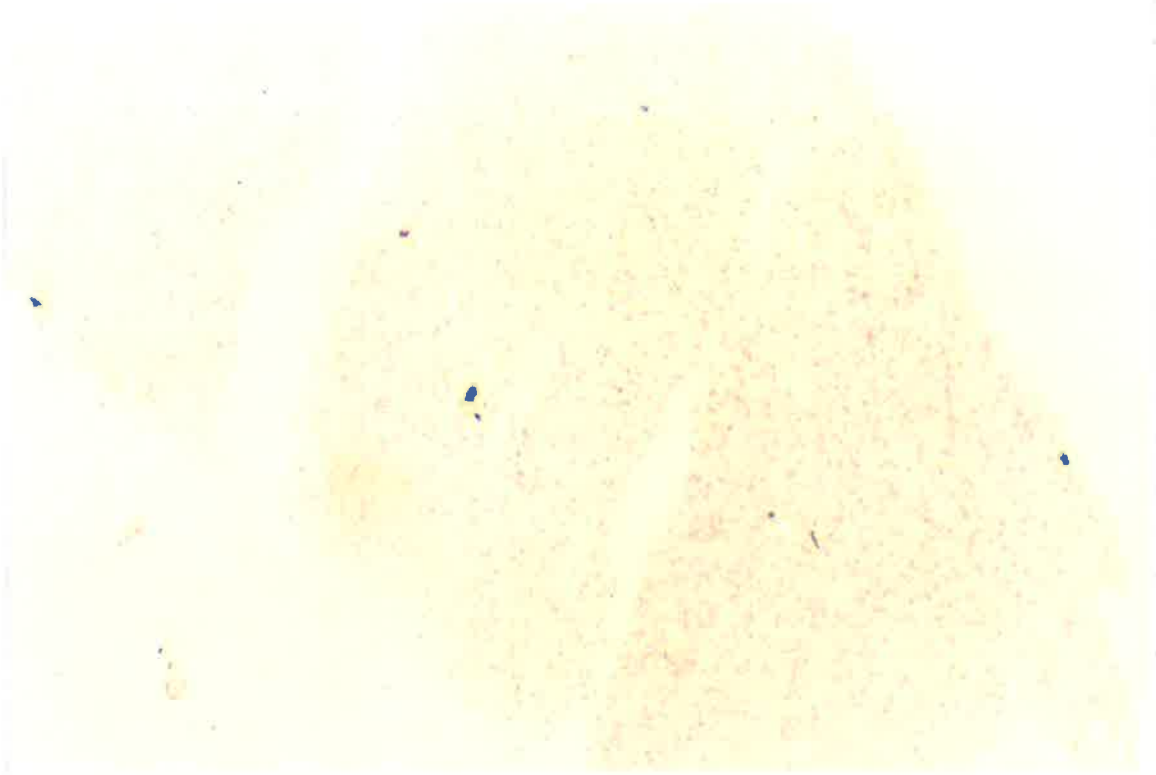
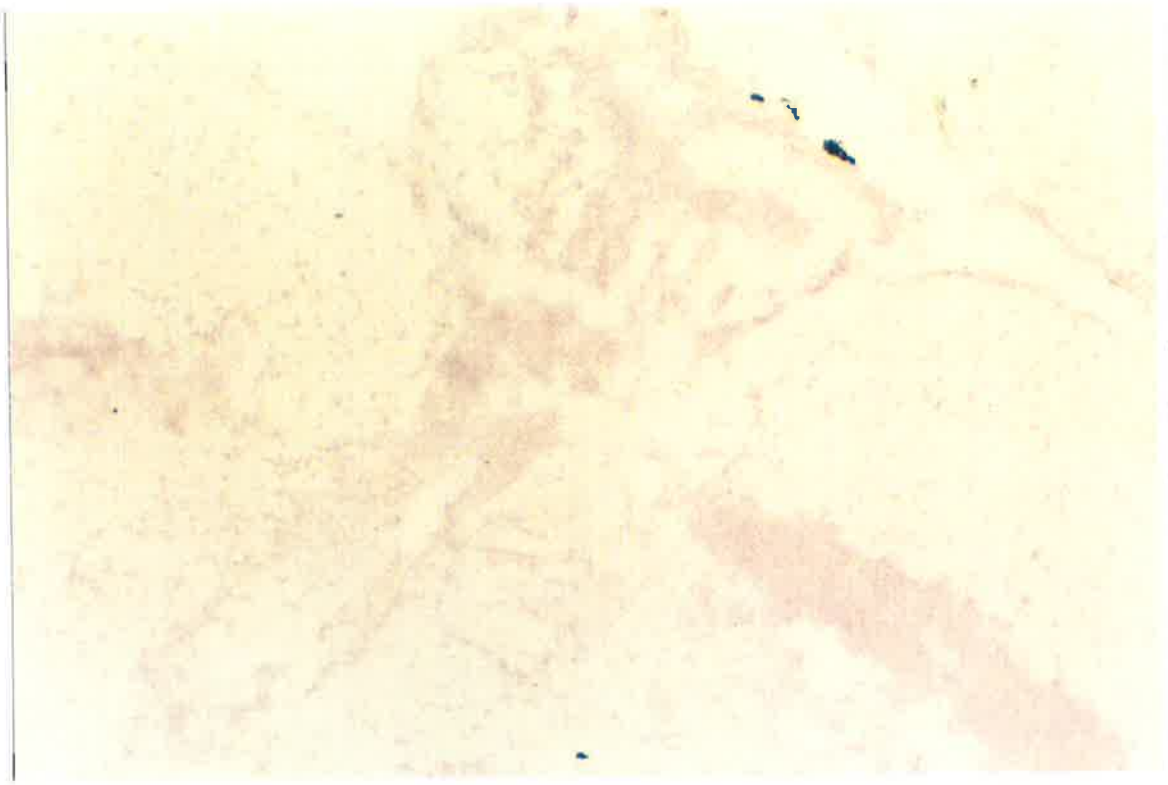


Figure 4.8. Immunohistochemical localisation of Vg in the brain tissue sections: Brain sections had shown endogenous peroxidase activity, therefore sections from the stimulated female fish were preincubated in 0.3% H_2O_2 for 30 min to eliminate this activity, the section were then incubated in the antiserum (1:100 in PBS) and after washing GAR-HRP was directed towards the rabbit immunoglobulin (1:50 in PBS). Immunopositive reactivity was visualised using DAB and hydrogen peroxide. Tissues were counter stained with Gill's haemotoxylin. **Top:** Section of the tissue stained in gill's haemotoxylin only. **Bottom:** Section completely processed as mentioned above resulted negative for the localisation of the antigen.



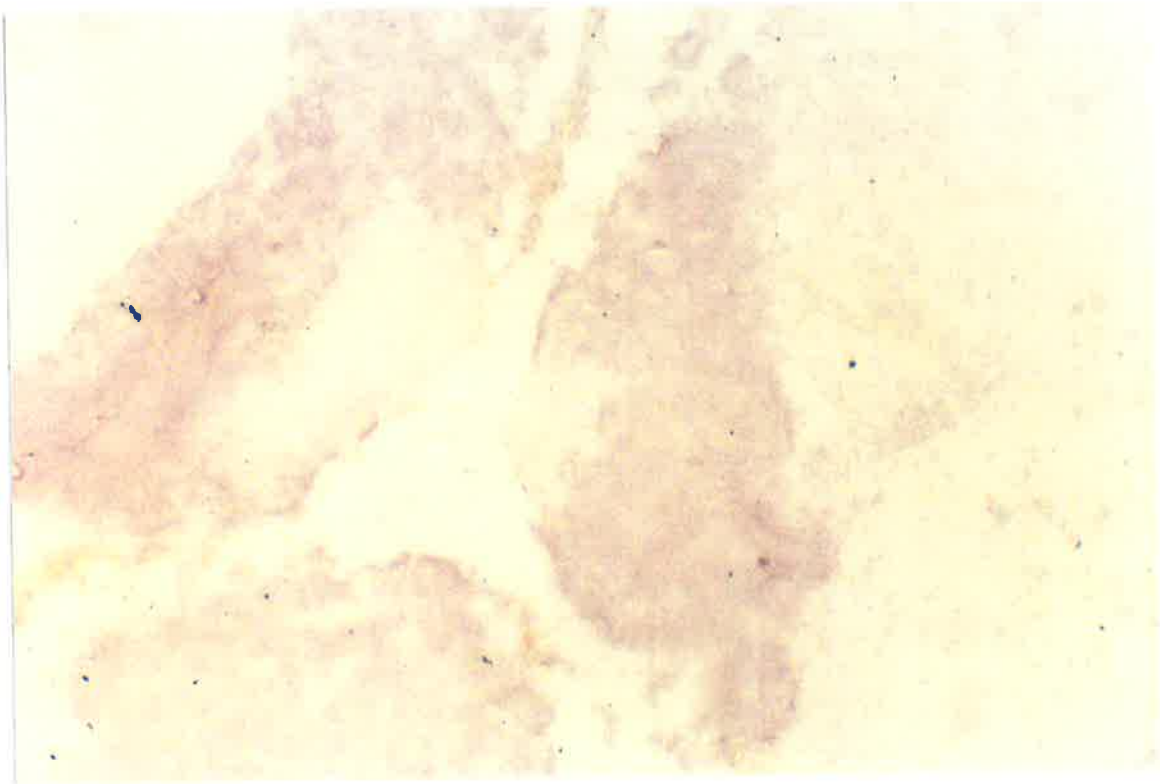
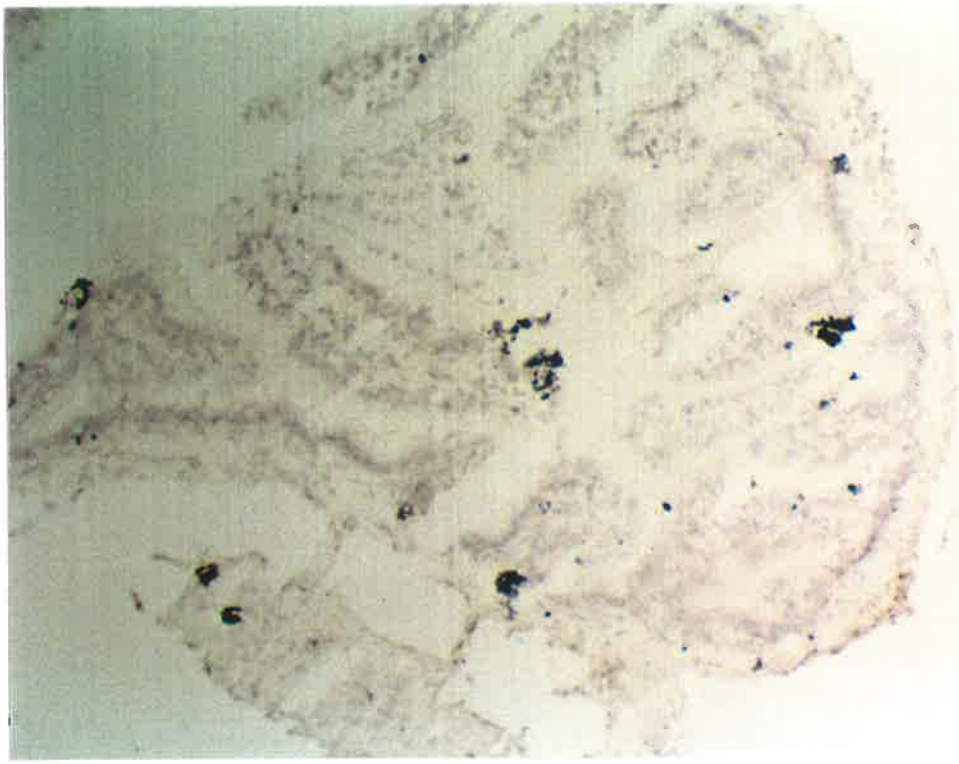


Figure 4.9. Immunohistochemical localisation of Vg in the gut tissue sections: Gut sections from the stimulated female fish were incubated in the antiserum (1:100 in PBS) and after washing GAR-HRP was directed towards the rabbit immunoglobulin (1:50 in PBS). Immunopositive reactivity was visualised using DAB and hydrogen peroxide. Tissues were counter stained with Gill's haemotoxylin. **Top:** Section of the tissue stained in gill's haemotoxylin only. **Bottom:** Section completely processed as mentioned above resulted negative for the localisation of the antigen.



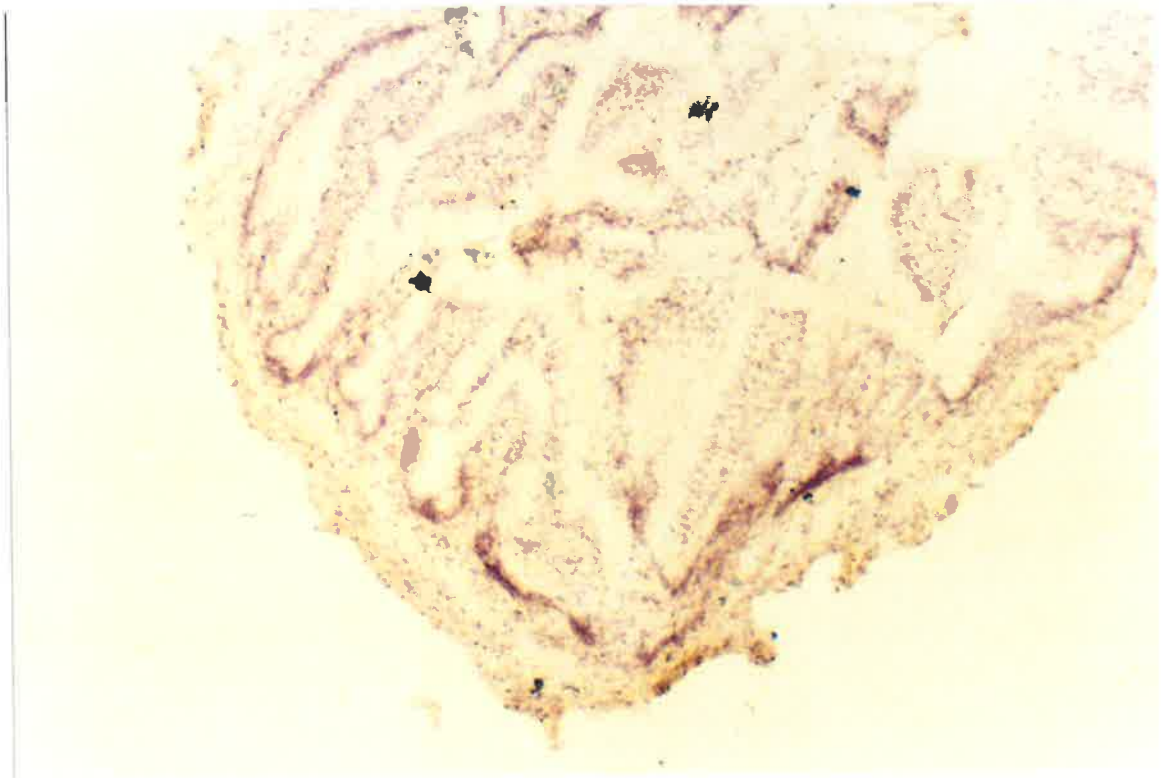


Figure 4.10. Concentration of antigen for coating the microtitre plates: ELISA microtitre plates were coated with antigen (Ag) (purified serum Vg) concentrations as shown, and 5 µg/ml of Ag concentration was chosen as the optimal coating concentration. At this concentration the antiserum gave a good titre and constituted a working dilution of 1:32000 (OD 1.5). BSA at 10 µg/ml gave a very low background

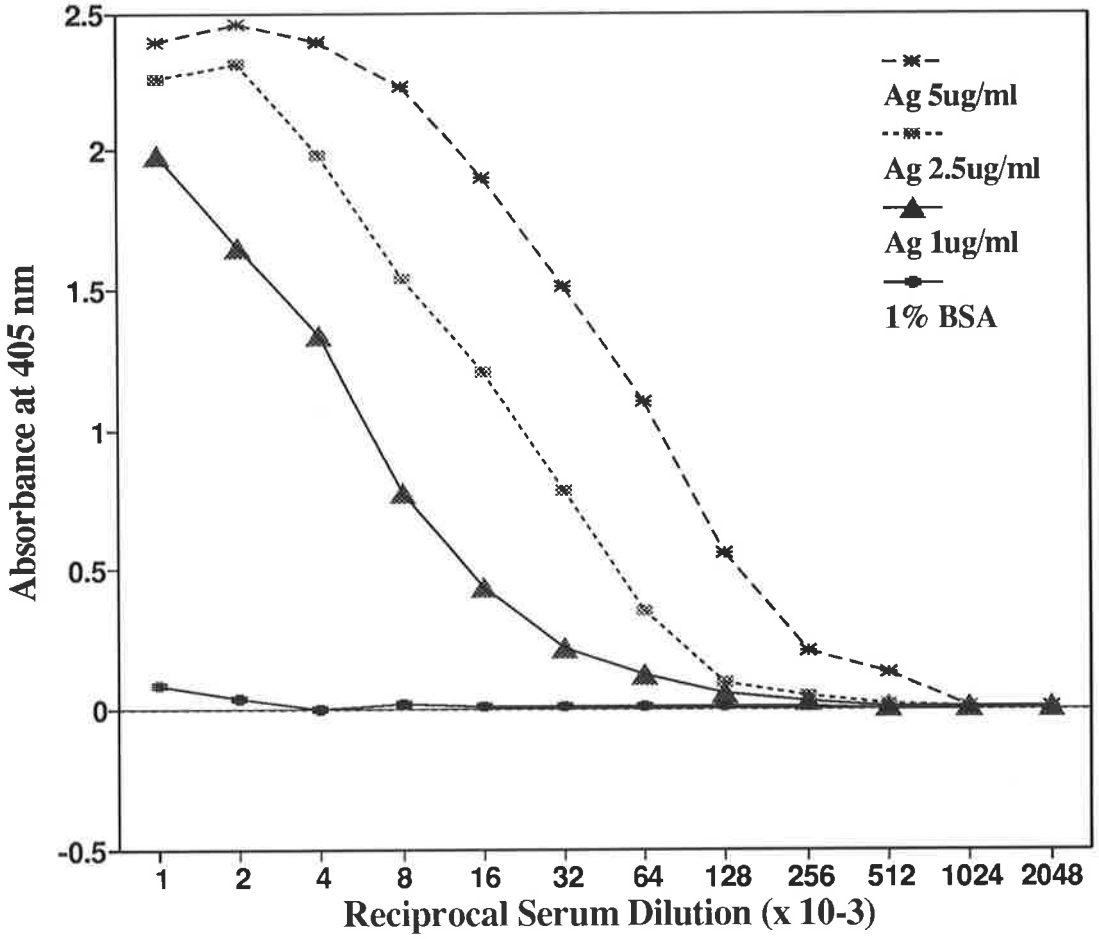

Absorbance at 405 nm
2.5
2
1.5
1
0.5
0
-0.5
1
2
4
8
16
32
64
128
256
512
1024
2048
Reciprocal Serum Dilution (x 10-3)
Ag 5ug/ml
Ag 2.5ug/ml
Ag 1ug/ml
1% BSA

Figure 4.11. Standardising the antibody working dilution: ELISA plates were coated with purified serum Vg as reference ligand (Vg 5 µg/ml) which was estimated by using BSA as reference. Ag was diluted serially with PBS-BSA. Antiserum (Ab) dilution were tested as shown, Ab dilution of 1:10000 gave high absorbance and low background against BSA (10 µg/ml)

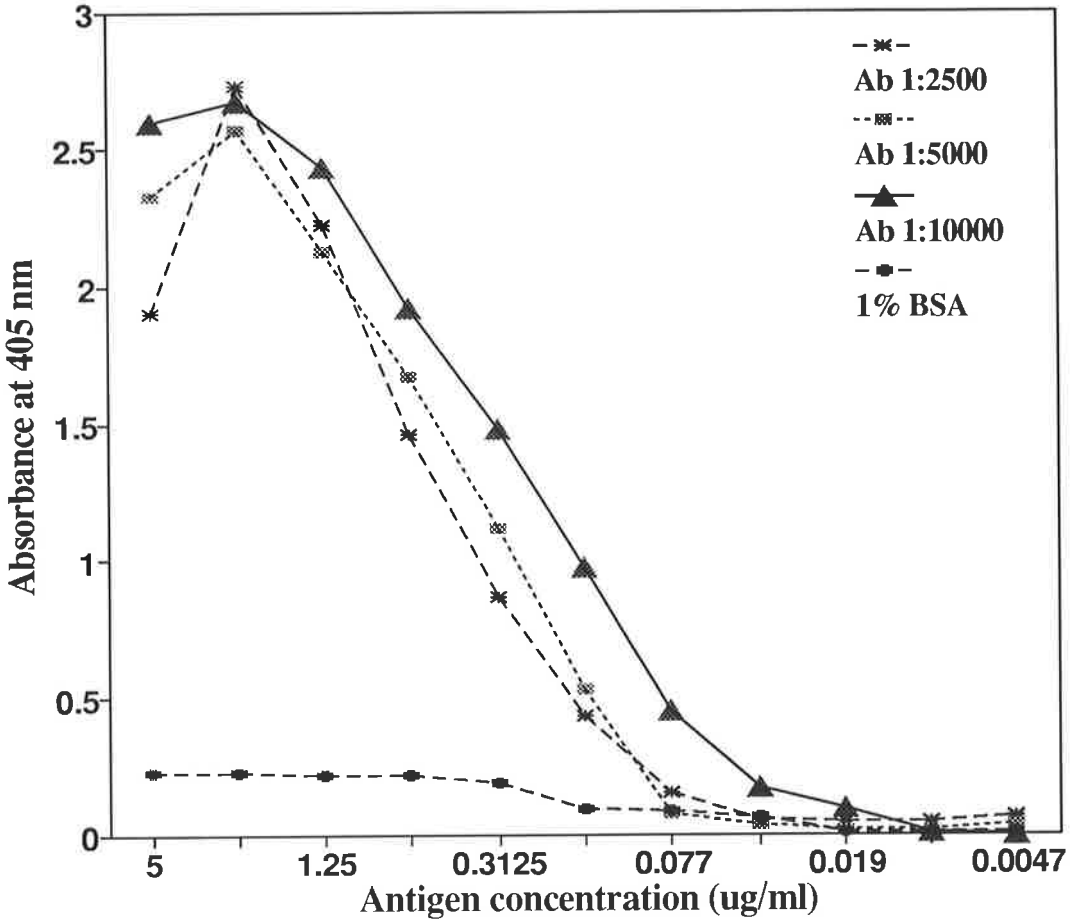

Absorbance at 405 nm
3
2.5
2
1.5
1
0.5
0
5
1.25
0.3125
0.077
0.019
0.0047
Antigen concentration (ug/ml)
Ab 1:2500
Ab 1:5000
Ab 1:10000
1% BSA

Figure 4.12. Verification of antigen specificity: Duplicate wells were coated with Ag (serum Vg 5 μg/ml) as positive control, BSA (10 μg/ml), male fish serum (CMS) (5 μg/ml), Ag coated wells with conjugate (C) GAR-AP (1:1000 in PBS), and Ag coated wells with NRS (instead of the rabbit antiserum) as negative controls. As shown the negative controls gave low absorbance values in comparison to the positive control.

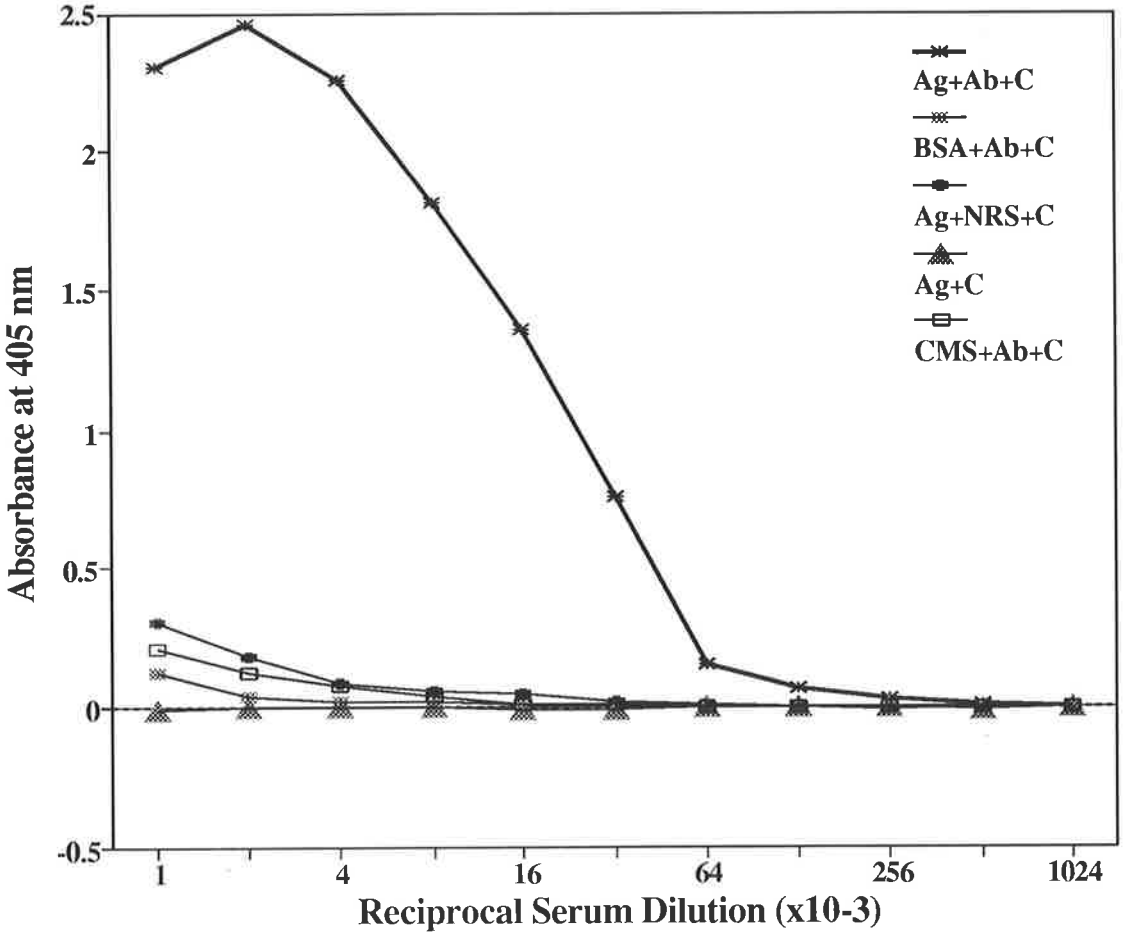

2.5
2
1.5
1
0.5
0
-0.5
Absorbance at 405 nm
1
4
16
64
256
1024
Reciprocal Serum Dilution (x10-3)
Ag+Ab+C
BSA+Ab+C
Ag+NRS+C
Ag+C
CMS+Ab+C

Figure 4.13. Verification of species specificity: Duplicate wells were coated with serially diluted zebra fish Vg (5 µg/ml), medaka Vg (5 µg/ml), chicken serum (10 µg/ml) and gecko serum (10 µg/ml) and tested against the ant-Vg antiserum at 1:10000 dilution. As shown there was no cross reaction between the anti-Vg antiserum from zebra fish and the material obtained from other species.

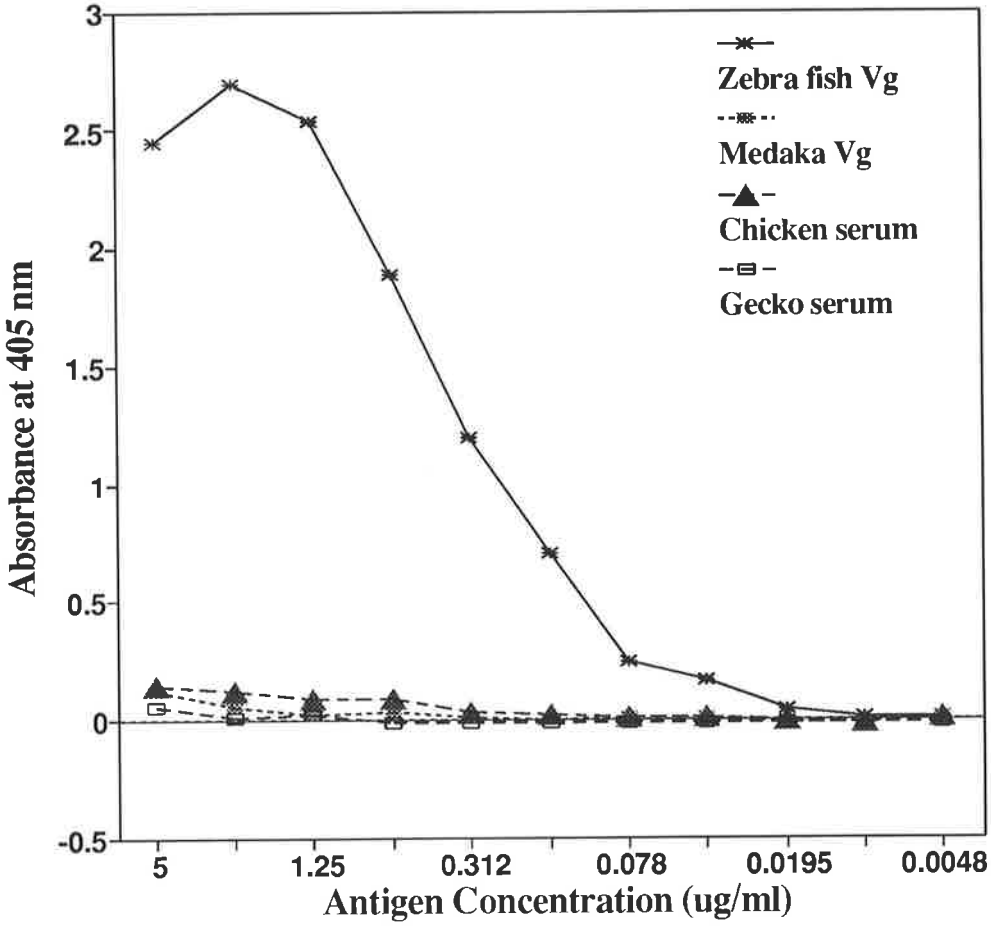

Absorbance at 405 nm
3
2.5
2
1.5
1
0.5
0
-0.5
5
1.25
0.312
0.078
0.0195
0.0048
Antigen Concentration (ug/ml)
Zebra fish Vg
Medaka Vg
Chicken serum
Gecko serum

Figure 4.14. Quantification of Vg in the E_2 stimulated and unstimulated male zebra fish: Duplicate wells were coated with serially diluted purified serum Vg (5 μg/ml) which was used as a reference standard (Std), serum collected from the males after first E_2 injection (MS-1), second E_2 injection (MS-2) third E_2 injection (MS-3) and unstimulated male (CMS) were also serially diluted in duplicate wells. Antiserum at 1:10000 dilution was used. Vg was not detected in the CMS and MS-1.

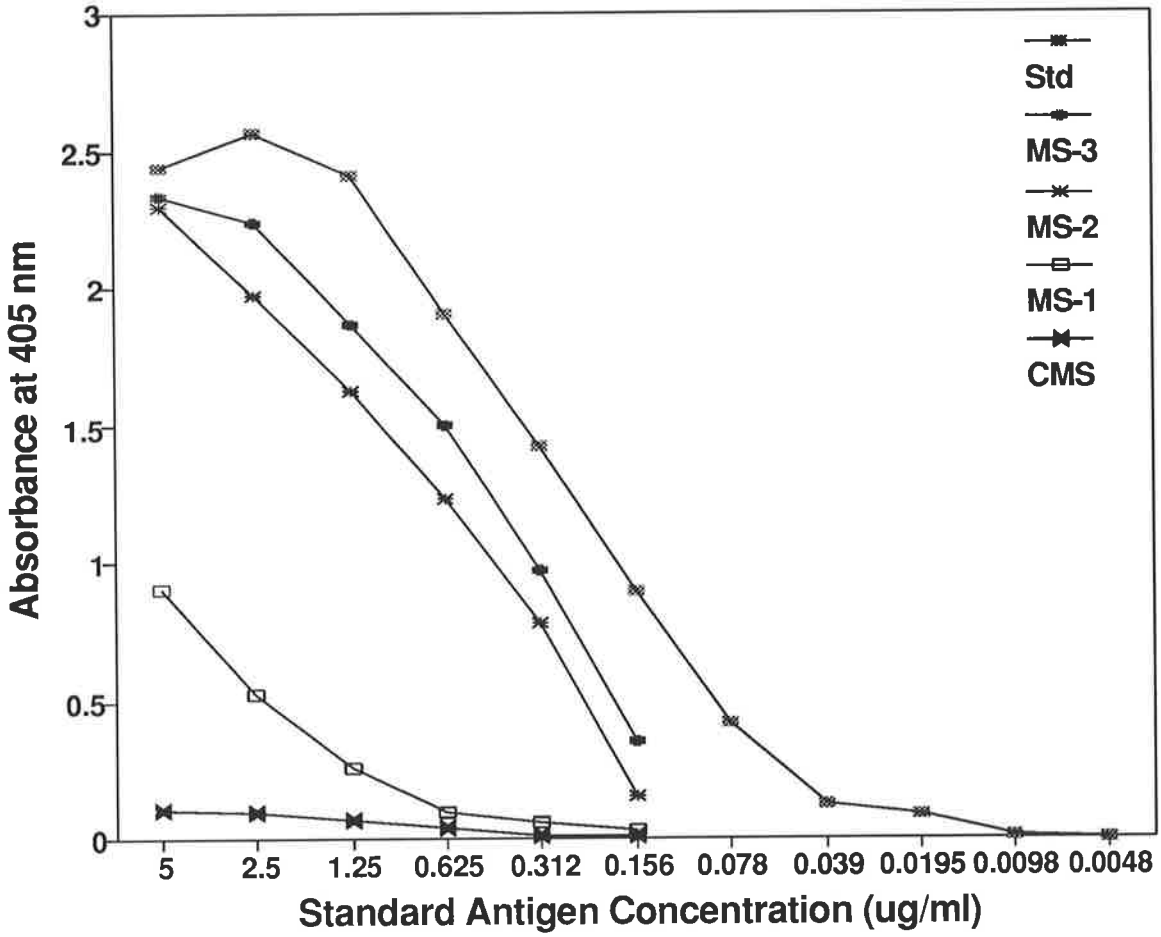
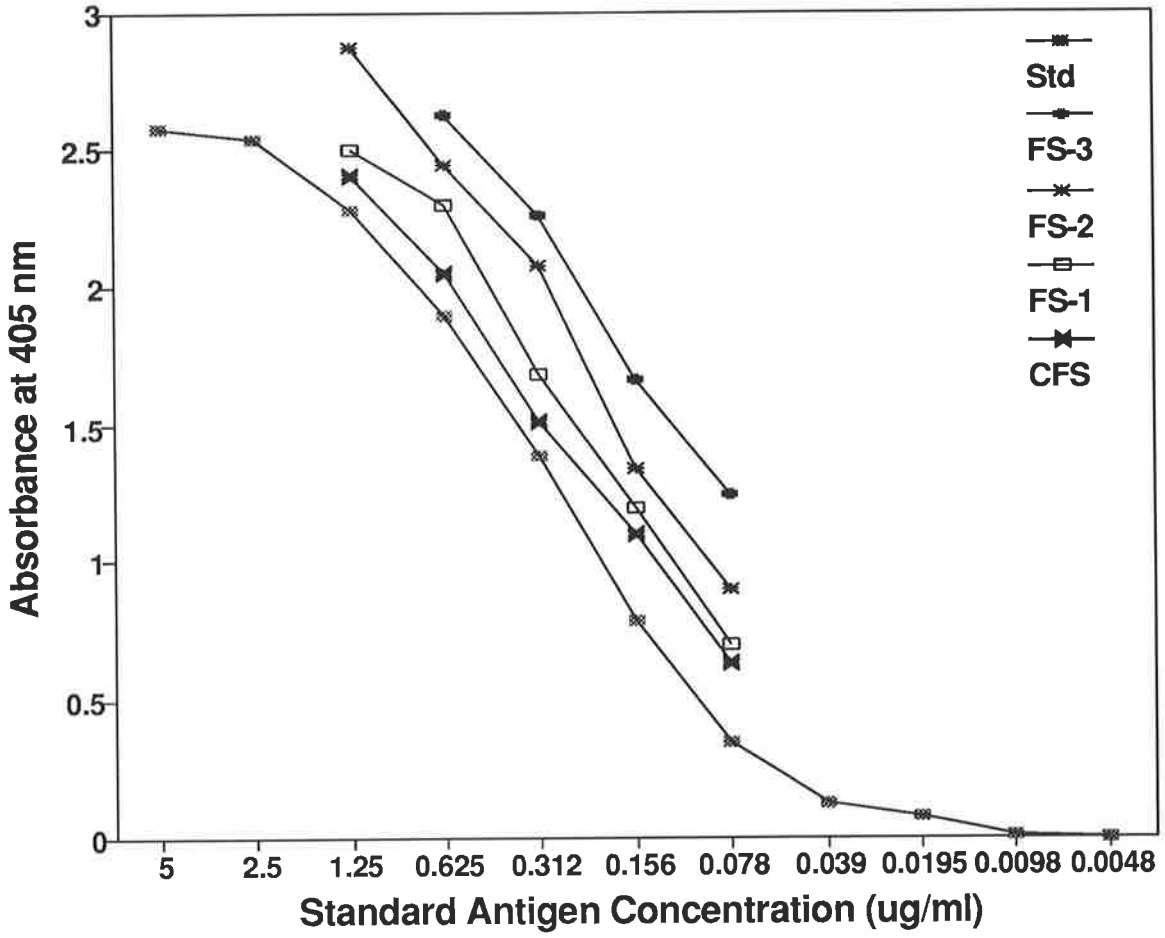

Absorbance at 405 nm
3
2.5
2
1.5
1
0.5
0
Std
MS-3
MS-2
MS-1
CMS
5
2.5
1.25
0.625
0.312
0.156
0.078
0.039
0.0195
0.0098
0.0048
Standard Antigen Concentration (ug/ml)

Figure 4.15. Quantification of Vg in the E_2 stimulated and unstimulated female zebra fish: Duplicate wells were coated with serially diluted purified serum Vg (5 μg/ml) which was used as a reference standard (Std), serum collected from the females after first E_2 injection (FS-1), second E_2 injection (FS-2) third E_2 injection (FS-3) and unstimulated mature female (CFS) were also serially diluted in duplicate wells. Antiserum at 1:10000 dilution was used.


Absorbance at 405 nm
3
2.5
2
1.5
1
0.5
0
5
2.5
1.25
0.625
0.312
0.156
0.078
0.039
0.0195
0.0098
0.0048
Standard Antigen Concentration (ug/ml)
Std
FS-3
FS-2
FS-1
CFS

CHAPTER 5

5. INDUCED OVULATION AND MATURATION OF OOCYTES *IN VITRO*

5.1 INTRODUCTION

In the teleost, post-vitellogenic oocytes remain arrested at the first meiotic prophase, until oocyte maturation is induced by the action of pituitary gonadotropins(Goetz 1983). Final oocyte maturation is characterised in the teleost by a complex series of cytoplasmic and nuclear changes, including morphological events such as lipid coalescence, germinal vesicle migration (GVM), germinal vesicle breakdown (GVBD), and clearing of the ooplasm (Wallace and Selman, 1981). In *vitro* GVBD has been used as the morphological criterion to assess the hormonal effect on final oocyte maturation, Stoeckel and Neves (1992) have recently reported methods of viewing GV in fish oocytes.

A variety of experimental approaches have been used to elucidate the maturation inducing steroid (MIS) in teleosts. These studies have shown that progestins are potent inducers of final oocyte maturation (Goetz, 1983; Degani and Boker, 1991; Jalabert, 1976), with 17α,20β-P being the most potent of the steroid tested (see review Nagahama et al.,1983; Scott and Canario,1987).

An increase in synthesis of 17α,20β-P *in vitro* from ovarian tissues during final oocyte maturation has been observed in *Brachydanio rerio* (Lambert et al.,1986) and *Cyprinus carpio* (Epler, 1987; Kime and Bieniarz, 1987). Further, the plasma levels

of 17α,20β-P was elevated in the plasma of *Cyprinus carpio* (Levavi-Zermonsky and Yaron, 1986), *Carrasius auratus* (Peter et al., 1984) and *Oncorhynchus rhodurus* (Young et al., 1983) around the time of maturation and ovulation. From these studies it has been concluded that 17α,20β-P is the natural MIS in salmonids and cyprinids (Scott and Canario,1987).

In *vivo* induction of maturation and ovulation in the teleost fish has been successfully achieved by injecting mammalian gonadotrophin either alone or in conjunction with fish gonadotropins (see review Donaldson and Hunter, 1983). Of the mammalian gonadotropin hCG has been used effectively on the widest range of fish species, for example in fresh water catfish, *Clarius macrocephalus* (Carreon et al., 1976; Mollah and Tan 1982), goldfish (Stacey et al., 1979), golden perch (Rowland 1988), and grey mullet (Alvarez-Lajonchere et al., 1988).

As part of a study on the regulation of breeding activity in the zebra fish the experiments conducted were carried out aimed at assessing the effect of growth (size) of oocytes and gonadosomatic index (GSI) (weight of gonad/body weight × 100) on *in vitro* maturation and ovulation, the potency of 17α,20β-P as a maturation inducing substance and finally to study, the efficacy of mammalian gonadotropins on *in vivo* induction, in this species.

5.2. MATERIALS AND METHODS

5.2.1. SPAWNING (BREEDING)

5.2.1.1. Fish

Adult mature fish acclimatised for at least one week in the stock tank were selected for spawning. Mature females with rounded abdomen and distinct gonopore which showed presence of whitish or yellowish plug on gentle pressure, and males

with slimmer body, and yellowish tinge on the fins were selected and introduced into the breeding tank and conditioned for breeding purpose for at least a week.

5.2.1.2. Breeding Tank Conditions

Water was changed regularly, the pH of the breeding tanks was maintained between 7.2-7.6. The temperature of the spawning tank was kept at 26^{o}C±1^{o}C, which was ideal for the breeding conditions. Temperatures above 30^{o}C and below 21^{o}C were found to inhibit spawning.

Photoperiod is an important factor for spawning. Zebra fish spawn or initiate breeding behaviour at the onset of light in the wild or under artificially controlled photoperiod in the laboratory conditions. Photoperiod of 14 h light and 10 h dark was maintained in the laboratory.

White gravel substrate was also found to inhibit spawning, so the spawning traps introduced contained a black mesh with pore size large enough to allow the eggs to pass through, and a green wool mop was introduced on the top of the mesh as a spawning substrate for nesting. The traps were made from either glass or plastic containers with a capacity of 2.5-5.0 litres (L). Selected males and females were transferred to the traps the night before and the ratio of male to female was maintained at 2:1.

5.2.1.3. Collection of Eggs

In the morning an hour after the onset of the light the traps were removed and the spawned eggs were collected by the help of pasture pipette, fertilised eggs were cleaned by rinsing them in filtered tank water and transferred to petridishes containing filtered tank water. Embryos were counted and then incubated on warming trays (Roteck Instruments, Australia) maintained at 26^{o}C±1^{o}C. Traps were cleaned and introduced into the breeding tanks.

5.2.2. *IN VITRO* FERTILIZATION (ARTIFICIAL INSEMINATION)

5.2.2.1. Collection Of Male and Female Gametes

Selected males and females were netted from the breeding tank at the onset of the photoperiod. All fish were anaesthetised in Benzocain (12.5 mg/L) prior to handling. Males were blotted dry with Kleenex tissue paper, the milt was collected by applying gentle pressure along the lateral side in the direction of the gonopore, and the milky secretion oozing out was collected by pasture pipette using gentle suction. Milt was pooled from several males and transferred to Hank's balanced salt solution (HBSS, Sigma) kept on ice.

For collection of the female gametes, the gonopore region was blot dried and sufficient pressure was applied to the abdominal area to strip the eggs. As the ovulated eggs were released, they were collected by using pasture pipette and transferred to the petridish as dry as possible.

5.2.2.2. Fertilization and Incubation of the Embryos

Eggs were placed in a petri dish and an aliquot of sperm milt was added and mixed gently, the gametes were activated by adding filtered tank water (approximately 0.5-1.0 ml). After few minutes 2-5 mls of filtered tank water was added and the petri dish was incubated at 26^{o}C on a warming tray. Fertilized eggs (embryos) were collected, counted and placed in freshly filtered tank water, dead and unfertilized eggs were removed and discarded. Embryos were left to incubate on the warming tray till they were hatched out, and hatched larvae were collected and transferred to 200 ml containers.

5.2.3. *IN VIVO* HORMONAL INDUCTION

5.2.3.1 Selection Of Fish

Female fish at two different stages were selected for hormonal induction; matured females, and post-spawning or over matured females (egg bound females with over matured eggs). Selected fish were acclimatised to the breeding tanks 24 h before induction.

5.2.3.2. Induction

Fish were anaesthetised in benzocaine, and 1-15 IU/gm body weight (b.w) of hCG (Chorulon, Intervet) was injected intraperitonealy at 4 h, 8 h, 12 h, and 24 h before the expected time of spawning. Fish were allowed to recover in fresh tank water before being reintroduced in the traps. Treated females were either kept isolated or with spermiating males in the traps.

5.2.3.3. Induced Spawning

Spawning was monitored by isolating the induced female fish in traps or by introducing spermiating males. After the onset of light the isolated females were checked for ovulated eggs by stripping, whereas the other group accompanied with males were allowed to undergo natural spawning. The stripped eggs obtained were artificially fertilized as mentioned above.

5.2.4. *IN VITRO* MATURATION

5.2.4.1. Collection Of Oocytes

Matured females from the stock or breeding tanks were netted and anaesthetised in benzocaine, each female was weighed individually after drying. Fish were sacrificed and both the ovary lobes were removed and weighed immediately to calculate the gonadosomatic index (weight of gonad/weight of fish × 100). Ovaries were rinsed in the fish Ringers solution , and placed in a petridish containing fresh fish Ringers. By the help of fine forceps the ovaries were teased open, and pasture pipette attached to a mouth sucker was utilised to isolate the oocytes from the ovarian tissues, which were then placed in a petridish containing fish Ringers. Small ovarian follicles attached to the oocytes were removed by forceps and discarded.

5.2.4.2. Culture Medium

Medium used for *in vitro* study of the oocytes included L-15 medium Leibovitz (L-15), Earl's medium 199 (E-199), Minimum essential medium Eagle (MEM) and Hanks Balanced salt solution (HBSS) (Sigma) supplemented with 10% Fetal Calf Serum (FCS; Commonwealth Serum Laboratories, Australia) or 2% BSA and antibiotics. The osmolarity of each medium was measured in an osmometer (Wescor, Logan, Utha), and adjusted to between 275 and 280 mOsm/kg with MQ H_2O prior to supplementation with serum, antibiotics or BSA and the pH adjusted to 7.4. All the media prepared were sterilised by filtration through 0.22 μm filters.

5.2.4.3. Steroids

Progesterone (P), 17α,20β-P and 20β-hydroxy-4-pregnene-3-one (20β-P) were obtained from Sigma. Stocks were made in ethanol and added to cultures at a final concentration of 0.1% ethanol.

5.2.4.4. Culture of oocytes

The size of the oocytes collected ranged from 50 μm to 900 μm in diameter. Oocytes were divided into individual groups according to their sizes. Oocytes were cultured in disposable plastic petridishes in culture media containing desired concentration of steroids and hormones. Controls were cultured in medium containing 0.1% ethanol. All the cultures were incubated at 26°C on a warming tray.

5.2.5. CLEARING OF THE OOCYTES

The presence of the germinal vesicle (GV) was assessed by incubating the fish oocytes in the clearing solution (Methanol:Acetic acid:Formaldehyde; 6:3:1), diluted five times in fish Ringers solution. Oocytes were selected before and after the experiment to locate GV.

5.2.6. *IN VITRO* OVULATION AND FERTILIZATION

Matured oocytes were selected from the cultures and incubated with media containing 5 μg/ml of Prostaglandin F2α (PGF2α; Upjohn Pty. Ltd. NSW). Ovulated oocytes were artificially inseminated (see 5.2.2) or activated by placing them in tank water.

5.3. RESULTS

5.3.1. INDUCED OVULATION AND SPAWNING

5.3.1.1. Effects of hCG Dose and Time of Induction on Ovulation

The first set of experiments was conducted using different dosages of hCG (1-15 IU), injected at different time (h) before the expected time of spawning. The hCG dose of 10 IU proved to be most effective dose in inducing ovulation (Table 5.1). The percentage of ovulation in 10 IU hCG treatment increased from 40% (24 h) to 100% (8 h). The material obtained by stripping the female zebra fish injected 24 h and 12 h before the expected time of spawning, consisted of a mixture of egg membranes, yolk fluid, and partly ruptured eggs. Very few eggs appeared morphologically viable, and they failed to fertilize. Similar results were obtained in the ovulated fish given 15 IU. No ovulation was seen in the group injected 4 h prior to stripping.

5.3.1.2. Effect of hCG in Presence of Male fish

The second set of experiment was performed to verify the effect of hCG observed above. Matured fish spawn naturally in the presence of males under optimal condition. Matured and overmatured females (egg bound females with overmatured eggs) were injected 8 h before the expected time of spawning with 10 IU/gm b.w of hCG, and either kept isolated or with males. Isolated females were checked for ovulated eggs by stripping at the onset of light, and those with the males were allowed to spawn naturally. This dose of hCG successfully induced ovulation in both the groups of matured and overmatured females (Table 5.2). The stripped eggs from matured females kept in isolation, and in presence of males, both gave a high

percentage of fertilization rate. In overmatured females the material obtained on stripping consisted of mainly yolk fluid and egg membranes along with few intact ovulated eggs that failed to fertilize, and those with males spawned producing very few eggs, these eggs collected from the traps were unfertilized, opaque and whitish in appearance.

5.3.2. *IN VITRO* MATURATION AND OVULATION

5.3.2.1. Effect of size of oocytes on maturation

Preliminary experiment showed that the oocytes isolated and cultured in presence and absence of ovarian tissue in fish Ringers solution resulted in spontaneous GVBD, but maturation was only observed in the larger oocytes, which was assessed by change in the opacity (Fig 5.1).

This experiment was set up to verify the oocytes size capable of *in vitro* maturation. Oocytes ranging from 300 µm to 900 µm in diameter were isolated from the ovarian tissue and cultured in fish Ringers solution containing 10 µg/ml P. Table 5.3 illustrates that P has no effect on oocytes less then 600 µm in diameter on continuous exposure, but maximum response was observed in the oocyte size ranging from 700 µm to 900 µm in diameter. Spontaneous ovulation was assessed by counting the presence of follicular envelops which are detached after the expulsion of the oocytes in the culture dish. Oocytes were checked every hour following incubation. After 6 h exposure to the steroids the large (800-900 µm diameter) oocytes were first to show the maturational effect.

5.3.2.2. Effect of Incubation Medium on Maturation

The effect of different medium on the maturation of the oocytes in the presence of 10 µg/ml P is as shown in Table 5.4. Media supplemented with 2% BSA displayed a higher maturation rate then those supplemented with 10% FCS. Medium MEM plus

2% BSA was found to be the most effective culture medium with 80% maturation and 21% ovulation. The first signs of maturation were observed after 3 h of exposure to steroid as opposed to 6 h in the previous experiment. This medium was selected for all the incubations in the following experiments.

5.3.2.3. Effect of Concentrations of Various Steroids

Progesterone and its derivatives have been proven to be the most effective maturational steroids in most teleost fish. When P, 17α,20β-P, and 20β-P were tested at different concentration (Table 5.5). All were effective in inducing maturation and initiating the maturational process even at the lowest dose tested. The most potent was 17α,20β-P however it was closely followed by 20β-P and P.

5.3.2.4. Effect of Time Exposure to 17α,20β-P

Oocytes ranging from 700 μm to 900 μm in diameter were incubated for appropriate time in the presence of the steroid, after which the oocytes were rinsed and transferred to medium without steroid (Table 5.6). Results indicate that the minimum exposure time required to stimulate maturation process was 10 mins. Percentage of matured oocytes was almost same in all groups. Visual checking showed that the maturation in all the groups had taken place after 3 h, whereas oocytes cultured in the absence of steroids showed maximum maturation after 24 h incubation.

5.3.2.5. Effect of Gonadosomatic Index on Steroid Maturation

Oocytes from females with GSI more than 16% and less than 16% were collected and incubated in presence of steroid (Table 5.7). Results indicate that in female with a higher GSI contained more oocytes ranging from 700μm to 900 μm in diameter

which were ideal for invitro maturation. Oocytes collected from females with GSI greater than 16% were more responsive to 17α,20β-P then those below 16% where the maturation droped from 94% to 50%.

5.3.2.6. Effect of Prostaglandin F2α on Ovulation

PGF2α was not found to be very effective on the oocytes matured under the influence of the steroid (Table 5.8). Though ovulation was observed in the cultured oocytes in presence of PGF2α, the result was not significant when compared to those ovulating in the continuous presence of maturational steroids

5.4. CONCLUSIONS AND DISCUSSION

Although zebra fish can spawn easily in captivity, studies described in this chapter idicates that hCG can be used for maturation or ovulation to facilitate the availability of viable oocytes and initiate spawning in matured and overmatured females. Secondly 17α,20β-P has been identified as possible maturation inducing steroid.

A single dose of hCG was shown to be effective in inducing ovulation in the zebra fish when given at appropriate time. This gonadotropin surge is presumably necessary in the plasma during natural spawning and ovultion. Gonadotropin induces the production of steroid (estrogen) which recruits a new clutch of vitellogenic oocytes, but at the same time causing atresia and hydration of mature oocytes (Wallace and Selman 1981). After hCG administration stripping resulted in the release of ovulated but non-viable and ruptured eggs resulting possibly, from hydration of ovulated oocytes in overmatured females.

In vitro maturation experiments in the zebra fish have indicated that the oocytes need to have achieved a particular size before the maturation process can be activated. From the previous chapters it is evident that the growth of the oocytes is dependent on the process of vitellogenesis, therefore those oocytes that respond to the steroidal maturation must be vitellogenic or post-vitellogenic oocytes, which is also suggestive that the developmental stage and the size of the oocytes is relevant to the maturation process.

Spontaneous ovulation has been observed *in vitro* studies on the oocytes of yellow perch (Goetz 1983) and medaka (Iwamatshu 1974, Hirose 1971) but very few of the oocytes undergo spontaneous meiotic maturation. This is contrary to the observation with the oocytes of zebra fish, where 10-20% of the oocytes showed maturation after incubation in a control medium and an increasing percentage of GVBD correlated with an increasing period of incubation. These interspecific difference can be attributed to the duration in the reproductive cycle, zebra fish being a continuous breeder. In a certain number of follicles maturation may have been initiated and the process is continued in the control medium, but ovulation was not observed in these oocytes.

Incubation medium supplemented with 2% BSA increased the number of oocytes undergoing maturation. Similarly van Ree et al.,(1977) found that omission of embryonic extracts or glucose led to decrease in GVBD of zebra fish oocytes. This seems contrary to the study conducted on cat fish (Goswami and Sundararaj,1971) where omission of vitamins, amino acids, FCS, and whole egg ultrafiltrate from the medium failed to affect the maturation process *in vitro*.

Of the progestational steroids tested 17α,20β-P proved to be the most potent and was effective after as little as 10 min exposure time. In a previous study van Ree et al.,(1977) found that progestational steroids were less effective then DOCA in the zebra fish, but they noted that in contrast to 17α,20β-P, DOCA was effective after 20 min exposure time but DOCA was not tested in these studies. The potency of 17α,20β-P is consistent with studies in other species where it has been shown to be

the most effective steroid for final oocyte maturation in most teleosts examined (Goetz,1983), the Atlantic croaker (Trant et al.,1986) being a notable exception.

Vitellogenesis is responsible for the growth of oocytes, which is concomitant with the increase in GSI. Increased GSI is known to elicit reproductive maturation of the females and this study confirms that maximum number of post-vitellogenic and vitellogenic oocytes are present in the fish with higher GSI and maximum maturational response can be evoked in them.

TABLE 5.1. Effect of hCG dose and time of induction on ovulation in zebra fish. Fish were injected with hCG at mentioned time (h) before the time of expected spawning, and were kept isolated at 26°C. Ovulation was assessed by stripping the fish at the onset of light. N represents the number of fish.

Treatment	24 h		12 h		8 h		4 h	
hCG dose IU/gm	N	% of ovulation (N)	N	% of ovulation (N)	N	% of ovulation (N)	N	% of ovulation (N)
1	4	0	5	0	4	0	4	0
5	5	20 (1)	5	20 (1)	5	0	5	0
10	5	40 (2)*	5	60 (3)*	5	100 (5)	5	0
15	4	50 (2)*	5	80 (4)*	5	80 (4)*	4	0
Saline	4	0	4	25 (1)	4	0	4	0

* Fish in the treatment group yielding ruptured non viable eggs

TABLE 5.2. Effect of hCG (10 IU/gm b.w) on spawning in matured and overmatured zebra fish. All the females were injected 8 h before the expected time of spawning, and were either kept isolated or with males. Isolated females were checked for ovulation by stripping and the eggs obtained were artificially fertilised, females with males were allowed to spawn. Viable eggs were assessed after 24 h. N represents the number of fish

Groups	N	Males	% of ovulation	% fertilization
Matured females	4	+[a]	100	92±3
	4	–	75	90±3
Over matured females	4	+	100*	0
	4	–	100*	0

* Ovulated eggs were ruptured and few intact were not viable for fertilization.
[a]. + (present) and – (absent)
Fertilization data expressed as the mean ± S.D.

TABLE 5.3. Effect of oocyte size on *in vitro* maturation (GVBD). Oocytes of different size were cultured in fish Ringers solution at 26°C for 24 h in the presence of progesterone (10 µg/ml). Data analysed using Chi-square analysis ($p<0.05$).

Size of oocytes (µm in diameter)	n*	n GVBD	% of GVBD
300-425	130	0	0
450-575	125	0	0
600-675[a]	135	37	28
700-800[b]	145	94	64
825-900[c]	141	72	51

* represents the number of oocytes.
a,b $p<0.001$
a,c $p<0.001$
b,c $p<0.02$

TABLE 5.4. Effect of culture medium on the maturation and ovulation.
Oocytes of zebra fish were incubated in continuous presence of 10 µg/ml progesterone (P). The number of oocytes (n) ranging from 700µm-900µm in diameter were cultured at 26°C. GVBD was assessed by clearing the oocytes after the incubation period, and ovulation by counting the spent follicular layer in the culture dish. Culture medium (CM) used were Hank's balanced salt solution (HBSS), L-15 medium Leibovitz (L-15), minimum essential medium eagles (MEM) and medium-199 (E199) with BSA or FCS. Data analysed using Chi-square analysis ($p<0.05$).

CM	n	% of GVBD	% Ovulation
HBSS+10%FCS	141	46	5
HBSS+2%BSA[a]	132	51	8
L-15+10% FCS	134	57±	9
L-15+2%BSA[b]	140	65	11
MEM+10%FCS	147	73	19
MEM+2%BSA[c]	132	80	21
E-199+10%FCS	133	64	18
E-199+2%BSA[d]	136	76	20

a,b $p<0.02$; a,c $p<0.001$; a,d $p<0.001$
b,c $p<0.005$; b,d $p<0.02$; c,d no significant difference.

TABLE 5.5. Effect of progesterone (P), 17α,20β-P, and 20β-P on oocytes maturation *in vitro*. Oocytes were cultured in medium MEM+2%BSA in the continuos presence of the steroids and the numbers shown and GVBD noted. Control cultures were incubated in the presence of ethanol not exceeding 0.1% in the culture medium. Data analysed using Chi-square analysis ($p<0.05$).

Treatment	1 µg/ml		5 µg/ml		10 µg/ml		25 µg/ml	
	n	%GVBD	n	%GVBD	n	%GVBD	n	%GVBD
P	131[a]	47	125[e]	51	125[h]	80	129[k]	74
17α,20β-P	140[b]	46	153[f]	73	125[i]	91	150[l]	72
20β-P	140[c]	45	153[g]	70	162[j]	81	149[m]	71
Control	152[d]	21						

Each value represents mean±standard error.
$p>0.05$ not mentioned.
a,d $p<0.001$; b,d $p<0.001$; c,d $p<0.001$
e,f $p<0.001$; e,g $p<0.001$; h,i $p<.008$; i,j $p<0.03$
a,h $p<0.001$; a,k $p<0.001$; e,h $p<0.001$; e,k $p<0.001$
b,f $p<0.001$; b,i $P<0.001$; b,l $P<0.001$; i,l $P<0.001$
c,g $p<0.001$; c,j $p<0.001$, c,m $p<0.001$, g,j $p<0.03$; j,m $p<0.05$

TABLE 5.6. Effect of time of exposure on oocyte maturation to 17α20β-P (10 µg/ml). Oocytes were exposed to the steroid for the time indicated and then transferred to medium without the steroid. Controls were cultured in MEM+2% BSA containing less then 0.1% ethanol. Data analysed using Chi-square analysis ($p<0.05$).

Time of Exposure	Controls		Treated	
	n	% GVBD	n	% GVBD
10 min	121	0	135	88
1 h	143	9.7	129	90
3 h	129	13	140	91
6 h	131	17	131	90
12 h	132	19	142	92
24 h	140	21	136	91

TABLE 5.7. Effect of GSI on *in vitro* maturation and total number of oocytes (700-900 µm in diameter). Individual females were selected and weighed and the ovaries were dissected out and weighed to calculate the GSI. Oocytes greater than 700 µm in diameter were selected and placed in the MEM+2%BSA containing 10 µg/ml of 17α,20β-P.

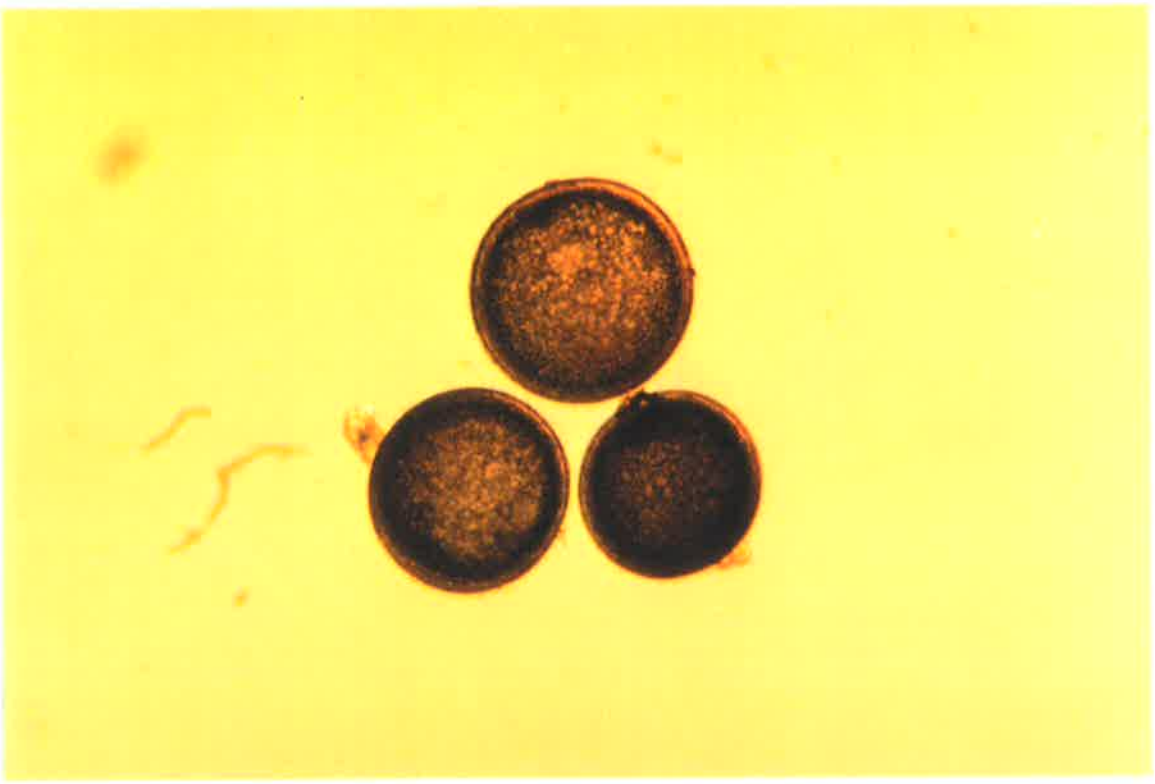
GSI	n*	%GVBD
16 to 19	96	94
13 to 16	35	48

*Average number of oocytes from 6 different females.

TABLE 5.8. Effect of prostaglandin F2α (PGF2α) (5 µg/ml) on ovulation *in vitro* . Oocytes were cultured either in continuos presence of 17α,20β-P (10 µg/ml) and PGF2α. Oocytes matured in the presence of 17α,20β-P were selected (oocytes showing translucent appearance see fig 5.1 bottom) and transferred to medium containing PGF2α. Ovulated oocytes were activated with water or sperm suspension diluted in tank water. Activation was assessed by the formation of animal pole or hydration of the oocyte.

Treatment	% Ovulation	% Activation
17α,20β-P+PGF2α	25	20
PGF2α	27	23
Control	0	0

Figure 5.1. Zebra fish oocytes, before and after *in vitro* maturation. Top: Freshly isolated oocytes of zebra fish showing the dark and opaque appearance of immature oocytes (400µm -700µm in diameter). Position of the GV is difficult to locate due to the opacity. **Bottom:** Oocytes cultured in the presence of steroids showing typical appearance of immature (opaque) and matured (translucent) oocytes, these characteristics was used to select the matured oocytes after treatment.



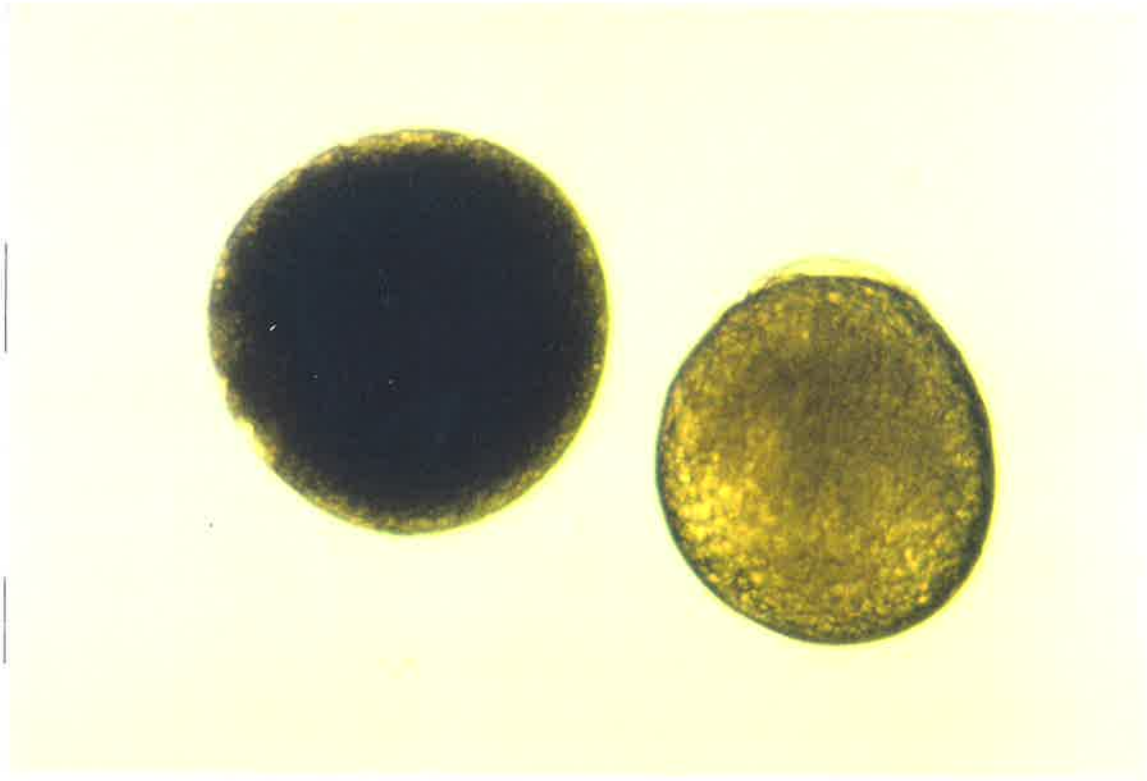
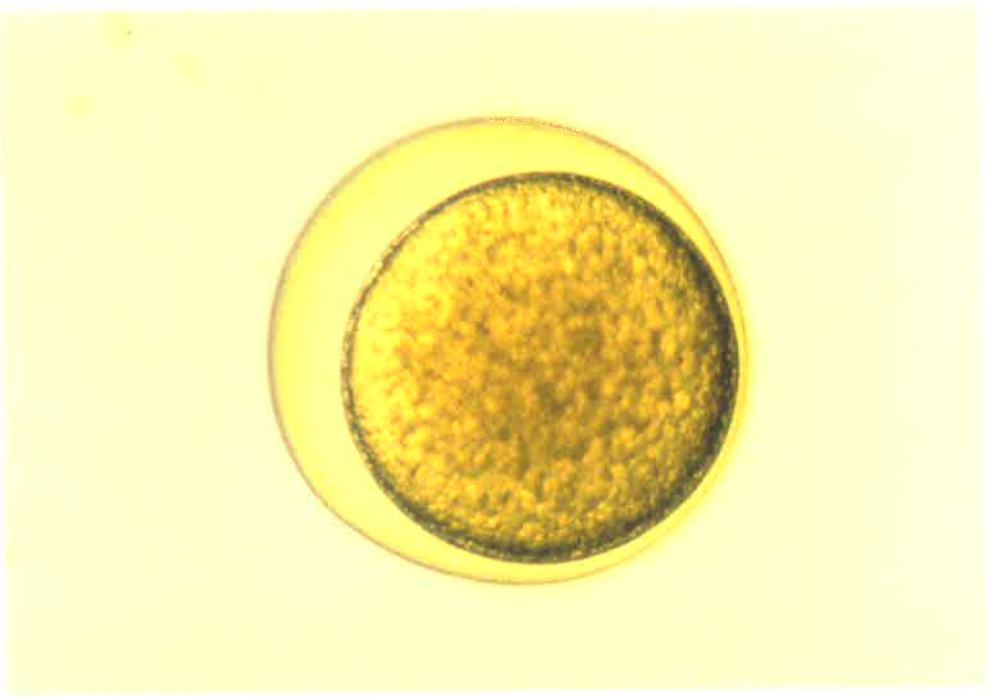
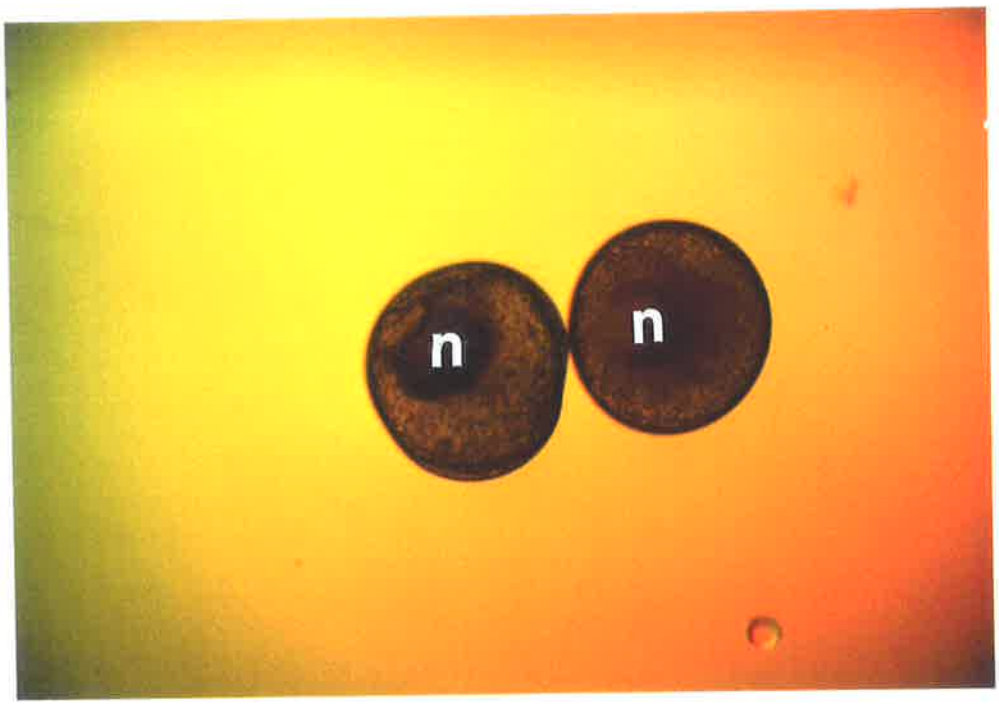


Figure 5.2. **Activated and immature oocytes of zebra fish: Top:** A typical ovulated oocyte showing activation after being placed in water, the transparent chorion layer becomes distinctly separated from the yolk mass. **Bottom:** Immature oocytes showing the presence of GV (n) after incubating in the clearing solution (Methanol:Acetic acid:Formaldehyde; 6:3:1). GV appears as a dark brown spot.

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CHAPTER 6

6. GENERAL DISCUSSION AND CONCLUSIONS

Vitellogenesis is an important integral component of the reproductive physiology of all oviparous vertebrates and is necessary to ensure that adequate nutritive material is packed into the oocyte before ovulation.

These studies confirm that, as with other teleosts, in the zebra fish Vg is the major protein synthesised by the liver in response to estradiol-17β, and that, consistent with Baileys (1957) hypothesis, following synthesis, Vg serves as the major yolk protein and is secreted into the plasma to be sequestered into the growing oocytes.

The biochemical information concerning Vg synthesis in a range of other species most notably *Xenopus* and the chicken clearly indicate that Vg is subjected to a great deal of posttranslational modifications in the liver before it is finally secreted into the bloodstream. Vg is characteristically phosphorylated, but generally to a lesser extent in teleosts than in the other vertebrates (Mommesen and Walsh 1988), presumably reflecting a lower serine content. However we have found that the serine content of Vg in the zebra fish to be significantly higher than most of the other teleosts studied, and is more like that of the amphibian and chicken suggesting a similar high level of phosphorylation. If zebra fish Vg is highly phosphorylated it may be speculated that measurement of alkaline-labile protein phosphorous in serine could be used to provide an index of the degree of Vg synthesis in this species as in certain other teleosts such

as the flounder (Emmersen and Petersen 1976) and catfish (Nath and Sundararaj 1981).

The dosage of 4 μg E_2/gm body weight used to induce the production of Vg in these studies, was sufficient to evoke a significant increase in both the male and female zebra fish. Dose levels used in other teleost studies (Nath and Sundararaj 1981; Silversand and Haux 1989; Bradley and Grizzle 1989) were much higher and had different regime of sampling. However when compared to the study with tilapia (Ding et al. 1989) where a similar hormonal induction regime led to the doubling of the total serum protein content on the eighth dayof treatment, in the female zebra fish, the serum Vg level was doubled in 24 hrs after a single administration of E_2 compared to the controls.

Vg has been previously purified for a number of teleost species utilising simple precipitation methods followed by chromatographic procedures (see 1.4.1). The equally simple procedure developed in this study has proven to be robust and reliable and yields pure and undegraded preparations of Vg as verified by amino acid analysis and electrophoresis. Past reports on the absence of Vg in the male oviparous vertebrates have attributed to the general consensus that Vg is a female-specific protein. However findings of this protein in the low quantities in the male serum (Ding et al. 1989; Goodwin et al. 1992) and from E_2 stimulated males (Sumpter 1985; Carnevelli et al 1991) has proved otherwise. Although Vg has been purified from the serum of E2 stimulated male teleosts (Maitre et al. 1985; Campbell and Idler 1980; Le Guellec et al. 1988), in these studies, we report isolation and purification of Vg in E_2 stimulated male gonads as verified by western blotting analysis and immunodiffusion, unreported in any other teleost studies.

Antiserum prepared against the Vg purified from the liver provided a ready identification of the protein in the serum and gonads and allowed the effects of the E_2 stimulation to be studied. Ovarian extracts of matured zebra fish showed two major immunogenic polypeptides tentatively identified as lipovitellin and phosvitin by using western ligand blotting methods. This observation suggests that in this species the

proteolytic cleavage which accompanies the transformation of Vg into lipovitellin and phosvitin leaves many important antigenic determinants intact. This is in contrast to the experience gained with the whitespotted charr (Kwon et al., 1990) and rainbow trout (Hara and Hirai, 1978) where phosvitin could not be detected immunologically using polyvalent antibody raised against lipovitellin, indicating differing antigenic determinants of the two products. This seems to be in agreement with the data of Tata et al. (1979) on the existence of distinct genes responsible for the transcription of the different yolk proteins.

The high degree of species specificity is an interesting feature of the teleost Vg,. Campbell and Idler (1980) reported the specificity in the species as closely related as atlantic salmon and rainbow trout, and this was further confirmed in the radioimmunoassay for atlantic salmon where no parallel displacement was given by the plasma from three species of pacific salmon (chinook, sockeye and chum), rainbow trout and winter flounder. Redshaw and Follett (1976) reported the specificity of a radioimmunoassay for bird vitellogenin, the antiserum to fowl Vg showed no cross-reaction to sera from vitellogenic fish, amphibians or reptiles. Similarly antiserum to zebra fish Vg in this study was immunologically distinct showing no cross-reaction with the sera of the representative vitellogenic fish (medaka), reptile (marbled gecko) and bird (chicken) studied. In previous studies tetrapod, amphibian and reptilian, Vgs have been shown to bear both common and species specific antigenic determinants while carp Vg was found to be immunologically distinct (Carnevali and Belvedere 1991). Vertebrate Vgs, therefore, appear to contain significantly divergent immunodomains. This discrepancy may be due to the extensive post-translational modification of Vg which may vary in different species with dissimilar prosthetic groups, thus allowing immunological divergence more likely dependent on the tertiary, spatial configuration of the molecule rather than on the primary structure.

Liver was recognised as the only organ responding to estrogen by synthesising and secreting Vg. The development of procedure allowing the isolation and

establishment of hepatocytes in cultures is deemed as a necessary prerequisite to study cellular factors regulating the activation and expression of Vg gene. For the isolation of the zebra fish hepatocytes, we were unable to use the *in situ* perfusion technique which has been widely employed for the mammalian, avian, amphibian and teleost liver due to the small size of the zebra fish liver. However, the alternative method employed proved equally efficient and resulted in high yield of isolated viable hepatocytes. An improved method to ensure attachment of the cells to the culture vessel was also needed and coating the wells with gelatin proved useful for this purpose. The addition of insulin to the serum free medium proved necessary to maintain the protein concentration and the cell number constant, possibly acting in its own right and substituting for other growth factors such as IGF-1. Hepatocyte cultures were carried out in an air atmosphere. Inclusion of 5% CO_2 proved inhibitory as previously noted with coho salmon embryonic cell (Fryer et al. 1965).

The hepatocyte cultures retained their capacity to respond to E_2 and were found to be dose dependent. Secretion in the culture medium of the synthesised protein occurred after a lag period of 3 h after E_2 stimulation . Under continuous E_2 stimulation the concentration of Vg increased until it became the major protein synthesised and secreted by the hepatocytes. These data clearly show that estrogen is required to allow for hepatocytes to express the Vg gene, however, the precise mechanism of steroid action and other factors which modulate Vg expression at the cellular level need to be elucidated.

The ovulation induction study demonstrated that hCG can be used to induce maturation, ovulation and spawning in zebra fish when the fish are maintained under optimal conditions. Teleost fish have been previously shown to be responsive to various gonadotrophin preparations from different sources, with homologous hormones being most active. However in the absence of a pure zebra fish gonadotrophin preparation, hCG is to be recommended as a good substitute in inducing spawning.

The *in vitro* maturation (IVM) studies carried out with zebra fish oocytes indicate that the progestins act directly on the oocytes to induce maturation. Of the progestins tested 17α,20β-P proved to be the most effective and is a strong contender as the naturally occurring MIS in the zebra fish as in other species. The size of the oocytes selected for IVM was critical to invoke the maximum response to the steroid, inferring that only fully grown oocytes need to undergo hormonal stimulation then final maturation.

The study on the relationship of GSI to final oocyte maturation in the zebra fish, indicated that GSI increased in the teleost undergoing vitellogenesis and may be used as an indicator of the degree of maturation. Post-vitellogenic and vitellogenic oocytes were more prevalent in the fish with a GSI of more then sixteen percent. Oocytes collected from fish with the higher GSI gave up to 90% maturation rate when cultured in presence of 17α,20β-P as opposed 50% in fish with GSI less then 16%.

The availability of the purified Vg, antiserum, and hepatocyte cultures opens many fields for research into vitellogenesis in the zebra fish, most notably the hepatic events concerning the molecular biology, and the regulation and cellular control of the enzymatic machinery involved in the uptake, and breakdown of vitellogenin in the oocytes. With the increasing utilisation of the zebra fish as a model in vertebrate developmental embryology (Concordet and Ingam 1994) such tools will provide basis for obtaining useful information , and allow valuable insights from the comparative and evolutionary viewpoints.

CHAPTER 7

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