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**Vitellogenin and vitellogenesis in greenback  
flounder *Rhombosolea tapirina***

Sun Biao, B.Sc.

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School of Aquaculture  
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## **Declaration**

This thesis contains no material which has been accepted for the award of any higher degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

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## Co-authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

- Paper 1 Sun, B., Pankhurst, N.W., Watts, M., 2004. Development of an enzyme-linked immunosorbent assay (ELISA) for vitellogenin measurement in greenback flounder *Rhombosolea tapirina*. Fish Physiology and Biochemistry (in press).
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- Pankhurst, N.W. assisted with guidance and general supervision in all aspects of producing publishable quality manuscripts, including experimental design, interpretation of data, and writing and proof reading of manuscripts
  - Watts, M. technologically assisted with purification of vitellogenin and development of vitellogenin ELISA

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed



Prof. Ned. Pankhurst

5/7/04

Signed



Prof. Chris Carter

14.07.04

Supervisor

School of Aquaculture

Tasmanian Aquaculture and Fisheries  
Institute

University of Tasmania

Locked Bag 1370

Launceston TAS 7250, Australia

Head of School

School of Aquaculture

Tasmanian Aquaculture and Fisheries  
Institute

University of Tasmania

Locked Bag 1370

Launceston TAS 7250, Australia

Current address:

Faculty of Science,

Engineering and Information

Technology

James Cook University

Townsville QLD 4811, Australia.



## Abstract

The overall aim of this study was to obtain knowledge about the yolk precursor, vitellogenin (Vtg) and the process of vitellogenesis in female greenback flounder (*Rhombosolea tapirina*), including purification and characterization of Vtg; development of a specific Vtg assay; induction of Vtg by 17 $\beta$ -estradiol (E<sub>2</sub>) *in vivo* and *in vitro*; relationship between endocrine regulation of vitellogenesis and oocyte growth in mature females; and the possible role of Vtg feedback on ovarian steroid production.

Vtg purified from the plasma of E<sub>2</sub>-treated male animals by gel filtration chromatography had an estimated molecular weight (MW) of ~ 540 kD, and resolved into three bands with estimated MW of 152, 102, 80 kD after SDS-PAGE. Western blotting demonstrated that the Vtg consisted of one subunit (152 kD). A polyclonal antibody against the 152 kD band developed from sheep, was used to establish an indirect antibody-capture competitive enzyme-linked immunosorbent assay (ELISA). The validation of the ELISA was confirmed by the parallelism between the standard Vtg curve and serial dilutions of plasma from vitellogenic females, but no cross-reaction was found with the plasma of males, or plasma from a range of other species.

In E<sub>2</sub>-treated fish, detectable concentrations of plasma Vtg were first found at 48 h, and reached a peak at 96 h post-injection, and increased as the E<sub>2</sub> dose increased, with a threshold of ~ 0.1 mg E<sub>2</sub> Kg body weight<sup>-1</sup>. Multiple injections of E<sub>2</sub> increased the absolute concentrations of Vtg and extended the peak Vtg up to 288 h, but did not change the threshold dose. The time-course and dose-response for induction of Vtg by E<sub>2</sub> in the flounder was similar to that seen in other species, however, plasma concentrations of Vtg were generally lower than those found in other teleosts.

Oocyte size-frequency distributions from mature females showed that this species has multiple clutch group synchronous type of oocyte development. But that reproductive development is not synchronized within the population. Consistent

with this observation, there were no significant seasonal variations in gonadosomatic index ( $I_G$ ), hepatosomatic index ( $I_H$ ), or plasma concentrations of Vtg,  $E_2$  and testosterone (T). Significant increases in  $I_G$ ,  $I_H$  and plasma concentrations of Vtg,  $E_2$  and T were observed in vitellogenic fish, and in fish undergoing final maturation. Plasma concentrations of Vtg and  $E_2$  rose steadily across oocyte sizes from 100 to 450  $\mu\text{m}$ , and both reached a concentration plateau at oocyte sizes of around 450  $\mu\text{m}$ . In contrast, plasma concentrations of T showed no marked increase until oocytes grew beyond 400  $\mu\text{m}$ .

Isolated vitellogenic follicles were incubated with human chorionic gonadotropin (hCG), dibutyryl cyclic AMP (dbcAMP) and gonadal steroid precursors T, 17-hydroxyprogesterone (17P) and androstenedione (A) in the presence or absence of Vtg. High concentrations of Vtg suppressed the production of  $E_2$  and T, and the effect appeared to operate at several levels along the steroidogenic pathways. These observations combined with findings of other studies indicate that Vtg might regulate its own production by modulating ovarian steroid synthesis.

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# **Chapter 1**

## **General introduction**

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# 1. General introduction

## 1.1. Background

There are over 20 000 species of teleosts living in marine and freshwater environments on earth, with widely diverse reproductive strategies and an equally large range of habitats. However, only a very small number of species have been domesticated (reviewed in Wallace, 1987; Tyler and Sumpter, 1996; Pankhurst, 1998). Over recent decades, the global catch of wild fish has been declining and about 75% of wild fisheries are in some degree of depletion. In contrast, average fish consumption per person has almost doubled over the same period. Therefore, aquaculture is considered as the main solution to meet this gap between supply and demand (The Economist, Aug 9, 2003, Vol.368, p.20).

Either in terms of meeting the continuing increase in consumer demand or providing new species for the market, flatfish are becoming important candidates for aquaculture around the world. Cultured flatfish include Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*) in Europe (Methven et al., 1992; Björnsson et al., 1998; Imsland and Jonassen, 2003), Atlantic halibut, yellowtail flounder (*Pleuronectes ferruginea*), southern flounder (*Paralichthys lethostigma*), summer flounder (*Paralichthys dentatus*) and winter flounder (*Pleuronectes americanus*) in North America (Clearwater and Crim, 1998; Burton and Vokey, 2000; Douglas et al., 2000; Kim and Lall, 2000; Merson et al., 2000; Hendry et al., 2002; Luckenbach et al., 2003), and pointhead flounder (*Hippoglossoides pinetorum*), Japanese flounder (*Paralichthys olivaceus*) and starry flounder (*Platichthys stellatus*) in Asia (Furuita et al., 2000; Tominaga et al., 2000; Estévez et al., 2001; Lim et al., 2002; Moon et al., 2003).

In the southern hemisphere, the greenback flounder (*Rhombosolea tapirina* Günther) is the largest and most abundant flounder found in the waters of Australia and New Zealand, and is widespread from New South Wales to southern Western Australia, and around Tasmania (Last, 1983; Kuiter, 1993; Van den

Enden et al., 2000). This species is amongst the best eating fishes found in Australia (Last, 1983; Kuiter, 1993) and exhibits promising potential in the world market (Hart, 1991; Gorman, 1994). However, the capture fishery shows a declining trend over the last decade. For example, the catch of flounders including greenback flounder decreased from over 40 tonnes in 1990-91 to 10 tonnes in 2000-01 in Tasmania (Assessment of recreational fish species – part one, Sea fishing & aquaculture, Primary industry, water & environment, Tasmania), and the catch of greenback flounder and seven other species of flatfish fell to 2963 tonnes in 1999-2000 in New Zealand, the lowest catch on record since 1986-87 (Summary of the assessments of the sustainability of current TACCs and recent catch levels and status of the stocks for the 2003-04 fishing years, Environment & sustainability, Ministry of Fisheries, New Zealand).

A series of studies has been conducted to understand the mechanisms of endocrine control of reproduction in this species including assessment of reproductive and endocrine events in wild males and females (Barnett and Pankhurst, 1999) and the use of exogenous hormones to manipulate oocyte development and ovulation in females (Poortenaar and Pankhurst, 2000 a, b, c) and to control spermiation in males (Pankhurst and Poortenaar, 2000) respectively. The acquisition of high quality eggs is a prerequisite for successful propagation of any aquaculture species (reviewed in Pankhurst, 1998). In female teleosts, vitellogenesis is a major event responsible for the growth of oocytes and may account for over 90% of the final egg size in some species (reviewed in Specker and Sullivan, 1994; Tyler et al., 2000). However, knowledge of this process in fishes is mainly based on studies of salmonids (reviewed in Tyler, 1991; Tyler and sumper, 1996). As each species has its own particular reproductive pattern (reviewed in Pankhurst and Carragher, 1991), a thorough investigation of this process in greenback flounder will be essential for both the understanding of endocrine control of vitellogenesis in association with oocyte growth in this species, and for providing new information on vitellogenesis in teleosts generally.

## **1.2. Vitellogenin (Vtg)**

### 1.2.1. *Vtg* gene and multiple forms of *Vtg*

The *Vtg* gene belongs to a small gene family in oviparous vertebrates and invertebrates (reviewed in Wahli, 1988; Lazier and MacKay, 1993). The *Vtg* gene in vertebrates contains two domains encoding the heavy and light chains of the lipovitellins (Lv-I and Lv-II) and one domain encoding phosvitin (Pv) (reviewed in Specker and Sullivan, 1994). The first complete structure of a teleost *Vtg* gene was identified in rainbow trout (*Oncorhynchus mykiss*), which showed a high conservation compared with the *Vtg* gene of other vertebrates except for the lack of a single exon but little similarity in the number and organization of introns compared to that of invertebrates (Mouchel et al., 1997). More recently, a complete *Vtg* cDNA was cloned from tilapia (*Oreochromis aureus*), which contained a large Pv domain compared to *Vtgs* in other fishes (Lim et al., 2001).

Two forms of *Vtg* have been found in various species of tilapia e.g. *Oreochromis aureus* (Ding et al., 1989), *Oreochromis mossambicus* (Kishida and Specker, 1993; Kim and Takemura, 2003) and *Oreochromis niloticus* (Buerano et al., 1995), and white sturgeon (*Acipenser transmontanus*) (Bidwell et al., 1991), black porgy (*Acanthopagrus schlegeli*) (Chang et al., 1996), barfin flounder (*Verasper moseri*) (Matsubara et al., 1999), Indian major carp (*Cirrhinus mrigala*) (Nath and Maitra, 2001), haddock (*Melanogrammus aeglefinus*) (Reith et al., 2001), medaka (*Oryzias latipes*) (Shimizu et al., 2002), Japanese common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2003), and three forms of *Vtg* were identified in perch (*Morone americana*) (Hiramatsu et al., 2002c). The presence of multiple forms of *Vtgs* in plasma can occur for several reasons; different *Vtg* mRNAs such as in haddock (Reith et al., 2001); the same *Vtg* mRNA but different variations after post-translational modification as in white sturgeon (Bidwell et al., 1991); the same *Vtg* protein exists but as a dimer or a monomer in plasma as in black porgy (Chang et al., 1996) and river lamprey (*Lampetra fluviatilis*) (Mewes et al., 2002); and finally, differences in identification methods. For example, in tilapia (*Oreochromis niloticus*), two forms of *Vtg* were found in the study by Burerano et al. (1995) but only one of them was found in studies by Lee et al. (1992) and Chan et al. (1991) in the same species. The difference appeared to result from

different concentrations of acrylamide used in native polyacrylamide gel electrophoresis (PAGE) (Burerano et al., 1995).

### ***1.2.2. Biochemical characteristics of Vtg***

Vtg is a large glycolipophosphoprotein binding divalent cations (predominantly calcium) in teleosts, which widely exists as a dimer in the blood, with a molecular weight (MW) ranging from 300-640 KD in different species (reviewed in Mommsen and Walsh, 1988; Specker and Sullivan, 1994; Tyler et al., 2000). It should be noted that the MW of Vtg can be strongly affected by the estimation method used e.g. the MW of native Vtg in rainbow trout ranges from 340 to 600 kD in different studies (Mommsen and Walsh, 1988). The number and MW of Vtg subunits is variable among species, and multiple subunits have been found in some fish which, as noted in the previous section, is attributable to the presence of different Vtg genes, post-translational modification and proteolytical breakdown during handling (Lee et al., 1992, and reviewed in Mommsen and Walsh, 1988; Specker and Sullivan, 1994).

A comparison of amino acid composition of Vtgs among teleost species has been made in several studies (e.g. Silversand and Haux, 1989; Tyler and Sumpter, 1990a; Mañanós et al., 1994b; Roubal et al., 1997; Heppell and Sullivan, 1999; Parks et al., 1999), which suggested that the amino acid composition was highly conserved. The content of phosphorus in fish Vtg is around 0.6-0.7% (Norberg and Haux, 1985; Mommsen and Walsh, 1988; Norberg, 1995), which is lower compared with 1.4% in Vtg of the amphibian, *Xenopus laevis* (Wallace, 1985). The phosphorylation also accounts for a negative surface charge on the Vtg molecule (Mommsen and Walsh, 1988). In contrast, fish Vtg contains about 20% lipid (Norberg and Haux, 1985; Mommsen and Walsh, 1988; Matsubara and Sawano, 1995; Norberg, 1995) which is higher than that (12%) in *Xenopus laevis* (Wallace, 1985).

### ***1.2.3. Isolation of Vtg***



Fish Vtgs are high molecular weight proteins that contain a polypeptide backbone, carbohydrates, lipids, phosphate and ions e.g. calcium, magnesium and iron (reviewed in Mommsen and Walsh, 1988), which makes it possible to devise different techniques and procedures to isolate Vtg from plasma, including gel chromatography (Emmersen and Petersen, 1976), affinity chromatography (Covens et al., 1988), anion exchange chromatography (Silversand et al., 1993), hydroxylapatite column chromatography (Fukada et al., 2003) and membrane chromatography (Shi et al., 2003). In some studies, a purified Vtg can be obtained only by one procedure (e.g. Emmersen and Petersen, 1976; Covens et al., 1988; Silversand et al., 1993; Chang et al., 1996; Shi et al., 2003) whereas others need a combination of different techniques (e.g. Kishida and Specker, 1993; Roubal et al., 1997; Brion et al., 2000; Fukada et al., 2003). In some species, selective chemical precipitation has been used as a preliminary step to enrich Vtg concentration in plasma samples before isolation (Ding et al., 1989; Buerano et al., 1995).

Teleost Vtgs are highly susceptible to proteolytic degradation (Silversand et al., 1993). Normally, the whole purification procedure is performed at 4°C in the presence of a protease inhibitor e.g. phenylmethylsulfonyl fluoride (PMSF) or Aprotinin. Even with this precaution, proteolysis of Vtg could not be completely avoided in any prolonged handling of plasma (Kishida et al., 1992; Silversand et al., 1993). Some studies demonstrated that additional isolation procedures did not consequently improve the purity of Vtg (Kishida et al., 1992; Sehgal and Goswami, 2001). In contrast, further treatment might result in either the aggregation of different forms of Vtg (Ding et al., 1989) or the increase of Vtg proteolysis (Kishida et al., 1992). Both would complicate the understanding of vitellogenesis for each particular species.

#### ***1.2.4. Detection of Vtg***

Plasma concentrations of Vtg normally show a linear relationship with plasma content of calcium and phosphoprotein phosphorus during vitellogenesis (Emmersen and Petersen, 1976; Johnson et al., 1991; Madsen et al., 1997;

Verslycke et al., 2002; Linares-Casenave et al., 2003). Both plasma concentrations of calcium and phosphoprotein phosphorus have been used as indirect indicators of plasma Vtg concentrations in various studies (Emmersen and Petersen, 1976; Tinsley et al., 1985; Nagler et al., 1987; Bidwell et al., 1991; Johnson et al., 1991; Luizi et al., 1997; Rinchard et al., 1997; Roberts et al., 1999; Verslycke et al., 2002). However, some research suggested that their profiles did not always represent the real changes of plasma Vtg levels (Tinsley et al., 1985; Nagler et al., 1987). Several direct immunoassays have been developed for detecting of Vtg in teleosts e.g. rocket immunoelectrophoresis (Ding et al., 1989), single radial immunodiffusion (Tao et al., 1993; Hoque et al., 1998), radioimmunoassay (Norberg and Haux, 1988; Tyler and Sumpter, 1990b), enzyme-linked immunosorbent assay (ELISA) (reviewed in Specker and Sullivan, 1994), immunoblotting and densitometric scanning (Tolar et al., 2001) and chemiluminescent immunoassay (Fukada et al., 2001; Fukada et al., 2003).

Despite the range of methods available, the most common technique for Vtg measurement is by ELISA (reviewed in Specker and Sullivan, 1994). There are three basic types of ELISA i.e. sandwich ELISA, antibody-capture competitive ELISA and antigen-capture competitive ELISA. The sensitivity of ELISAs can be further improved by using enzyme cascades (Specker and Anderson, 1994). Various ELISAs have been established for teleost Vtgs and used widely to measure Vtg in plasma (reviewed in Specker and Sullivan, 1994), mucus (Kishida et al., 1992; Chang et al., 1996), muscle tissue or whole body homogenate (Heppell and Sullivan, 2000; Fenska et al., 2001; Holbech et al., 2001; Mylchreest et al., 2003) and culture medium (Takemura and Kim, 2001; Kim et al., 2003).

The establishment of an ELISA rests on the development of a specific antibody to Vtg for each species, which is usually derived from the plasma of animals injected with putative purified Vtg fractions after chromatography (Nunez Rodriguez et al., 1989; Mañanós et al., 1994a; Bon et al., 1997; Korsgaard and Pedersen, 1998; Mosconi et al., 1998; Johnsen et al., 1999; Fenska et al., 2001; Schafhauser-Smith and Benfey, 2002). However, the specificity of the antibody is dependent on the purity of Vtg fractions but the possibility of cross-reactivity with other serum

proteins cannot be excluded (Schafhauser-Smith and Benfey, 2002). To address this, pre-incubation of the antibody with male plasma (Nunez Rodriguze et al., 1989; Bon et al., 1997; Lomax et al., 1998; Parks et al., 1999) or further purification of the antibody by affinity chromatography (Korsgaard and Pedersen, 1998; Schafhauser-Smith and Benfey, 2002) was used to reduce or eliminate such cross-reactivity in several studies. Because of the common instability of Vtg among species (Silversand et al., 1993) and the limited plasma sample size in small fish e.g. zebrafish (Holbech et al., 2001), lipovitellin (Lv) has been purified from yolk protein and used to set up ELISAs for measurement of Vtg as well (Perez and Callard, 1993; Okumura et al., 1995; Hartling et al., 1997; Hiramatsu et al., 1997; Holbech et al., 2001).

### **1.3. Endocrine regulation of Vitellogenesis**

#### ***1.3.1. Steroidogenesis***

The endocrine regulation of reproduction is controlled via the hypothalamic-pituitary-gonadal axis, which is triggered by environmental factors such as photoperiod and water temperature. In response, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) (reviewed in Yu et al., 1997). During vitellogenesis, follicle-stimulating hormone (FSH) is released from the pituitary under the stimulation of GnRH, and binds to the FSH receptor located on the thecal cell of the ovarian follicle. Subsequently, FSH-receptor binding activates the *de novo* synthesis of testosterone (T) through a cyclic AMP/protein kinase A system (reviewed in Nagahama et al., 1995). T diffuses into the granulosa layer of the follicle where it is aromatised by cytochrome P450aromatase (P450arom) to 17 $\beta$ -estradiol (E<sub>2</sub>), which is in turn released to the circulation (reviewed in Nagahama et al., 1995; Pankhurst, 1998) (Fig. 1.1 on next page (not one after)).

#### ***1.3.2. Synthesis of Vtg in hepatocytes***

In the bloodstream, E<sub>2</sub> is carried to the liver attached to steroid-binding proteins (SBP), which serve to protect plasma-borne E<sub>2</sub> from conjugation and subsequent excretion (reviewed in Hobby et al., 2000). In the liver, E<sub>2</sub> combines with the

estrogen receptor (ER) after diffusing into hepatocytes. The E<sub>2</sub>-ER complex enters the nucleus and binds to estrogen response elements upstream of the Vtg gene, subsequently triggering its transcription. Vtg mRNA is transferred to the rough endoplasmatic reticulum (RER) in the cytosol where the Vtg protein is synthesized. After modification including lipidation, glycosylation and phosphorylation in the RER and packaging in the Golgi, Vtg is excreted into the bloodstream where it circulates as a dimer (reviewed in Mommsen and Walsh, 1988; Lazier and MacKay, 1993) (Fig. 1.1).

### ***1.3.3. Incorporation of Vtg into yolk proteins***

In the ovary, Vtg is selectively sequestered into growing oocytes via receptor-mediated endocytosis (reviewed in Specker and Sullivan, 1994) and FSH is involved in this process (Tyler et al., 1991b). The proteolytic cleavage of Vtg into yolk proteins occurs in the presence of enzymes from lysosomes (Mommsen and Walsh, 1988). Recently, cathepsin B, D and L were demonstrated to be involved in the processing of yolk proteins and their activities showed a vitellogenic stage-dependent relationship (Carnevali et al., 1999; Kwon et al., 2001). In oocytes, the Vtg-derived yolk proteins contain Lv and Pv (reviewed in Mommsen and Walsh, 1988), and a third yolk protein ( $\beta'$ -component) has also been identified in salmonids (Specker and Sullivan, 1994), barfin flounder (Matsubara and Sawano, 1995), white perch (*Morone americana*) (Hiramatsu et al., 2002a) and bester (*Huso huso x Acipenser ruthenus*) (Hiramatsu et al., 2002b). Recently, a fourth yolk protein by name C-terminal component has been confirmed in barfin flounder (Matsubara et al., 2003) (Fig. 1.1).

The cleavage of Vtg into yolk proteins has several significant effects on oocyte growth and maturation; i.e. providing nutrition sources for the sequent development of the embryo; generating osmotic pressure during oocyte maturation to produce hydration; as a major source of lipid in teleost eggs, and for transmission of other components e.g. minerals, vitamins and hormones into eggs (Silversand et al., 1996 and reviewed in Specker and Sullivan, 1994; Tyler et al., 2000).

### 1.4. Vitellogenesis induction *in vivo* and *in vitro*

Vtg is a female-specific protein that normally is present in the plasma of mature females. However, male and juvenile fishes do have the Vtg gene which is normally not expressed because of low estrogen titers in their bloodstream, but can be triggered by treatment with, or exposure to exogenous estrogens (reviewed in Specker and Sullivan, 1994; Tyler et al., 2000).

In the absence of ovarian tissue, induced Vtg can accumulate to high concentrations (Emmersen and Petersen, 1976; Chang et al., 1996) and be maintained for a long time in the plasma of males (Ding et al., 1989; Luizi et al., 1997; Sherry et al., 1999). This has led to the wide use of Vtg induction in male or immature fish to obtain Vtg for purification and characterization and to subsequently establish Vtg ELISAs. The close correlation between plasma Vtg and  $E_2$  also is helpful in investigating the kinetics of Vtg induction by exogenous  $E_2$  in relation to the time-course and dose-response in various species (Celius and Walther, 1998; Lomax et al., 1998; Panter et al., 1998; Bowman et al., 2000; Arukwe et al., 2001; Calson and Williams, 2001). Some environmental pollutants can interfere with the normal reproductive systems of fish by mimicking the effects of natural  $E_2$  through binding with ERs, and the abnormal presence of Vtg in male plasma can also be used as a sensitive bio-indicator to monitor natural or man-made estrogenic substances in the aquatic environment (reviewed in Sumpter and Jobling, 1995; Tyler et al., 2000).

Hepatocyte culture includes the isolation of viable hepatocytes from the liver, and their transfer to an artificial environment in which the cells may continue to survive and function (reviewed Segner, 1998). Compared with *in vivo* systems for assessing the estrogenic effects of various chemicals, *in vitro* hepatocyte culture has been considered as a useful approach with some advantages such as simplification of experimental conditions, consistency between experiments, and being cheap and rapid to perform (reviewed in Baksi and Frazier, 1990; Segner, 1998). This method has been employed to investigate factors regulating

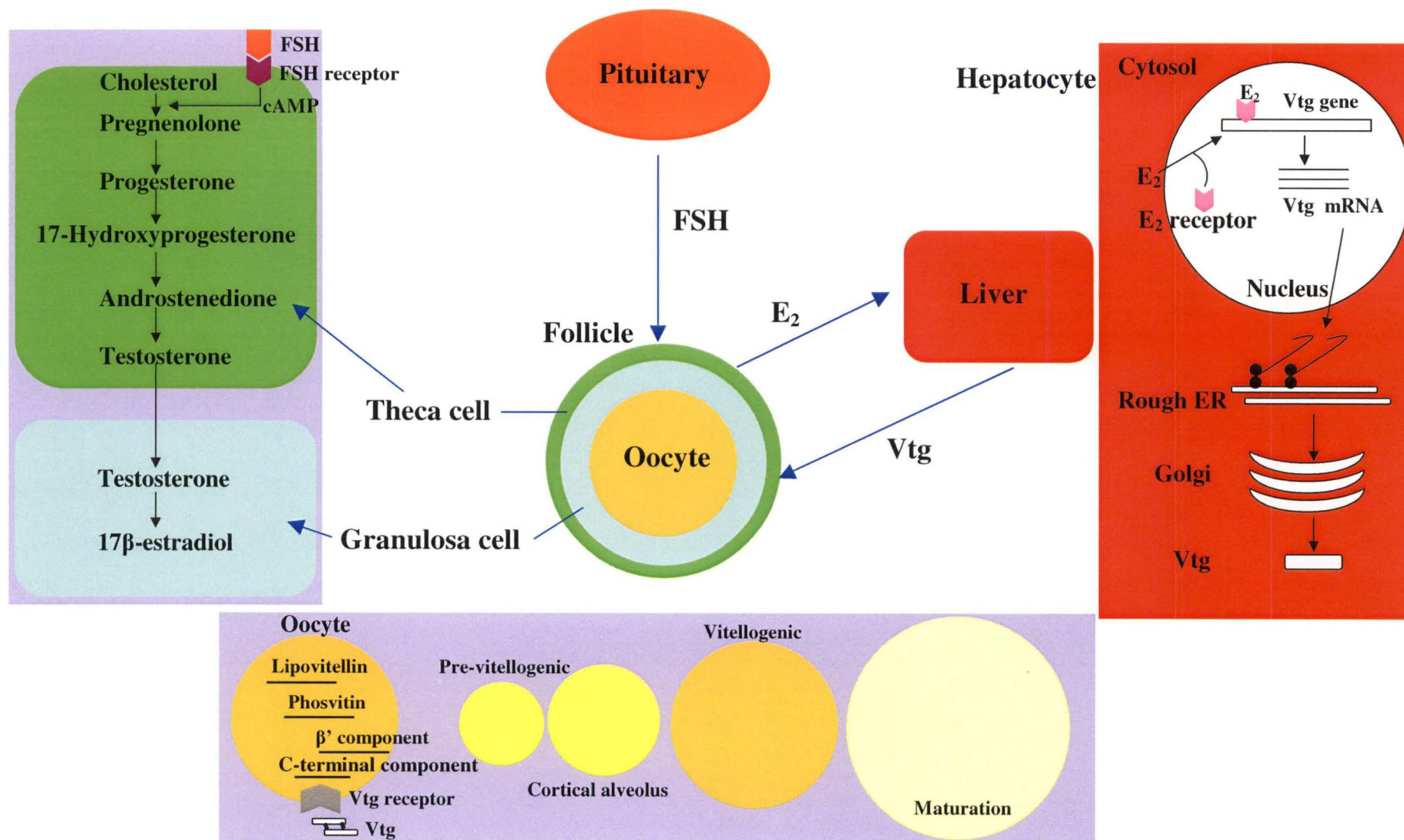


Fig. 1.1. Diagram of endocrine regulation of vitellogenesis (based on Lazier and MacKay, 1993 and Kagawa, 1994).

vitellogenesis in a number of species (Kwon et al., 1993; Kwon and Mugiya, 1994; Mori et al., 1998; Kim et al., 2003).

## **1.5. Vitellogenesis and Oocyte growth**

### ***1.5.1. Patterns of oocyte growth***

In teleosts, the patterns of oocyte growth have been classified into synchronous ovarian growth where there is only one clutch of oocytes in the ovary and the fish spawns once in a lifetime, group synchronous ovarian growth where at least two clutches of oocytes are present in the ovary and spawning occurs once per season, and multiple clutch, group synchronous or asynchronous ovarian growth where several clutches of oocytes are found in the ovary at once, and there are multiple spawning events over a reproductive season (reviewed in Wallace et al., 1987; Pankhurst, 1998).

### ***1.5.2. Oocyte growth***

The process of oocyte growth is normally divided into four main stages, primary growth, cortical alveoli stage, vitellogenesis and final maturation as reviewed in Wallace et al. (1987), Selman and Wallace (1989), and Tyler and Sumpter (1996). However, these authors emphasise that the nomenclature did not imply the sequential displacement of different stages one by one. In fact, in some species especially multiple spawners, the overlap of different ovarian stages was observed in one ovary (Pankhurst and Conroy, 1987; Johnson et al., 1991; Scott and Pankhurst, 1992; Clearwater and Pankhurst, 1994; Berlinsky et al., 1995). The incorporation of Vtg in oocytes does not start until oocytes reach a certain size (Tyler et al., 1991a). According to the appearance of yolk proteins in oocytes, the growth of oocytes is also divided into endogenous and exogenous vitellogenesis (e.g. Pankhurst and Conroy, 1987; Kestemont et al., 1999; Kokokoiris et al., 2000). However, this kind of classification has subsequently been questioned by some researchers, where vitellogenesis is considered to be totally exogenous and

is transferred from a phase dominated by lipid accumulation to a phase dominated by Vtg deposition (reviewed in Le Menn et al., 2000).

As yolk proteins contribute more than 90% of the content of the egg in some species, vitellogenesis represents a major event of oocyte growth (Tyler et al., 2000). This is accompanied by a simultaneous increase of metabolic activity in the liver and the ovary. Gonadosomatic index ( $I_G$ ), hepatosomatic index ( $I_H$ ) and plasma concentrations of Vtg and steroids (mainly  $E_2$  and T) are, therefore, considered as useful indicators for assessment of the ovarian development. These parameters have been extensively employed to describe reproductive events in teleosts and a positive relationship is observed between them and the growth of oocytes (Pankhurst and Conroy, 1987; Johnson et al., 1991; Scott and Pankhurst, 1992; Clearwater and Pankhurst, 1994; Thomas et al., 1994; Rinchard et al., 1997; Roberts et al., 1999; Kokokoiris et al., 2000; Kokokiris et al., 2001; Schafhauser-Smith and Benfey, 2002; King and Pankhurst, 2003).

#### ***1.5.3. Vtg feedback on ovarian steroid production***

In some multiple spawning species, a high concentration of plasma  $E_2$  was only present at the beginning of vitellogenesis despite vitellogenesis occurring over an extended period (Methven et al., 1992; Clearwater and Pankhurst, 1994; Koya et al., 2003). Studies by Resi-Henriques et al. (1997) and Lee et al. (2000) also suggested that high plasma concentrations of Vtg suppressed ovarian  $E_2$  production. An *in vitro* study in rainbow trout (Resi-Henriques et al., 1997, 2000) demonstrated that Vtg in the culture medium inhibited the activity of steroid-converting enzymes in follicles. All of these results suggest that Vtg may regulate its own production by modifying ovarian steroid production.

### **1.6. The present study**

Because of the limited information on vitellogenesis in flatfish generally and the variation of reproductive strategies among different species, it was considered



necessary to examine this process in greenback flounder. Accordingly, experiments were conducted with the following specific aims:

1. Purification and characterization of Vtg of greenback flounder, and establishment of Vtg ELISA for this species.

Gel chromatography was used to isolate Vtg from the plasma of E<sub>2</sub>-treated males. This method was chosen because it is simple and rapid, and has been successfully used to isolate Vtgs of high purity from rainbow trout and Atlantic salmon (*Salmo salar*) in a previous study (Watts et al., 2003). The highest MW band of putative Vtg after SDS-PAGE was excised and used as an antigen to produce a polyclonal antibody for use in ELISA. Subsequently, a competitive ELISA was developed using previously established techniques (Watts et al., 2003).

2. Investigation of the induction of vitellogenesis *in vivo* using male flounder, and *in vitro* by using hepatocytes from male and female flounder.

Various *in vivo* and *in vitro* studies have demonstrated that Vtg production induced by E<sub>2</sub> shows a time- and dose-dependent relationship (Bowman et al., 2000; Arukwe et al., 2001; Takemura and Kim, 2001). The purpose of the present study was to determine the role of E<sub>2</sub> in the synthesis of Vtg in regard to time-course and dose (threshold)-response in greenback flounder. This was then further investigated using an *in vitro* hepatocyte system.

3. Definition of the relationship between the endocrine regulation of vitellogenesis, and oocyte growth during the seasonal reproduction of females.

Vitellogenesis is one of the most significant event during oocyte growth and is very important for a successful reproduction (Tyler et al., 2000). However, knowledge of this process is still far from complete in greenback flounder

(Barnett and Pankhurst, 1999). Plasma concentrations of Vtg and gonadal steroids ( $E_2$  and T) are sensitive endocrine indicators of oocyte growth (Methven et al., 1992). In this study, the correlations between ovarian development and plasma concentrations of Vtg,  $E_2$  and T were investigated to further our understanding of endocrine control of reproduction in greenback flounder.

4. Investigation of the feedback effect of Vtg on ovarian  $E_2$  synthesis *in vitro* using isolated ovarian follicles.

Treatment of vitellogenic rainbow trout with Vtg results in the suppression of plasma concentrations of  $E_2$ , and the inhibitory effect is diminished after the termination of treatment (Resi-Henriques et al., 1997). Further, *in vitro* studies suggest that Vtg can inhibit the activity of steroid-converting enzymes in the follicle (Resi-Henriques et al., 1997, 2000). This present study investigated the possibility of Vtg feedback on ovarian steroid production in greenback flounder using isolated vitellogenic follicles.

This thesis consists of 8 chapters, each written as a potentially discrete publication. This has led to some planned overlap in introduction and discussion sections. Chapters 1 and 8 are the general introduction and general discussion of the thesis respectively, and Chapters 2-7 are:

**Chapter 2;** Induction, purification, and partial characterization of vitellogenin in greenback flounder, *Rhombosolea tapirina*

**Chapter 3;** Development of an enzyme-linked immunosorbent assay (ELISA) for vitellogenin in greenback flounder *Rhombosolea tapirina*

**Chapter 4;** Effects of  $17\beta$ -estradiol on vitellogenin induction *in vivo* in greenback flounder *Rhombosolea tapirina*

Chapters 2, 3 and 4 in combined form have been published as follows:

Sun B., Pankhurst N.W. and Watts M. (2003). Development of an enzyme-linked immunosorbent assay (ELISA) for vitellogenin measurement in greenback flounder *Rhombosolea tapirina*. *Fish Physiology and Biochemistry* 29, 13-21.

**Chapter 5;** Patterns of oocyte growth, vitellogenin and gonadal steroid concentrations in greenback flounder *Rhombosolea tapirina*

Chapter 5 has been written up for publication as follows:

Sun B. and Pankhurst N.W. (2004). Patterns of oocyte growth, vitellogenin and gonadal steroid concentrations in greenback flounder *Rhombosolea tapirina*. *Journal of Fish Biology* 64, 1399-1412.

Parts of this chapter were also presented in poster form at the 7th International Symposium on Reproductive Physiology of Fish, Mie, Japan, May 2003.

Sun B. and Pankhurst N.W. (2003). Correlation between oocyte development and plasma concentrations of steroids and vitellogenin in greenback flounder *Rhombosolea tapirina*. *Fish Physiology and Biochemistry* 28, 367-368.

**Chapter 6;** Vitellogenin induction *in vitro* by 17 $\beta$ -estradiol in primary cultures of greenback flounder (*Rhombosolea tapirina*) hepatocytes

**Chapter 7;** *In vitro* effect of vitellogenin on steroid production by ovarian follicles of greenback flounder *Rhombosolea tapirina*

This chapter is under review:

Biao Sun and Ned W. Pankhurst. *In vitro* effect of vitellogenin on steroid production by ovarian follicles of greenback flounder *Rhombosolea tapirina*. Submitted to *Comparative Biochemistry and Physiology*.

Purification and characterization of Vtg, identification of ELISA parameters and establishment of Vtg ELISA were performed with the assistance of Dr. Marianne Watts. Dr. Barry Munday assisted with animal management in production of the Vtg antiserum.

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**Chapter 2**

**Induction, purification, and partial  
characterization of vitellogenin in greenback  
flounder, *Rhombosolea tapirina***

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## **2. Induction, purification, and partial characterization of vitellogenin in greenback flounder, *Rhombosolea tapirina***

### **2.1. Introduction**

In teleosts, as in other oviparous species, the glycolipophosphoprotein vitellogenin (Vtg) is the precursor of yolk proteins. Vtg synthesis is a prerequisite for oocyte growth and contributes vitally to egg quality and reproductive success. The molecular weight of Vtg is high and varies from 300-640 kD in different species (reviewed in Specker and Sullivan, 1994; Tyler et al., 2000). Vtg is produced by the liver in response to stimulation by estrogens, principally 17 $\beta$ -estradiol (E<sub>2</sub>) (Van Bohemen et al., 1982; Norberg and Haux, 1985) and is transported in the blood to the ovary (Copeland et al., 1986), where it is specifically sequestered by growing oocytes and proteolytically cleaved into two major yolk proteins: lipovitellin and phosvitin (Tyler et al., 1988). Exogenous E<sub>2</sub> can induce Vtg synthesis in juveniles of both sexes and also in adult males (Specker and Sullivan, 1994).

The greenback flounder, *Rhombosolea tapirina* is the most common pleuronectid in temperate Australian waters (Van den Enden et al., 2000) and is widespread from New South Wales to southern Western Australia, and is especially common in Tasmania (Last, 1983; Kuitert, 1993). There is a high demand for flatfishes including flounder in southern Australia, New Zealand, Europe and South East Asia (Hart, 1991; Gorman, 1994). Although greenback flounder has many features that make it suitable as an aquaculture species (Hart, 1991), egg production and egg quality in this species are unreliable, and the development of a flounder industry will require better availability of viable eggs (Pankhurst, 1998).

Substantial progress has been made in a number of areas; such as feeding behavior of larvae (Cox and Pankhurst, 2000), reproductive biology and endocrinology (Barnett and Pankhurst, 1999), genetic variation (Van den Enden et al., 2000) and the use of exogenous hormones to manipulate reproduction in greenback flounder



(Poortenaar and Pankhurst, 2000 a, b; Pankhurst and Poortenaar, 2000). However, there is still little understanding of the process of vitellogenesis in this species, and of its role in determining egg quality and viability.

The aim of the present study was to develop a simple method for purification of an intact and homogenous Vtg fraction from blood samples from E<sub>2</sub>-treated mature male fish, and to partially characterize the Vtg fraction. Briefly, plasma was fractionated by gel filtration chromatography and the putative Vtg fraction further purified using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting purified Vtg was then characterized using two different techniques for the estimation of molecular weight. The resulting product will be used in the next phase of the study - development of an enzyme-linked immunosorbent assay (ELISA) for the measurement of greenback flounder Vtg.

## 2.2. Materials and Methods

### 2.2.1. *Fish and fish treatment*

Two + year old maturing male fish were obtained from the School of Aquaculture aquatic facility at University of Tasmania in Launceston. Fish were maintained in the aquatic facility in 4000-l recirculating systems incorporating a biofilter, coarse solids filter and aeration system, and connected to 'Aquahort' temperature control units. Fish used for experiment were fed at ~ 3% body weight daily with a mixture of chopped fish and dry pellets, with body weights ranging from 100 to 170 g. Water temperature was  $12 \pm 1^{\circ}\text{C}$  and photoperiod 10: 14 (light: dark) during the experiment.

Fish were divided into two treatment groups ( $n = 4$ ), anaesthetised in 0.05% 2-phenoxyethanol (Sigma), weighed, fin-clipped for identification and treated with either saline or 17 $\beta$ -estradiol (E<sub>2</sub>) suspension (ethanol: saline = 1: 9) at 2.0 mg kg<sup>-1</sup> body weight (Pankhurst et al., 1986). Saline controls were included to demonstrate that the putative E<sub>2</sub>-inducible Vtg fraction was not present in fish that

were not injected with E<sub>2</sub>. All treatments were administered by intraperitoneal injection (ipi) in an injection volume of 1.0 ml kg<sup>-1</sup>. Fish were injected three times at an interval of seven days.

### ***2.2.2. Blood sample collection and treatment***

Seven days after the third injection, fish were anaesthetised as before, and bled by caudal puncture using preheparinised 3 ml syringes. Tubes filled with blood were kept on ice before centrifuging at 12000 g for 3 min at 4°C. The plasma fraction was collected and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma) was added (plasma: PMSF = 2: 1 v/v) to avoid proteolysis of Vtg in the plasma samples, and then the mixture was kept at 4°C until purification.

### ***2.2.3. Purification of vitellogenin***

Two millilitres of sample (plasma/PMSF) were applied onto a Sephacryl HR 300 (Amersham Pharmacia Biotech AB) 90 × 1.6 cm column at 4°C. The sample was eluted with 0.05 M Tris base-0.15 M NaCl (pH 8.0), at a flow rate of 10 ml h<sup>-1</sup>, 2 ml fractions were collected and their absorbances were read at 280 nm, using a UNICAM 8625 UV/VIS spectrometer and quartz cuvettes.

### ***2.2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)***

Putative Vtg peak fractions obtained from gel filtration chromatography (identified by presence in E<sub>2</sub> but not saline injected male fish) were further purified by SDS-PAGE (4% stacking gel and 10% resolving gel) prepared according to Laemmli (1970). Fixation and visualization of proteins were conducted with 0.025% Coomassie Brilliant Blue R-250 (CBB), followed by 50% methanol, 10% acetic acid for 1 h, and 7% acetic acid and 5% methanol for another 1 h.

### ***2.2.5. Estimation of Vtg molecular weight***

Two techniques were used to estimate the molecular weight of Vtg. The molecular weight of native Vtg was estimated from elution position on the Sephacryl HR 300 column, calibrated with the elution positions of markers of known molecular weight. The molecular weight standards used were blue dextrin (2000 kD), thyroglobulin (669 kD), apoferritin (443 kD),  $\beta$ -amylase (200 kD), alcohol dehydrogenase (150 kD), albumin (66 kD), and carbonic anhydrase (29 kD) (Sigma).

The molecular weight of the Vtg subunits was estimated from migration on SDS-PAGE. SDS-PAGE protein standards of molecular weight 200 kD, 116 kD, 97 kD, 66 kD, 55 kD, 37 kD, 31 kD, and 22 kD (BioRad) were employed as molecular weight markers.

### ***2.2.6. Stability of vitellogenin***

To test the stability of Vtg in plasma following storage at 4°C, a plasma sample from one E<sub>2</sub>-treated fish was stored for 8 and 17 days respectively. Then samples from the two different storage times were run on the same column as before and main fractions corresponding to different Vtg peaks were pooled separately, and loaded in two different wells of the same SDS-PAGE gel as before.

## **2.3. Results**

### ***2.3.1. Induction and purification of vitellogenin***

Figure 2.1 shows the isolation of plasma protein on a Sephacryl HR 300 column from eight male fishes after treatments of saline or E<sub>2</sub> respectively. A large elution peak was observed in the plasma from two E<sub>2</sub>-treated fishes (Fig. 2.1: E<sub>2</sub>-2 and E<sub>2</sub>-3) and this peak was completely absent in all control animals (Fig. 2.1: S-1, S-2,

S-3, and S-4), and also did not appear in another two E<sub>2</sub>-treated male fishes (Fig. 2.1: E<sub>2</sub>-1 and E<sub>2</sub>-4).

### ***2.3.2. Molecular weight estimation of Vtg***

The molecular weight of native Vtg was estimated by comparing elution positions of sample and protein standards of known molecular weight (Fig. 2.2). The calibration curve was prepared by plotting the logarithms of the known molecular weights of protein standards versus their respective  $V_e/V_o$  values ( $V_e$  is the elution volume and  $V_o$  is the void volume, and  $V_o$  was calculated from the elution volume of Blue Dextran) (Fig. 2.3).

In addition to the putative Vtg peak, three small peaks (labelled A, B, and C in Fig. 2.2) were observed. Peak A eluted at the same position as Blue Dextran, which indicated a large molecular weight. Peaks B and C eluted at around 195 and 103 kD (Table 1.1) respectively and appeared in the plasma of most fish (Fig. 2.1).

The main fractions corresponding to the putative Vtg and the peak fraction of the small peaks A, B and C in samples E<sub>2</sub>-2 and E<sub>2</sub>-3 were selected and further purified by SDS-PAGE. Vtg separated into three bands, and peaks A, B and C generated 1, 5 and 3 bands respectively after SDS-PAGE (Fig. 2.4). The molecular weights of these subunits were estimated from a comparison of positions of different bands and protein standards of known molecular weight. The logarithm of the protein standards of known molecular weight was plotted against the relative mobility ( $R_f$  = distance between the origin and positions of protein bands / distance between the origin and dye front), and linear regression was used to calculate the molecular weight of different bands (Fig. 2.5). These molecular weight estimations are shown in Table 2.2.

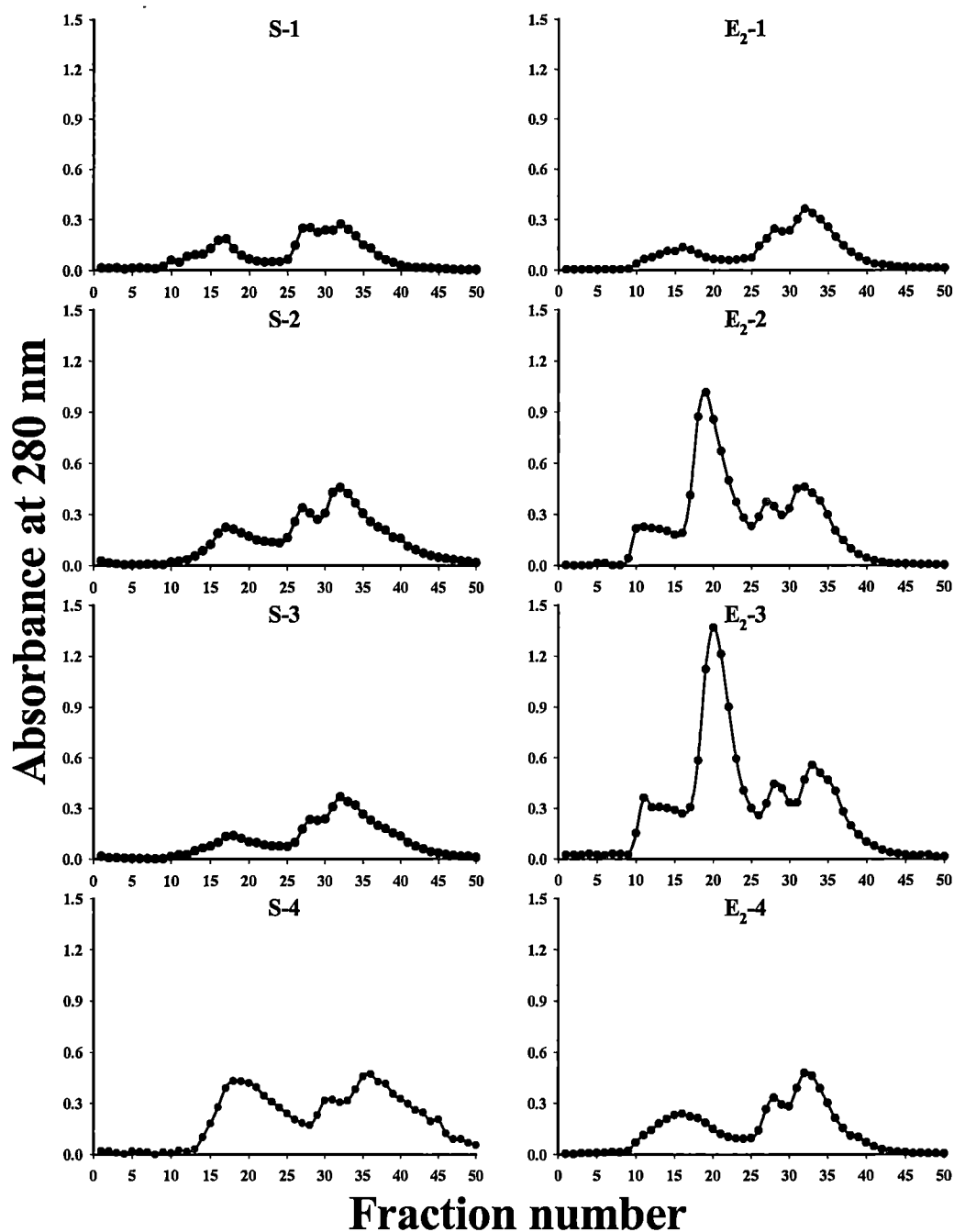


Fig. 2.1. Purification of flounder vitellogenin by gel filtration chromatography. Elution profiles of fractions from gel filtration chromatography eluted on a Sephacryl HR 300 (90  $\times$  1.6 cm). S-1, S-2, S-3 and S-4 are profiles of four saline-treated fishes; and  $E_2$ -1,  $E_2$ -2,  $E_2$ -3 and  $E_2$ -4 are profiles of four  $E_2$ -treated fishes.

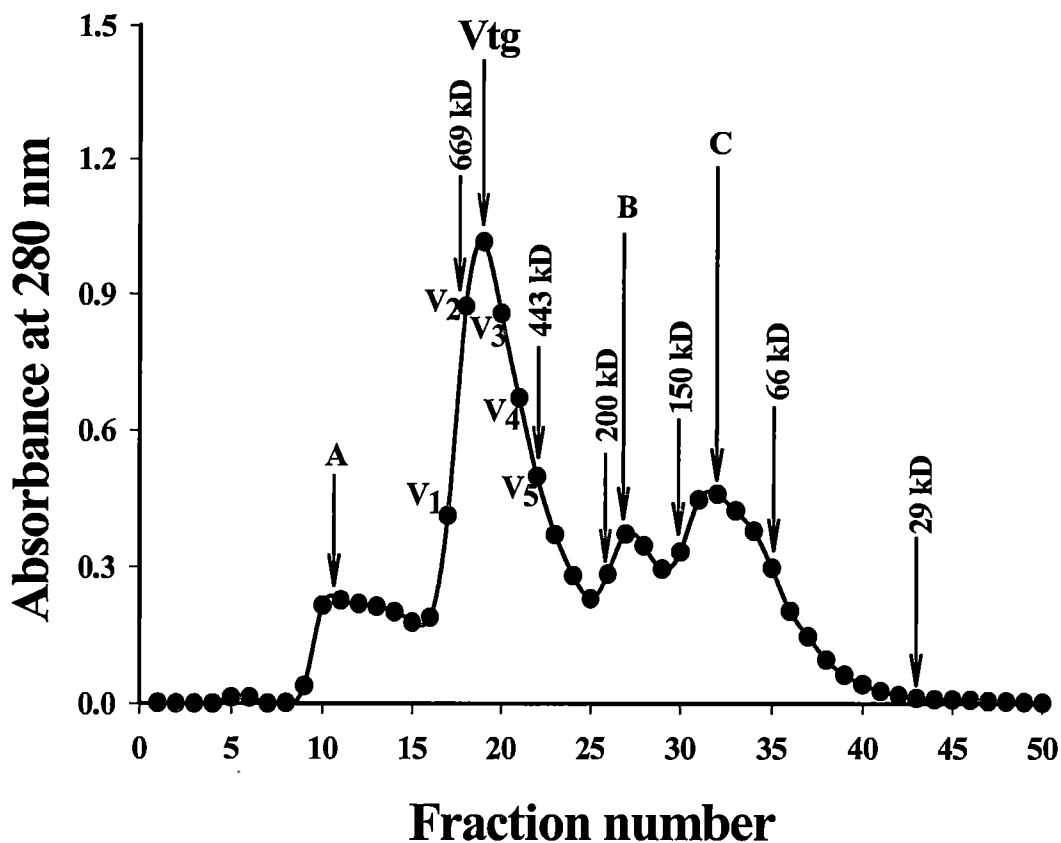


Fig. 2.2. Molecular weight estimation of native flounder Vtg on Sephacryl HR 300 ( $90 \times 1.6$  cm). Flow rate,  $10 \text{ ml h}^{-1}$ ; fraction size, 2 ml. Arrows indicate elution positions of different markers (kD), putative vitellogenin (Vtg) and three small peaks (A, B and C).  $V_1$ - $V_5$  are different fractions of Vtg peak.

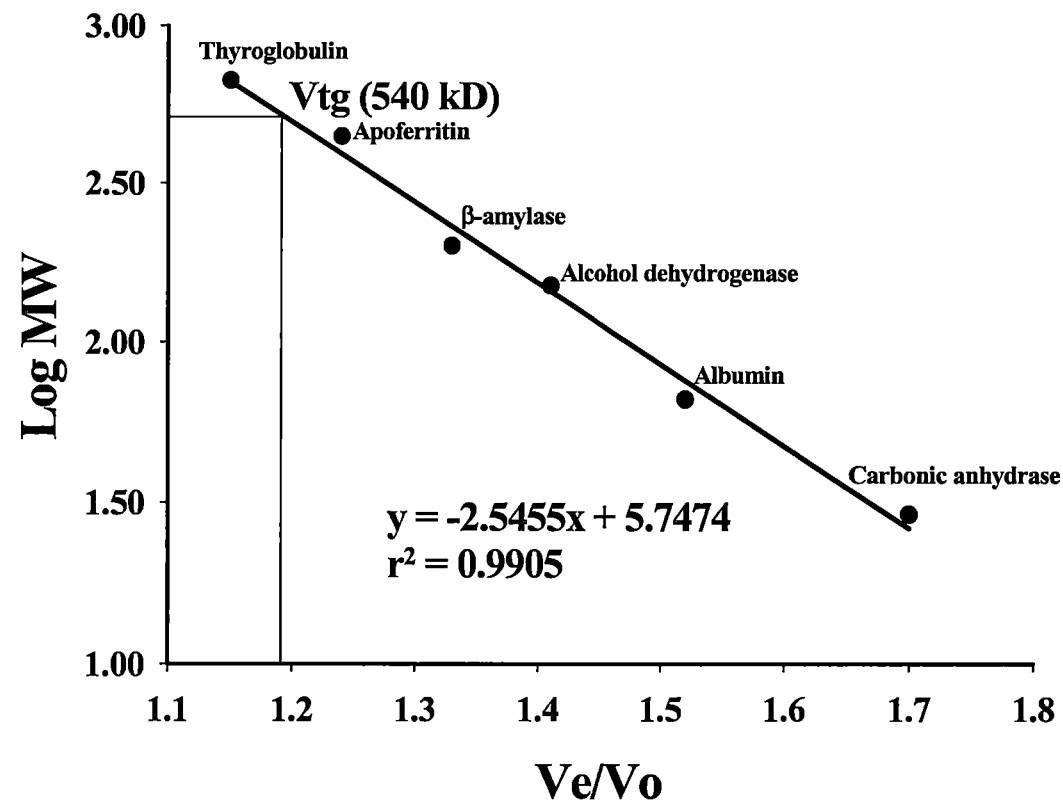


Fig. 2.3. Calibration curve for calculation of peak protein molecular weight after gel filtration chromatography.

Table 2.1 Molecular weight (kD) of different peak proteins estimated from gel filtration chromatography

Peaks	Vtg	B	C
Sample-1 (E <sub>2</sub> -2)	574	207	110
Sample-1 (E <sub>2</sub> -3)	505	182	96
Mean MW	540	195	103

Table 2.2 Estimated molecular weights (kD) of subunits after SDS-PAGE

	Bands	Sample-1 (E <sub>2</sub> -2)	Sample-2 (E <sub>2</sub> -3)	Subunit mean MW
Vtg	1	153.3	149.6	151.5
	2	104	100.8	102.4
	3	82.7	77.4	80.1
Peak B	1	155	144.1	149.5
	2	109.1	104.6	106.9
	3	89	83.5	86.3
	4	51.2	44.9	48
	5	31.1	27	29
Peak C	1	94.1	83.5	88.8
	2	66.3	55.2	60.7
	3	33.4	28	30.7



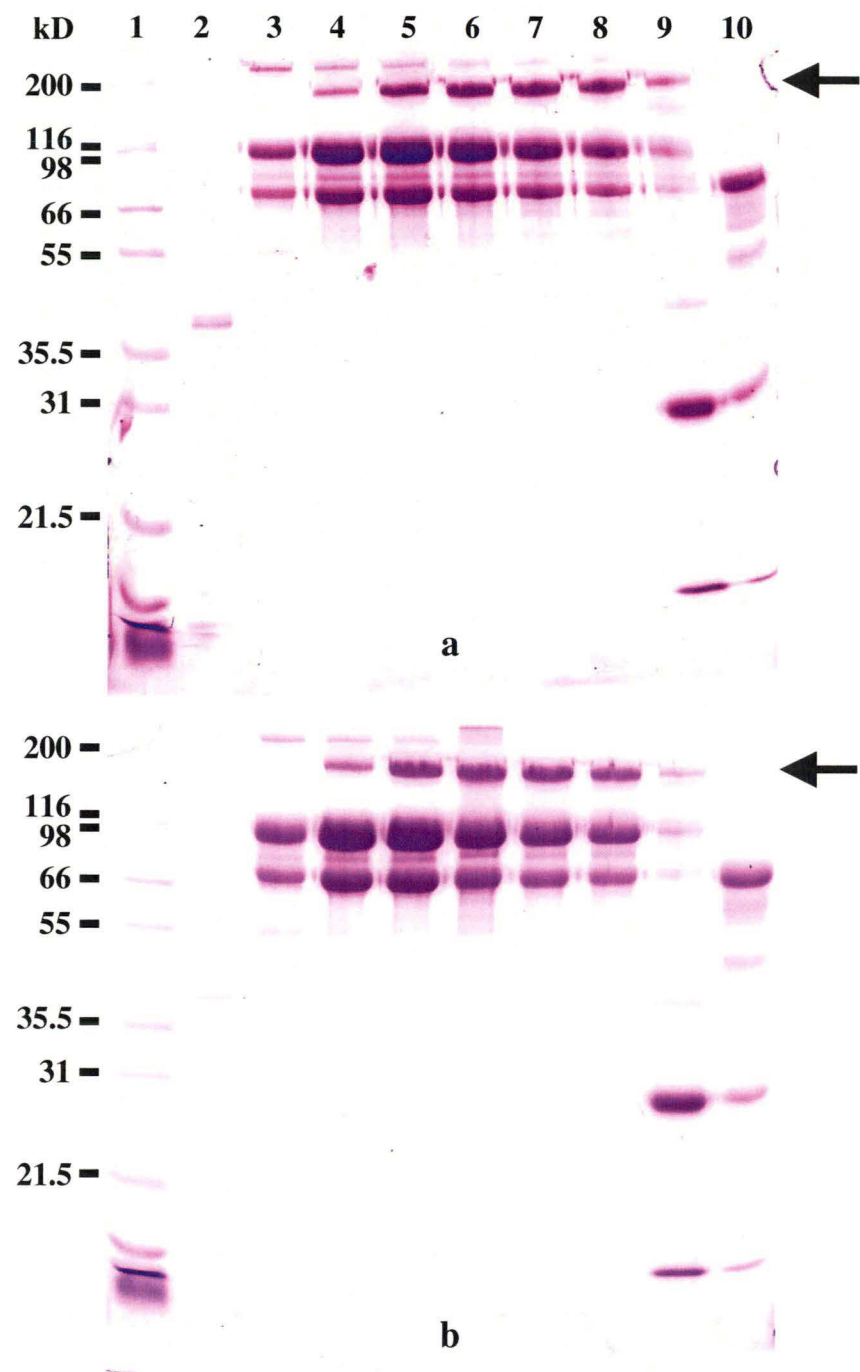


Fig. 2.4. SDS-PAGE profiles of fractions from plasma of E<sub>2</sub>-treated male fish after gel filtration chromatography. For a) is sample-1 (E<sub>2</sub>-2) and b) is sample-2 (E<sub>2</sub>-3). Lane 1: standard molecular weight markers. Lane 2: peak A fraction. Lane 3: fraction V<sub>1</sub>. Lane 4: fraction V<sub>2</sub>. Lane 5: peak Vtg fraction. Lane 6: fraction V<sub>3</sub>. Lane 7: fraction V<sub>4</sub>. Lane 8: fraction V<sub>5</sub>. Lane 9: peak B fraction. Lane 10: peak C fraction. The numbers on the left indicate molecular weight (kD) of standard markers. Arrows indicate position of Vtg subunit.

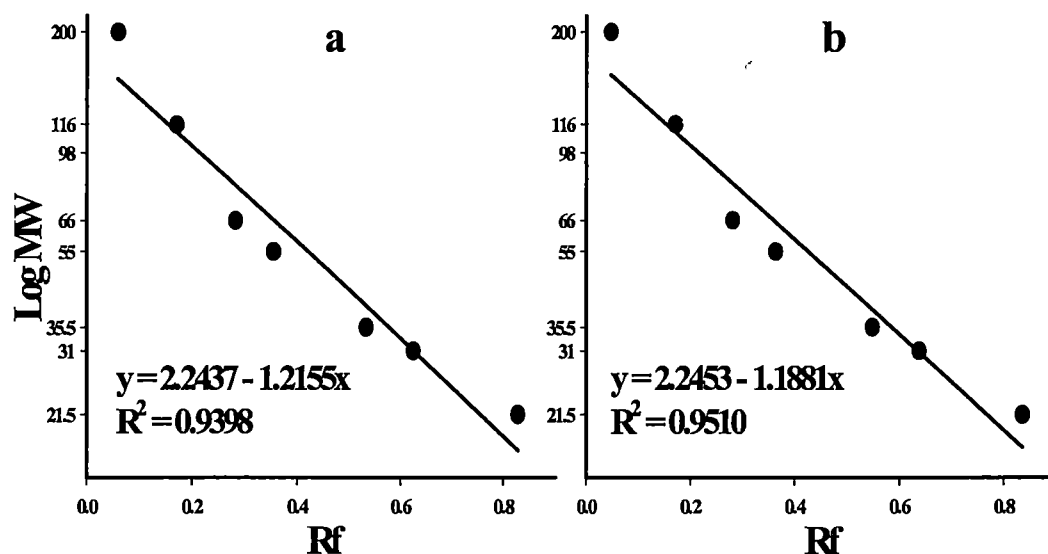


Fig. 2.5. Calibration curve for calculation of subunit molecular weight after SDS-PAGE, for a) sample-1 (E<sub>2</sub>-2) and b) sample-2 (E<sub>2</sub>-3).

### 2.3.3. Stability of vitellogenin under storage

Plasma kept for 8 days had a steep and narrow peak and the absorbance of the peak fraction at 280 nm was 1.367. In contrast, the peak of plasma kept for 17 days was broad and the absorbance of the peak fraction at 280 nm was 0.853, indicating that some degree of proteolysis had occurred in the sample compared with 8 days (Fig. 2.6). The main fractions of Vtg peaks were pooled separately (Fig. 2.6) and further checked by SDS-PAGE, and both samples had three bands at 152, 102 and 80 kD positions, and no new bands appeared in the 17-day sample (data not shown).

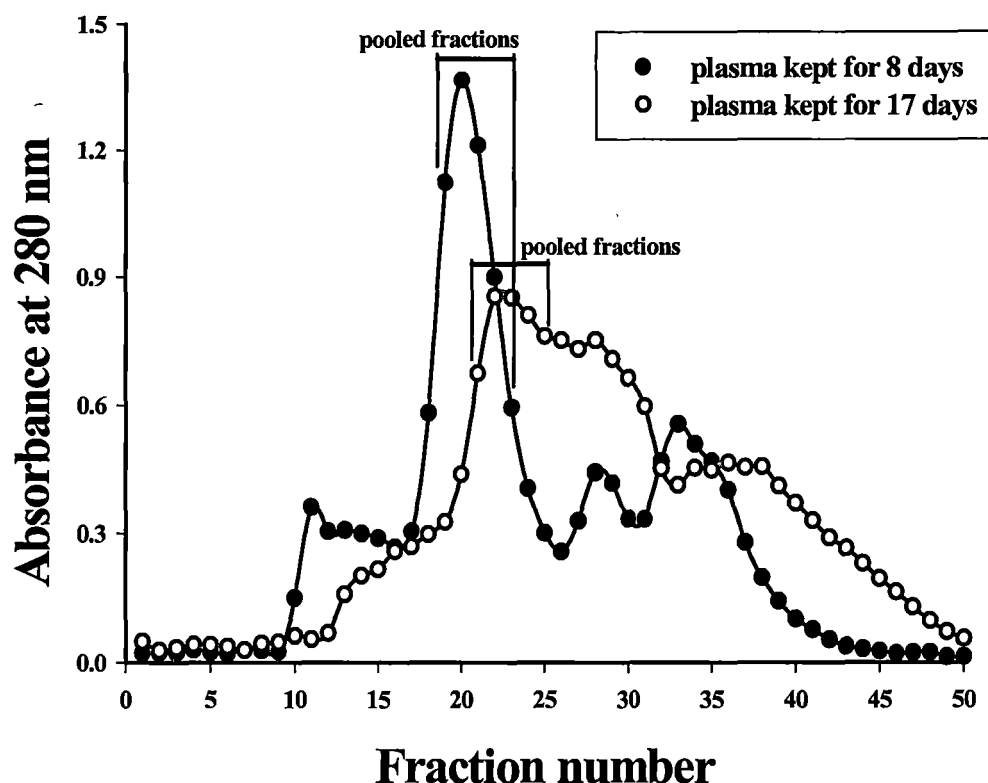


Fig. 2.6. Plasma samples from the same fish but stored for 8 or 17 days at 4°C, then chromatographed on a Sephacryl HR 300 column (90 × 1.6 cm).

## 2.4. Discussion

The major protein appearing in plasma of greenback flounder after  $E_2$  administration was identified as Vtg. The criteria used for the tentative identification of Vtg were that this protein was induced by  $E_2$ , had a high molecular weight (540 kD or so) and that this high molecular weight protein was completely absent from male fish treated with saline. In the present experiment, two  $E_2$ -treated males were induced to produce Vtg but there was no response in two others. Vtg is normally present only in the plasma of female fish, but males do have the Vtg gene, the expression of which can be triggered by exogenous  $E_2$  (Tyler et al., 2000). In the liver,  $E_2$  activates the hepatocyte cells via a receptor-mediated process to trigger the production of Vtg (Lazier and MacKay, 1993). Estrogen receptor (ER) levels in fish liver fluctuate depending on season, sex,

sexual maturity and endogenous E<sub>2</sub> level (Smith and Thomas, 1990, 1991). E<sub>2</sub> stimulation can increase the number of ER in the liver, which further increases the responsiveness and capacity of the system (Valotaire et al., 1993). Temperature is also an important regulator of the ER accumulation pattern (MacKay and Lazier, 1993; MacKay et al., 1996). The apparent absence of Vtg from plasma of males and juveniles is attributed to the lack of sufficient E<sub>2</sub> titers compared with mature females (Specker and Sullivan, 1994). In the present study, the E<sub>2</sub> dose of 2.0 mg kg<sup>-1</sup> was suitable for hepatic stimulation of flounder as shown by the induction of Vtg. The reason that two males were not induced to produce Vtg is not clear but may be the loss of the injection bolus to other organs or tissues such as gut and fat.

In male summer flounder (*Paralichthys dentatus*), there was no significant difference between concentrations of plasma Vtg in fish treated with 1.0 or 10 mg kg<sup>-1</sup> E<sub>2</sub> (Folmar et al., 2001). Moreover, the plasma Vtg concentration of fish treated with E<sub>2</sub> at 2.0 mg kg<sup>-1</sup> was a little higher than that of fish treated with E<sub>2</sub> at 20 mg kg<sup>-1</sup> in juvenile summer flounder (Mills et al., 2001). Mills et al. (2001) also suggested that coconut oil as a carrier may affect the actual exposure levels of E<sub>2</sub>. Pankhurst et al. (1986) showed that saline as a carrier generated a high E<sub>2</sub> peak in serum but it was cleared rapidly. The issue may as much be that E<sub>2</sub> levels were not maintained high enough for long enough. Therefore, the possibility of injection loss combined with rapid clearance may have lead to insufficiently high levels of E<sub>2</sub> in the two non-responsive males.

Teleost Vtg is temperature-sensitive and is easily denatured during isolation procedures (Norberg and Haux, 1985; Silversand and Haux, 1989; Silversand et al., 1993; Norberg, 1995; Johnsen et al., 1999). Addition of the protease inhibitor PMSF or Aprotinin and low temperature purification are typically used to prevent the disintegration of Vtg (Norberg and Haux, 1985; Silversand and Haux, 1989; Silversand et al., 1993; Johnsen et al., 1999). However, even when these precautions were taken, proteolysis of Vtg could not be completely avoided (de Vlaming et al., 1980; Silversand and Haux, 1989; Silversand et al., 1993). Rainbow trout (*Oncorhynchus mykiss*) Vtg is less sensitive to proteolysis than sea

trout (*Salmo trutta*) Vtg under identical isolation and purification procedures (Norberg and Haux, 1985). The degradation of Arctic charr (*Salvelinus alpinus*) Vtg without prior treatment with Aprotinin was negligible (Johnsen et al., 1999). However, Atlantic halibut (*Hippoglossus hippoglossus*) Vtg became increasingly susceptible to proteolysis with prolonged storage at  $-20^{\circ}\text{C}$  (Norberg, 1995). The stability of Vtg from different species also varied during purification (Silversand et al., 1993). In order to obtain intact Vtg, it was usually necessary that the plasma be directly loaded onto a column (Norberg and Haux, 1985; Silversand et al., 1993; Lomax et al., 1998) or deep frozen and stored at an extreme low temperature before subsequent purification and analysis (Nunez-Rodriguez et al., 1989; Tyler and Sumpter, 1990; Norberg, 1995; Johnsen et al., 1999). Our whole purification process was performed under  $4^{\circ}\text{C}$ , with gel filtration chromatography being a one-step and relatively quick procedure. In contrast to the sample subjected to 17 days of storage at  $4^{\circ}\text{C}$ , the Vtg sample stored for only 8 days at this temperature eluted in a steep, narrow and symmetric peak from gel filtration chromatography. As different fractions were further checked by SDS-PAGE, the first three bands of peak B (lane 9) had the same molecular weight and electrophoresis pattern as that of the main peak Vtg fraction of peak Vtg (lane 4-lane 8). This means that some degree of degradation of Vtg had already occurred, and that flounder Vtg is quite unstable during extended storage at  $4^{\circ}\text{C}$  even in the presence of PMSF.

The molecular weight of teleost Vtg varies between 300 and 640 kD (Tyler et al., 2000). Different analytical methods may have an influence on the estimation of molecular weight (Norberg and Haux, 1985). In the present experiment, gel filtration chromatography was used to purify Vtg and estimate its molecular weight. The molecular weight of Vtg estimated from gel filtration chromatography has a lower precision than other available methods (Work and Burdon, 1980). However, the mean molecular weight of Vtg obtained from this experiment is very close to other known molecular weight Vtgs from flatfishes (Table 3). Firstly, it may be that the molecular weights of these species are indeed very close because they are very closely related species. Secondly, the purification and estimation

method is the same, except for turbot. In the salmon family, molecular weights of Vtgs range between 440-600 kD, but this is thought to result from the different separation methods that were employed in various studies (Tyler, 1991). The molecular weight of Vtg was 440 kD for both rainbow trout and sea trout when an identical purification procedure was used (Norberg and Haux, 1985).

The molecular weight of flounder Vtg subunits estimated by SDS-PAGE was 152 kD, which is among the range of the subunit molecular weights reported for other flatfishes e.g. 130 kD in English sole (*Pleuronectes vetulus*) (Roubal et al., 1997), 160 kD in Atlantic halibut (Norberg, 1995), 175 kD in winter flounder (*Pleuronectes americanus*) (Hartling et al., 1997) and 185 kD in turbot (Silversand and Haux, 1989). Vtg usually exists as a dimer in blood (Specker and Sullivan, 1994). The apparent difference between the molecular weight from gel filtration chromatography and from SDS-PAGE is probably because the lipid content of Vtg in teleost is about 20%, and is removed during SDS treatment (Silversand and Haux, 1989). In addition to the main Vtg subunit, there were also two smaller bands (102 and 80 kD) that appeared on SDS-PAGE. Western blots revealed that they were immunologically identical to the Vtg subunit and resulted from different degrees of degradation of the parent molecule (data shown in the following chapter). After SDS treatment, Vtg will change from three-dimensional structure into a linear shape, and the epitope is determined by the primary structure of Vtg polypeptide. As the antibody is only produced against the 152 kD band, the result of Western blotting suggests that the two minor bands come from Vtg. Except for fraction V<sub>1</sub>, the main fractions of the Vtg peak (fractions V<sub>2</sub>-V<sub>5</sub>) all had the same pattern on SDS-PAGE, indicating that the Vtg obtained from gel filtration chromatography was of high purity.

The molecular weight of peak A was much larger than Vtg (judged from the elution position on gel filtration chromatography), but its subunit was only about 47 kD on SDS-PAGE. One of the possible explanations is that a low molecular weight serum globulin aggregated to a larger complex, which eluted earlier than Vtg.

Peaks B and C appeared in saline-treated fish as well as E<sub>2</sub>-treated fish, and this observation agrees with findings reported by Hobby et al. (2000). Comparison with Hobby's study suggests that peaks B and C represent albumin-like protein and sex steroid binding protein (SBP) respectively. Albumin-like protein has been found in some other species (Davidson et al., 1989; Tyler and Sumpter, 1990; Maillou and Nimmo, 1993 a, b). The molecular weight of albumin-like protein in flounder is about 195 kD, and close to that in rainbow trout (Davidson et al., 1989).

The molecular weight of peak C (putative SBP) is around 103 kD, and it is within the range of known molecular weights of SBP in teleost species (reviewed in Hobby et al., 2000). This protein cleaved into three bands on SDS-PAGE with the first one the strongest - its molecular weight (89 kD) being quite close to that of native SBP (Hobby et al., 2000). It is possible that SBP in flounder is composed of one subunit, and the appearance of the other two bands may be because of different degrees of degradation.

A small peak appeared in four saline-treated and two E<sub>2</sub>-treated fish (E<sub>2</sub>-1 and E<sub>2</sub>-4) at elution position of fractions 16-18 but was not observed in fish E<sub>2</sub>-2 and E<sub>2</sub>-3. As noted earlier this peak was also probably agglutinated globulin, and may have been covered by the Vtg peak in the two responsive fish, which eluted at fraction 19 in fish E<sub>2</sub>-2 and 20 in fish E<sub>2</sub>-3.

It is reported that E<sub>2</sub> can induce the liver to produce eggshell zona radiata proteins (ZRPs) in some teleost fishes (reviewed in Hyllner and Haux, 1995; Tyler et al., 2000). ZRPs have species-specific molecular weights and consist of three proteins components (Oppen-Berntsen et al., 1991), e.g. Atlantic cod (*Gadus morhua*) (78, 54 and 47 kD), rainbow trout (60, 55 and 50 kD) and Atlantic salmon (*Salmo salar*) (66, 61 and 55 kD) (Tyler et al., 2000). ZRPs were not found in the plasma of E<sub>2</sub>-treated flounder in the present study. One explanation for this is that ZRPs appeared in the plasma of flounder at a low concentration and co-eluted with SBP (peak C).

Table 2.3 Molecular weight of native Vtg from four species of flatfishes

	Molecular weight	Method	Reference
Greenback flounder ( <i>Rhombosolea tapirina</i> )	540 kD	Gel filtration chromatography, Sephacryl HR 300, 90 × 1.6 cm column (Pharmacia), 4°C	Present study
Barfin flounder ( <i>Verasper moseri</i> )	520 kD	Gel filtration chromatography, Superose 6 HR 10/30 (Pharmacia), 4°C	Matsubara and Sawano, 1995
Turbot ( <i>Scophthalmus maximus</i> )	530 kD	Purified by high-performance anion-exchange chromatography, Mono Q HR 5/5, 50 × 5 mm I.D. (Pharmacia), 4°C and estimated by native PAGE	Silversand and Haux, 1989
Flounder ( <i>Platichthys flesus</i> )	550 kD	Gel filtration chromatography, Ultrogel 34 LKB, 92 × 1.5 column, 4°C	Emmersen and Petersen, 1976

Several different separation techniques have been developed for the isolation and purification of Vtg from fish, amphibians, reptiles, and birds (Wallace, 1965; Wiley et al., 1979; Wallace, 1985; Mommsen and Walsh, 1988). However, it is usually difficult to get entirely pure Vtgs using a single technique (Selman and Wallace, 1983; Kanungo et al., 1990) and a more successful approach is to combine different separation techniques to obtain a higher purity of Vtg (Norberg and Haux, 1985; Silversand and Haux, 1989; Tyler and Sumpter, 1990). In the present study, flounder Vtg was isolated by gel filtration chromatography with a



large molecular weight as in other teleosts. SDS-PAGE revealed that this protein consists of one subunit and was of high purity.

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## **Chapter 3**

# **Development of an enzyme-linked immunosorbent assay (ELISA) for vitellogenin in greenback flounder *Rhombosolea tapirina***



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### **3. Development of an enzyme-linked immunosorbent assay (ELISA) for vitellogenin in greenback flounder *Rhombosolea tapirina***

#### **3.1. Introduction**

Globally, flatfish are considered to be important commercial fishes in terms of their contribution fisheries and to meeting the market demand for white fleshed fishes (Gorman, 1994; Bolker and Hill, 2000; Kim and Lall, 2000; Mugnier et al., 2000). The greenback flounder *Rhombosolea tapirina* is the most common flatfish in temperate Australian waters (Van den Enden et al., 2000) and is regarded as a potentially valuable species for aquaculture in Australia (Hart, 1991). Although considerable knowledge of reproduction in females has been acquired in a series of prior studies (Barnett and Pankhurst, 1999; Poortenaar and Pankhurst, 2000 a, b), the success of flounder farming is still limited by some technical issues, including the instability of egg production and the unreliability of egg quality. Effects to address this problem are confounded by the fact that our understanding of vitellogenesis (yolk deposition) in this species is limited.

Teleost vitellogenin (Vtg) is a female specific glycolipophosphoprotein that is synthesized in the liver under the influence of  $17\beta$ -estradiol ( $E_2$ ). During vitellogenesis, Vtg is transported to the ovarian follicles via the bloodstream and is taken up by oocytes and used as the precursor of the yolk protein, which are essential for the developing embryo. Therefore, vitellogenesis is a crucial period of the reproductive cycle, which contributes to the availability of high quality eggs (reviewed in Specker and Sullivan, 1994; Tyler et al., 2000). An essential component of understanding the process of vitellogenesis is the capacity to measure Vtg in the blood. As plasma Vtg levels are a direct indicator of oocyte growth in females, the quantification of Vtg levels in the blood can also be a useful approach to assessing reproductive status in female teleosts (Bon et al.,

1997; Heppell et al., 1999). Compared with routine methods such as histological examination used to evaluate maturational processes in females, measurement of Vtg levels in blood or scale mucus is a reliable, rapid, and non-lethal and minimally damaging approach (Gillis et al., 1990; Kishida et al., 1992; Chang et al., 1996; Mosconi et al., 1998; Heppell et al., 1999).

An increasing amount of scientific evidence suggests that many environmental pollutants (collectively termed xenoestrogens), which can mimic the effects of native endogenous estrogens and disrupt the endocrine system of wildlife, have a potentially harmful influence on normal reproductive development (reviewed in Kime, 1998; Tyler et al., 2000). Fish live in seas, rivers and lakes where such pollutants tend to accumulate and, therefore, fish are particularly vulnerable to their effects. Vtg is a female specific protein, but estrogens or xenoestrogens can trigger the expression of the Vtg gene in males and immature animals. The abnormal vitellogenic response in males exposed to xenoestrogens can be used as a bio-indicator of environmental exposure (reviewed in Kime, 1998; Sherry et al., 1999a; Tyler et al., 2000). As a group of demersal species, flatfish commonly lives in coastal waters, estuaries and lagoons (Gorman, 1994; Hartling et al., 1997; Winzer et al., 2001), so they are frequently considered as a sentinel species for monitoring biological impact of xenoestrogens in aquatic environments (Hartling et al., 1997; Roubal et al., 1997; Folmar et al., 2001; Mills et al., 2001; Winzer et al., 2001; Zarogian, et al., 2001). In order to utilize flatfish in this way, it is necessary to be able to quantify their plasma levels of Vtg.

Several indirect and direct methods have been developed for determination of Vtg levels in teleosts. Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are two typical methods used to quantify Vtg in plasma and mucous samples as a result of their specificity and sensitivity. ELISA can offer a number of advantages over RIA, such as less equipment, easier development and validation, and safety while preserving the advantages of sensitivity and specificity (Nunez Rodriguze et al., 1989; Bon et al., 1997; Lomax et al. 1998). The antibody-capture competitive ELISA is typically used, as it can measure

different size antigens, and is easy to perform. It has been employed widely by various researchers for the measurement of Vtg in fishes (reviewed in Specker and Anderson, 1994; Specker and Sullivan, 1994). The purpose of the present study was to describe the development and validation of an indirect antibody-capture competitive ELISA for greenback flounder Vtg, and this method will be used to determine seasonal changes of plasma Vtg levels and the threshold and time-course for the induction of Vtg by estrogens in a later work.

## 3.2. Materials and Methods

### 3.2.1. Assay Reagents

#### 3.2.1.1. Vitellogenin (Vtg)

Standard flounder Vtg was obtained from the plasma of E<sub>2</sub>-treated males by gel filtration chromatography, as described in Chapter 2. Vtg was stored in 1 ml aliquots (1.0 mg ml<sup>-1</sup>) at -20°C.

#### 3.2.1.2. Anti-Vtg antibody

Putative Vtg identified from gel filtration was run on SDS-PAGE as described in Chapter 2. The gel bands containing Vtg subunits (152 kD) were cut out and homogenized in PBS: Freund's complete adjuvant = 1: 1 v/v, and the antigen was kept at -20°C until immunizations. Two sheep were then injected 4 times subcutaneously into the neck with 2 ml of the antigen at intervals of 4 weeks, 2 weeks and 2 weeks, respectively. Blood samples were collected at the first and third injections, and two weeks after the fourth injection. The blood sample allowed to clot at 4°C overnight. The serum was collected by centrifugation at 1000 g for 5min and stored at -20°C after freeze-drying.

#### 3.2.1.3. Conjugated antibody (DASC-AP)

Donkey anti-sheep IgG conjugated to alkaline phosphatase (DASC-AP) was purchased from Sigma, diluted 1:1000 (diluted in 20 mM Tris, 0.5 M NaCl pH 7.2) and kept at -20°C before use.

#### 3.2.1.4. Substrate

p-Nitrophenyl phosphate (pnpp, Sigma) 1.0 mg ml<sup>-1</sup> in 0.01% MgCl<sub>2</sub> (Sigma) and 10% diethanolamine (Sigma) was made fresh before use.

#### 3.2.1.5. Buffers

Chemicals were purchased from Sigma except where noted. All buffers were made with autoclaved RO deionized water.

Coating buffer: 50 mM sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) pH 9.6

TBS buffer: 20 mM Tris, 0.5 M NaCl pH 7.2

Washing buffer (TBST): TBS buffer containing 0.05% Tween-20

Blocking buffer (TBSB): TBS buffer containing 5% skim milk (SM)  
(Bonlac foods limited, Australia)

Diluent buffer (TBSD): TBS buffer containing 3% SM

All buffers were stored at 4°C until use.

#### 3.2.2. Western blotting

Ten micro-litres of reduced pooled Vtg sample (fractions V1-V5 in Chapter 2, from which the same reduced bands were used as the antigen to raise the antiserum in sheep) were loaded into each well on SDS-PAGE gel and pre-stained SDS-PAGE protein standards (BioRad) were employed as molecular weight markers, then samples were electrophoresed as before. Protein bands separated on SDS-PAGE were transferred to a 0.45 µm nitro-cellulose membrane (BioRad) by equilibration in a collection bath containing 48 µM Tris, 39 mM glycine, 20% methanol, pH 9.2 (Schaefer and Bjerum-Neilsen transfer buffer), then electro-transferred with a Hoefer Semi-Phor Semi-Dry transfer unit at 0.8 mA cm<sup>-2</sup> for 2 h. Blots on membranes were stained with Ponceau Red (Sigma) to confirm the transfer, then de-stained in water and unoccupied sites on the membranes were blocked by soaking in TBSB overnight. Following blocking, the membrane was cut into strips, and transferred to separate containers and rinsed in TBS, then covered with antiserum from each sheep, diluted to 1:100 in TBSD, for 1 h. The strips were washed three times over a 15 min period, first in TBS, then in TBST,

and then in TBS again. The membrane was then incubated with a 1:10,000 dilution of DASC-AP in TBSD. After 1 h incubation, the membrane was rinsed three times as before. The complex was detected with BCIP/NBT (BioRad substrate kit), and then rinsed copiously in distilled water. All incubations were carried out at ambient temperature. Pre-immune sheep serum (from the blood sample before the first injection) was employed as a negative antibody control and used at the same dilution.

### ***3.2.3. Assay protocol***

An indirect antibody-capture competitive ELISA was developed for measuring Vtg in plasma samples. In this assay, a known quantity of antigen (purified Vtg) is adsorbed to the wells of 96-well micro-titer plates. A fixed amount of the first antibody and a variable amount of antigen (unknown samples or purified Vtg used as a standard) are incubated and then distributed to the micro-titer plate. The antibody binds to the antigen in solution or on the well of plate depending on the relative amount of antigen present in the sample. After appropriate washing, unbound antibodies are removed, the amount of antibody adhering to the plate being determined by adding an enzyme-labelled secondary antibody. Therefore, the amount of antibody bound to the plate is inversely proportional to the amount of antigen in the sample. The standard curve made with purified Vtg is used to extrapolate the quantity of Vtg in the sample (Specker and Anderson, 1994; Lomax et al. 1998).

#### ***3.2.3.1. Determination of optimal assay conditions***

Four plates (coated plates) were coated with purified Vtg at concentrations of 2.5, 1.25, 0.625, or 0.31  $\mu\text{g ml}^{-1}$  respectively in 100  $\mu\text{l}$  coating buffer. In separate plates (competition plates), Vtg was diluted across the plate one way (left to right), using 1 + 1 serial dilution with TBSD to give a dilution range from 100  $\mu\text{g ml}^{-1}$  in column 1, to 0.08  $\mu\text{g ml}^{-1}$  in column 11, and antibody was diluted across the plate in an orthogonal direction (top to bottom), to give a dilution range of antibody in TBSD from 1: 100 in row A, to 1: 6400 in row G. Thus, a chequerboard was

obtained with serial dilutions of purified Vtg in each of columns 1-11, excluding column 12 wells, and with serial dilutions of antibody in each of rows A-G, excluding row H wells (Fig. 3.1). After incubation, the contents of wells on competition plates were distributed to the corresponding positions on coated plates as described below. The chequerboard was used to determine the working concentrations of the antibody and the coating Vtg, and the working range of the standard curve. When displacement curves from combinations of various dilutions of antibody and Vtg are compared, three points should be considered; first, that the linear part of micro-titer plate reader is normally between 0 and 2 absorbance units, second, that a more sensitive ELISA can be obtained by trying to reach a higher maximum absorbance, and third, that the steepest portion of the curve which indicates the most sensitive and precise part of the displacement curve should be selected as the standard curve (Specker and Anderson, 1994).

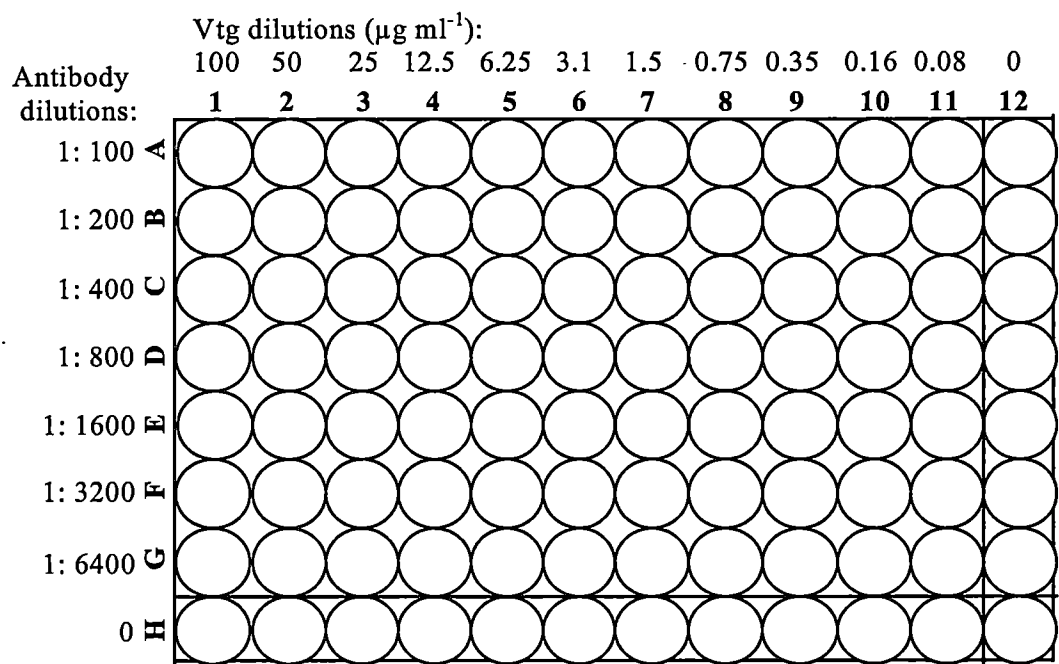


Fig. 3.1. Layout of chequerboard. Vtg diluted from left to right and antibody diluted from top to bottom on the plate. Wells of column 12 did not contain Vtg, and were used as the zero standard (OS), and wells of row H did not contain antibody and were used to measure non-specific binding (NSB).

3.2.3.2. ELISA procedure

**3.2.3.2.1. Antigen coating**

The coating was performed in 96-well micro-titer plates with a 100  $\mu\text{l}$  coating buffer containing 2.5  $\mu\text{g ml}^{-1}$  of Vtg. The plates were then covered, and incubated with shaking overnight at 4°C.

**3.2.3.2.2. Preparation of standard curve and samples**

Serial of dilutions of purified Vtg ranging from 20 to 0.16  $\mu\text{g ml}^{-1}$  in TBSD were prepared in separate tubes, and 100  $\mu\text{l}$  of each standard was transferred in triplicate to the wells in competition plates. Plasma samples from males and females were diluted from 1: 200 to 1: 12800 separately and added to the competition plates as before.

**3.2.3.2.3. First antibody incubation**

One hundred micro-litres of sheep anti-Vtg diluted 1: 100 in TBSD were added to each well on competition plates. Three wells were selected for non-specific binding (NSB) values and did not contain the first antibody, and another three wells without purified Vtg or plasma samples were used for zero standard (OS) values. The plates were then incubated with shaking overnight at 4°C.

**3.2.3.2.4. Blocking and competition**

The contents of the wells on coated plates were discarded by inverting the plates, followed by 4 wash cycles with 300  $\mu\text{l well}^{-1}$  TBST, using a Model 1250 Immunowash plate washer (BioRad). The blocking of non-specific binding sites was achieved by incubating the plates with TBSB (300  $\mu\text{l well}^{-1}$ ) at 37°C for 1 h, after which the TBSB was discarded. The contents of the competition plates were then transferred to the corresponding wells on the coated plates (100  $\mu\text{l well}^{-1}$ ) and the plate was incubated at 37°C for 1 h, then washed as before.

**3.2.3.2.5. Second antibody incubation**

Each well received 100  $\mu\text{l}$  of DASC-AP diluted 1: 10,000 in TBSD. The plate was incubated at 37°C for 1 h and washed as before.

#### 3.2.3.2.6. *Substrate addition*

Each well received 100  $\mu$ l of pnpp ( $1.0 \text{ mg ml}^{-1}$ ) and was incubated at  $37^{\circ}\text{C}$  for 30 min.

#### 3.2.3.2.7. *Absorbance measurement*

The absorbance of each well was measured at 405 nm using SPECTRA Rainbow Thermo plate reader.

#### 3.2.3.3. *Expression and treatment of data*

The relationship between absorbance values and the proportion of antibody bound in standards and samples was expressed by the following equation:  $\%B = [(OD - NSB) (OD_0 - NSB)^{-1}] \times 100$ , where  $\%B$  is the relative binding, OD the absorbance of a standard or unknown sample,  $OD_0$  the absorbance of the zero standard (maximum binding), and NSB the absorbance of the non-specific binding. Logit  $\%B = \text{Ln } [\%B (1 - \%B)^{-1}]$  was plotted against log standard or plasma sample dilution.

#### 3.2.4. *Plasma samples*

For validating this assay, three-year-old vitellogenic F1 females ( $n = 3$ ) and spermiated males ( $n = 2$ ) were sampled from the School of Aquaculture aquatic facility at University of Tasmania in Launceston. The maintenance of fish and the collection and treatment of blood samples were the same as described in Chapter 2. Plasma samples (labelled  $F_1$ ,  $F_2$  and  $F_3$  for females, and  $M_1$  and  $M_2$  for males respectively) were kept at  $4^{\circ}\text{C}$  before assay. All samples and standards were run in triplicate. Samples were diluted in the range of 1: 200 to 1: 12800 in TBSD to ensure that readings fell within the range of the standard curve. Parallelism of Vtg in plasma samples against purified Vtg was tested in this working range.

### 3.3. Results

#### 3.3.1. *Western blotting*



Serum collected after two injections of antigen, and serum from before the first injection (negative antibody control) were tested against Vtg standard SDS-PAGE bands by Western blotting. Three bands were all recognized by the antiserum (from two injections) (Fig. 3.2a) and no immunological cross-reaction was observed in the negative control serum (Fig. 3.2b). This indicated that there was an antigenic response in the sheep after Vtg injections, and also that the two minor bands on SDS-PAGE (Chapter 2) were degradation products from the first (strongest) Vtg band as a result of different degrees of reduction. This Ag-Ab response observed after 2 injections was very weak with only a faint reaction colour observed on blots. Accordingly, another two inoculations were subsequently performed in order to increase the antibody titre.

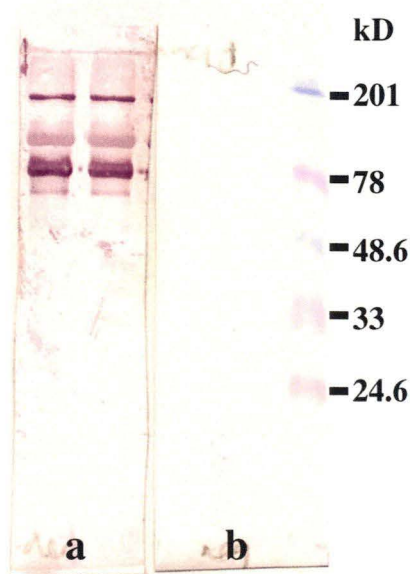


Fig. 3.2. Western blotting using homologous antiserum against greenback flounder Vtg. For a) using antiserum from sheep after two injections, b) using serum from the same sheep before the first injection. The numbers on the right indicate molecular weight (kD) of standard markers.

### 3.3.2. Establishing Vtg ELISA parameters

A competition ELISA was used to determine the optimal assay conditions and different displacement curves were obtained (Fig. 3.3). Fig. 3.3a shows the

various displacement curves from serial dilutions of antibody at a Vtg coating concentration of  $2.5 \mu\text{g ml}^{-1}$ . The curve from 1: 100 antibody dilution was steeper and had a higher absorbance than any other curves in the linear range. Fig. 3.3b describes displacement curves at four different Vtg coating concentrations with the antibody dilution fixed at 1: 100. When the coating concentration was  $2.5 \mu\text{g ml}^{-1}$ , the displacement curve was much steeper and the absorbance much higher than in the other curves within the linear range. Thus, a coating concentration of Vtg at  $2.5 \mu\text{g ml}^{-1}$  and an antibody dilution of 1: 100 were subsequently employed for this assay. A working range for the standard curve of  $0.16\text{-}20 \mu\text{g ml}^{-1}$  was chosen on the basis of the linear portion of the displacement curve (Fig. 3.3c).

### 3.3.3 Assay validation

The specificity of the antibody was verified by using a series of dilutions of plasma samples from naturally matured males and females. The binding displacement curves from plasma dilutions of females were shown against the Vtg standard curve. No significant displacement was observed in the plasma of males until plasma dilutions exceeded 1: 200 (Fig. 3.4a). Linearization of the binding curves was obtained by logit transformation (Fig. 3.4b). Parallelism of the regression curves was assessed by the F-test on mean squares. No significant differences were observed between the standard preparation ( $y = 0.3826 - 1.2727x$ ;  $r^2 = 0.9873$ ) and vitellogenic females: F1 ( $y = -3.6623 - 0.8166x$ ;  $r^2 = 0.9785$ ), F2 ( $y = -4.1957 - 1.0084x$ ;  $r^2 = 0.9835$ ) and F3 ( $y = -3.6697 - 0.9432x$ ;  $r^2 = 0.9758$ ), which indicated that the antibody recognized Vtg in different plasma samples in the same way as it did in the Vtg standard preparation.

Plasma samples were diluted to at least 1: 200 to avoid the effects of other plasma proteins, and to ensure that displacement values fell within the range of the standard curve ( $0.16\text{-}20 \mu\text{g ml}^{-1}$  Vtg). This gives a minimum effective detection limit of Vtg in plasma of  $32 \mu\text{g ml}^{-1}$  at a plasma dilution of 1: 200. The measured Vtg level in the three vitellogenic females F1, F2 and F3 was 26, 19 and  $9 \text{ mg ml}^{-1}$  respectively.

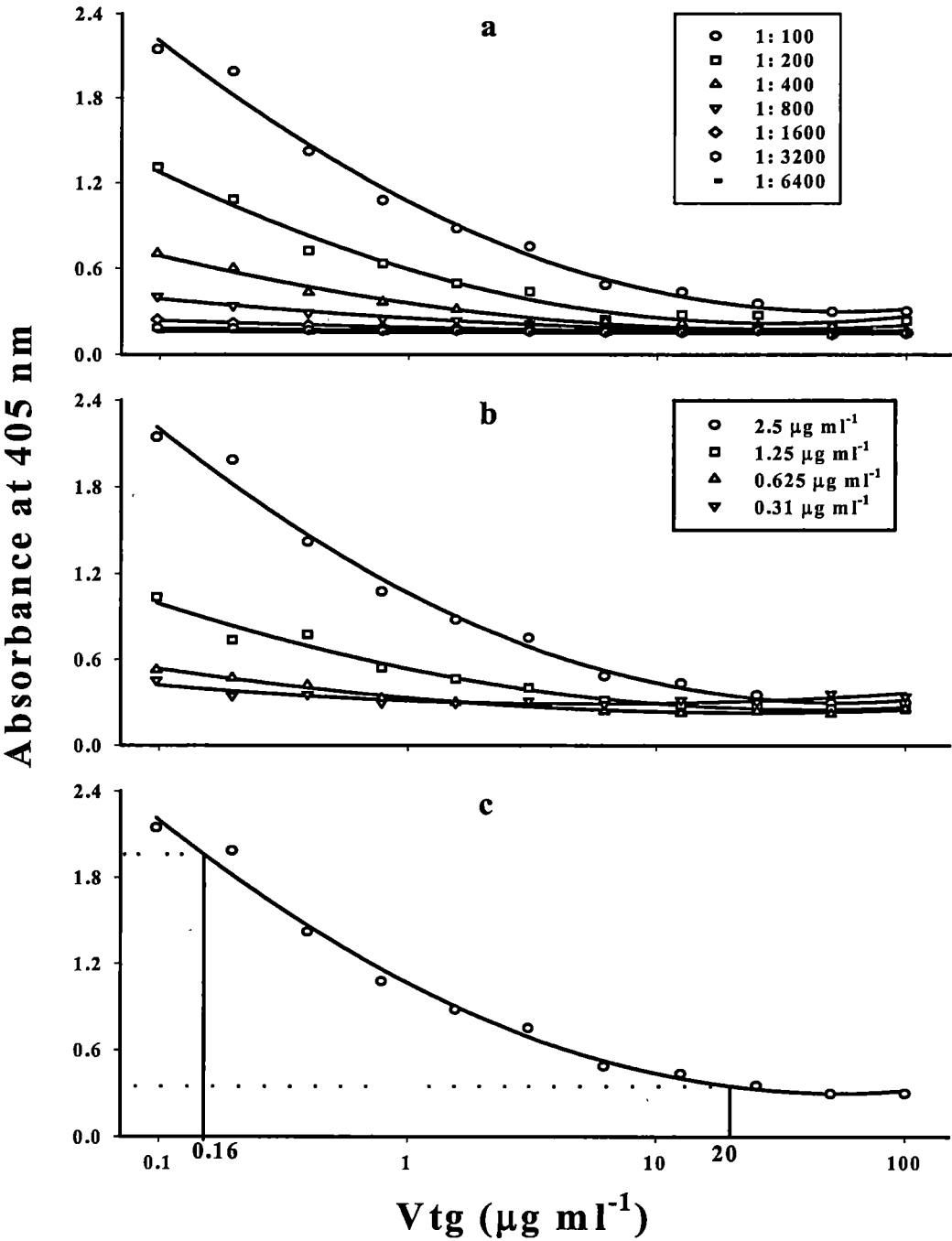


Fig. 3.3. Determination of optimal assay conditions. (a) Displacement curves of different antibody dilutions (1: 100 to 1: 6400) at  $2.5 \mu\text{g ml}^{-1}$  Vtg coating concentration. (b) Displacement curves of different Vtg coating concentrations ( $0.31$ - $2.5 \mu\text{g ml}^{-1}$ ) at 1: 100 antibody dilution. (c) Displacement curve of  $2.5 \mu\text{g ml}^{-1}$  Vtg coating concentration and 1: 100 antibody dilution.

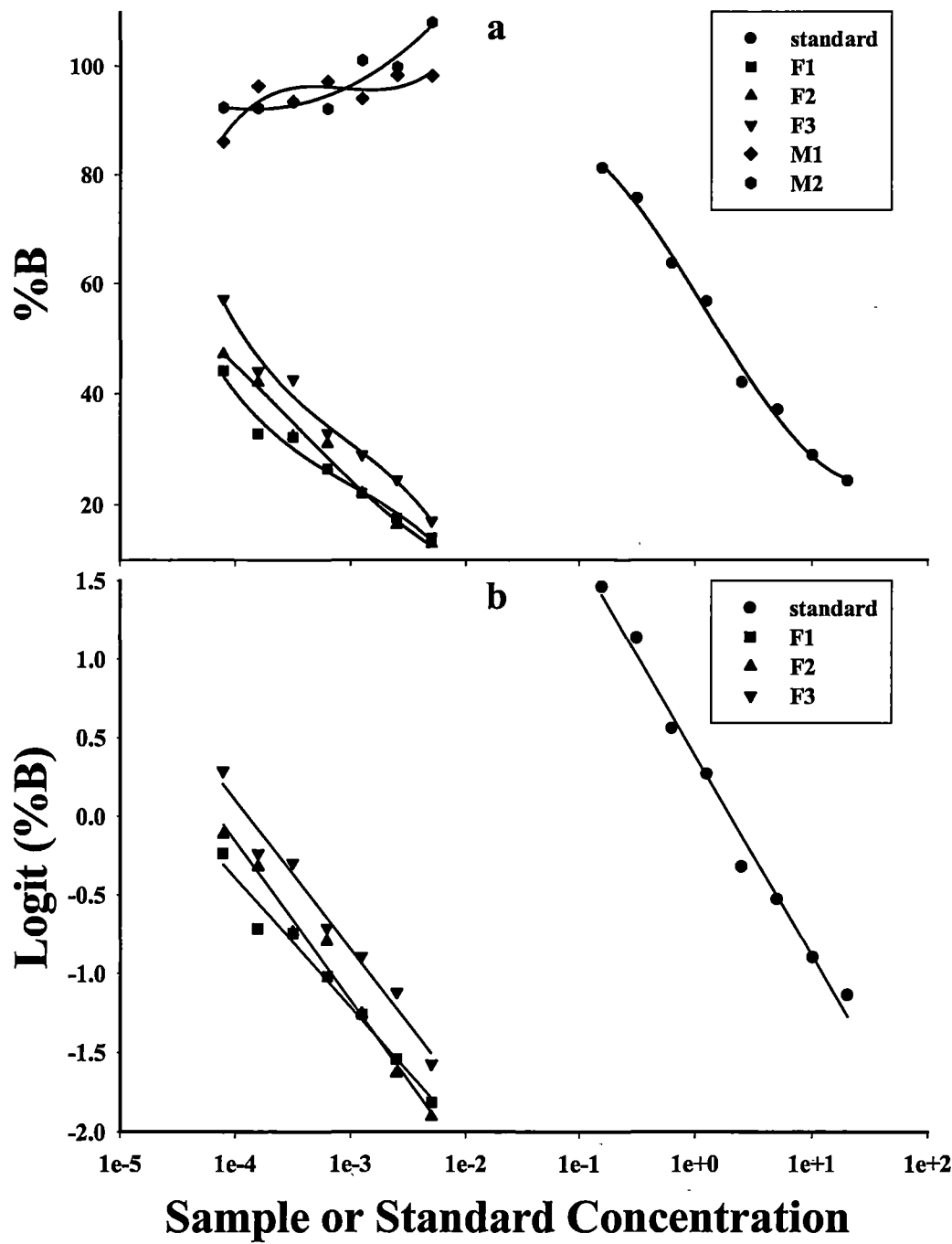


Fig. 3.4. (a) Binding displacement curves obtained with Vtg standards and serial dilutions of plasma from three vitellogenic females (F1, F2, and F3), and two spermiated males (M1 and M2). (b). Linearization of binding curves by logit transformation. All points are the mean of triplicate determinations.

### 3.4. Discussion

The purpose of this present study was to develop a competitive ELISA for measuring Vtg levels in greenback flounder. The sensitivity and reliability of the assay was further assessed by comparing the displacement curves between plasma samples and Vtg standards. The parallelism between the different samples from females and the standards demonstrates that the specific antibody recognizes the standard preparation and the native Vtg in the plasma in a similar manner. This indicates that the ELISA is suitable for detecting Vtg in greenback flounder plasma. The first step in the development of a Vtg ELISA is to obtain a purified Vtg and a specific antibody. The Vtg obtained by gel filtration chromatography is of high purity (Chapter 2). The specificity of the antibody in this assay was demonstrated by the fact that it did not show significant cross-reaction with any protein in the plasma of males.

A checkerboard was used to determine the optimal assay conditions in the present study. The final assay parameters employed in this ELISA are  $2.5 \mu\text{g ml}^{-1}$  Vtg coating concentration, 1: 100 antibody dilution and a range of standard concentrations between  $0.16\text{--}20 \mu\text{g ml}^{-1}$ . A comparison of these parameters with those used in other competitive ELISAs is shown in Table 3.1. The coating concentration of Vtg in greenback flounder was very close to that used in striped bass (*Morone saxatilis*) (Heppell et al., 1999), but much higher than the Vtg coating concentration in other species shown in Table 3.1. The 1: 100 antibody dilution chosen in this study was close to 1: 125 used for channel catfish (*Ictalurus punctatus*) where a monoclonal antibody was employed (Goodwin et al., 1992), but two-three orders of magnitude higher than that used for other species, as shown in Table 3.1. In optimising the present assay, absorbance values in the range 0–2 (the range of linear response of the micro-plate EIA reader) were sought. As shown in Fig. 3b, when the Vtg coating concentration was less than  $2.5 \mu\text{g ml}^{-1}$ , the absorbance values fell markedly and the displacement curves became flat. A similar trend also was observed when the antibody dilution was greater than 1: 100. This dilution is generally higher than antibody concentrations used

elsewhere (Table 3.1). Such a dilution was necessary due to the low titre of the antibody as indicated by the results of Western blotting, and was demonstrated to be feasible for the assay.

Table 3.1 Assay parameters of antibody-capture competitive ELISA for different teleost Vtgs

Species	Coating Vtg (ng ml <sup>-1</sup> )	Antibody dilution	Standard curve range (ng ml <sup>-1</sup> )	Sensitivity (ng ml <sup>-1</sup> )	Reference
<i>Solea vulgaris</i>	125	1: 100,000	12.5-1600	30 (90% binding)	Nunez Rodriguez et al.; 1989
<i>Morone saxatilis</i>	100	2.5 µg ml <sup>-1</sup>	8-1000	8 (91.4 ± 4.1% binding)	Kishida et al.; 1992
<i>Dicentrarchus labrax</i>	100	1: 100,000	1-60	1 (85% binding)	Mañanós et al., 1994
<i>Oncorhynchus mykiss</i>	100	1: 100,000	33-1473	9.4	Mourot and Le Bail, 1995
<i>Oncorhynchus mykiss</i>	100	1: 100,000	20-320	9 (92% binding)	Bon et al., 1997
<i>Sparus aurata</i>	100	1: 6000	3.9-2000	8 (90% binding)	Mosconi et al., 1998
<i>Pleuronectes vetulus</i>	200	1: 100,000	10-450	10 (85% binding)	Lomax et al., 1998
<i>Salmo trutta</i>	125	1: 240,000	25-500	10.5	Sherry et al., 1999b
<i>Mycteroperca microlepis</i>	500	1: 20,000	19-1452	19 (90% binding)	Heppell and Sullivan, 1999
<i>Salvelinus alpinus</i>	200	1: 25,000	2-125	2 (90% binding)	Johnsen et al., 1999
<i>Morone saxatilis</i>	3500	1: 20,000	33-1188	33 (90% binding)	Heppell et al., 1999
<i>Thunnus thynnus</i>	500	1: 7500	2.4-1250	8 (90% binding)	Susca et al., 2001
<i>Danio rerio</i>	150	1: 20,000	3-500	2-3 (85% binding)	Fenske et al., 2001

The maximum sensitivity of the ELISA for greenback flounder is  $160 \text{ ng ml}^{-1}$  of standard or a plasma equivalent of  $32 \text{ } \mu\text{g ml}^{-1}$ . The sensitivity of competitive ELISAs established by different researchers varies from  $33 \text{ ng ml}^{-1}$  in striped bass (Heppell et al., 1999) to  $1.0 \text{ ng ml}^{-1}$  in sea bass (*Dicentrarchus labrax*) (Mañanós et al., 1994) (Table 3.1). The detection limit for the flounder Vtg in plasma samples is  $32 \text{ } \mu\text{g ml}^{-1}$  at the chosen plasma dilution of 1: 200. A lower plasma dilution was used in some other studies, e.g. 1: 16 in rainbow trout (*Oncorhynchus mykiss*) (Mouroto and Le Bail, 1995). It is possible that using plasma at a higher concentration could lower the detection limit, e.g. a 1: 50 dilution has been used in some prior studies of fish Vtg ELISAs (Bon et al., 1997; Johnsen et al. 1999); however, this increases the risk of non-specific binding by other plasma proteins in the assay.

The estimation of Vtg levels in the three vitellogenic female greenback flounder used here gave similar values for all three fish. The absolute values were similar to those reported in other teleost species during vitellogenesis,  $10 \text{ mg ml}^{-1}$  in sole (*Solea vulgaris*) (Nunez Rodriguez et al., 1989),  $3.0 \text{ mg ml}^{-1}$  in sea bass (Mañanós et al., 1994),  $60 \text{ mg ml}^{-1}$  in rainbow trout (Bon et al., 1997),  $17 \text{ mg ml}^{-1}$  in English sole (*Pleuronectes vetulus*) (Lomax et al., 1998),  $1.5 \text{ mg ml}^{-1}$  in gilthead seabream (*Sparus aurata*) (Mosconi et al., 1998),  $22 \text{ mg ml}^{-1}$  in Arctic charr (*Salvelinus alpinus*) (Johnsen et al. 1999) and  $18 \text{ mg ml}^{-1}$  in bluefin tuna (*Thunnus thynnus*) (Susca et al., 2001). It remains to be demonstrated whether the range of values reported for various teleost species reflects real phylogenetic differences or the differing status of maturation of fish in the various studies. However, all species appear to have Vtg levels in the  $\text{mg ml}^{-1}$  range during vitellogenesis.

Common problems encountered when establishing ELISAs for teleost Vtgs include the need to develop a specific, homologous antibody for Vtg from each species and the low level immunological response seen on some occasions (Heppell et al., 1999). It was reported that the antibody of English sole Vtg could recognize Vtg-like material in related species (Lomax et al., 1998). Furthermore, a

gag (*Mycteroperca microlepis*) Vtg ELISA could be used to detect Vtg in other two grouper species (Heppell and Sullivan, 1999), and a striped bass Vtg ELISA was validated for measuring Vtg in two related species (Heppell et al., 1999). In contrast, a rainbow trout Vtg ELISA could not be used to quantify Vtg levels in other species of salmonids (Mourot and Le Bail, 1995). This apparent specificity suggests the necessity to develop a specific Vtg ELISA for each species or at least genus of fish under investigation (Mourot and Le Bail, 1995; Heppell et al., 1999). The potential cross-reactivity of flounder anti-Vtg with the Vtg of other species has yet to be assessed.

Several methods alternative to ELISA have been developed to measure the Vtg levels in fishes. For example, measurements of calcium and phosphoprotein phosphorous levels in fish plasma. But such methods were proven to be non-specific (reviewed in Tyler and Sumpter, 1990; Mourot and Le Bail, 1995). In contrast, immunoagglutination, immunoelectrophoresis and radial immunodiffusion, which exploit the specific reaction between antibody and antigen, showed high specificity but low sensitivity for measurement of fish Vtgs (reviewed in Mourot and Le Bail, 1995; Johnsen et al., 1999). Although radioimmunoassay can overcome these problems, it presents new problems such as the instability of radiolabeled Vtg (reviewed in Kishida et al., 1992; Mañanós et al., 1994) and the problem of management of radioactive waste (Specker and Anderson, 1994). Because of the evident advantages of ELISA over other methods, a competitive ELISA was developed in this study. The ELISA described here is an easy and quick method which can be used to assay multiple samples at once. The method is specific for greenback flounder Vtg and has a suitably high sensitivity.

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## **Chapter 4**

### **Effects of 17 $\beta$ -estradiol on vitellogenin induction *in vivo* in greenback flounder *Rhombosolea tapirina***

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## 4. Effects of 17 $\beta$ -estradiol on vitellogenin induction *in vivo* in greenback flounder *Rhombosolea tapirina*

### 4.1. Introduction

The accumulation by growing oocytes of vitellogenin (Vtg), a large yolk precursor protein, through the process of vitellogenesis is a common characteristic of all egg-laying vertebrates and is vital for the success of reproduction in fishes (Specker and Sullivan, 1994). Vitellogenin synthesis is under control of the hypothalamus-pituitary-gonad axis. Pituitary release of follicle-stimulating hormone (FSH) stimulates the release of 17 $\beta$ -estradiol (E<sub>2</sub>) from the ovarian tissue of maturing female fish. The E<sub>2</sub> is carried in the circulation to the liver where it triggers the production of Vtg which is, in turn, transported via the bloodstream to the ovary. At the ovary, Vtg is taken up by the growing oocytes and converted into final egg yolk (lipovitellins, phosvitins and phosvettes,  $\beta'$ -component, and C-terminal component) as nutrients for normal development of the embryo (reviewed in Specker and Sullivan, 1994; Van Der Kraak et al., 1998; Tyler et al., 2000; Matsubara et al., 2003).

It is becoming increasingly apparent that many environmental pollutants in the aquatic environment can interfere with the normal reproductive systems of fish by mimicking the effects of natural E<sub>2</sub> through binding with estrogen receptors (Sherry et al., 1999a). Male and juvenile fishes do have the Vtg gene, but it is normally not expressed because of low estrogen titers in the bloodstream. Vitellogenin production can be induced in males and immature animals upon their exposure to exogenous estrogens or estrogen mimics. The abnormal presence of Vtg in the plasma of males or juvenile fish can be used as a bio-indicator for the presence of estrogenic chemicals in the aquatic environment (reviewed in Specker and Sullivan, 1994; Sumpter and Jobling, 1995; Tyler et al., 2000).

While the role of E<sub>2</sub> in stimulating vitellogenesis is well understood (reviewed in Specker and Sullivan, 1994; Tyler et al., 2000), there is less knowledge of either the specific dose thresholds or the time-course of Vtg induction by exogenous E<sub>2</sub>. The investigation of these areas will help us to understand the role of E<sub>2</sub> in regulating Vtg gene expression and protein synthesis, and the stability of Vtg in plasma over time. As different studies have demonstrated that this induction process is affected by fish phylogeny, physiological status, and environmental factors (Mackay and Lazier, 1993; Pawlowski et al., 2000; Bowman et al., 2002; Hotta et al., 2003), i.e. the induction pattern is different among different species, it is necessary to characterise this process for each particular species.

The time-course of Vtg induction has been examined in rainbow trout (*Oncorhynchus mykiss*) (Arukwe et al., 2001) and sheepshead minnow (*Cyprinodon variegatus*) (Bowman et al., 2000) in terms of synthesis of both Vtg mRNA and protein. The relationship between doses of E<sub>2</sub> and Vtg levels has been described in fathead minnows (*Pimephales promelas*) (Panter et al., 1998), English sole (*Pleuronectes vetulus*) (Lomax et al., 1998), Atlantic salmon (*Salmo salar*) (Celius and Walther, 1998) and rainbow trout (Calson and Williams, 2001). The kinetics of Vtg induction appear to be different, and therefore difficult to compare, among various species (Lazier and MacKay, 1993). It is clear, however, that E<sub>2</sub> is the most potent estrogen for inducing vitellogenesis *in vivo*. For example, the ability of E<sub>2</sub> to induce Vtg is much stronger than that of *p*-nonylphenol and bis-phenol-A via water exposures (Tabata et al., 2001), and plasma Vtg concentrations induced by injection are higher for E<sub>2</sub> than from other natural or man-made estrogens (Tilton et al., 2001).

Because males have negligible E<sub>2</sub> titres but are estrogen-responsive in terms of Vtg production, and as Vtg has a longer residence time in the blood and can accumulate to very high levels in males (Specker and Anderson, 1994), male fish are good models for investigating the E<sub>2</sub>-Vtg relationship. The present study examines this relationship with respect to the time-course and dose-response for induction of vitellogenesis as assessed in greenback flounder (*Rhombosolea tapirina*) using the Vtg ELISA described in chapters 2 and 3 of this thesis.



Injection of E<sub>2</sub> in saline vehicle was employed, as this approach makes it possible to track the subsequent Vtg induction resulting from stimulation by an initial E<sub>2</sub> peak, because E<sub>2</sub> administration in saline enters and is cleared quickly from the bloodstream (Pankhurst et al., 1986). Three successive experiments were performed to examine the influences of exogenous E<sub>2</sub> on Vtg induction. The first experiment examined the time-course of Vtg induction. Male flounder received a single injection of E<sub>2</sub> at a dose previously demonstrated to induce vitellogenesis (Chapter 2), and the fish were serially sampled at various intervals after the injection. In the second experiment, fish were injected with a range of doses of E<sub>2</sub>, and were bled at sampling times, predicted on the basis of results of the first experiment, to produce maximum Vtg levels. Based on the results of the first two experiments, a third experiment was designed and conducted to assess the effect of multiple injections of E<sub>2</sub> on induction of vitellogenesis.

## 4.2. Materials and Methods

### 4.2.1. Fish treatment

Fourteen-month old male flounder were obtained from stocks grown at the School of Aquaculture, University of Tasmania in Launceston. Fish were maintained in the School aquatic facility in recirculating systems incorporating a biofilter, coarse solids filter and aeration system, and connected to 'Aquahort' temperature control units, and fed at ~ 3% body weight daily with commercial dry feed pellets (Pivot Aquaculture, Hobart). Water temperature was  $15 \pm 1^{\circ}\text{C}$  and photoperiod 14: 10 (light: dark) during experiments. Before each experiment, the gender of the fish was verified by visual observation the fish under background lighting, which makes the shape of the gonad apparent, and by manual application of gentle pressure to their abdomen in order to extrude milt from the males. Fish with body weights that ranged from 40-80 g were selected and released into 200-l holding bins for the duration of experiments. One quarter of the water volume was changed daily during the period of experiments.

For experiment, fish were anaesthetised in 0.05% 2- phenoxyethanol (Sigma), weighed, fin-clipped for identification and treated with either saline solution (ethanol: saline = 1: 9) as a control or E<sub>2</sub> suspension in saline solution (Pankhurst et al., 1986). All treatments were administered by intraperitoneal injection (ipi) in an injection volume of 1.0 ml kg<sup>-1</sup>. Blood samples were taken at each sampling time by caudal puncture using preheparinised 1 ml syringes and 25 G needles. Plasma was separated from blood samples by centrifuging at 12000 g for 3 min at 4°C, divided into two aliquots (10 µl for Vtg ELISA and 25 µl for steroid assay) and then stored frozen at -20°C until analysis. At the end of each experiment, fish were killed by spinal transection and macroscopic examination of the gonad was used to confirm gender classification.

#### **4.2.2. Experimental protocols**

Experiment one: Time-course of Vtg induction by E<sub>2</sub>

Fish were divided into two groups (n = 8), and injected with either saline or E<sub>2</sub> at a dose of 5.0 mg kg<sup>-1</sup> body weight (BW). The dose was chosen on the basis of a dose previously confirmed to stimulate Vtg production in male flounder (Chapter 2, this volume). Blood samples were collected at 0, 6, 24, 48, 96 and 144 h post-injection (p.i.).

Experiment two: Dose-response of Vtg induction by E<sub>2</sub>

Groups of fish (n = 7) were treated with either 1) saline; 2) E<sub>2</sub> at 0.5 mg kg<sup>-1</sup> BW; 3) E<sub>2</sub> at 1.0 mg kg<sup>-1</sup> BW; or 4) E<sub>2</sub> at 5.0 mg kg<sup>-1</sup> BW. Blood samples were collected at 0, 48 and 96 h p.i..

Experiment three: Vtg induction by serial injections of E<sub>2</sub>

Three groups of fish (n = 7) received three injections at an interval of 96 hours of either 1) saline; 2) E<sub>2</sub> at 0.1 mg kg<sup>-1</sup> BW; or 3) E<sub>2</sub> at 1.0 mg kg<sup>-1</sup> BW. Fish were bled at 0 h and 96 h after each injection.

#### **4.2.3. Vtg measurement**

Plasma samples were diluted to 1: 200 in 20 mM Tris-0.5 M NaCl buffer (pH 7.2) containing 3% skim milk, and Vtg was measured using the ELISA described in Chapter 3. The detection limit for Vtg in plasma is 32  $\mu\text{g ml}^{-1}$ . Inter-assay variability (%CV) was measured using aliquots of a pooled internal standard and was 12.8% (n = 10).

#### ***4.2.4. E<sub>2</sub> measurement***

Plasma E<sub>2</sub> levels were measured by radioimmunoassay using the reagents and protocol described in Pankhurst and Conroy (1987). Briefly, 25  $\mu\text{l}$  of plasma were extracted with 250  $\mu\text{l}$  ethyl acetate, and 100  $\mu\text{l}$  extract was used for each assay tube. The detection limit of E<sub>2</sub> in plasma was 0.3  $\text{ng ml}^{-1}$ , and inter-assay variability (%CV determined from repeat measurement of a pooled internal standard) was 14.3% (n = 5).

#### ***4.2.5. Statistical analysis***

Data were analyzed by one-way ANOVA or T-Test followed where appropriate by a Student-Newman-Keuls means comparison test, using the computer package SPSS 10.0 for Windows. Except where indicated, a significant difference of  $p < 0.05$  was used for all tests.

### **4.3. Results**

There was some fish mortality during the experiments and the gender of some fishes could not be ascertained because of the immature state of their gonads. Therefore, n values were lower in some experiments than described in Materials and Methods. The n values are noted below on the individual.

#### ***4.3.1. Experiment one***

Treatment of male flounder with E<sub>2</sub> at a dose of 5.0  $\text{mg kg}^{-1}$  caused a rapid and significant increase of E<sub>2</sub> level in plasma within 6 h (Fig. 4.1a). After that, E<sub>2</sub>

levels started to decrease, but significant elevations of  $E_2$  were maintained beyond 48 h p.i..  $E_2$  levels were not elevated above controls at 96 and 144 h p.i.. Vtg appeared in the plasma at 48 h p.i. and measurable levels were also present at 96 and 144 h p.i. (Fig. 4.1b). Compared with the control group, a significant difference of plasma Vtg level was only seen in  $E_2$ -treated fish at 48 h p.i. despite the highest mean value at 96 h p.i. where the t value approached significance ( $P < 0.075$ ). The lack of significant difference at 96 h p.i. is attributable to the high variability of Vtg level in  $E_2$ -treated fish at this sample time.

#### **4.3.2. Experiment two**

There was no significant difference of plasma  $E_2$  levels between the control group and any  $E_2$ -treated group at 0 and 96 h p.i., nor was there any difference among  $E_2$ -treated groups at different doses. However, plasma  $E_2$  levels in  $5.0 \text{ mg kg}^{-1}$  group were significantly higher than those in other groups at 48 h p.i., but there was no difference between the saline group and the two lower  $E_2$  dose groups (Fig. 4.2). Plasma Vtg levels were elevated over the control at 48 h p.i. in response to treatment with  $E_2$  at a dose of  $5.0 \text{ mg kg}^{-1}$ , but not other doses. At 96 h p.i., plasma Vtg levels were significantly elevated over the control in all  $E_2$ -treated groups, and levels in fish treated with  $E_2$  at  $5.0 \text{ mg kg}^{-1}$  were significantly higher than in fish treated with  $E_2$  at  $0.5$  and  $1.0 \text{ mg kg}^{-1}$  (Fig. 4.2).

#### **4.3.3. Experiment three**

Plasma  $E_2$  levels showed no difference among different treatments at any sample time (Fig. 4.3). There was no detectable Vtg in the plasma of fish in all treatments at 0 h. In contrast, there were significant increases in plasma Vtg levels in fish treated with  $E_2$  at  $1.0 \text{ mg kg}^{-1}$  at all subsequent sampling times. Vtg levels were detectable in fish treated with  $0.1 \text{ mg kg}^{-1}$   $E_2$  at 96, 192 and 288h whereas there was no detectable Vtg in controls, but values were not significantly different from control values at any time (Fig. 4.3).

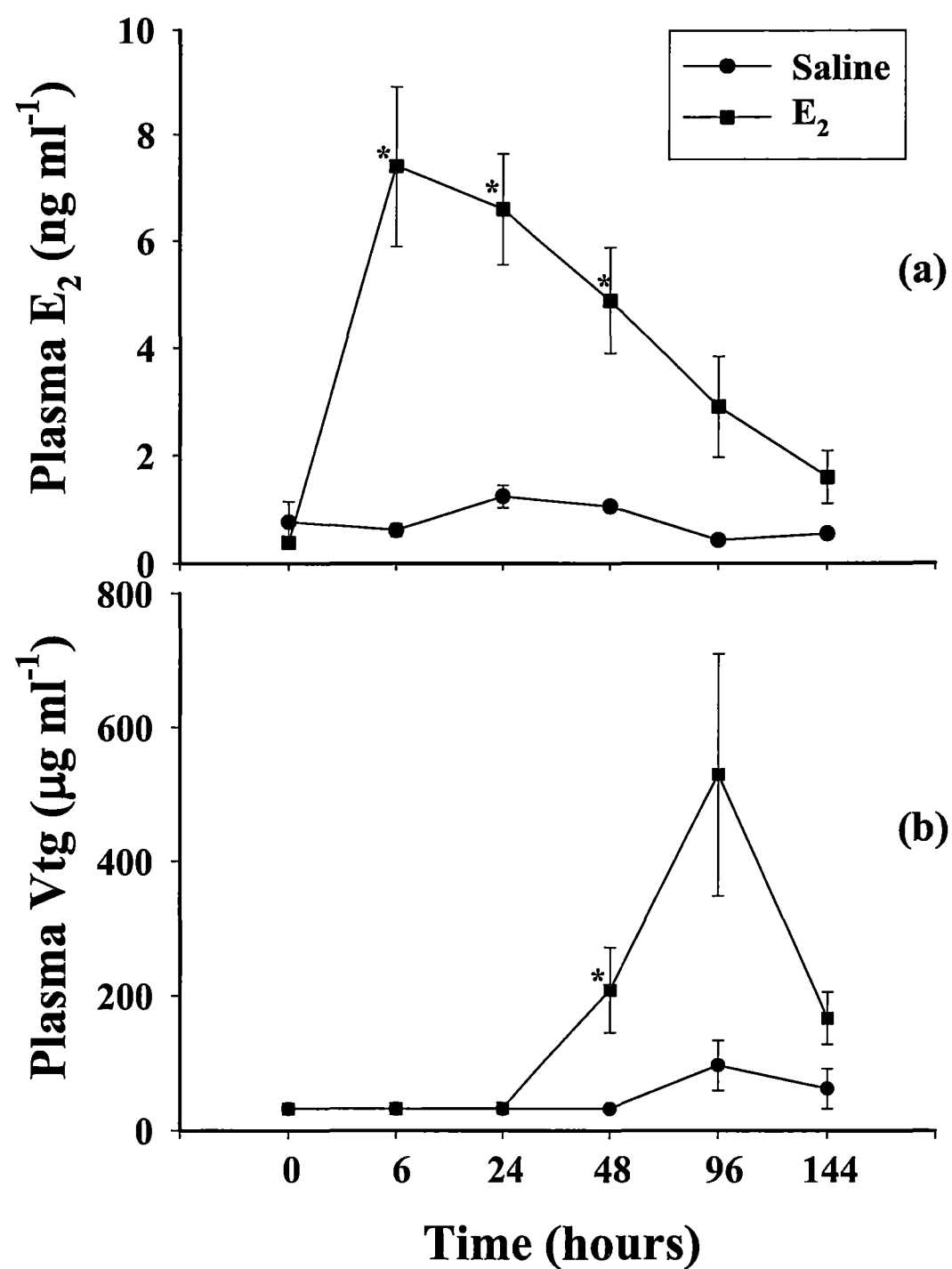


Fig. 4.1. Plasma levels of E<sub>2</sub> and Vtg in male flounder injected with saline or E<sub>2</sub> (5.0 mg kg<sup>-1</sup>). Values are mean ± SE (n = 4 in the saline-treated group; n = 5 in the E<sub>2</sub>-treated group). \* = difference between E<sub>2</sub> and saline treatments at that sample time (P < 0.05).

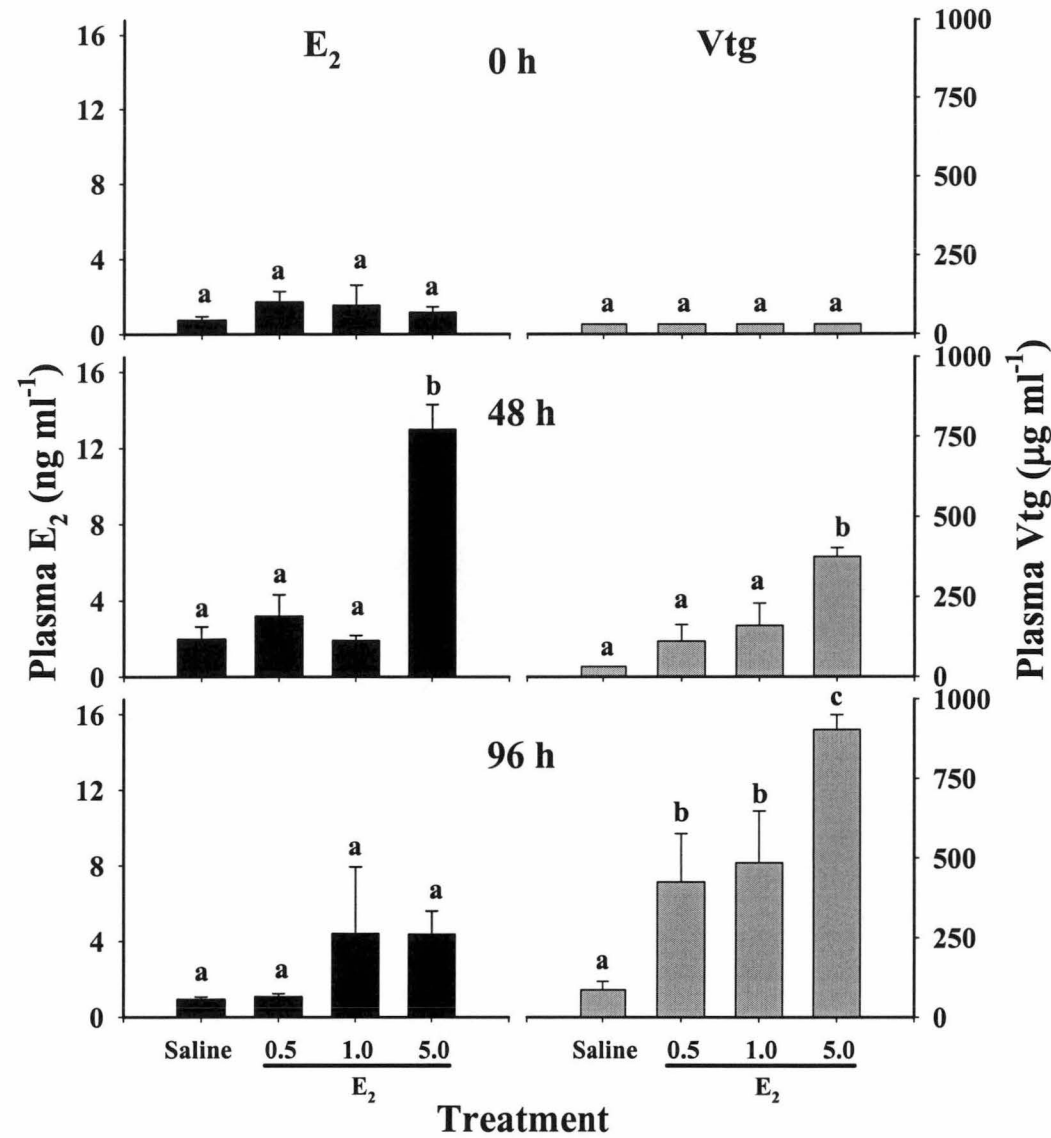


Fig. 4.2. Plasma levels of  $E_2$  and Vtg in male flounder injected with saline, or  $E_2$  at 0.5, 1.0 or 5.0 mg kg<sup>-1</sup>. Values are mean + SE (n = 6 in saline, 0.5 and 5.0 groups; n = 4 in 1.0 group). Common superscripts show values that are not significantly different (P > 0.05); comparisons are between treatments at different times.

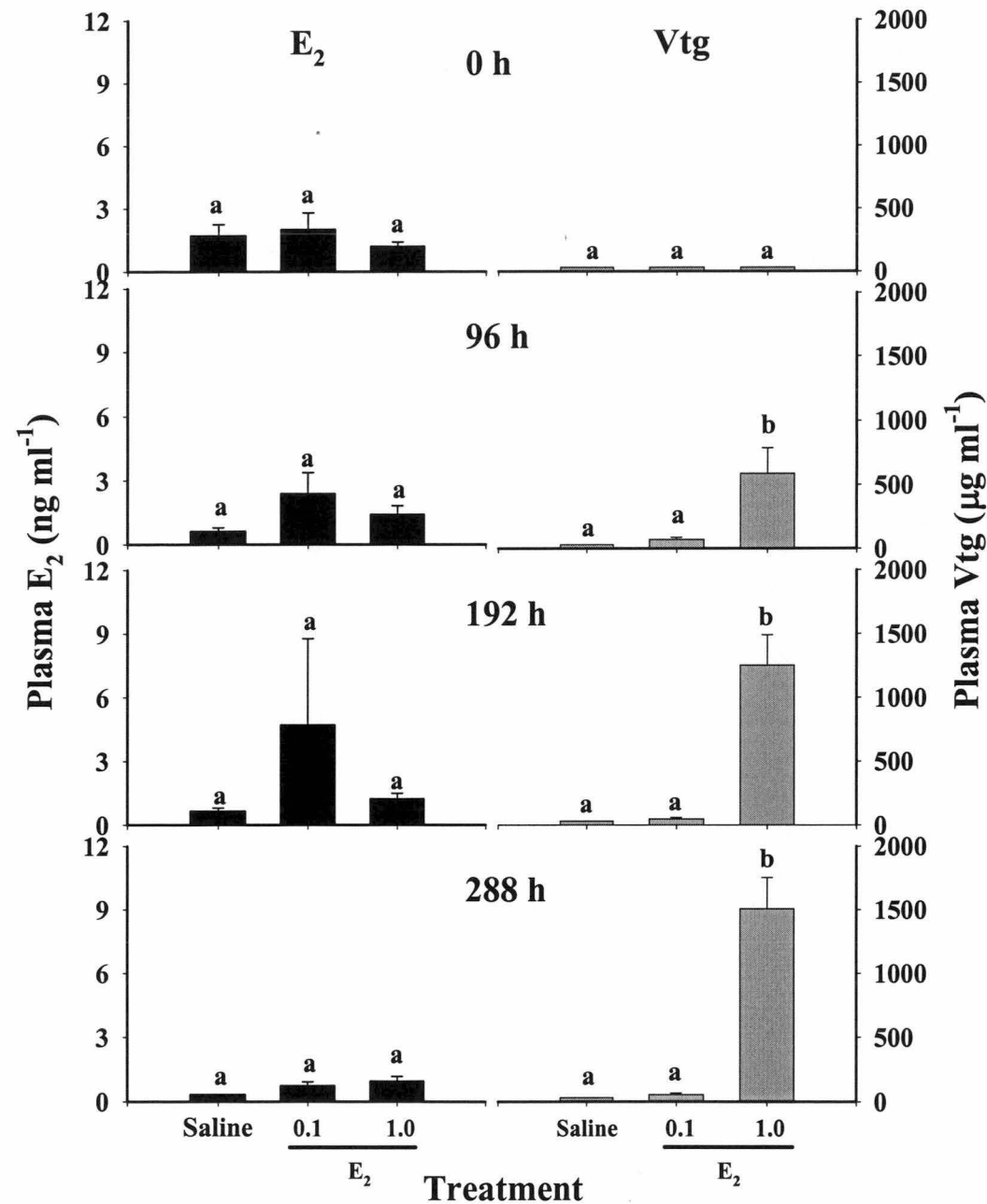


Fig. 4.3. Plasma levels of  $E_2$  and Vtg in male flounder injected with 3 injections of saline, or  $E_2$  at 0.1 or 1.0 mg kg<sup>-1</sup> at an interval of 96 hours. Values are mean + SE (n = 5). Other details are same as for Fig. 4.2.

#### 4.4. Discussion

In the present study, three consecutive experiments were carried out to assess the effects of E<sub>2</sub> on vitellogenin induction *in vivo* in greenback flounder. In the time-course experiment, the treatment of male greenback flounder with E<sub>2</sub> at a dose of 5.0 mg kg<sup>-1</sup> resulted in a rapid elevation of serum E<sub>2</sub> level in 6 h p.i., and the increase was maintained for at least 48 h. A similar trend was observed in goldfish (*Carassius auratus*) where injection with E<sub>2</sub> in saline at 2.0 or 20 mg kg<sup>-1</sup> resulted in peak E<sub>2</sub> levels at 1 h p.i. (Pankhurst et al. 1986). Plasma E<sub>2</sub> levels may also have peaked at an earlier time than the first sample at 6 h p.i. in greenback flounder. Compared with flounder, E<sub>2</sub> levels in goldfish fell more quickly, with E<sub>2</sub> levels falling from over 150 ng ml<sup>-1</sup> at 1 h p.i. to 8.0 ng ml<sup>-1</sup> at 24 h p.i.. The difference may relate to specific variations in clearance rate, or the fact that goldfish experiments were conducted at 20°C compared with 15°C in flounder. The present study does confirm the findings of Pankhurst et al. (1986) that E<sub>2</sub> in saline is released very rapidly to the plasma.

In the present study, treatment with E<sub>2</sub> at 5.0 mg kg<sup>-1</sup> resulted in the successful induction of Vtg production in all experimental animals, but it took at least 48 h for Vtg to appear in the plasma. In contrast, the initial high pulse of E<sub>2</sub> appeared within 6 h p.i. and the extended elevation of E<sub>2</sub> levels lasted over 48 h p.i.. Because there was only a single peak in Vtg production, and this was not sustained beyond 96 h p.i., it is probable that the initial high E<sub>2</sub> pulse rather than the extended elevation of E<sub>2</sub> induced the subsequent production of Vtg. This would suggest that there is a time-lag of about 40 h between the elevation of E<sub>2</sub> and the peak of Vtg levels. A similar effect was found in squirrelfish (*Holocentrus ascensionis*) where the highest plasma Vtg level occurred 24 h after peak E<sub>2</sub> levels (Thompson et al., 2001). In the bloodstream, E<sub>2</sub> first combines with serum steroid-binding proteins, before entering hepatocytes in the liver by diffusion, where it binds with the estrogen receptor, triggering expression of Vtg genes. This initiates a series of steps including transcription, translation and post-translational modification, before completed Vtg is secreted into the blood (reviewed in Lazier and MacKay, 1993). In both sheepshead minnow and rainbow trout, Vtg mRNA



levels increased in a time-dependent manner and accumulated to a maximum level at 48 and 72 h p.i. respectively, after E<sub>2</sub> treatment (Bowman et al., 2000; Arukwe et al., 2001). These observations suggest that the time delay between E<sub>2</sub> peak and Vtg levels reflects the time-course for the combination of events described above.

A single injection of E<sub>2</sub> at a high dose (e.g. 5.0 or 10 mg kg<sup>-1</sup>) has also proven to be an effective method for Vtg induction *in vivo* in other species (Roubal, et al., 1997; Arukwe et al., 1999; Arukwe et al., 2001) with the time-course of Vtg induction being affected by a number of factors. Firstly, E<sub>2</sub> concentration can affect the time of appearance of Vtg in the plasma. Folmar et al. (2001) proposed that Vtg levels following treatment with high E<sub>2</sub> doses usually increased Vtg faster and to higher levels than the increase in plasma Vtg following lower E<sub>2</sub> dose. MacKay and Lazier (1993) also suggested that the induction of the hepatic translational and secretory apparatus was accelerated by high doses of E<sub>2</sub>. High-dose E<sub>2</sub> treatment led to earlier detection of Vtg in plasma than seen after administration of lower E<sub>2</sub> doses to European eels (*Anguilla anguilla*) (Luizi et al., 1997) and fathead minnows (Parks et al., 1999). Similarly, in tilapia (*Oreochromis mossambicus*), the appearance of the Vtg peak in plasma following treatment with 10 mg kg<sup>-1</sup> E<sub>2</sub> was 48 hours earlier than that following treatment with 0.1 mg kg<sup>-1</sup> E<sub>2</sub> (Takemura and Kim, 2001). Secondly, species sensitivity to E<sub>2</sub> administration combined with the temperature at which experiments were conducted may be responsible for different time-courses for induction of vitellogenesis among species. Increased temperature usually increases the rate of uptake, metabolism and clearance of E<sub>2</sub> which accelerates the appearance time of the initial high pulse and the following rate of decline of E<sub>2</sub> in the plasma (Pankhurst et al. 1986). Increased temperature also accelerates the rate of Vtg gene expression and subsequent protein synthesis and release to plasma (MacKay and Lazier, 1993). When a similar treatment as in the present study was employed in rainbow trout, the first observation of Vtg and the appearance of peak Vtg levels in the plasma were at 48 and 72 h p.i. respectively, even though the temperature was lower (10-11°C) than that used in the present study (Arukwe et al., 2001). In brown trout (*Salmo trutta*) held at 8-10°C, the highest Vtg level was present in plasma 11 days after a single injection of E<sub>2</sub> in saline (Sherry et al.,

1999b). These various results suggest that the time-course of Vtg induction may be species-specific as well as temperature-dependent.

Funkenstein et al. (2000) proposed that the E<sub>2</sub> injection vehicle had an influence on the time-course of Vtg induction. Compared with saline as an injection vehicle, E<sub>2</sub> in vehicles such as cocoa butter was released at a much slower rate which may enhance the persistence of a relatively high level of E<sub>2</sub> (Pankhurst et al. 1986). As a consequence, it may take longer for the development of elevated Vtg to occur but Vtg also will persist longer in the plasma. For example, where cocoa butter was used as vehicle, Vtg was detected in gilthead seabream (*Sparus aurata*) as early as 3 days after E<sub>2</sub> treatment but the increase was sustained for 30 days (Funkenstein et al., 2000).

There can be a small amount of E<sub>2</sub> in the plasma of male and immature fish, but normally such a level of E<sub>2</sub> does not trigger Vtg induction in the liver (Specker and Sullivan, 1994). In the present study, a measurable level of E<sub>2</sub> was found in all control fish and E<sub>2</sub>-treated fish prior to injection, and a low level of Vtg was detected in the plasma of control fish sampled at 96 and 114 h p.i. in Experiment 1 and 96 h p.i. in Experiment 2. A similar finding was also observed in lamprey (*Lampetra fluviatilis*) where the possibility of phyto- or xenoestrogenic effects of the E<sub>2</sub> vehicle (walnut oil) was excluded (Mewes et al., 2002). In contrast, no measurable induction of Vtg was found in the plasma of saline-treated brown trout (Sherry et al., 1999b). Budworth and Senger (1993) found that there was a transfer of testosterone from hormone-treated fish to control fish in a recirculating-water system. E<sub>2</sub>-treated and control fish were held in the same container in the present study, with the result that high-dose E<sub>2</sub> treatment could result in the release of E<sub>2</sub> into water through gills, and subsequent absorption by control fish, resulting in detectable Vtg levels in control fish in some experiments. The fact that Vtg was never detectable in controls at the beginning of the experiments indicates that the low levels of E<sub>2</sub> found in the plasma of males are insufficient to stimulate Vtg production.

In Experiment 2 in the present study, there was no significant elevation of plasma  $E_2$  levels in response to  $E_2$  at 0.5 and 1.0 mg kg<sup>-1</sup>, but there was with the 5.0 mg kg<sup>-1</sup> treatment, as in the first experiment. This is probably due to lower doses being cleared more quickly. In goldfish, treatment with  $E_2$  at 20 mg kg<sup>-1</sup> resulted in a much higher plasma  $E_2$  level than treatment with 0.2 mg kg<sup>-1</sup>, and plasma  $E_2$  levels fell more quickly following the low dose treatment (Pankhurst et al., 1986). The lack of an elevation of plasma  $E_2$  levels in all treatments at 96 h p.i. in the present study is consistent with that in Experiment 1. The highest dose of  $E_2$  (5.0 mg kg<sup>-1</sup>) led to a significant increase in Vtg levels in plasma at 48 h p.i.. In contrast, the two lower dose treatments needed 96 h to produce an increase in Vtg. The effect of dose of  $E_2$  on the time-course of Vtg induction has been discussed earlier and the results in this experiment are consistent with that effect. As 0.5 mg ml<sup>-1</sup>  $E_2$  produced significantly elevated Vtg levels compared with the control at 96 h p.i., this dose was above the threshold for inducing Vtg in greenback flounder. A treatment of 0.1 mg kg<sup>-1</sup> was added to Experiment 3 to determine the threshold dose of  $E_2$  required for Vtg induction in this species. A small amount of Vtg was detected in plasma at 96 h p.i., but this was not significant relative to control values. Combined results from the two experiments show that plasma levels of Vtg increased in flounder in a dose-dependent manner in response to  $E_2$  treatment with a threshold  $E_2$  dose for Vtg induction of about 0.1 mg kg<sup>-1</sup>. In other species, the threshold of  $E_2$  dose that induced Vtg production was 8.0 µg kg<sup>-1</sup> in English sole (Lomax et al., 1998) and 10 µg kg<sup>-1</sup> in Atlantic salmon (Celius and Walther, 1998). Different threshold responses may be attributable to treatment with  $E_2$  in different vehicles e.g. coconut oil in English sole (Lomax *et al.*, 1998), soybean oil in Atlantic salmon (Celius and Walther, 1998) and saline in greenback flounder. A second possibility is the difference of detection limits of Vtg ELISAs used in the different studies, e.g. 1.0 µg ml<sup>-1</sup> in English sole (Lomax et al., 1998) and 32 µg ml<sup>-1</sup> in flounder.

Other studies also have shown that there is a dose-response relationship between  $E_2$  and Vtg concentration. In English sole, when fish received an injection of  $E_2$  in coconut oil at doses ranging from 8.0 to 5000 µg kg<sup>-1</sup>, Vtg levels in the plasma rose from 1.0 to 600 µg ml<sup>-1</sup> (Lomax et al., 1998). When fathead minnows were

exposed to E<sub>2</sub> from 10 to 1000 ng ml<sup>-1</sup> through a continuous flow exposure system, plasma Vtg levels increased from < 100 ng ml<sup>-1</sup> to > 0.5 mg ml<sup>-1</sup> (Panter et al., 1998). In rainbow trout exposed to E<sub>2</sub> via diet, plasma Vtg levels increased from around 0.1 to over 1.0 mg ml<sup>-1</sup> as E<sub>2</sub> doses increased from 0.05 to 12.5 mg kg<sup>-1</sup> (Carlson and William, 2001). A similar situation was also observed *in vitro* using cultured tilapia hepatocytes and measuring levels of the two tilapia Vtgs (Vtg210 and Vtg140) in the culture medium. In this case, as E<sub>2</sub> concentration increased from 10<sup>-7</sup> to 10<sup>-5</sup> M, levels of Vtg210 and Vtg140 increased from around 40 to 1000 ng ml<sup>-1</sup> and from 23 to over 500 ng ml<sup>-1</sup> respectively (Takemura and Kim, 2001). Le Guevel and Pakdel (2001) further demonstrated that E<sub>2</sub> strongly stimulated Vtg gene expression in rainbow trout hepatocyte cultured at a concentration of 1.0 µmol l<sup>-1</sup> and reached a maximum level at 10 µmol l<sup>-1</sup>. Although different injection vehicles, different exposure routes and different models (whole fish *in vivo* or hepatocytes *in vitro*) were employed in these studies, a positive correlation between E<sub>2</sub> doses and Vtg or Vtg mRNA levels existed in all of them.

In Experiment 3 in the present study, a measurable level of Vtg was detected following treatment with 0.1 mg kg<sup>-1</sup> E<sub>2</sub> but there were no differences from controls in all three sample times. This suggests that Vtg induction could not be increased at a low E<sub>2</sub> dose even with multiple injections. In contrast, fish treated with E<sub>2</sub> at 1.0 mg kg<sup>-1</sup> showed plasma Vtg levels at 192 h that were twice as high as those at 96 h and this was maintained at 288 h. A much higher Vtg level obtained after a second injection of E<sub>2</sub> was also observed in lampreys (*Lampetra fluviatilis*) with plasma Vtg levels after the second injection being 800 times higher than that after the first injection. This was attributed to a ‘memory’ effect with multiple injections (Mewes et al., 2002). After E<sub>2</sub> exposure, the number of E<sub>2</sub> receptors (ER) increases, and as the expression of Vtg genes directly depends on the number of ER, this further improves the sensitivity of E<sub>2</sub>-response elements located upstream of Vtg genes (Lazier and MacKay, 1993; Valotaire et al., 1993; Flouriot et al., 1997). Both the ER and Vtg gene transcripts and mRNA stability are enhanced in the presence of E<sub>2</sub> (Flouriot et al., 1996). Bowman et al. (2000) also found that double injections could increase the half-life and stability of Vtg

mRNA and protein compared with a single injection. In the studies by Bowman et al. (2000) and Mewes et al. (2002), the second injection was applied during the period when Vtg levels were declining. In contrast, repeated injections of greenback flounder in the present study occurred while Vtg levels were still high. There was an increase in Vtg levels 96 h after a second injection, but the magnitude of the increase was much smaller than that in lamprey (Mewes et al., 2002). This may indicate that the increase of plasma Vtg levels after serial injections of E<sub>2</sub> in flounder is not a memory effect of the type described above but an additive effect of the extended elevation of plasma E<sub>2</sub>.

In summary, the induction of Vtg was demonstrated to be a time- and dose-dependent process in greenback flounder as in other species, and it seemed that multiple injections with moderate rather than high doses of E<sub>2</sub> were the most effective method for inducing Vtg production.

#### 4.5. References

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## **Chapter 5**

### **Patterns of oocyte growth, vitellogenin and gonadal steroid concentrations in greenback flounder *Rhombosolea tapirina***

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## 5. Patterns of oocyte growth, vitellogenin and gonadal steroid concentrations in greenback flounder *Rhombosolea tapirina*

### 5.1. Introduction

The patterns of oocyte growth in teleosts can be divided into three basic types depending on the range of developmental stages of oocytes present in ovaries during maturation, and the spawning frequency in each single reproductive season. These patterns are: (1) *synchronous ovarian* growth where there is only one clutch of oocytes in the ovary and the fish spawns once in a lifetime, (2) *group synchronous ovarian* growth where at least two clutches of oocytes are present in the ovary and spawning occurs once per season, and (3) *multiple group synchronous* or *asynchronous ovarian* growth where several clutches of oocytes are found in the ovary at once and there is multiple spawning over a reproductive season (reviewed in Wallace et al., 1987; Pankhurst, 1998). Previous studies on wild populations of the Australian pleuronectid *Rhombosolea tapirina* Günther (greenback flounder) have shown that there is multiple group synchronous ovarian development (Barnett and Pankhurst, 1999) and the capacity for multiple spawning. Females have a distinct separation of the developing oocyte clutches and can be induced to ovulate daily by the treatment with human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone analogue (LHRH-a) (Poortenaar and Pankhurst, 2000a). Both plasma concentrations of 17 $\beta$ -estradiol (E<sub>2</sub>) and testosterone (T) are generally elevated in association with vitellogenesis (Barnett and Pankhurst 1999), but the specific relationship between oocyte growth and plasma steroid concentrations remains to be described. In particular there is little information about maturational synchrony among individuals either in wild or in cultured populations.

Vitellogenesis is a particularly significant period of reproductive development and is characterized by rapid growth of oocytes and may be responsible for accumulation of up to 90 % of the final egg size (Tyler et al., 2000). As in other

oviparous vertebrates, the synthesis of vitellogenin (Vtg) in teleosts is regulated by estrogens, principally E<sub>2</sub> (Specker and Sullivan, 1994). In the ovarian follicles of maturing females, T is produced in the thecal layer under the stimulation of follicle stimulating hormone (FSH), then it is converted to E<sub>2</sub> in the presence of aromatase in granulosa layer (Nagahama, 1994). After formation, Vtg is secreted into the bloodstream by which it is transported to the ovary, where it is incorporated into growing oocytes as a necessary nutrient for the development of embryos (Specker and Sullivan, 1994; Tyler et al., 2000). Hence, plasma profiles of Vtg, E<sub>2</sub> and T are sensitive indicators for detecting the onset and progression of vitellogenesis and oocyte growth.

A wide range of studies shows that there is a positive correlation between plasma concentrations of Vtg, E<sub>2</sub> and T and the growth of oocytes in teleost fishes (e.g. Thomas et al., 1994; Rinchard et al., 1997; Kokokiris et al., 2001; Schafhauser-Smith and Benfey, 2002; King and Pankhurst, 2003). Various aspects of ovarian development and its endocrine correlates have been described in some flatfishes as well. For example, there are histological studies of oocyte development in winter flounder *Pseudopleuronectes americanus* (Walbaum) (Burton and Idler, 1984), European flounder *Pleuronectes flesus* L. (Janssen et al., 1995) and American plaice *Hippoglossoides platessoides* (Fabricius) (Maddock and Butrton, 1999), measurement of oocyte size in sole *Solea solea* (L.) (Ramsay and Witthames, 1996), histological assessment in association with plasma concentrations of E<sub>2</sub> in *Pleuronectes americanus* Walbaum (Harmin et al., 1995) and summer flounder *Paralichthys dentatus* (L.) (Merson et al., 2000), plasma concentrations of Vtg and steroids during seasonal reproduction in Atlantic halibut *Hippoglossus hippoglossus* (L.) (Methven et al., 1992), and seasonal changes of plasma steroids (Wingfield and Grimm, 1977) and plasma concentrations of steroids in relation to oocyte final maturation and ovulation (Scott et al., 1998) in plaice *Pleuronectes platessa* L.. However, a detailed investigation of plasma concentrations of Vtg and E<sub>2</sub> in association with the development of oocytes has only been performed in English sole *Parophrys vetulus* Girard (Johnson et al., 1991). The greenback flounder (*R. tapirina*) is considered as a potential new aquaculture species in Tasmania (Hart, 1991). A

thorough understanding of endocrine changes correlated to oocyte growth in this species is useful and, in fact, necessary both for solving the bottlenecks in reproductive development usually met in cultured fish (Pankhurst, 1998) and for providing more information about this biological process in flatfishes generally.

In the present study, an intense strategy of fortnightly sampling was performed on previously mature greenback flounder over the autumn period of normal reproductive development. Gonadosomatic index ( $I_G$ ), hepatosomatic index ( $I_H$ ), plasma Vtg and steroid concentrations and oocyte size were measured at each sampling time. The  $I_G$  and  $I_H$  are two useful indices for the assessment of anabolism in the gonad and the liver during ovarian growth (Scott and Pankhurst, 1992). Measurements of plasma steroid concentrations combined with the oocyte size can provide an articulate description of ovarian development (Pankhurst, 1998), and, as noted above, elevated plasma Vtg concentrations are indicative of periods of oocyte growth. We attempted to understand the changing trend of  $I_G$ ,  $I_H$  and plasma concentrations of Vtg,  $E_2$  and T in terms of sampling time, and the stage of ovarian development to determine the precise relationship between plasma concentrations of Vtg,  $E_2$  and T and the growth of oocytes. Fish were held on natural photoperiod but with artificially declining temperatures to mimic the natural autumn temperature regime. Previous studies (Poortenaar 1998; Barnett and Pankhurst, 1999) and our unpublished data indicated that spawning is usually concentrated in winter with recrudescence apparently occurring through the autumn.

## **5.2. Materials and Methods**

### ***5.2.1. Fish maintenance***

Reproductively developing, previously mature female flounder were obtained from stocks grown at the School of Aquaculture, University of Tasmania in Launceston. Fish were maintained in the School aquatic facility in recirculating systems incorporating a biofilter, coarse solids filter and aeration system, and connected to 'Aquahort' temperature control units. Fish were fed with commercial

dry feed pellets (Pivot Aquaculture, Hobart) at ~ 3% body weight daily. Water temperature was initially  $17 \pm 1^\circ\text{C}$  and fish were held under natural photoperiod ( $42^\circ 25'\text{S}$ ).

### 5.2.2. Sample collection

Fish were sampled fortnightly during the period from February to June 2002 (hereafter referred to as samples A to I). The reproductive season in this species normally starts in March, which is covered by the present sampling regime. A drop in water temperature was initiated from the third sampling point (C) with a stepwise drop of  $1^\circ\text{C}$  at every sampling time that followed. This was to produce a simulated natural autumn fall in temperature (Fig. 5.1). At each sampling time, seven fish were randomly selected and the sex of fish was confirmed by visual observation under background lighting to show the shape of the gonad. Fish were anaesthetised in 0.05% 2- phenoxyethanol (Sigma), blood samples taken by caudal puncture using preheparinised 1 ml syringes and 25 G needles, and stored in Eppendorf tubes on crushed ice before centrifuging at 12000 g for 3 min at  $4^\circ\text{C}$  to separate plasma. Plasma samples were divided into two aliquots (10  $\mu\text{l}$  for Vtg ELISA and 100  $\mu\text{l}$  for steroid assay) and then stored frozen at  $-20^\circ\text{C}$  until analysis. Fish were killed by spinal transection and kept in labelled plastic bags on crushed ice before dissection.

### 5.2.3. Whole animal and gonadal measurements

The weight of whole fish (BW), gonads (GW) and livers (LW) and the length of fish (L) were measured; condition factor (CF) ( $\text{CF} = \text{BW} \times 100 / \text{L}^3$ ),  $I_G$  ( $I_G = \text{GW} \times 100 / \text{BW}$ ) and  $I_H$  ( $I_H = \text{LW} \times 100 / \text{BW}$ ) were calculated. For each fish, the diameters of 100 oocytes were measured from preparations of fresh tissue dispersed in saline as described in Barnett and Pankhurst (1999), with the size frequency determined at an interval of 50  $\mu\text{m}$  at sizes from 50 to 1000  $\mu\text{m}$ . The number of oocytes measured had been employed previously to determine oocyte size-frequency distribution and classification of oocyte stages in greenback flounder (Barnett & Pankhurst, 1999; Poortenaar & Pankhurst, 2000a) and other

species (Thomas et al., 1994; Cerdá et al., 1996; Asturiano et al., 2000; Poortenaar et al., 2001; Arocha, 2002). The highest frequency of the larger oocytes present in each fish was defined as the modal maturing oocyte size. The development of the gonad was classified into four stages according to oocyte diameter based on Barnett and Pankhurst (1999), which was verified by using standard paraffin wax histology for ovarian examination (Table 5.1).

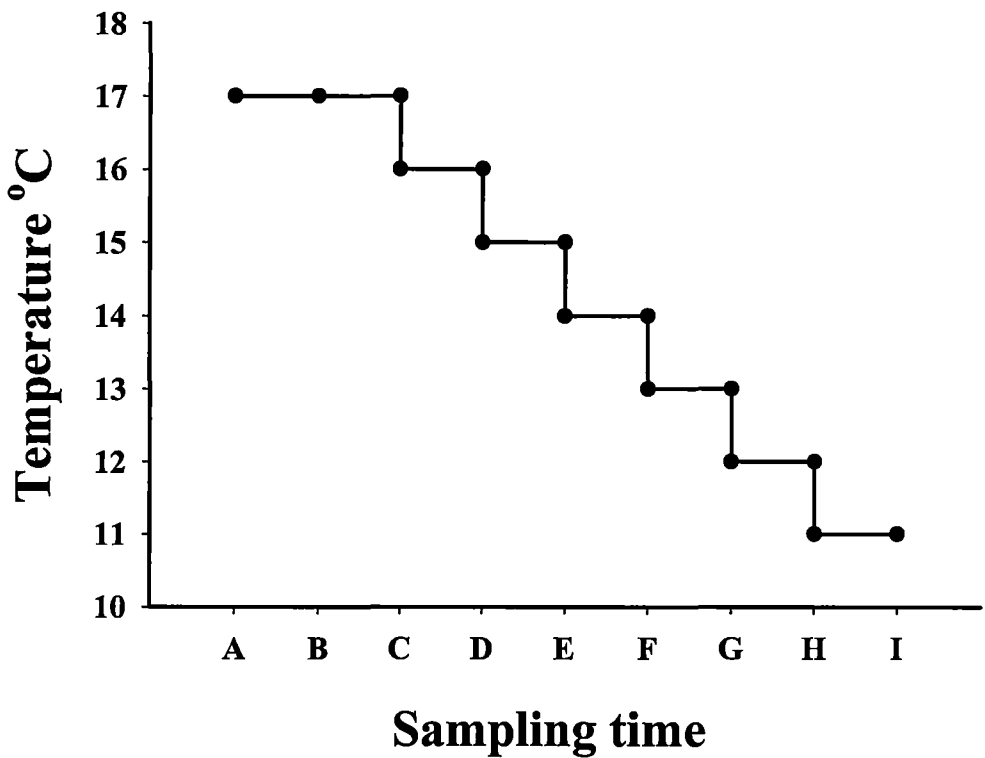


Fig. 5.1. Water temperature profile during the period of sampling. Letters from A (February) to I (June) are different sampling times at an interval of two weeks.

Table 5.1. Criteria for classification of *R. tapirina* gonad stages modified from Barnett and Pankhurst (1999)

Gonad stage	Oocyte classification	Oocyte diameter (D) (µm)
1	Previtellogenic	D < 150
2	Cortical alveolar	150 ≤ D < 250
3	Mid-late vitellogenic	250 ≤ D ≤ 450
4	Final maturation	> 450



#### **5.2.4. *Vtg measurement***

Plasma samples were diluted to 1: 200 in 20 mM Tris-0.5 M NaCl buffer (pH 7.2) containing 3% skim milk and Vtg was measured using the ELISA described in Chapter 3. The detection limit of Vtg in plasma is 32  $\mu\text{g ml}^{-1}$ . Inter-assay variability (%CV) was measured using aliquots of a pooled internal standard and was 3.45% (n = 3).

#### **5.2.5. *Steroid measurement***

Plasma concentrations of  $\text{E}_2$  and T were measured by radioimmunoassay using the reagents and protocols described in Pankhurst and Conroy (1987). Briefly, 100  $\mu\text{l}$  of plasma were extracted with 1 ml ethyl acetate, and 200  $\mu\text{l}$  extract was used for each assay tube, and samples were measured in duplicate. The detection limit of  $\text{E}_2$  and T in plasma was 0.15  $\text{ng ml}^{-1}$ , and all samples were measured in a single assay.

#### **5.2.6. *Statistical analysis***

First, second, third or fourth order regressions analyses were conducted using SigmaPlot 4.0 to investigate the relationship between plasma concentrations of Vtg,  $\text{E}_2$  and T and oocyte modal size for each fish. Analyses of  $I_G$ ,  $I_H$ , BW, CF and plasma concentrations of Vtg,  $\text{E}_2$  and T in relation to both sampling time and oocyte stages were performed by one-way ANOVA followed where appropriate by a Duncan's means comparison test, using the computer package SPSS 10.0 for Windows. Unless otherwise noted, a significant difference of  $P < 0.05$  was used for all tests. All proportion data were arcsine transformed prior to analysis but untransformed data are shown on figures.

### **5.3. Results**

Fish were sampled on the basis of being presumptive females based on external examination, however, one male was found in samples A and H. A significant

difference in BW was only found between sample C and sample I. However, there was no significant change in CF with sampling time (Table 5.2). A significant difference in BW was seen between gonad stage 1 and stages 3 and 4, and a significant difference of CF was observed between gonad stages 1 and 2 and stages 3 and 4 (Table 5.3).

The growth pattern of oocytes is described by the oocyte size-frequency distributions at different sampling times shown in Fig. 5.2. Oocytes of a wide range of sizes were found in fish from most samples with the exception of sample A where only oocyte stages 1 and 2 were present. At other times, there were 1 to 2 fish with mature ovaries (oocyte stage 4) but no evidence of an increase in the proportion of mature fish as sampling progressed, nor was development synchronized among fish at any sample time.

There were no significant changes of  $I_G$  among fish from different sampling times (Fig. 5.3a) whereas a significant difference in  $I_H$  was observed between sample A and samples C, G and H, and between sample I and samples C and H (Fig. 5.3b). There were, however, no statistically significant differences in the plasma concentrations of Vtg (Fig. 5.4a) and T (Fig. 5.4c); a significant difference in plasma  $E_2$  concentrations was only seen between sample B and I during the sampling period (Fig. 5.4b).

Significant changes of  $I_G$  and  $I_H$  were observed in relation to oocyte growth (Fig. 5.5).  $I_G$  at stage 4 was significantly higher than that at any other stage, and  $I_G$  at stage 3 was also significantly higher than at stages 1 and 2, which in turn were not different from each other (Fig. 5.5a). Significantly higher  $I_H$  was seen at stage 3 in relation to stages 1 and 2, but there was no difference between stages 3 and 4, or among stages 1, 2 and 4 (Fig. 5.5b).

Fish at stages 3 and 4 had significantly higher plasma concentrations of Vtg compared with those at stages 1 and 2, but there was no significant difference in plasma Vtg concentrations between stages 1 and 2, nor stages 3 and 4 (Fig. 5.6a). The changing pattern of plasma  $E_2$  concentrations among stages was similar to

that of Vtg concentrations, with elevated plasma E<sub>2</sub> concentrations at stage 3 relative to stages 1 and 2, but not stage 4 (Fig. 5.6b). Significant differences in plasma T concentrations were only observed between stages 1 and 4 (Fig. 5.6c).

The relationship between plasma concentrations of Vtg, E<sub>2</sub> and T and oocyte modal size was analyzed by regression of various orders (Table 5.4) and 4th order regression gave the highest R<sup>2</sup> values for all plasma parameters. No measurable plasma Vtg was found when the oocyte size was smaller than 150 µm. Plasma Vtg concentrations were positively correlated with oocyte size from over 150 to 300 µm, and reached a plateau across oocyte sizes from 300 to 450 µm (Fig. 5.7a). Plasma E<sub>2</sub> concentrations were low at oocyte sizes of less than 150 µm, then increased gradually to oocyte sizes of 500 µm (Fig. 5.7b). There was no change of plasma T concentrations at oocyte sizes below 300 µm, after which an elevation was observed which became more evident at oocyte sizes beyond 400 µm (Fig. 5.7c).

Table 5.2. Body weight (BW) and condition factor (CF) of flounder during the sampling period. Values are mean ± SE (n = 6-7).

Sampling time	BW	CF
A	113.5 ± 11.2 <sup>ab</sup>	1.55 ± 0.06
B	107.3 ± 14.2 <sup>ab</sup>	1.59 ± 0.12
C	94.0 ± 5.7 <sup>a</sup>	1.46 ± 0.05
D	112.6 ± 9.0 <sup>ab</sup>	1.52 ± 0.09
E	118.4 ± 18.0 <sup>ab</sup>	1.48 ± 0.08
F	125.0 ± 9.8 <sup>ab</sup>	1.54 ± 0.1
G	121.1 ± 16.5 <sup>ab</sup>	1.43 ± 0.06
H	107.2 ± 8.6 <sup>ab</sup>	1.36 ± 0.03
I	143.0 ± 13.7 <sup>b</sup>	1.53 ± 0.07

Common superscripts show values that are not significantly different ( $P > 0.05$ ). There were no difference among CF values.

Table 5.3. Body weight (BW) and condition factor (CF) of flounder in different gonad stages. Values are mean  $\pm$  SE (n shown in Fig. 5.5).

Gonad stage	1	2	3	4
BW	84.8 $\pm$ 6.2 <sup>a</sup>	107.3 $\pm$ 7.4 <sup>ab</sup>	126.6 $\pm$ 5.9 <sup>b</sup>	118.6 $\pm$ 11.0 <sup>b</sup>
CF	1.28 $\pm$ 0.09 <sup>a</sup>	1.39 $\pm$ 0.03 <sup>a</sup>	1.55 $\pm$ 0.03 <sup>b</sup>	1.64 $\pm$ 0.08 <sup>b</sup>

Common superscripts show values that are not significantly different ( $P > 0.05$ ).

Table 5.4. Comparison of  $R^2$  from different order regression of modal oocyte size against Vtg and steroid concentrations

Regression order	$R^2$		
	Vtg	E <sub>2</sub>	T
1	0.5827	0.4235	0.5239
2	0.5985	0.4261	0.6698
3	0.6076	0.4382	0.6905
4	0.6565	0.4385	0.6983

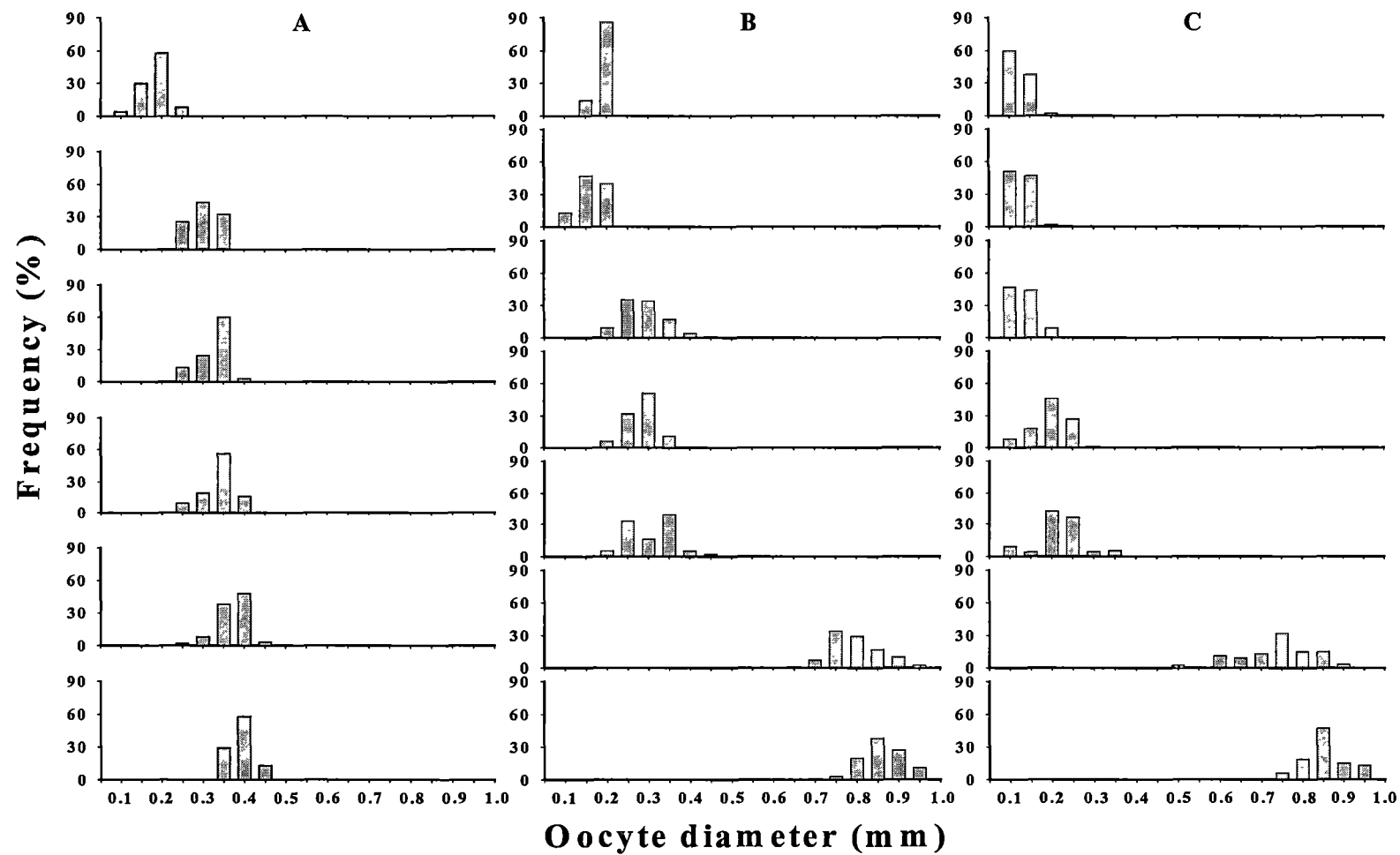


Fig. 5.2. Frequency distribution of oocyte diameters during the period of sampling. Each histogram shows the data from an individual fish.

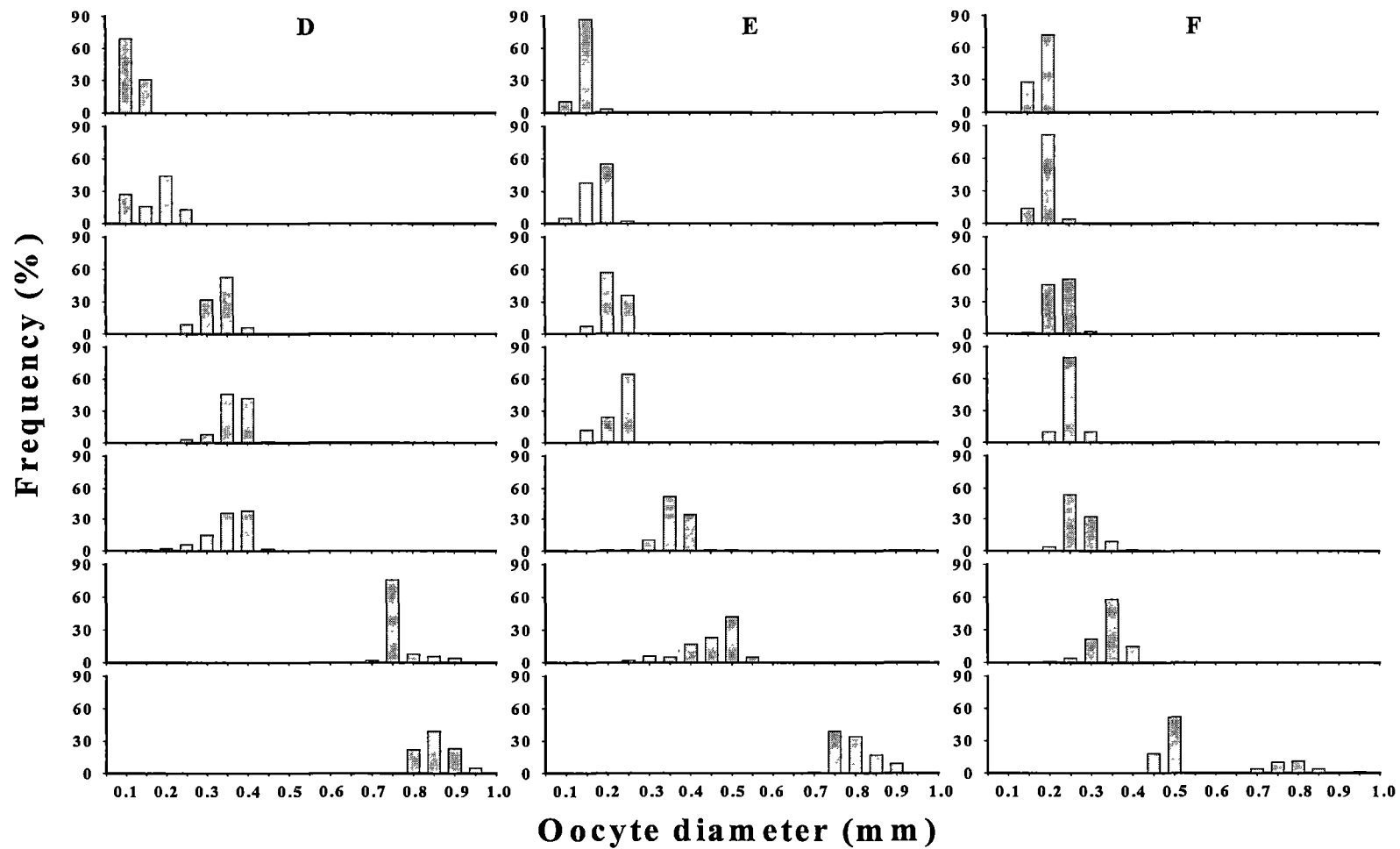


Fig. 5.2. Continued-1

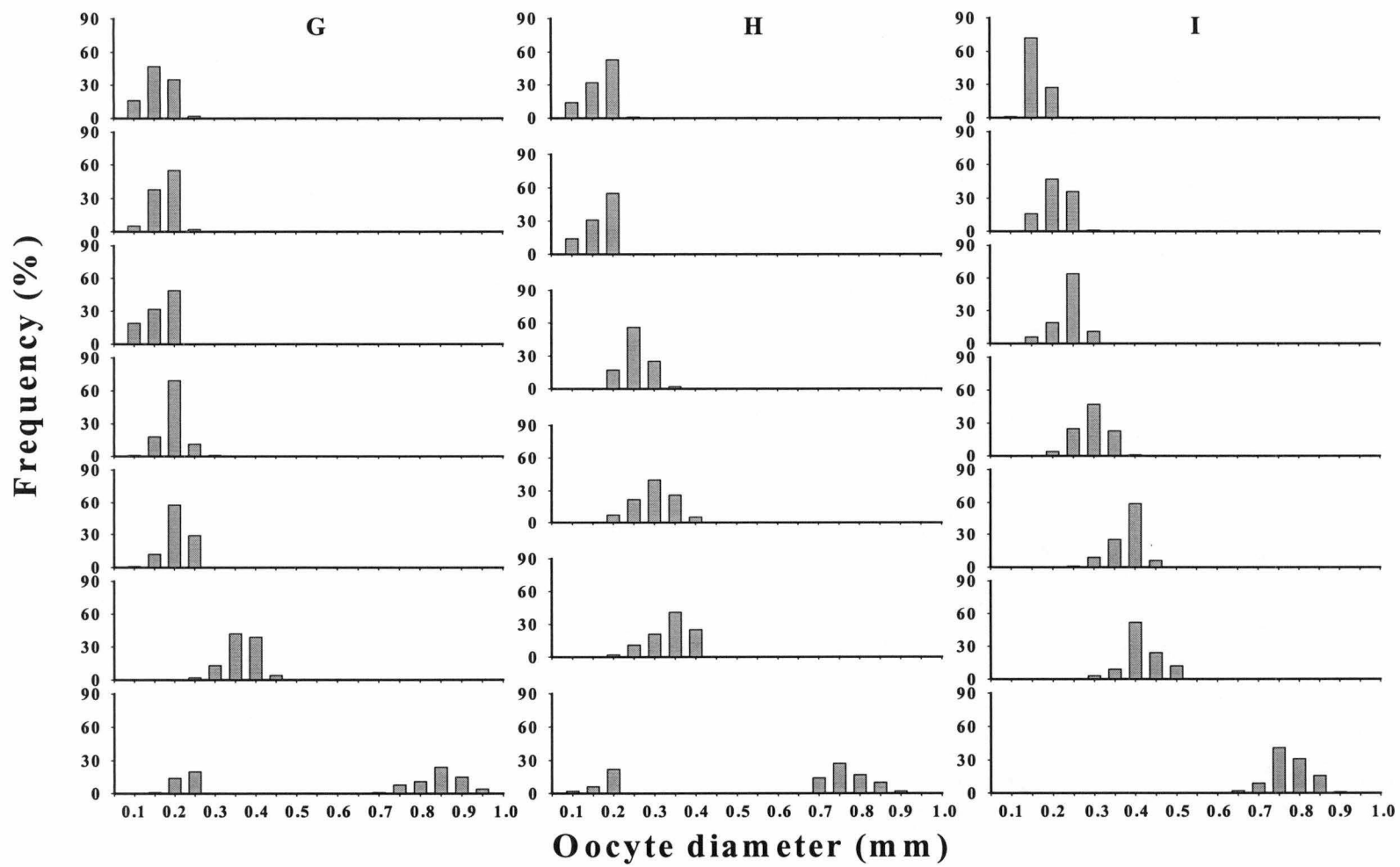


Fig. 5.2. Continued-2

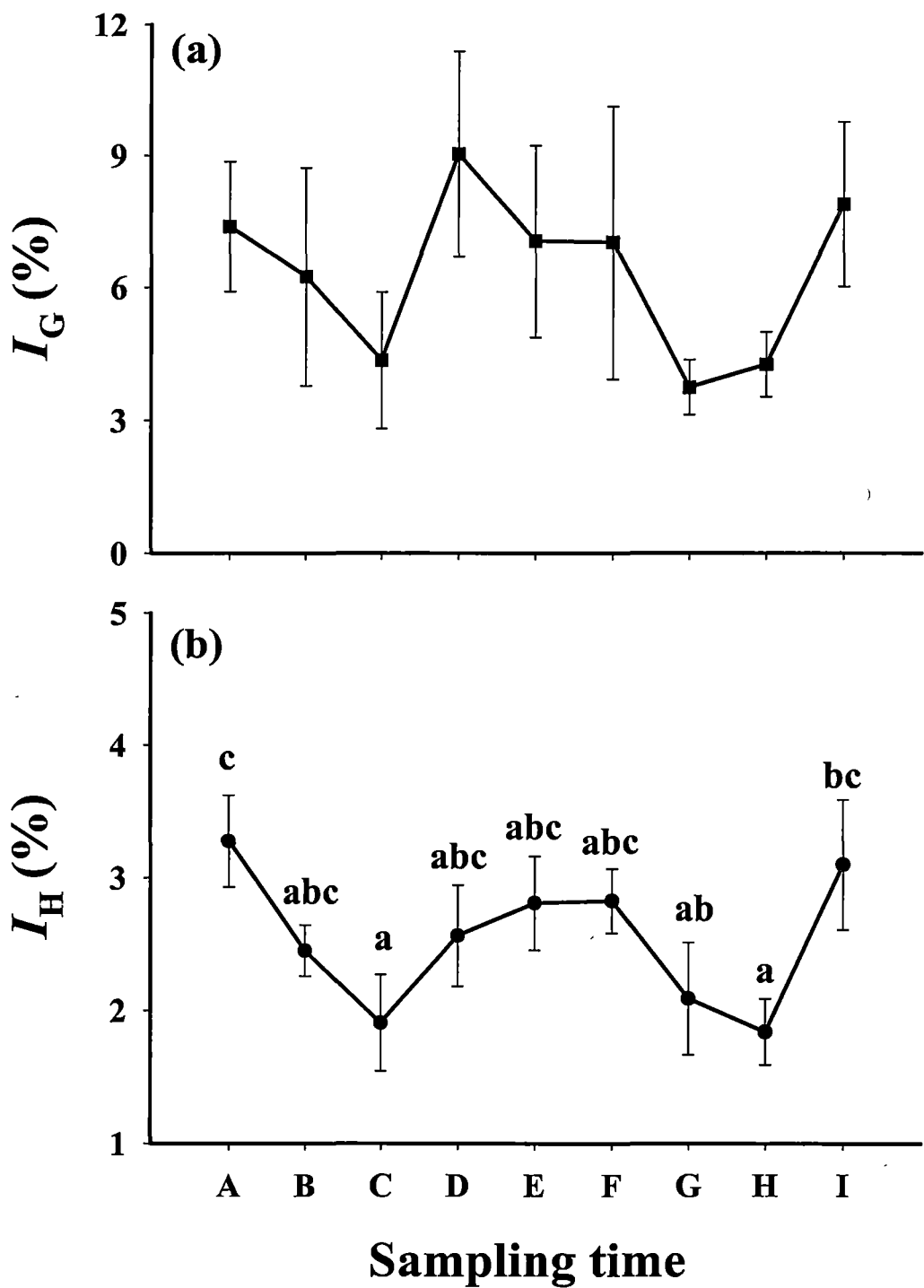


Fig. 5.3. Fortnightly changes of (a)  $I_G$  and (b)  $I_H$  during the sampling period. Values are mean  $\pm$  SE (n = 6-7). Common superscripts show values that are not significantly different ( $P > 0.05$ ).



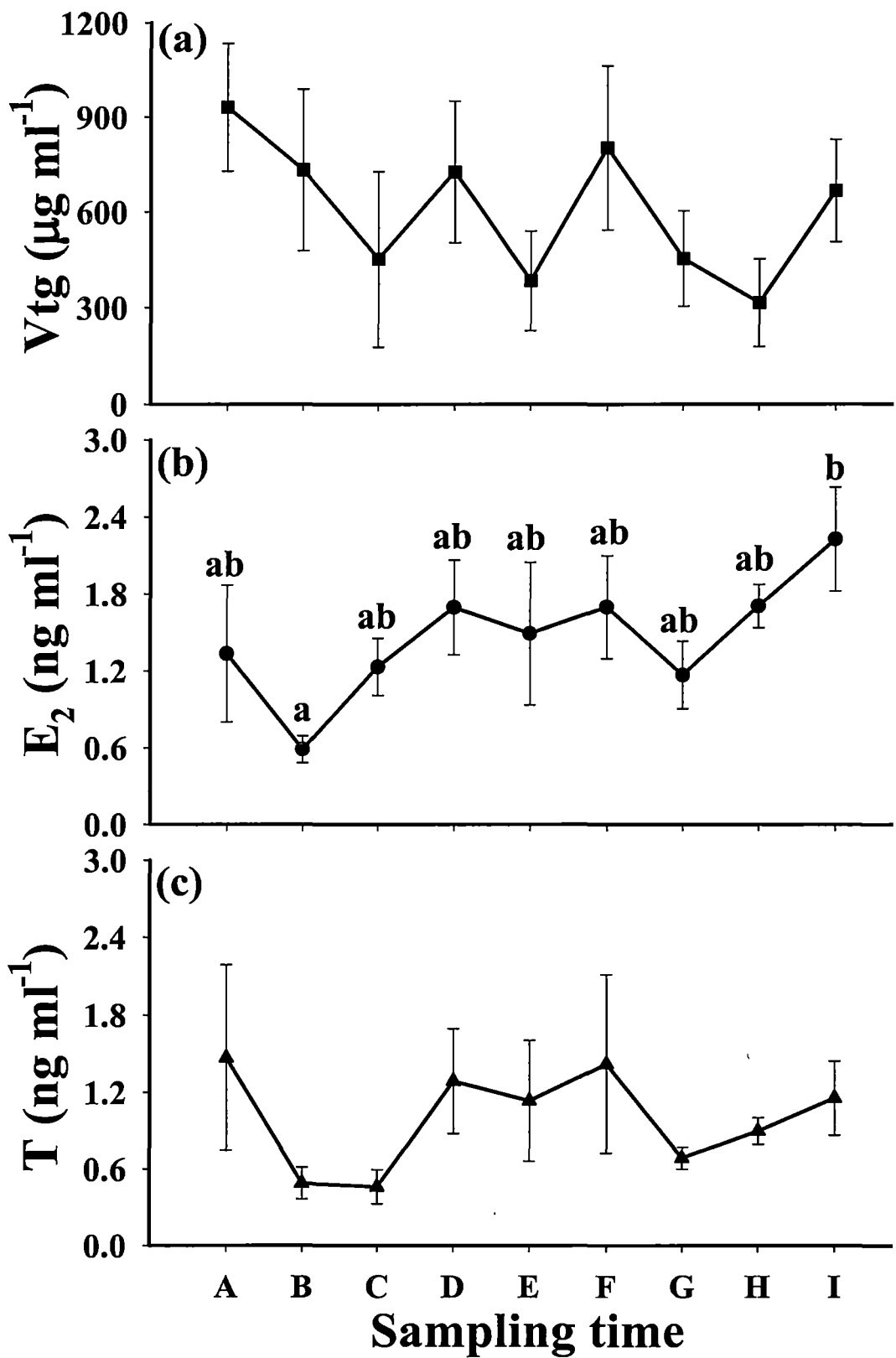


Fig. 5.4. Fortnightly changes of plasma concentrations of (a) Vtg, (b) E<sub>2</sub> and (c) T during the period of sampling. Other details are the same as for Fig. 5.3.

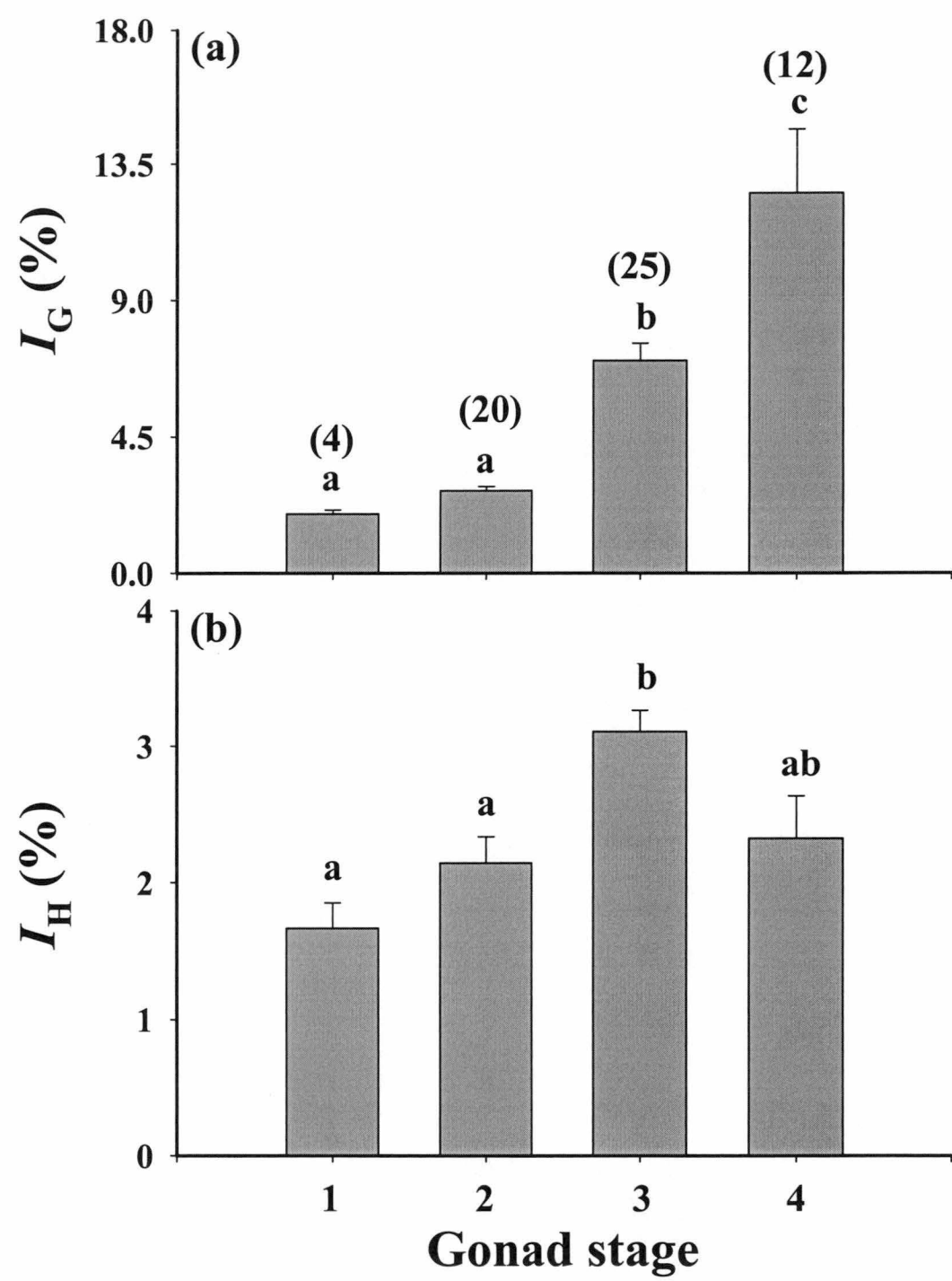


Fig. 5.5. Changes in (a)  $I_G$  and (b)  $I_H$  in relation to gonad stage. Values are mean + SE (n). Gonad stages are described in Table 5.1. Common superscripts show values that are not significantly different ( $P > 0.05$ ).

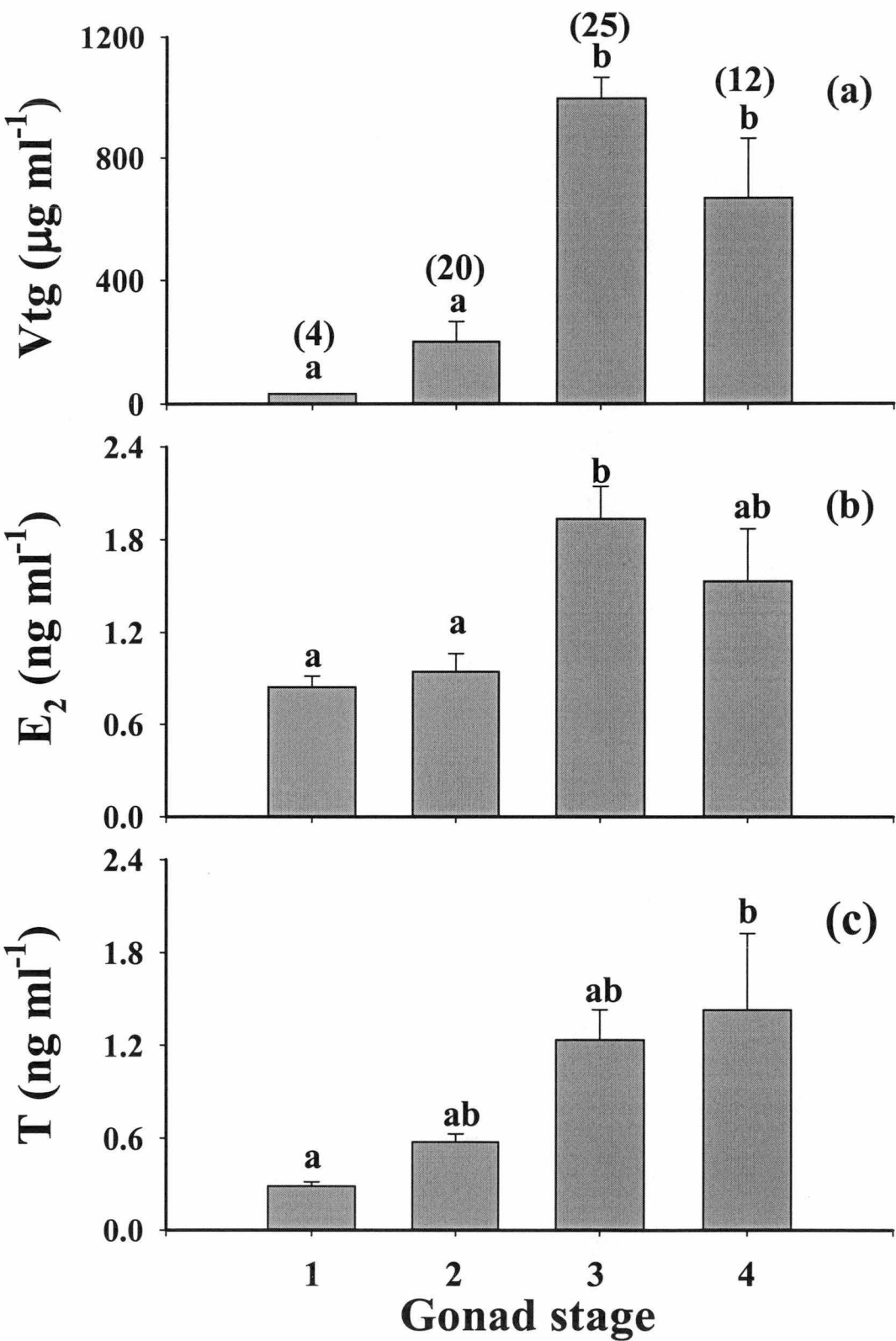


Fig. 5.6. Plasma concentrations of (a) Vtg, (b) E<sub>2</sub> and (c) T in relation to gonad stage. Other details are same as for Fig. 5.5.

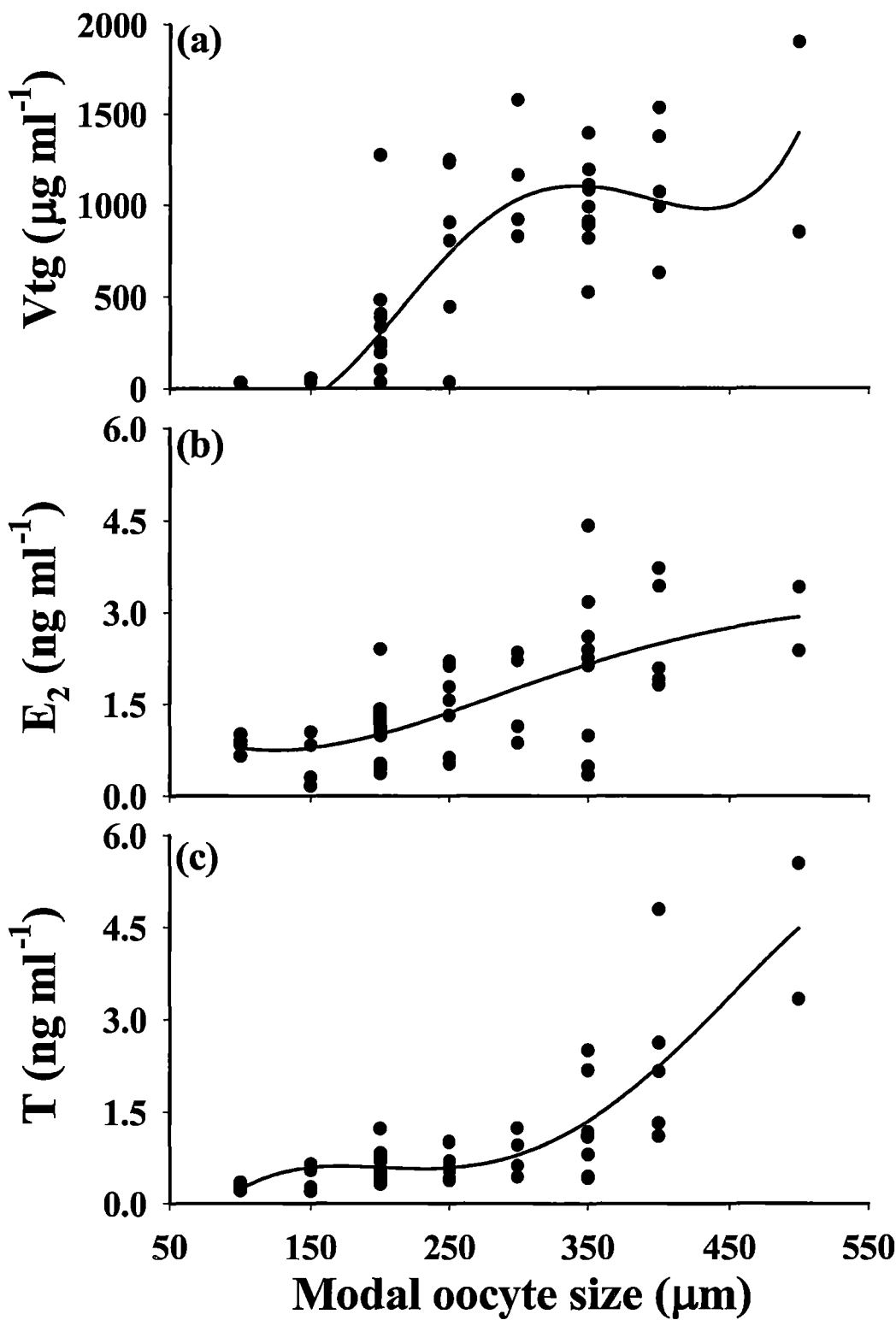


Fig. 5.7. Plasma concentrations of (a) Vtg, (b) E<sub>2</sub> and (c) T in relation to modal oocyte size. Curves show forth order regression: (a)  $R^2 = 0.657$ ; (b)  $R^2 = 0.439$ ; (c)  $R^2 = 0.698$ .

## 5.4. Discussion

The presence of a wide range of vitellogenic oocytes in individual ovaries, and the coexistence of vitellogenic oocytes and oocytes undergoing final maturation in some ovaries confirm that the oocyte developmental pattern in *R. tapirina* is multiple group synchrony, and that this species has the capacity for multiple ovulations in a single reproductive season. This is consistent with earlier findings in both wild and hormone-treated fish (Barnett and Pankhurst, 1999; Poortenaar and Pankhurst, 2000a). The ovulatory periodicity of fish in the wild is unknown; however, captive fish ovulate daily for several days following treatment with exogenous hormones (Poortenaar and Pankhurst, 2000a). Natural spawning of groups of captive broodstock occurs every 1-2 days during peak spawning periods (unpublished data) but the contribution of individual fish to this pattern is not known. The latency of ~ 96 h between exogenous hormone treatment and ovulation in *R. tapirina* (Poortenaar and Pankhurst, 2000b) indicates that the transition through final maturation is relatively rapid, and this is consistent with the low frequency of fish with oocytes in the 500 – 700 µm size range in the present study.

CF is a useful index that can reflect reproductive status and is influenced by a number of factors including season and developmental stages of gonad (Barnham and Baxter, 1998; Lizama and Ambrósio, 2002). In the present study, the lack of temporal change of CF and BW indicated that there is no synchronised seasonal shift of somatic condition during sampling period. Significant increase in BW and CF at stages 3 and 4 demonstrated that individual gonad stage was related to the somatic condition in *R. tapirina*. A significant increase of CF was also found in vitellogenic fish in *P. vetulus* (Johnson et al., 1991). It should be noted that fish in the present study were all vitellogenic at the time of first sampling. It is possible that the difference in CF might have been more marked if sampling had also included the period prior to vitellogenesis. In any event, it appears that the ‘decision’ to enter vitellogenesis is partly dependent on accumulating suitable size and condition.

No mature fish were found in February (sample A) which suggests that reproductive development is depressed in summer. Thereafter, maturing fish appeared in every sample but there was not any population synchrony in oocyte development as sampling progressed, and this appears to be the situation in wild fish as well (Barnett and Pankhurst, 1999). This suggests that there is probably a broad but not very strong seasonal effect on *R. tapirina* reproduction and that this species shows a pattern of scattered spawning over most of the year. This is also consistent with the absence of temporal differences in all reproductive parameters measured. A more significant seasonal impact was present in other flatfishes during the reproductive period, with a seasonal shift of ovarian stages in *P. vetulus* (Johnson et al., 1991), *P. flesus* (Janssen et al., 1995), *P. americanus* (Harmin et al., 1995) and *P. dentatus* (Merson et al., 2000), seasonal fluctuations of plasma Vtg and steroids in *P. platessa* (Wingfield and Grimm, 1977), *H. hippoglossus* (Methven et al., 1992), and *P. americanus* (Harmin et al., 1995), and seasonal changes of  $I_G$  and  $I_H$  in *P. platessa* (Wingfield and Grimm, 1977) and *P. flesus* (Janssen et al., 1995); *H. platessoides* in contrast did not show any seasonal variation in  $I_G$  (Maddock and Buttrton, 1999). Collectively, these results highlight the variety of seasonal reproductive strategies in multiple spawning species, even among closely related taxa.

In the present study, oocytes were classified into 4 stages according to their sizes, on the basis of size-developmental stage data from a previous study (Barnett and Pankhurst, 1999). The developmental stages chosen reflect transistion through the main events involved in teleost oocyte growth and development (Wallace et al., 1987; Tyler and Sumpter, 1996).  $I_G$  in the present study increased gradually from ovaries with stage 1 to stage 3 oocytes, indicating that as in other teleosts, Vtg deposition accounts for the main increase in  $I_G$  prior to ovulation. For example, in brook trout *Salvelinus fontinalis* (Mitchell),  $I_G$  increased from around 1.0% to 22.0% accompanied by a rise in plasma Vtg concentrations from 3.0 mg ml<sup>-1</sup> to 94.0 mg ml<sup>-1</sup> during vitellogenesis (Schafhauser-Smith and Benfey, 2002).  $I_G$  in ovaries at stage 4 (final maturation) in the present study was significantly higher than that at stage 3, and this is in agreement with a similar finding in wild fish by Poortenaar (1998). A higher  $I_G$  at final maturation was also found in *P. dentatus*

(Merson et al., 2000), kingfish *Seriola lalandi* Valenciennes (Poortenaar et al., 2001) and red porgy *Pagrus pagrus* (L.) (Kokokiiris et al., 2000). This is probably due to the increase in oocyte volume and weight as a result of hydration during final maturation. In *H. hippoglossus*, the wet weight of oocytes increased in concert with hydration, whereas the dry weight of oocytes was the same as in vitellogenic oocytes (Norberg, 1987). Similarly, Pacific herring *Clupea pallasii* Valenciennes ovarian water content increased by 70% during hydration (Gillis et al., 1990), and the oocyte size of swordfish *Xiphias gladius* L. increased 150% during hydration (Arocha, 2002). Therefore, the increase of  $I_G$  at stage 4 in the present study is most likely due to the uptake of water by maturing oocytes.

$I_H$  showed a similar pattern of increase as  $I_G$  from ovarian stage 1 to stage 3. The liver is the site for Vtg synthesis, and a series of metabolic activities will be enhanced during vitellogenesis (Gillis et al., 1990; Lazier and MacKay, 1993). A significant increase of water, protein and RNA contents was observed in the liver of E<sub>2</sub>-treated murrelet *Channa punctatus* (Bloch) (Sehgal and Goswami, 2001). A similar pattern of increasing  $I_G$  and  $I_H$  from stage 1 to stage 3 in *R. tapirina* is probably a reflection of the synchrony of the successive processes of synthesis of Vtg in liver, and subsequent sequestration of Vtg into the ovary. The lack of statistical difference of  $I_H$  between stage 4 and stage 3 ovaries indicates that ovaries at stage 4 still contains clutches of oocytes undergoing vitellogenesis as shown in oocyte size-frequency distributions, and this also is consistent with elevated plasma concentrations of Vtg and E<sub>2</sub> at this stage.

Plasma concentrations of Vtg and E<sub>2</sub> rose steadily with the advancing gonad stage. A similar positive relationship between plasma Vtg and E<sub>2</sub> concentrations is also present in salmonids (Copeland et al., 1986; King and Pankhurst, 2003), *H. hippoglossus* (Methven et al., 1992), common snook *Centropomus undecimalis* (Bloch) (Roberts et al., 1999), *C. pallasii* (Koya et al., 2000), *Dicentrarchus labrax* (Linnaeus) (Asturiano et al., 2000) and *P. dentatus* (Merson et al., 2000). This is consistent with the regulation of Vtg synthesis by E<sub>2</sub> in *R. tapirina* as in other teleost species (Specker and Sullivan, 1994). Plasma concentrations of both Vtg and E<sub>2</sub> reached a peak in *R. tapirina* with stage 3 ovaries. The significant

increase of Vtg and E<sub>2</sub> at ovarian stage 3 is consistent with findings from other multiple spawning species during vitellogenesis such as gag *Mycteroperca microlepis* (Goode and Bean) (Heppell and Sullivan, 1999) and *S. lalandi* (Poortenaar et al., 2001). Plasma concentrations of Vtg and E<sub>2</sub> were not different between ovarian stages 3 and 4 in *R. tapirina* although E<sub>2</sub> concentrations were no longer different at stage 4 relative to stages 1 and 2. As discussed earlier, *R. tapirina* is a multiple spawner and the coexistence of vitellogenic and maturing oocytes occurs during the whole spawning season (Barnett and Pankhurst, 1999; this study). The result is that the ovary at stage 4 still contains substantial numbers of the vitellogenic follicles that characterize stage 3 ovaries.

As a precursor of E<sub>2</sub>, T is converted into E<sub>2</sub> through the activity of aromatase (reviewed in Nagahama, 1994). The increase in plasma T concentrations at stage 4 in the present study may be due to the decrease of aromatase activity during final oocyte maturation. In the ovary of tilapia *Oreochromis niloticus* (L.), aromatase mRNA expression and aromatase concentrations and enzymatic activity were low in early vitellogenic follicles, increasing with advancing vitellogenesis, then declining in post-vitellogenic follicles (Chang et al., 1997). However, plasma concentrations of E<sub>2</sub> in fish in the present study were still relatively high at stage 4. The presence of high concentrations of E<sub>2</sub> and T during final maturation was also observed in another multiple group synchronous species, spiny damselfish *Acanthochromis polyacanthus* (Bleeker). This was attributed to stimulation of remaining vitellogenic follicles by a preovulatory GtH surge (Pankhurst et al., 1999). The moderate elevation of plasma E<sub>2</sub> concentrations in stage 4 greenback flounder seen here probably also accounts for the continued elevation of plasma Vtg concentrations in the present study.

Absolute plasma concentrations of E<sub>2</sub> and T in the present study (~ 2.0 and 1.5 ng ml<sup>-1</sup> respectively) were slightly lower than those recorded from wild fish (~ 3.0 ng ml<sup>-1</sup> for both steroids; Barnett and Pankhurst, 1999) but similar to cultured fish (~ 2.0 ng ml<sup>-1</sup>) that subsequently ovulated in response to exogenous hCG and LHRH-a treatments (Poortenaar and Pankhurst, 2000a). This suggests that endocrine processes were essentially the same as in wild fish and is consistent with stress-



acclimation to captivity that usually occurs over several culture generations (Pankhurst, 1998).

The profiles of plasma Vtg, E<sub>2</sub> and T all showed an increase as a function of oocyte size in *R. tapirina*, but the pattern of increase was variable. At oocyte sizes smaller than 150 µm, there was no detectable Vtg in plasma, consistent with the fact that oocytes were at the previtellogenic stage, when the content of the oocyte is derived completely from non-Vtg sources and the formation of the Balbiani body is mainly responsible for any size increase (Wallace et al., 1987; Tyler and Sumpter, 1996). Vtg was detectable in plasma at oocyte sizes of greater than 150 µm, and increased to a plateau at oocyte sizes beyond 250 µm, as vitellogenesis was initiated. The increase in oocyte size at this stage is due to the presence of glycoproteins and lipid uptake from Vtg (Wallace et al., 1987; Tyler and Sumpter, 1996). Plasma concentration of E<sub>2</sub> showed a more gradual but sustained increase as vitellogenesis proceeded, suggesting that a high plasma E<sub>2</sub> concentration is not necessary to trigger vitellogenesis in this species. Elevated plasma concentrations of Vtg and E<sub>2</sub> were sustained at oocyte sizes of over 250 µm reflecting the high steroidogenic activity in ovarian follicles during vitellogenesis (Tyler and Sumpter, 1996).

In contrast to E<sub>2</sub>, there was no increase in plasma concentrations of T until oocyte sizes over 350 µm were attained, at which stage plasma concentrations of Vtg and E<sub>2</sub> appeared to plateau. This is consistent with the role of T as substrate for E<sub>2</sub> production during the vitellogenic growth of oocytes as discussed earlier, and also with decreased aromatase activity as follicles complete vitellogenesis. Increases in plasma T stimulate luteinizing hormone (LH) synthesis during the late stages of gonadal development (reviewed in Van Der Kraak et al., 1998), and exogenous T increases LH mRNA levels in goldfish *Carassius auratus* (L.) pituitary (Huggard et al., 1996), and enhances pituitary responsiveness to LHRH-a in *R. tapirina* (Poortenaar and Pankhurst, 2000b). In the present study, the high plasma T concentrations in fish with larger oocytes may increase positive feedback on the pituitary and facilitate a pre-ovulatory LH surge. The present results indicate that increased plasma concentrations of Vtg and E<sub>2</sub> are robust indicators of active

vitellogenesis in *R. tapirina* whereas elevated plasma T concentrations are indicative of impending oocyte maturation.

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**Chapter 6**

**Vitellogenin induction *in vitro* by 17 $\beta$ -  
estradiol in primary cultures of greenback  
flounder (*Rhombosolea tapirina*) hepatocytes**

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## 6. Vitellogenin induction *in vitro* by 17 $\beta$ -estradiol in primary cultures of greenback flounder (*Rhombosolea tapirina*) hepatocytes

### 6.1. Introduction

Vitellogenin (Vtg) is a large glycolipophosphoprotein which is synthesized in the liver of mature females in response to circulating 17 $\beta$ -estradiol (E<sub>2</sub>), and of males or immature females treated with exogenous estrogens (reviewed in Mommsen and Walsh, 1988; Specker and Sullivan, 1994). Generally, E<sub>2</sub> is considered as the most important steroid hormone for stimulation of the production of Vtg in hepatocytes. However, other hormones may be involved in Vtg synthesis as well, for example, homologous pituitary homogenate and growth hormone (GH) induced Vtg production in liver slices of the frog (*Rana esculenta*) (Carnevall et al., 1992), and the implantation of cortisol lead to a rapid and transient transcription of Vtg gene in the liver of tilapia (*Oreochromis aureus*) (Ding et al., 1994). These results suggest that the control of synthesis of Vtg *in vivo* is a multi-hormone process.

*In vitro*, a large population of hepatocytes can be obtained from a single liver or pooled livers, providing a consistent cell population for use in different experiments, and as the isolated hepatocytes are maintained under defined conditions, the complexity of multi-factors present *in vivo* can be avoided (reviewed in Baksi and Frazier, 1990; Segner, 1998). These features make hepatocyte culture a useful system to elucidate the mechanism of individual factors or combination of different factors potentially involved in the regulation of vitellogenesis. For example, the combination of E<sub>2</sub> with GH and/or prolactin stimulated Vtg synthesis in hepatocytes of eel (*Anguilla japonica*) (Kwon and Mugiya, 1994); and co-incubation of E<sub>2</sub> and progesterone, or cortisol enhanced the expression of Vtg genes in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Mori et al., 1998); E<sub>2</sub> and/or testosterone (T), and E<sub>2</sub> and GH

stimulated the production of Vtg in primary cultures of hepatocytes in European eel (*Anguilla anguilla*) (Peyon et al., 1997, 1998); and E<sub>2</sub> and/or T increased Vtg synthesis in hepatocytes of tilapia (*Oreochromis mossambicus*) (Kim et al., 2003).

The successful establishment of an *in vitro* culture system for hepatocytes involves the isolation of high viability cells, and the maintenance of a high survival rate of cells during the period of experiment. As *in vitro* hepatocytes are under somewhat unnatural conditions, it is essential to determine optimum conditions for the isolation and culture of each particular species (reviewed in Mommsen et al., 1994; Segner, 1998). *In vitro* hepatocyte culture systems have been set up for many different teleosts; however, only a few are for flatfishes (reviewed in Baksi and Frazier, 1990; Segner, 1998). A previous study (Chapter 4) on greenback flounder (*Rhombosolea tapirina*) demonstrated that Vtg induction *in vivo* by E<sub>2</sub> is time- and dose- dependent. The aim of this chapter is to develop an *in vitro* primary culture of hepatocytes for this species and to further assess the effects of E<sub>2</sub> and other factors on Vtg synthesis *in vitro* using this system.

## 6.2. Materials and methods

### 6.2.1. Fish

Sexually maturing male greenback flounder (2-year-old), used in the present study were collected from stocks grown at the School of Aquaculture, University of Tasmania in Launceston. They were held at  $13 \pm 1^{\circ}\text{C}$  and ambient photoperiod in recirculation systems and fed at ~ 3% body weight daily with commercial dry feed pellets (Pivot Aquaculture, Hobart). Fish were not fed for 24 h before each experiment. Sex was determined by visual observation of the shape of the gonad under back light, and the presence of milt expressed by gentle abdominal pressure.

### 6.2.2. Hepatocyte isolation and culture

Fish were anaesthetised in 0.05% 2- phenoxyethanol (Sigma) with the surface of the abdomen sterilized with 70% ethanol. Because the hepatic veins of greenback flounder are too small to perfuse, a perfusion from the heart was tried to flush the blood from the liver. However, the process of perfusion was slow and also did not significantly increase the yield of hepatocytes or reduce the contamination with erythrocytes in the final hepatocyte preparation compared with the bleeding from the caudal vein (detail as follows). Therefore, no perfusion of the liver was performed in the present study. Instead, as much blood as possible was extracted by caudal puncture using preheparinised syringes and 25 G needles. The liver was carefully removed from the abdominal cavity and transferred into an aseptic petri dish, then washed 2 to 3 times with cold Cortland salt solution (Wolf, 1963). The following process of hepatocyte isolation was modified from Mommsen et al. (1994). Briefly, the liver was cut into small pieces and washed in Cortland salt solution several times, and small blood clots were removed with forceps. The tissue was then transferred to a sterilized tube containing  $2.5 \text{ mg ml}^{-1}$  collagenase (Sigma) in Cortland salt solution, which was capped and spun for 20 min at room temperature. The resulting cell suspension was decanted to another sterilized tube and washed 3 times with Cortland salt solution by centrifugation at 100 g for 10 min at  $4^{\circ}\text{C}$ . The final cell pellet was resuspended in Leibowitz-15 medium (L15, Invitrogen) supplemented with defatted bovine serum albumin (BSA) (Sigma) or foetal calf serum (FCS) (concentration varied in different trials; for details see Results), 1% v/v penicillin streptomycin solution ( $5000 \text{ IU ml}^{-1}$  Penicillin and  $5.0 \text{ mg ml}^{-1}$  Streptomycin, Sigma), 2.0 mM L-glutamine (Sigma), and the pH was adjusted to 7.6. Hepatocytes were counted using a hemocytometer and viability was determined by the trypan blue exclusion method (Klaunig et al., 1985). Hepatocytes obtained using this method had viability of over 90% and the yield of cells from a single liver was sufficient for each independent experiment.

The number of hepatocytes harvested following digestion varied in different experiments, largely as a result of a difference in liver weight. In order to keep the number of hepatocytes in each incubation consistent, the final hepatocyte pellet was usually resuspended in a small volume of L15 and adjusted to a cell density of  $5.5 - 6.0 \times 10^6 \text{ ml}^{-1}$  by dilution after counting. Cells were plated to 24-well

tissue culture microplates (Iwaki) and cultured at 12°C for 24 h, then the media replaced and E<sub>2</sub> in ethanol was added to the fresh media. The final concentration of ethanol in the media never exceeded 0.1%. Following incubation, the media were collected from the plates, frozen and stored at – 20°C before the measurement of Vtg by using the ELISA described in Chapter 3.

### **6.2.3. Experimental protocols**

#### **6.2.3.1. Experiment-1: threshold and dose-response**

L15 alone (control) or L15 containing 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup> or 10<sup>-4</sup> M E<sub>2</sub> (doses based on effective doses used by Takemura and Kim, 2001) was added to pre-cultured cells with 4 replicates for each treatment. The plates were cultured at 12°C for 24, and 48 h, respectively in an initial experiment, but this was later extended to 48 and 72 h. The experiment was repeated five times (one at 24 and 48 h, four at 48 and 72 h).

#### **6.2.3.2. Experiment-2: time course of Vtg induction**

Phenol-free L15 containing 2% BSA with or without E<sub>2</sub> (10<sup>-6</sup> M) was added to pre-cultured male and vitellogenic female hepatocytes respectively and cells incubated for 24, 48, 72, 96, 120 or 144 h. Phenol-free L15 was used here in order to rule out the possible photometric interference by phenol red in the Vtg ELISA.

#### **6.2.3.3. Experiment-3: assessment of media effects on Vtg measurement**

Because Vtg was not detectable in cultures of viable cells incubated at E<sub>2</sub> doses, and for times which stimulate Vtg production in other species (e.g. Takemura and Kim, 2001), a series of assays was conducted to assess whether components of the incubation medium itself were interfering with the assay. Vtg standards from 0.16 to 20 µg ml<sup>-1</sup> were prepared in 20 mM Tris-0.5 M NaCl buffer containing 3% skim milk, pH 7.2 (3% TBSD), 3% TBSD containing 1% or 10% FCS, 3% TBSD containing 0.1 or 2% BSA, or 3% TBSD : L15 (phenol red) at 1: 1 or 4: 1, respectively. An attempt was also made to assess the possibility of measuring Vtg in culture media without dilution to increase the assay detection level to 0.16 µg ml<sup>-1</sup>. Vtg standards were directly prepared in L15 with or without phenol red

respectively from 0.16 to 20  $\mu\text{g ml}^{-1}$ . All assays were then run as described in Chapter 3.

### 6.3. Results

Cells were pre-cultured for 24 h to allow recovery from any damage resulting from tissue digestion and cell dissociation. After pre-culture, cells could be maintained for at least one week without significant loss of cell viability, as determined by microscopic observation of trypan blue exclusion.

#### 6.3.1. Experiment-1

##### 6.3.1.1. Trial one

Cells were seeded at a concentration of  $10^6$  well<sup>-1</sup> cultured in L15 with phenol red supplemented with 10% FCS. After pre-culture, media were replaced by fresh L15, and E<sub>2</sub> ranging from 0.1 to 1000 ng ml<sup>-1</sup> (i.e.  $\sim 10^{-10}$  to  $10^{-6}$  M) was added as described in experiment protocols. For assay, media were diluted to 2 or 5 times in 3% TBSD. All values were below the assay detection limit of 0.16  $\mu\text{g ml}^{-1}$ .

##### 6.3.1.2. Trial two

On the basis of trial one, it was considered that either the incubation time was not long enough for accumulation of detectable Vtg in the media, or that the E<sub>2</sub> dose was not high enough to trigger Vtg induction in hepatocytes. As a result, the incubation time was extended to 72 h and the E<sub>2</sub> dose range changed to  $10^{-8}$  -  $10^{-4}$  M. Vtg remained undetectable in the media.

##### 6.3.1.3. Trial three

In this trial, cells were incubated with or without 1% FCS for 48 and 72 h to determine whether the higher level of FCS used in earlier cultures was causing non-specific binding in the assay. Vtg still could not be detected in media. However, cell quality was not as good as in the previous trials and some cells in serum free media were stripped from the bottom of wells at the end of culture.

**6.3.1.4. Trial four**

This trial investigated the possibility that phenol red affected the ELISA by changing absorbance readings of media independently of Vtg-antibody binding. Cortland salt solution containing 0.1% BSA was used as culture media in this experiment. Again, no measurable Vtg was found after 48 and 72 h incubation. Both quantity and quality of cells were found to be decreased at the end of the culture compared with cells cultured in L15 with either 1% or 10% FCS.

**6.3.1.5. Trial five**

Phenol free L15 with 2% BSA was used as culture media in this experiment because 2% BSA had been used elsewhere as a supplement in teleost hepatocyte culture (Mommensen et al., 1994) and also as blocking solution in ELISAs (Susca et al., 2001). The purpose of this trial was to attempt to improve cell quality, and to reduce possible interference from culture media in the ELISA. An improvement in cell quantity and quality was obtained compared to trials 3 and 4 but no Vtg was measured in media. A comparison of cell quantity and quality is shown in Table 6.1.

**6.3.2. Experiment-2**

Induction on time course studies (Chapter 3) demonstrated that it takes 96 h for Vtg to reach peak levels in plasma of greenback flounder, and another study (Peyon et al., 1993) suggested that Vtg induction *in vitro* might require a preliminary E<sub>2</sub>-priming *in vivo*. Therefore, the purpose of this experiment was to determine whether the lack of measurable Vtg in media in previous trials was due to insufficient incubation time or no induction of Vtg without E<sub>2</sub>-priming. No measurable Vtg was found in any treatment.

**6.3.3. Experiment-3**

FCS at either 1 or 10% did not affect the ELISA (Fig. 6.1). BSA appeared to make the standard curve flatter which might reduce the sensitivity of the ELISA but both curves were still linear (Fig. 6.2). Standards in L15 at 1: 1 produced

better linear displacement compared with L15 at 4: 1 (Fig. 6.3). In contrast, neat L15 interfered with specific binding in the ELISA (Fig. 6.4). Parallellism of different regression curves was assessed by the F-test on mean squares. No significant differences were observed between the standard curve and curves with FCS, BSA and L15 dilutions (Figs. 6.1, 6.2 and 6.3) whereas a significant difference ( $F_{(2,21)} = 4.246$ ,  $p < 0.028$ ) was found between the standard curve and curves from Vtg standards in neat L15 (Fig. 6.4).

Table 6.1 Comparison of hepatocyte quality and quantity<sup>a</sup> in Experiments 1 and 2

		Experiment 1		Experiment 2
Trial	1	++++ <sup>b</sup> (10% FCS)	Male	+++ (2% BSA)
	2	++++ (10% FCS)	Female	+++ (2% BSA)
	3	++ <sup>d</sup> (1% FCS)		
		+ <sup>e</sup> (no FCS or BSA)		
	4	+ (0.1% BSA)		
	5	+++ <sup>c</sup> (2% BSA)		

<sup>a</sup> An initial experiment demonstrated that only viable cells adhered to the bottom of wells after several washes at the end of culture. Cell density was  $10^6 \text{ ml}^{-1}$  i.e.  $10^6 \text{ well}^{-1}$ , for all trials here. Symbols (++++, +++, ++ and +) were used to describe the development of hepatocytes for each incubation.

<sup>b</sup> Cells were fully developed, very refractive and bright, large and nearly round, and covered > 90% area of well, contacting tightly with neighbouring cells.

<sup>c</sup> Morphological characteristics of cells were similar to those of +++++, covered > 80% area of well, most cells contacted tightly but small gaps were observed between some cells.

<sup>d</sup> Not all cells were fully developed, cell sizes were variable, covered 60 – 70 % area of well, with gaps seen among cells.

<sup>e</sup> Most cells were not fully developed and dull, covered < 50% area of well with large gaps between cell clumps.



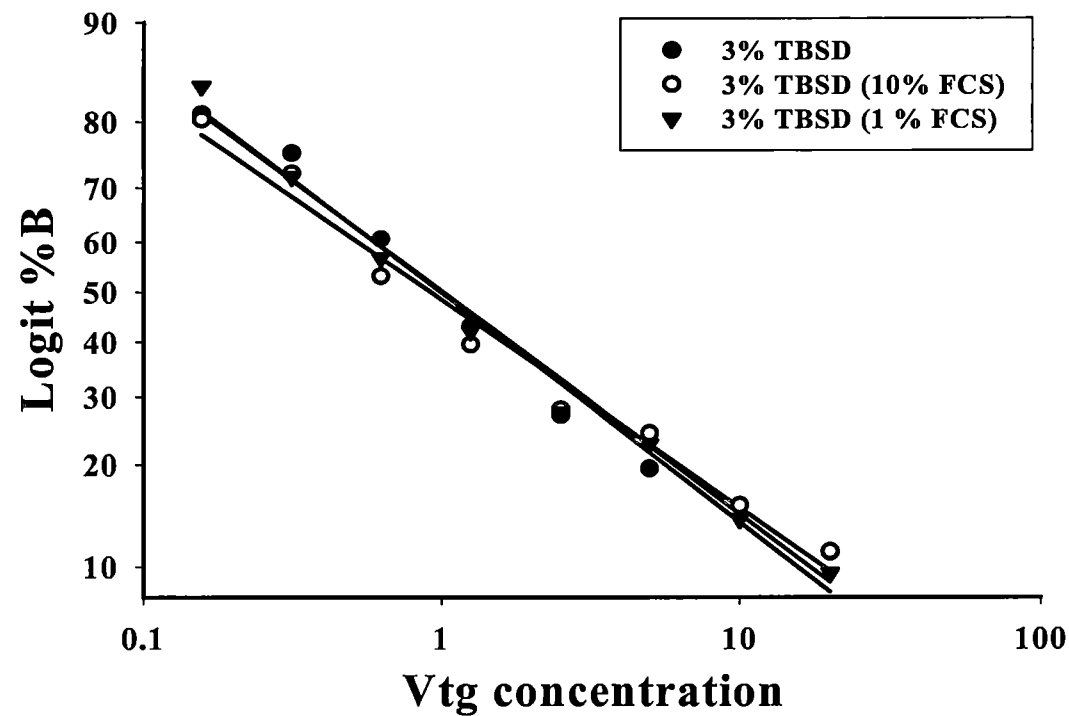


Fig. 6.1. Curves from Vtg standards (0.16 to 20  $\mu\text{g ml}^{-1}$ ) diluted in 3% TBSD with or without 1% or 10% FCS.

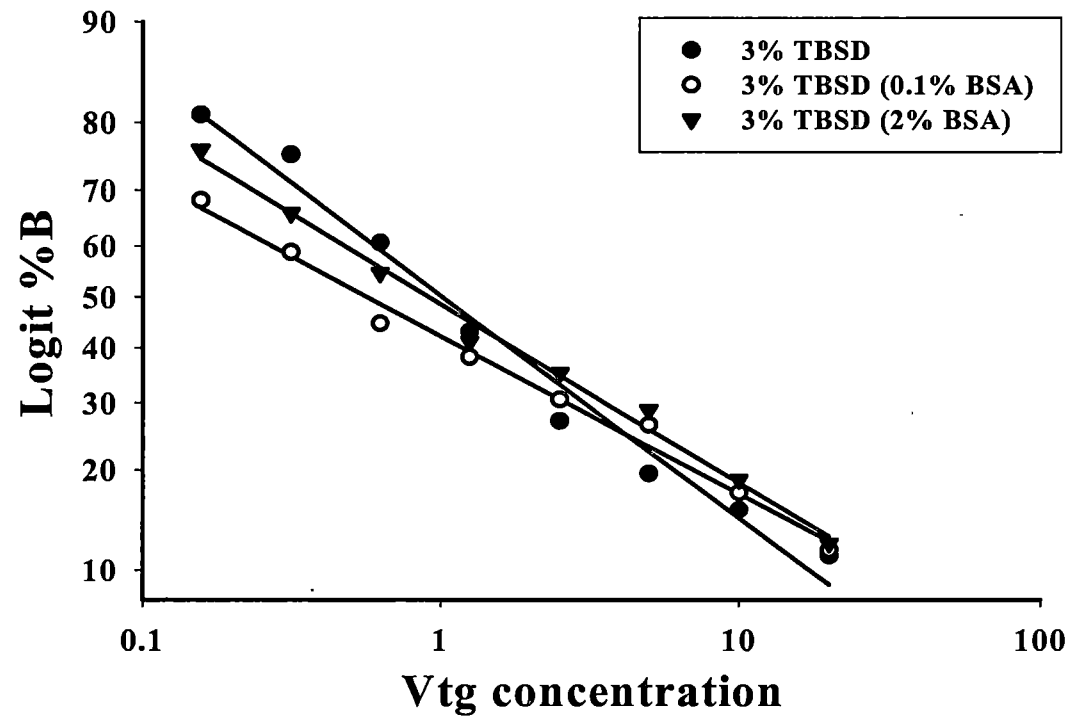


Fig. 6.2. Curves from Vtg standards (0.16 to 20  $\mu\text{g ml}^{-1}$ ) diluted in 3% TBSD with or without 0.1% or 2% BSA.

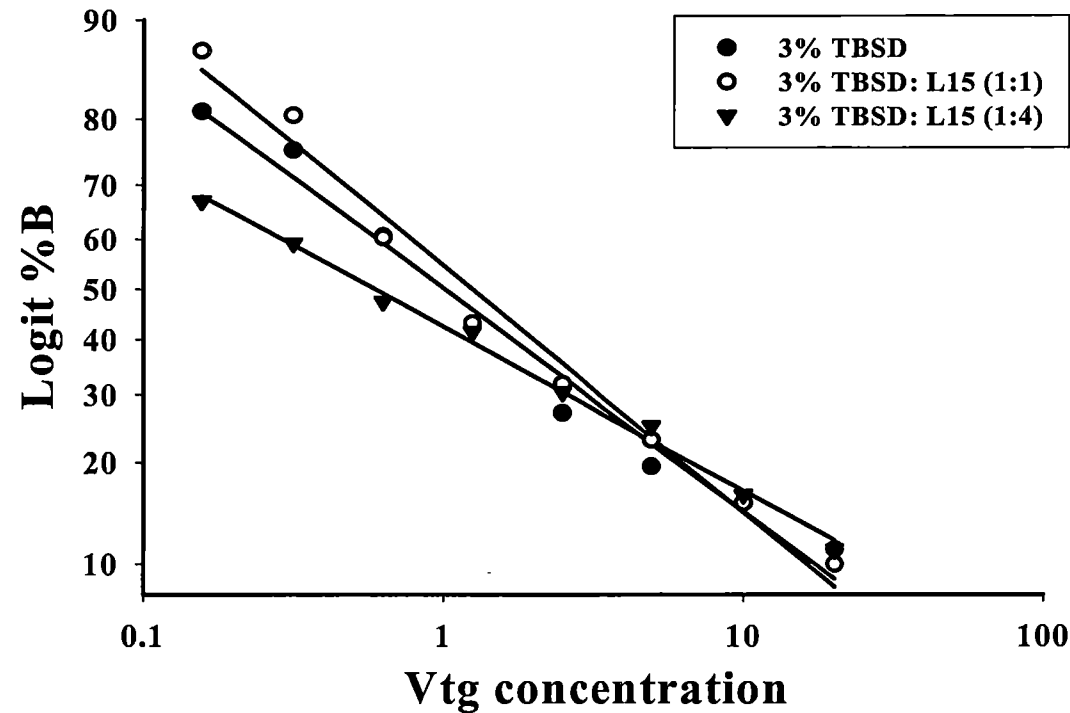


Fig. 6.3. Curves from Vtg standards ( $0.16$  to  $20 \mu\text{g ml}^{-1}$ ) diluted in 3% TBSD alone or with L15 phenol red at 1: 1 or 4: 1.

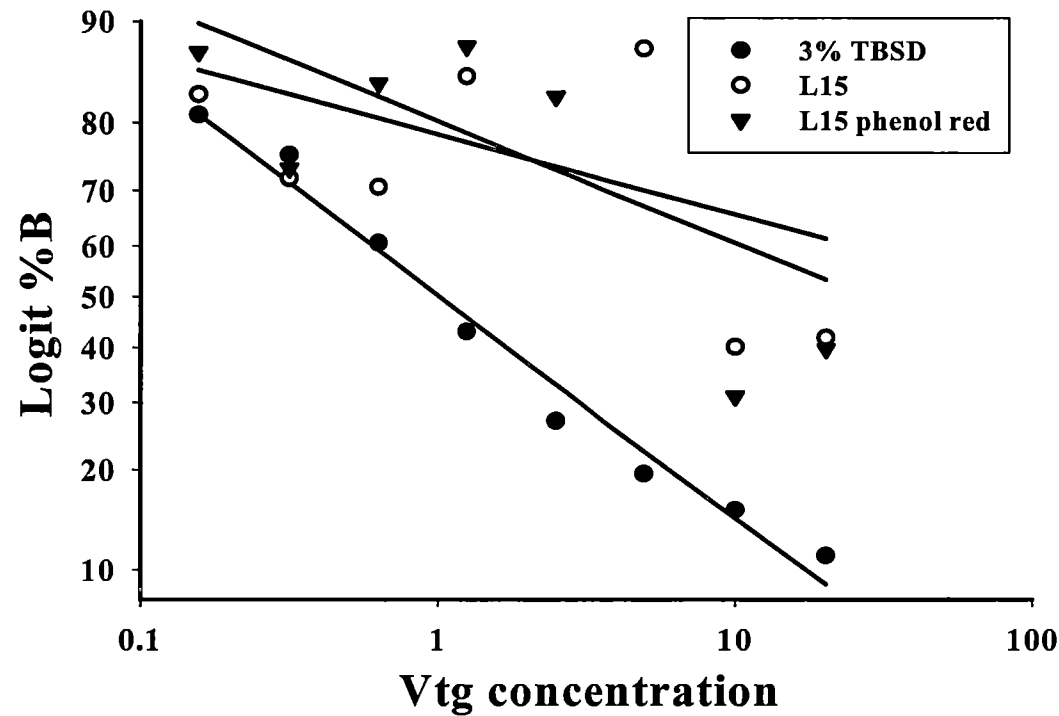


Fig. 6.4. Curves from Vtg standards ( $0.16$  to  $20 \mu\text{g ml}^{-1}$ ) diluted in 3% TBSD, L15 or L15 phenol red respectively.

## 6.4. Discussion

The isolation of hepatocytes from greenback flounder without a perfusion process is feasible as demonstrated in the present study by the high rate of survival during a long period of primary cell culture. The perfusion technique is quite difficult to perform in some flatfish species as a result of their small hepatic veins (reviewed in Baksi and Frazier, 1990; Mommsen et al., 1994). However, the viability of isolated hepatocytes in the present study is comparable to that of hepatocytes obtained by perfusion in other studies (e.g. Flouriot et al., 1993; Kwon et al., 1993; Celius and Walther, 1998; Peyon et al., 1998; Smeets et al., 1999; Kim and Takemura, 2003). In other studies, isolated hepatocytes were usually cultured for 24 to 48 h before exposure to E<sub>2</sub> (Mommsen and Lazier, 1986; Vaillant et al., 1988; Monteverdi and Di Giulio, 1999; Takemura and Kim, 2001), which helps hepatocytes to repair any injury from isolation, and return cellular function to original levels. Under these conditions, hepatocytes can generally be maintained for one week in the following culture (Mommsen and Lazier, 1986; Monteverdi and Di Giulio, 1999; Ferraris et al., 2002).

Different media e.g. L15, Medium 199, Dulbecco's modified medium, Eagle's Medium, Williams' medium E, modified Waymouth's medium, RPMI 1640 medium and Krebs-Henseleit buffer have been used in various species for hepatocyte culture (Morrison et al., 1985; Mommsen and Lazier, 1986; Baksi and Frazier, 1988; Flouriot et al. 1993; Peyon et al., 1993; Ferraris et al., 2002; Winzer et al., 2002; Kim and Takemura, 2003). A medium with high osmolarity is recommended for hepatocyte culture in salt-water teleosts. L15 contains a high concentration of free base amino acids resulting in a high osmolarity and also is suitable for the long-term culture of teleost hepatocytes (reviewed in Mommsen et al., 1994). Hepatocytes in L15 synthesize more Vtg compared with other media (Kim and Takemura, 2003). These results combined with good viability of hepatocytes in the present study indicate that L15 was an appropriate choice of medium for use with greenback flounder.

Serum supplements such as FCS and BSA are usually added to the medium to help the adhesion and growth of hepatocytes, and improve the rate of cell survival (reviewed in Mommsen et al., 1994; Segner, 1998). Various concentrations of FCS and BSA had been employed in different studies e.g. 10% FCS (Flouriot et al., 1993) and 1% BSA (Sathiyaa et al., 2001) respectively in rainbow trout, and 2% BSA (Mommsen and Lazier, 1986) and 0.5% FCS (Celius and Walther, 1998) in Atlantic salmon (*Salmo salar*). In contrast, hepatocytes of channel catfish (*Ictalurus punctatus*) (Monteverdi and Di Giulio, 1999) and tilapia (*Oreochromis mossambicus*) (Kim and Takemura, 2003) grew well in L15 in the absence of serum supplements. The addition of 10% FCS in culture medium also enhanced the expression of estrogen receptor (ER) and Vtg mRNA in hepatocytes of rainbow trout (Flouriot et al., 1993), and L15 containing 0.5% BSA increased Vtg mRNA levels in liver slices of Japanese eel (*Anguilla japonica*) (Adachi et al., 2000). In the present study, both 10% FCS and 2% BSA significantly improved the growth of hepatocytes. Two percent BSA was subsequently used in most experiments to avoid the variation of serum from different batches.

A wide range of E<sub>2</sub> doses ( $10^{-10}$  to  $10^{-4}$  M) was used in the present study, which covered the range of doses of E<sub>2</sub> effective in other studies, e.g.  $4 \times 10^{-8}$  to  $7 \times 10^{-6}$  M (Pelissero et al., 1991) and  $10^{-9}$  to  $10^{-5}$  M (Vaillant et al., 1988; Kwon et al., 1993) in rainbow trout,  $10^{-8}$  to  $10^{-5}$  M in European eel (Peyon et al., 1997),  $10^{-10}$  to  $10^{-8}$  M in Atlantic salmon (Celius and Walther, 1998),  $5 \times 10^{-8}$  to  $10^{-5}$  M in tilapia (Takemura and Kim, 2001),  $6 \times 10^{-10}$  to  $1.1 \times 10^{-6}$  M in carp (*Cyprinus carpio*) (Smeets et al., 1999). There was a dose-response relationship between E<sub>2</sub> concentrations and Vtg production in these studies at E<sub>2</sub> concentrations in medium up to a certain level. Beyond this level, increases in E<sub>2</sub> concentration did not result in further increases of Vtg in media (Pelissero et al., 1991; Kwon et al., 1993; Smeets et al., 1999). This was attributed to the saturation of ER at high doses of E<sub>2</sub> (Pelissero et al., 1991; Kwon et al., 1993). These results indicate that the failure to detect Vtg in the present study was unlikely to have been due to an inappropriate choice of E<sub>2</sub> dose.

The hepatocyte concentration used in the present study was  $10^6 \text{ ml}^{-1}$  which is within the range of  $10^5$  to  $2 \times 10^7 \text{ ml}^{-1}$  reported by other studies (reviewed in Baksi and Frazier, 1990). A comparison of Vtg production in hepatocytes of different species is shown in Table 6.2. The amount of Vtg in all these studies was less than  $1.0 \mu\text{g ml}^{-1}$  in the medium. Typical Vtg concentrations in the plasma of some species were  $81 \text{ mg ml}^{-1}$  in  $\text{E}_2$ -treated male rainbow trout (Bon et al., 1997),  $> 2.0 \text{ mg ml}^{-1}$  for Vtg210 in  $\text{E}_2$ -treated male tilapia (Takemura and Kim, 2001), and over  $4.0 \text{ mg ml}^{-1}$  in some untreated male channel catfish (Goodwin et al., 1992). In contrast, the maximum concentration of plasma Vtg in  $\text{E}_2$ -treated greenback flounder was around  $500 \mu\text{g ml}^{-1}$  (Chapter 4). A comparison between the levels of Vtg mRNA indicates that the effect of  $\text{E}_2$ -stimulation *in vitro* is 10 times less than that *in vivo* (Vaillant et al., 1988), which suggests that a more sensitive analysis method is needed for detecting Vtg *in vitro*. The concentrations of plasma Vtg in some species (e.g. Table 6.2) are over 2000 times higher than those in *in vitro* media. The detection limit of the ELISA used in the present study is  $320 \text{ ng ml}^{-1}$  at 1: 1 dilution of medium and it is impossible to improve the limit without interfering with the ELISA. These results suggest that the ELISA could not detect any Vtg in the medium if the production of Vtg from hepatocytes of greenback flounder was below  $320 \text{ ng ml}^{-1}$  in the present study. If this is the case, then greenback flounder hepatocytes produce substantially lower amounts of Vtg *in vitro* at cell densities where other species produced Vtg concentrations that would have been above the detection limit of the assay used here (Table 6.2).

The time-course induction of Vtg *in vitro* varies among different species. The optimal induction of Vtg occurred at 24 to 72 h after the seeding of hepatocytes in channel catfish (Monteverdi and Di Giulio, 1999). Vtg was detected in the medium 72 h after  $\text{E}_2$  exposure and reached a peak concentration at 96 h in Atlantic salmon (Celius and Walther, 1998). Significant increase of Vtg in the medium was observed 48 h after  $\text{E}_2$  treatment in tilapia (Takemura and Kim, 2001), whereas Vtg was present in medium 24 h after  $\text{E}_2$  exposure, and the accumulation of Vtg was sustained for 5 days in rainbow trout (Kwon et al., 1993). The difference may be species specific as well as resulting from variable culture conditions such as differences in cell density, incubation temperature and

medium E<sub>2</sub> concentrations. In the present study, a wide range of culture times and E<sub>2</sub> doses were employed, and the number of hepatocytes is within the range of those used in other studies. It seems that it was probably not the choice of experimental protocol that resulted in failure to detect Vtg in the medium.

Table 6.2 Comparison of Vtg production in hepatocytes *in vitro* of three species

Species	Cell (ml <sup>-1</sup> )	E <sub>2</sub> (M)	Incubation time	Vtg (ng ml <sup>-1</sup> )	References
Rainbow trout	2 – 4 × 10 <sup>6</sup>	3 × 10 <sup>-7</sup>	48 h	800	Pelissero et al., 1991
Channel catfish	10 <sup>6</sup>	10 <sup>-8</sup> 10 <sup>-9</sup>	48 h	500 430	Monteverdi and Di Giulio, 1999
Tilapia	10 <sup>6</sup>	10 <sup>-5</sup>	48 h 72 h	650 (Vtg210) 750 (Vtg210)	Takemura and Kim, 2001

Male or immature fish normally have lower levels of ER which can be up-regulated by the stimulation with estrogens (Mommensen and Lazier, 1986). In rainbow trout hepatocytes, the production of Vtg after a second E<sub>2</sub> stimulation was proportional to the number of ER synthesized in the first stimulation, which indicated that the production of Vtg may be limited by ER levels in hepatocytes (Vaillant et al., 1988). This was further confirmed by the fact that E<sub>2</sub> did not stimulate hepatocytes of silver eels to produce Vtg whereas significant increases of Vtg production were observed in hepatocytes from eels which had received *in*

*vivo* E<sub>2</sub>-priming (Peyon et al., 1993). In order to assess whether there was a similar effect in greenback flounder that accounted for the lack of responsiveness *in vitro*, hepatocytes from females were tested in the present study. Vtg production in female hepatocytes is much higher than that in male e.g. 10 times in sea bass (*Dicentrarchus labrax*) (Carnevali et al., 1995) and 25 times in carp (Smeets et al., 1999) presumably as a result of E<sub>2</sub>-priming during *in vivo* exposure. The lack of measurable Vtg in female hepatocytes in the present study probably indicated that the amount of Vtg produced by hepatocytes of greenback flounder is very low which is in agreement with the low concentrations of Vtg (~ 1.0 mg ml<sup>-1</sup>) in plasma of vitellogenic fish (Chapter 5), compared to 60 mg ml<sup>-1</sup> in vitellogenic rainbow trout (Bon et al., 1997).

An *in vitro* hepatocyte culture system had been developed in greenback flounder which was used to assess the effect of E<sub>2</sub> on Vtg induction in hepatocytes. However, no measurable Vtg was found in all experiments under different conditions. The possible reasons were demonstrated to be the unexpectedly low production of Vtg in hepatocytes of this species and the relatively high detection limit of the ELISA used in the present study.

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## **Chapter 7**

***In vitro* effect of vitellogenin on steroid  
production by ovarian follicles of greenback  
flounder *Rhombosolea tapirina***

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## **7. *In vitro* effect of vitellogenin on steroid production by ovarian follicles of greenback flounder *Rhombosolea tapirina***

### **7.1. Introduction**

Oocyte growth (vitellogenesis) is a major event in teleost oogenesis, involving the secretion of vitellogenin (Vtg) by the liver and the processing of this precursor into yolk proteins by the oocyte (reviewed in Wallace, 1985; Specker and Sullivan, 1994). Pituitary follicle-stimulating hormone (FSH) is released and travels via the circulation to the ovary where it binds to the FSH receptor located on the thecal cell, and sequentially triggers the *de novo* synthesis of testosterone (T) through a receptor-mediated adenylate cyclase-cAMP system. T diffuses into the granulosa cell and is converted into 17 $\beta$ -estradiol (E<sub>2</sub>) in the presence of aromatase cytochrome P-450 (P-450arom) (reviewed in Nagahama et al., 1995). E<sub>2</sub> is secreted into the bloodstream, transported to the liver by steroid-binding proteins where E<sub>2</sub>-receptor interaction stimulates the synthesis of Vtg production by hepatocytes. Vitellogenin is, in turn, transported via the circulatory system to the ovary where it is taken up by developing oocytes via receptor-mediated endocytosis (reviewed in Mommsen and Walsh, 1988; Specker and Sullivan, 1994).

In some teleosts, plasma E<sub>2</sub> concentrations are usually elevated during vitellogenesis and decline in response to the surge of luteinizing hormone (LH) as oocytes begin maturation (Devlin and Nagahama, 2002). However, in some other species, it appears that once vitellogenesis is triggered, high plasma E<sub>2</sub> concentrations are not necessary for sustaining the ongoing production of Vtg (Methven et al., 1992; Clearwater and Pankhurst, 1994; Koya et al., 2003). Treatment of vitellogenic rainbow trout (*Oncorhynchus mykiss*) with exogenous Vtg results in a suppression of plasma concentrations of E<sub>2</sub>, which return to normal levels after the termination of Vtg administration (Resi-Henriques et al., 1997). Collectively, these results suggest the possibility that, Vtg may execute

negative feedback control on ovarian E<sub>2</sub> production. Further evidence for an inhibitory role for Vtg in the regulation of follicular E<sub>2</sub> synthesis is provided by the *in vitro* study of rainbow trout vitellogenesis by Resi-Henriques et al. (1997, 2000). When isolated follicles from vitellogenic females were incubated with Vtg in the presence of various steroid substrates, the increase in culture medium levels of E<sub>2</sub> was attenuated. This observation indicates that the presence of high concentrations of Vtg may inhibit the activities of steroid converting enzymes in the ovarian follicles.

*In vitro* approaches have several attributes that make them suitable for investigating the possible effects of Vtg on sex steroid biosynthesis and secretion. Isolated ovarian follicles maintain the capacity to synthesize steroids under defined culture conditions for periods from 30 min to a few days, which makes it possible to establish the optimum *in vitro* parameters for each species, and accordingly, to investigate the ovarian steroidogenic activity by the measurement of steroids released by isolated follicles in the culture medium (e.g. Pankhurst, 1997; Haddy and Pankhurst, 1998; Pankhurst and Riple, 2000). This technique has been widely used in different species to investigate the mechanisms of follicular signal transduction in amago salmon (*Oncorhynchus rhodutus*) (Kanamori and Nagahama, 1988), medaka (*Oryzias latipes*) (Nagahama et al., 1991) and Atlantic salmon (*Salmo salar*) (Watts et al., 2003), and the effects of various GtHs, and steroidogenic enzymes in striped trumpeter (*Latris lineata*) (Pankhurst, 1997), rainbow trout (Haddy and Pankhurst, 1998) and greenback flounder (*Rhombosolea tapirina*) (Pankhurst and Riple, 2000). The systematic application of GtHs, agents which modulate intracellular levels of cAMP, and steroid precursors allows the investigation of various levels in the steroid synthesis pathway, and the possible levels of effect of Vtg on steroidogenic processes.

The good understanding of dynamics of steroidogenesis in isolated follicles of greenback flounder (Pankhurst and Riple, 2000), and the extended reproductive season and wide size range of vitellogenic follicles usually present in this species (Barnett and Pankhurst, 1999; Chapter 5 this study) make greenback flounder a

good model for further investigating hormone-mediated vitellogenesis *in vitro*. Dibutyryl cyclic AMP (dbcAMP) is a useful agent for increasing effective intracellular cAMP levels in the follicles and for assessing its role as second messenger in cellular signal transduction (Kanamori and Nagahama, 1988; Nagahama et al., 1991; Watts et al., 2004). Human chorionic gonadotropin (hCG) is an effective substitute in assessment of the activity of native GtH in stimulating steroidogenesis in greenback flounder, and the provision of steroid substrates is a useful tool for investigating the activity of specific steroidogenic enzymes in follicles of this species as well (Pankhurst and Ripley, 2000). Accordingly, three successive experiments were performed in this study to estimate the effects of Vtg on basal and hCG-, dbcAMP- and steroid precursor-stimulated E<sub>2</sub> and T production by greenback flounder follicles *in vitro*, which will allow assessment of possible pathways by which Vtg may affect control of gonadal steroidogenesis.

## 7.2. Materials and methods

### 7.2.1. Fish and Fish maintenance

Sexually maturing 3 + -year-old female flounder were acquired from stocks grown at the School of Aquaculture aquatic facility at the University of Tasmania in Launceston. Fish were held in a 1000-l tank connected to a semi-closed sea water recirculation system at  $12 \pm 1^\circ\text{C}$  and natural photoperiod, and fed daily with dry pellets (Pivot Aquaculture, Hobart, Australia) at 3% body weight. Fish with vitellogenic ovaries were selected for experiments. These fish were characterised by a large ovary bulging above the dorsal musculature without detectable extrusion of eggs following manual application of pressure to the abdomen. After anaesthesia in 0.05% 2- phenoxyethanol (Sigma), fish were killed by spinal transection and ovaries were excised. Body weight (BW) and gonad weight (GW) were recorded for each fish.

### 7.2.2. Vtg preparation



Maturing males from the same source were implanted with an appropriate length of silastic pellet containing E<sub>2</sub> using the method described in Pankhurst et al. (1986), to give a dose of 50 mg kg<sup>-1</sup> BW. Blood samples were taken weekly, by caudal puncture using preheparinised 1 ml syringes and 25 G needles, beginning two weeks after the E<sub>2</sub> administration. Separation of plasma and isolation of Vtg were performed immediately after each sampling according to the method described in Chapter 2. Purified Vtg was frozen and stored at -20°C until use. Before each experiment, Vtg samples were defrosted and pooled into an Amicon Ultra-15 Centrifugal Filter Device (Millipore) and concentrated by centrifuging at 3000 g and 4°C to give a final concentration of 50 mg ml<sup>-1</sup>.

### ***7.2.3. Follicle preparation***

Ovarian follicles were dispersed and washed in ice cold Leibowitz-15 medium (L15, Sigma) adjusted to 405 mOs kg<sup>-1</sup>, and 100-µl aliquots of final follicle suspension were added to each well of 24-well tissue culture microplates (Iwaki) as described in Pankhurst and Ripley (2000). Four additional 100-µl aliquots were used to determine the number of vitellogenic follicles present in each well. Twenty follicles from each fish were measured to determine the mean maximum follicle size.

### ***7.2.4. Incubation protocols***

Follicles were incubated in a final volume of 1-ml of L15 either alone or containing Vtg added as 100 µl of 1.0, 10 or 50 mg ml<sup>-1</sup> in L15 to give final concentrations of 0.1, 1.0 or 5.0 mg ml<sup>-1</sup>; dbcAMP (Sigma) added as 100 µl of 1.0, 10 or 100 mM in L15 to give final concentrations of 0.1, 1.0 or 10 mM; hCG (Sigma) added as 100 µl of 100 or 1000 U ml<sup>-1</sup> in L15 to give final concentrations of 10 or 100 U ml<sup>-1</sup>; T added as 10 µl of 1.0 or 10 µg ml<sup>-1</sup> in ethanol to give final concentrations of 10 or 100 ng ml<sup>-1</sup>; 17-hydroxyprogesterone (17P) added as 10 µl of 10 µg ml<sup>-1</sup> in ethanol to give final a concentration of 100 ng ml<sup>-1</sup>; androstenedione (A) added as 10 µl of 2.0 µg ml<sup>-1</sup> in ethanol to give a final concentration of 20 ng ml<sup>-1</sup>. All wells without steroids received 10 µl of ethanol.

Follicles were incubated at 12°C for 18 h after which culture media were aspirated into Eppendorf tubes and stored frozen until analysis.

### **7.2.5. Experiment protocols**

Three experiments were carried out with three different fish in Experiment 1 and two different fish in Experiment 2 and Experiment 3 respectively, and four replicate wells for each treatment within fish. The reproductive parameters of fish used in experiments are shown in Table 7.1.

#### **7.2.5.1. Experiment 1**

This experiment was designed to assess the effect of physiological concentrations of Vtg on basal-, hCG- and steroid precursor-stimulated E<sub>2</sub> and T production. Follicles were incubated with L15 alone (control) or containing hCG at 10 or 100 U ml<sup>-1</sup> (hCG10, hCG100), T at 10 or 100 ng ml<sup>-1</sup> (T10, T100) and 17P at 100 ng ml<sup>-1</sup> (17P100), with or without Vtg at 0.1 or 1.0 mg ml<sup>-1</sup> (Vtg0.1, Vtg1.0).

#### **7.2.5.2. Experiment 2**

This experiment was based on the results of Experiment 1 to examine the effect of higher concentrations of Vtg on basal-, hCG- and steroid precursor-stimulated E<sub>2</sub> and T production. Follicles were incubated with L15 alone or containing hCG at 10 U ml<sup>-1</sup>, 17P at 100 ng ml<sup>-1</sup> and A at 20 ng ml<sup>-1</sup> (A20), with or without Vtg at 1.0 or 5.0 mg ml<sup>-1</sup> (Vtg1.0, Vtg5.0).

#### **7.2.5.3. Experiment 3**

This experiment was used to detect the effect of various concentrations of Vtg on cAMP-stimulated E<sub>2</sub> and T production. Follicles were incubated with L15 alone or containing dbcAMP at 0.1, 1.0 or 10 mM (dbcAMP0.1, dbcAMP1.0, dbcAMP5.0) with or without Vtg at 0.1, 1.0 or 5.0 mg ml<sup>-1</sup>. There was no Vtg alone treatment in this experiment because of insufficient supplies of Vtg.

### **7.2.6. Steroid measurement**

Medium concentrations of E<sub>2</sub> and T were measured by radioimmunoassay as described in Pankhurst (1997) using the reagents and protocol in Pankhurst and Conroy (1987). All samples were measured in duplicate and the detection limit of E<sub>2</sub> and T in media was 30 pg ml<sup>-1</sup>. Inter-assay variability (%CV) measured using aliquots of a pooled internal standard was 12.3% (n = 9) and 18.3% (n = 7), for E<sub>2</sub> and T respectively.

### 7.2.7. Statistical analysis

All values were expressed as means ± standard error (S.E.). Data were analysed by one-way ANOVA followed by Duncan's test for comparisons among treatments with a significant level of  $P < 0.05$ .

Table 7.1 Reproductive parameters of individual fish used in different experiments

Experiment	Fish	BW (g)	GW (g)	GSI (%) <sup>a</sup>	FD (μm) <sup>b</sup>	FN per well <sup>c</sup>
1	1	288	44	15.28	740 ± 9	492 ± 7
	2	330	31.7	9.61	510 ± 11	852 ± 13
	3	193	24	12.44	578 ± 9	545 ± 20
2	1	329	35.8	10.88	675 ± 9	656 ± 40
	2	204	10.71	5.25	693 ± 8	553 ± 12
3	1	212.5	74.6	35.11	843 ± 20	312 ± 9
	2	220.6	37.68	17.08	622 ± 11	672 ± 23

<sup>a</sup> GSI (gonadosomatic index) =  $GW \times 100/BW$ .

<sup>b</sup> FD (follicle diameter) = Mean ± S.E. (n = 20).

<sup>c</sup> FN (follicle number) = Mean ± S.E. (n = 4).

## 7.3. Results

### 7.3.1. Experiment 1

Testosterone (both doses), 17P and the high dose hCG treatments resulted in significant increases of E<sub>2</sub> production by follicles from all three fish (Figs. 7.1, 7.2 and 7.3), whereas the low dose hCG treatment increased E<sub>2</sub> production by follicles from only one fish (Fig. 7.2) but not by follicles from the other two animals (Figs. 7.1 and 7.3). The 17P treatment led to an increase in T production by follicles from three fish whereas hCG treatments had no such effect (Figs. 7.1, 7.2 and 7.3). Administration of Vtg at 0.1 mg ml<sup>-1</sup> had no effect on basal E<sub>2</sub> and T production by follicles from any fish (Figs. 7.1, 7.2 and 7.3). However, administration of Vtg at 1.0 mg ml<sup>-1</sup> increased basal E<sub>2</sub> production by follicles from one fish (Fig. 7.1), but had no effect either on basal E<sub>2</sub> production by follicles from the other two fish (Figs. 7.2 and 7.3) or on basal T production by follicles from all three fish (Figs. 7.1, 7.2 and 7.3). In response to the low dose hCG treatment, administration of Vtg (both doses) increased E<sub>2</sub> production by follicles from one fish (Fig. 7.1), but had no effect on either E<sub>2</sub> production by follicles from the other two fish (Figs. 7.2 and 7.3) or T production by follicles from all three fish (Figs. 7.1, 7.2 and 7.3). In response to the high dose hCG treatment, administration of Vtg at both doses had no effect on T production and administration of Vtg at 0.1 mg ml<sup>-1</sup> had no effect on E<sub>2</sub> production, by follicles from all three fish (Figs. 7.1, 7.2 and 7.3). However, administration of Vtg at 1.0 mg ml<sup>-1</sup> increased E<sub>2</sub> production by follicles from one fish (Fig. 7.1) and decreased E<sub>2</sub> production by follicles from the other two fish (Figs. 7.2 and 7.3). Administration of Vtg at both doses had no effect on E<sub>2</sub> production by follicles from all three fish in response to low dose T treatment (Figs. 7.1, 7.2 and 7.3); however, in response to high dose T treatment, administration of Vtg at both doses decreased E<sub>2</sub> production by follicles from one fish (Fig. 7.2) but had no effect in another one (Fig. 7.1), administration of Vtg at 1.0 mg ml<sup>-1</sup> but not 0.1 mg ml<sup>-1</sup> decreased E<sub>2</sub> production by follicles from the third fish in response to high dose T treatment (Fig. 7.3). In response to 17P treatment, administration of Vtg (both doses) decreased E<sub>2</sub> production by follicles from one fish (Fig. 7.2) but had no effect in the other two fish (Figs. 7.1 and 7.3), and administration of Vtg at 1.0 mg ml<sup>-1</sup> reduced T production by follicles from all three fish (Figs. 7.1, 7.2 and 7.3) whereas administration of Vtg at 0.1 mg ml<sup>-1</sup> decreased T production by follicles from one fish (Fig. 7.1).

### 7.3.2. Experiment 2

Treatment of follicles with 17P increased E<sub>2</sub> and T production by follicles from both fish as in Experiment 1 (Figs. 7.4 and 7.5). Treatment with A resulted in an increase in T production by follicles from both fish (Figs. 7.4 and 7.5) but E<sub>2</sub> production by follicles from one fish (Fig. 7.4). The hCG treatment stimulated an increase in E<sub>2</sub> not T production by follicles from one fish (Fig. 7.4), and T but not E<sub>2</sub> production by follicles from the other one (Fig. 7.5). Administration of Vtg (both doses) had no effect on basal T production by follicles from either fish whereas administration of Vtg at 5.0 mg ml<sup>-1</sup> inhibited basal E<sub>2</sub> production by follicles from both fish (Figs. 7.4 and 7.5), and administration of Vtg at 1.0 mg ml<sup>-1</sup> decreased basal E<sub>2</sub> production by follicles from one fish (Fig. 7.4). Administration of Vtg at 5.0 mg ml<sup>-1</sup> also decreased E<sub>2</sub> and T production in response to 17P treatment, and E<sub>2</sub> production in response to hCG treatment by follicles from both fish (Figs. 7.4 and 7.5), and T production in response to hCG treatment by follicles from one fish (Fig. 7.5). Administration of Vtg at 1.0 mg ml<sup>-1</sup> reduced E<sub>2</sub> and T production in response to 17P and A treatments, and E<sub>2</sub> production in response to hCG treatment by follicles from one fish (Fig. 7.4), but only T production in response to 17P and A treatments by follicles from the other fish (Fig. 7.5).

### 7.3.3. Experiment 3

The dbcAMP treatments reduced E<sub>2</sub> production by follicles in a dose-dependent manner, whereas the highest dose dbcAMP treatment increased T production by follicles from both fish (Figs. 7.5 and 7.6). The T production by follicles from one fish also decreased in response to 0.1mM dbcAMP treatment (Fig. 7.6). In response to 0.1mM dbcAMP treatment, administration of Vtg at 0.1 mg ml<sup>-1</sup> had no effect on E<sub>2</sub> production and T production by follicles from both fish (Figs. 7.6 and 7.7) and administration of Vtg at 1.0 mg ml<sup>-1</sup> only reduced E<sub>2</sub> production by follicles from one fish (Fig. 7.7). In response to 1.0 mM dbcAMP treatment, administration of Vtg at 0.1 mg ml<sup>-1</sup> reduced E<sub>2</sub> production but not T production, whereas administration of Vtg at 1.0 mg ml<sup>-1</sup> had no effect on E<sub>2</sub> production and T

production by follicles from both fish (Figs. 7.6 and 7.7). In response to 10 mM dbcAMP treatment, administration of Vtg at 0.1 and 1.0 mg ml<sup>-1</sup> had no effect on E<sub>2</sub> production by follicles from both fish (Figs. 7.6 and 7.7) whereas administration of Vtg at 0.1 mg ml<sup>-1</sup> increased T production by follicles from one fish (Fig. 7.6) and administration of Vtg at 1.0 mg ml<sup>-1</sup> decreased T production by follicles from the other fish (Fig. 7.7). Administration of Vtg at 5.0 mg ml<sup>-1</sup> reduced T production by follicles from both fish (Figs. 7.6 and 7.7) and E<sub>2</sub> production by follicles from one fish (Fig. 7.6). A summary of the significant effects in all 3 experiments is shown in Tables 7.2 to 7.4.

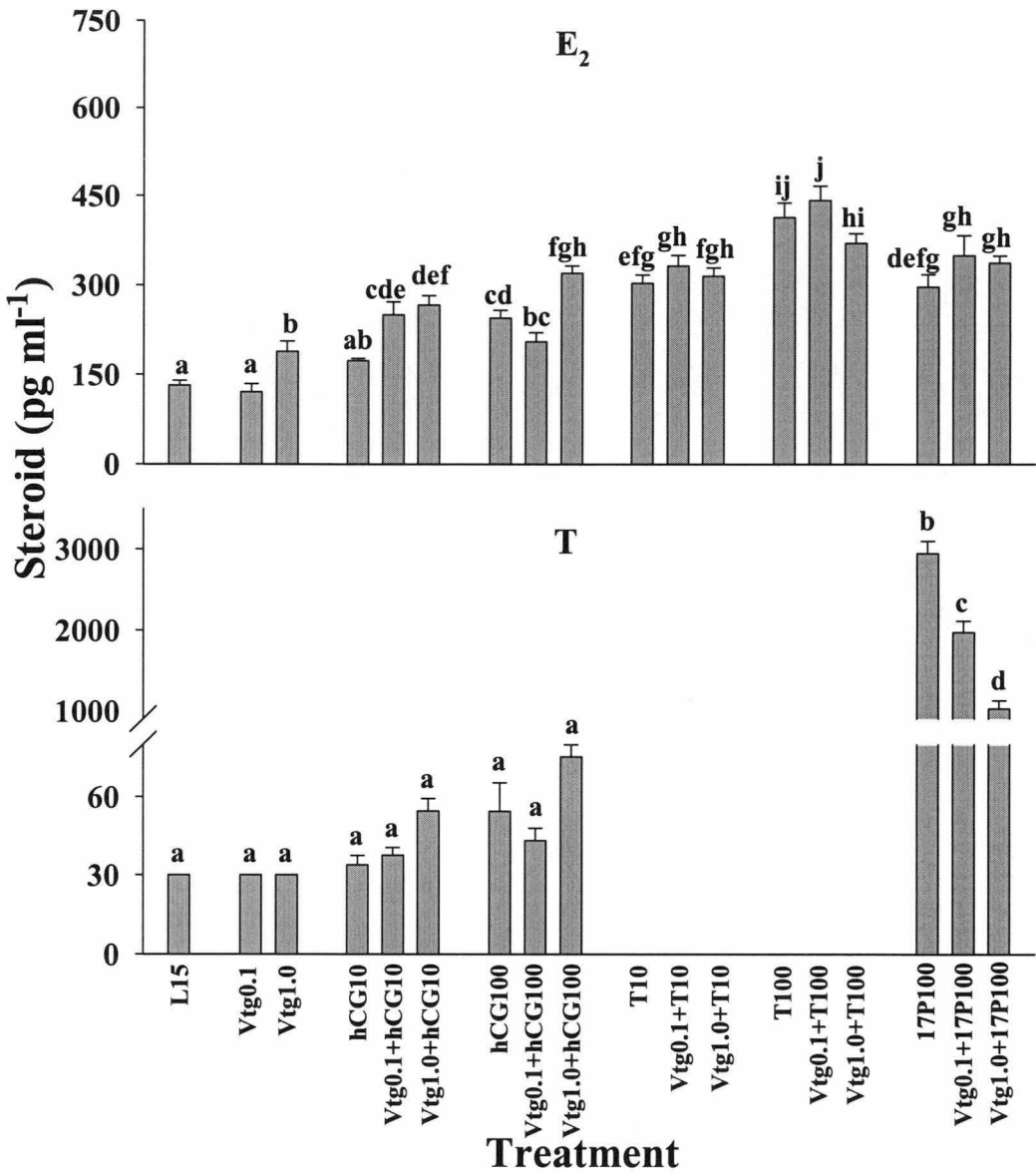


Fig. 7.1. Production of E<sub>2</sub> and T by isolated ovarian follicles from fish 1 (experiment 1) incubated with Vtg alone or hCG, T and 17P with or without Vtg (details of treatments are given in Materials and Methods). Values are mean + S.E. (n = 4). Values with the same superscript are not significantly different (P > 0.05).

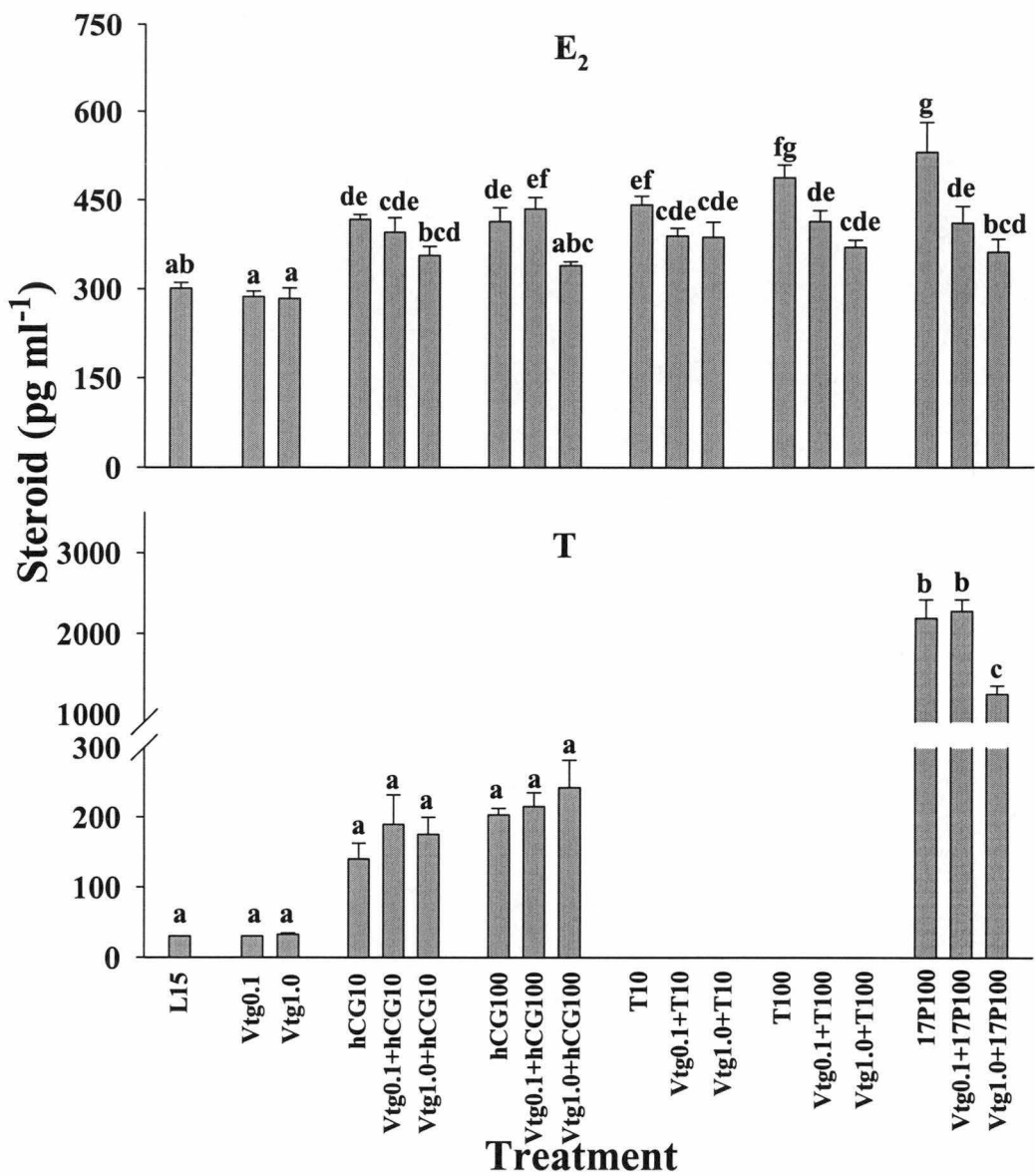


Fig. 7.2. Production of E<sub>2</sub> and T by isolated ovarian follicles from fish 2 (experiment 1). Other details as for Fig. 7.1.



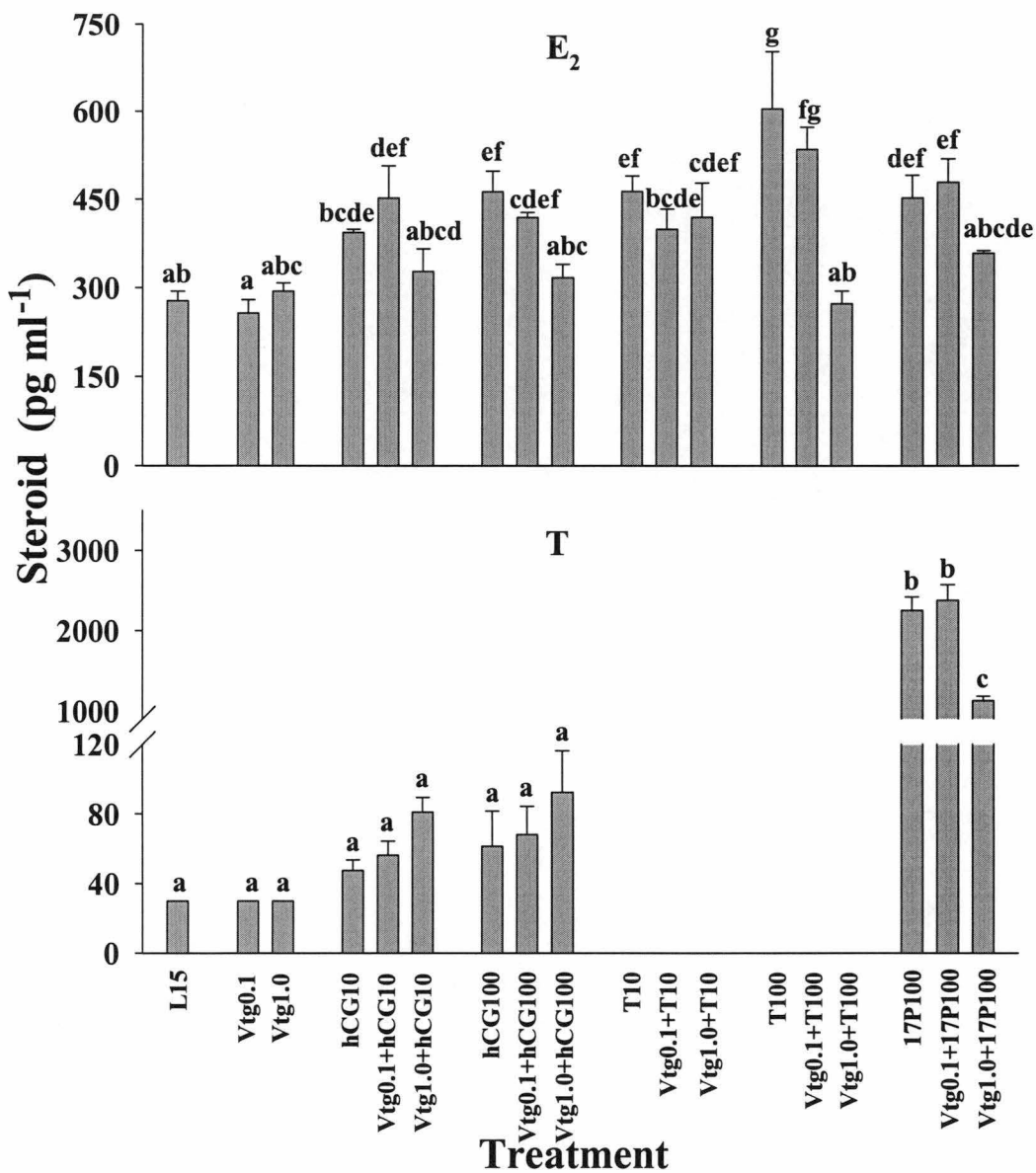


Fig. 7.3. Production of E<sub>2</sub> and T by isolated ovarian follicles from fish 3 (experiment 1). Other details as for Fig. 7.1.

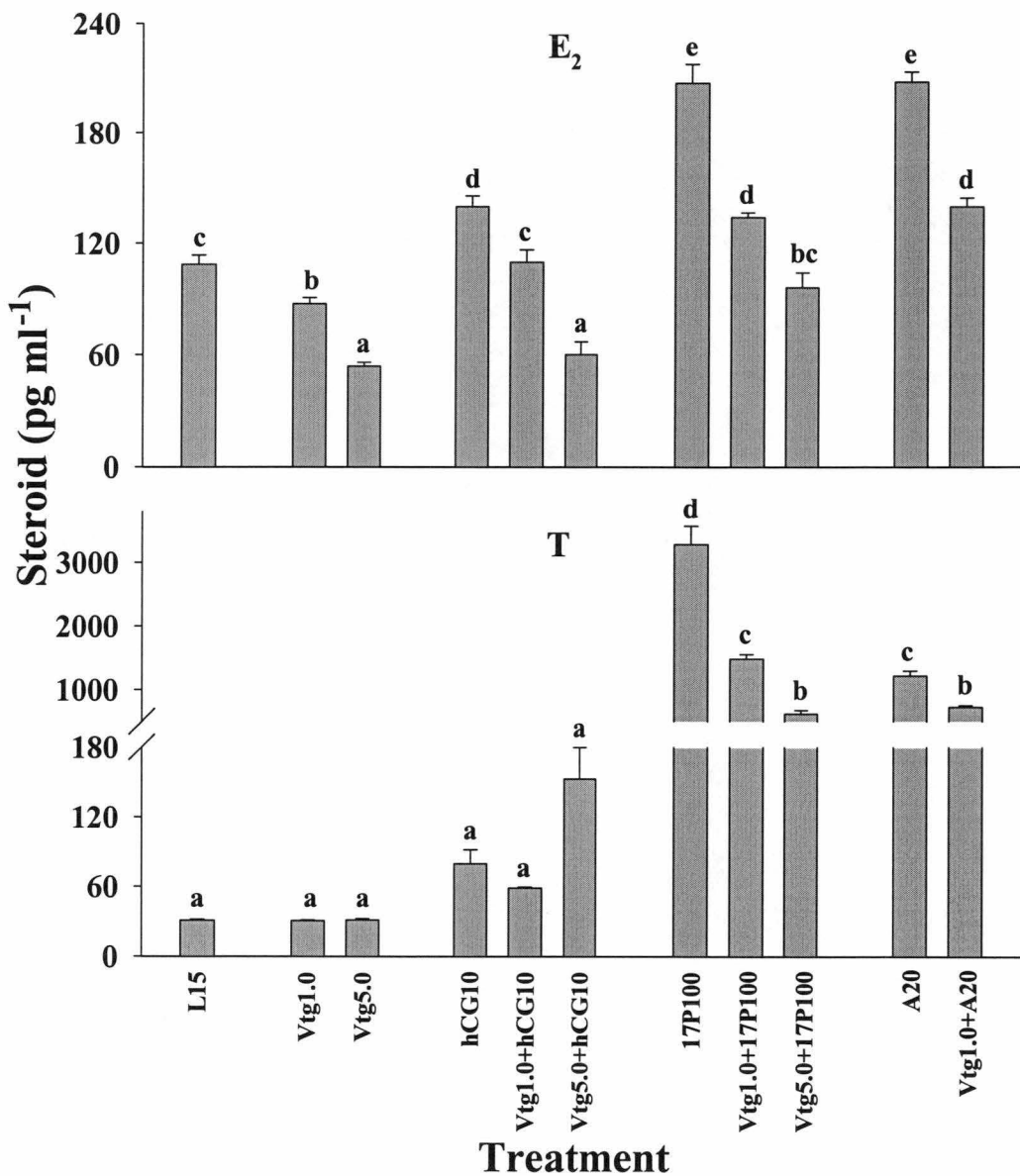


Fig. 7.4. Production of E<sub>2</sub> and T by isolated ovarian follicles from fish 1 (experiment 2) incubated with Vtg alone or hCG, 17P and A with or without Vtg (details of treatments are given in Materials and Methods). Other details as for Fig. 7.1.

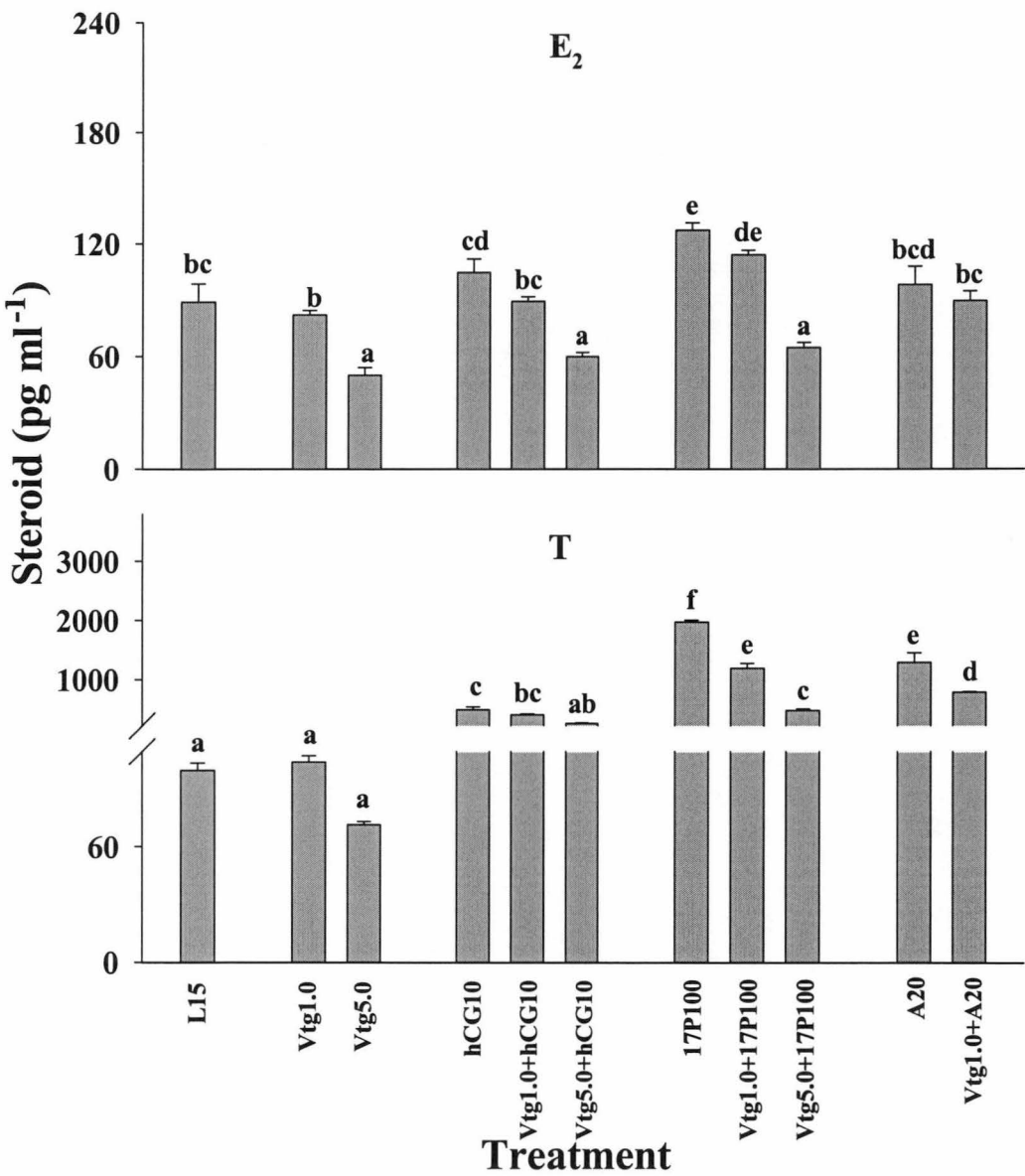


Fig. 7.5. Production of E<sub>2</sub> and T by isolated ovarian follicles from fish 2 (experiment 2). Other details as for Fig. 7.4.

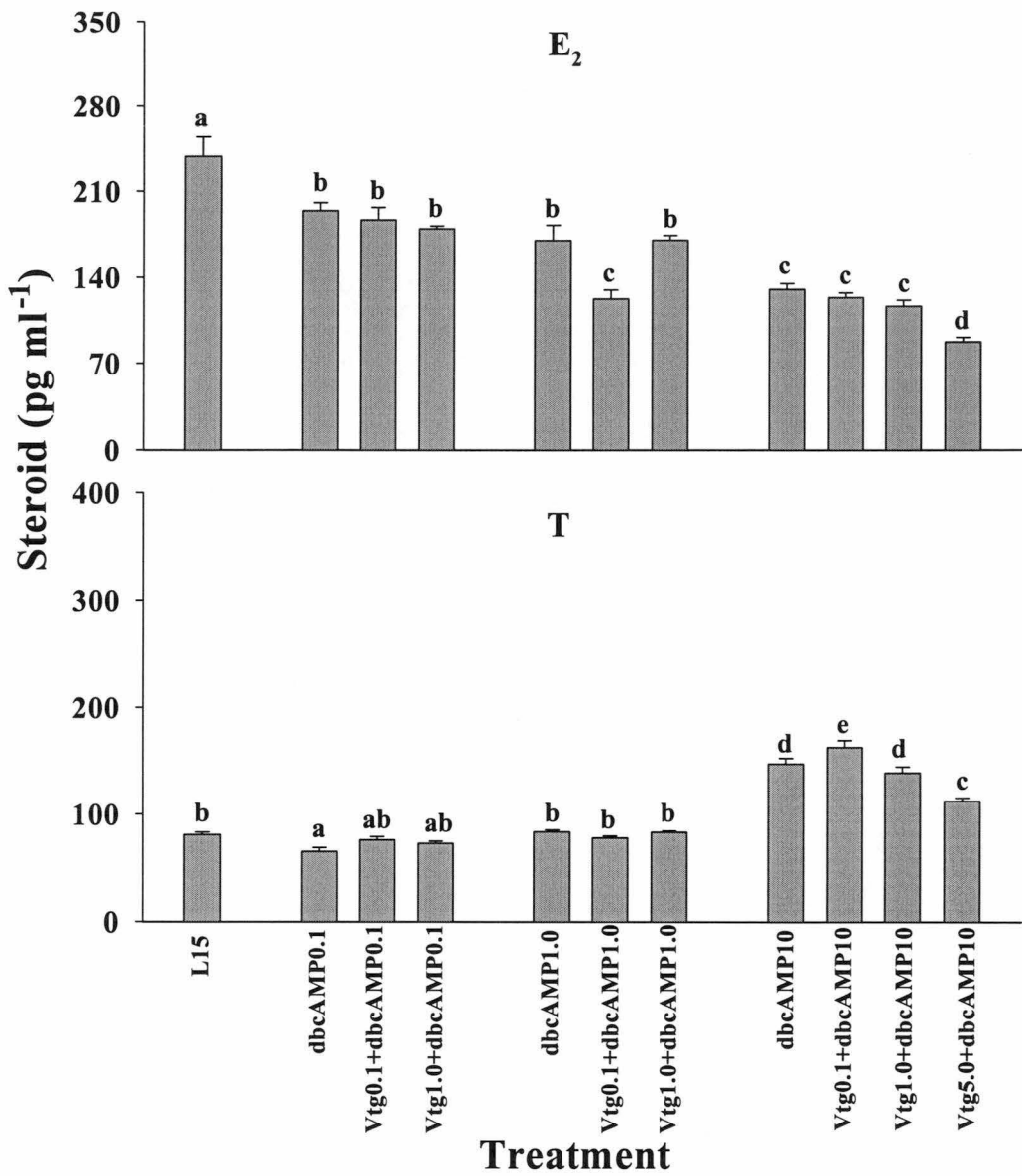


Fig. 7.6. Production of E<sub>2</sub> and T by isolated ovarian follicles from fish 1 (experiment 3) incubated with dbcAMP with or without Vtg (details of treatments are given in Materials and Methods). Other details as for Fig. 7.1.

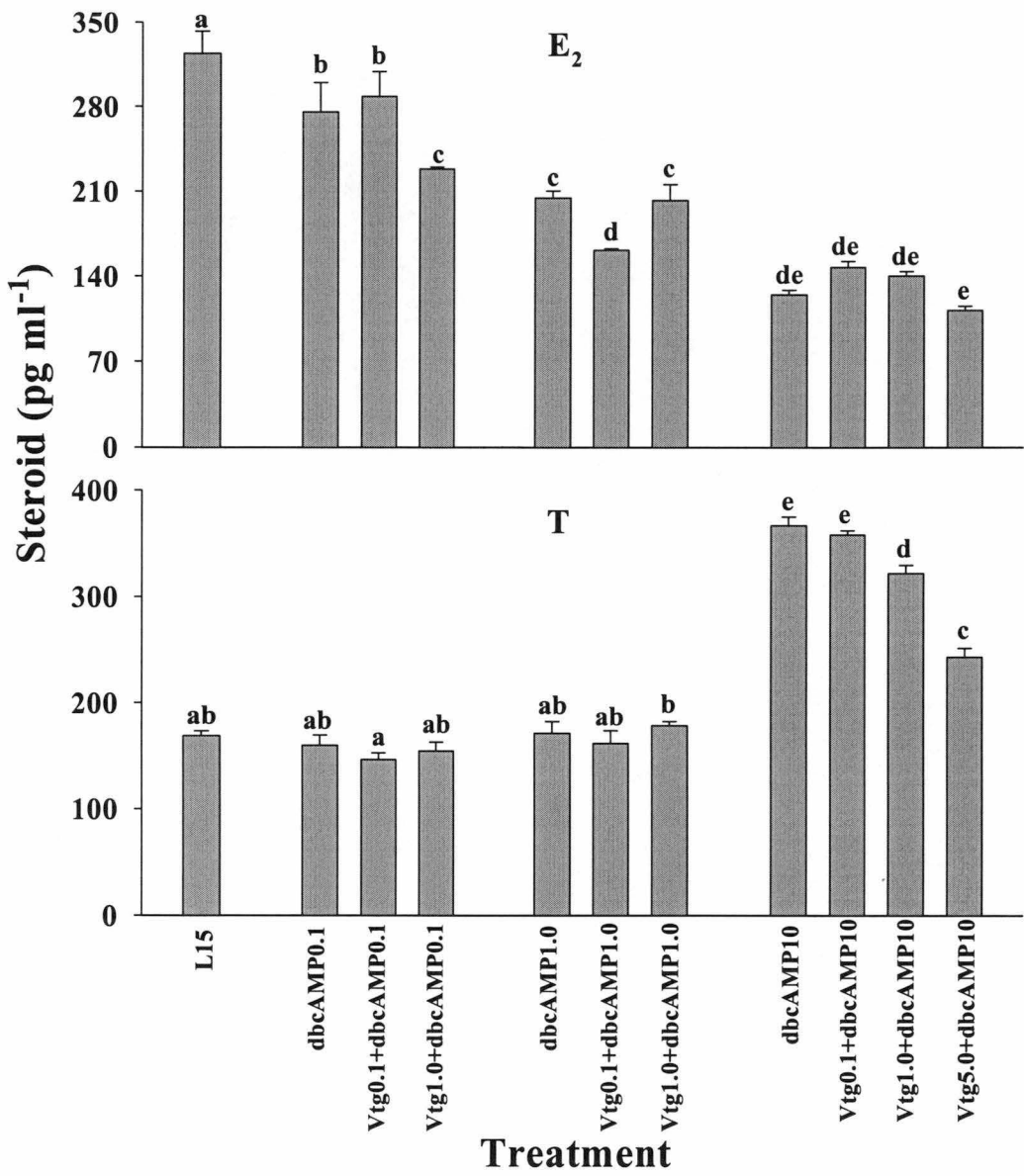


Fig. 7.7. Production of  $E_2$  and T by isolated ovarian follicles from fish 2 (experiment 3). Other details as for Fig. 7.6.

Table 7.2 Significant effects of different treatments on E<sub>2</sub> and T production in experiment 1

Treatment	E <sub>2</sub>			T		
	L15 <sup>1</sup>	Vtg0.1 <sup>2</sup>	Vtg1.0 <sup>2</sup>	L15 <sup>1</sup>	Vtg0.1 <sup>2</sup>	Vtg1.0 <sup>2</sup>
L15			+			
hCG10	+	+	+			
hCG100	+++		+--			
T10	+++					
T100	+++	-	--			
17P100	+++	-	-	+++	-	---

+ = stimulatory effect; - = inhibitory effect. Each + or - indicates the result from a separate fish.

<sup>1</sup> Responses relative to L15.

<sup>2</sup> Responses relative to that treatment without Vtg.

Table 7.3 Significant effects of different treatments on E<sub>2</sub> and T production in experiment 2

Treatment	E <sub>2</sub>			T		
	L15 <sup>1</sup>	Vtg1.0 <sup>2</sup>	Vtg5.0 <sup>2</sup>	L15 <sup>1</sup>	Vtg1.0 <sup>2</sup>	Vtg5.0 <sup>2</sup>
L15		-	--			
hCG10	+	-	--	+		-
17P100	++	-	--	++	--	--
A20	+	-		++	--	

Symbols are as described in Table 7.2.

Table 7.4 Significant effects of different treatments on E<sub>2</sub> and T production in experiment 3

Treatment	E <sub>2</sub>				T			
	L15 <sup>1</sup>	Vtg0.1 <sup>2</sup>	Vtg1.0 <sup>2</sup>	Vtg5.0 <sup>2</sup>	L15 <sup>1</sup>	Vtg0.1 <sup>2</sup>	Vtg1.0 <sup>2</sup>	Vtg5.0 <sup>2</sup>
dbcAMP0.1	--		-		-			
dbcAMP1.0	--	--						
dbcAMP10	--			-	++	+	-	--

Symbols are as described in Table 7.2.

## 7.4. Discussion

Microscopic observation demonstrated that the majority of follicles were at the vitellogenic growth stage with a very small portion of follicles undergoing final oocyte maturation or hydration in an individual ovary. This was further confirmed by a high basal E<sub>2</sub> production by follicles from all fish, which also was considerably higher than basal T production except for fish 2 in Experiment 2. A comparison of these results with those of a previous study (Pankhurst and Riple, 2000) indicated that follicles obtained via manual dispersion procedures in the present study were intact and maintained steroidogenic function *in vitro* during the culture period.

A previous study (Pankhurst and Riple, 2000) showed that hCG stimulated both E<sub>2</sub> and T production in greenback flounder follicles. The observations of follicular E<sub>2</sub> production in the present study are consistent with that finding. However, medium T concentrations were not increased by treatment of the follicles with hCG in the present study, except in a single case. A comparison between the two studies indicated that basal T concentration in incubations of responsive follicles in the present study had values similar to those in the study by Pankhurst and Riple (2000). This presumably indicates a high activity of P-450arom in these follicles, resulting in a high conversion of T to E<sub>2</sub>. The stimulation of T production *in vitro* by hCG also was maturation stage dependent in goldfish (*Carassius auratus*) (Kagawa et al., 1984). Treatment of follicles from vitellogenic striped trumpeter with hCG also increased *in vitro* E<sub>2</sub> production without changing T production (Pankhurst, 1997).

Isolated follicles of greenback flounder were capable of converting exogenous steroid precursors into E<sub>2</sub> and T after 18 h of culture (Pankhurst and Riple, 2000). In the present study, 4 out of 5 incubations presented the same pattern as that seen in a prior study by Pankhurst and Riple (2000). In the one exceptional incubation where basal T concentration was higher than that of E<sub>2</sub>, 17P at 100 ng ml<sup>-1</sup> was converted into E<sub>2</sub> and T whereas A at 20 ng ml<sup>-1</sup> was converted into T but not E<sub>2</sub>. Post-vitellogenic striped trumpeter follicles also had the capacity of converting

exogenous 17P (100 ng ml<sup>-1</sup>) into T but not E<sub>2</sub> (Pankhurst, 1997). This may suggest that the conversion of steroid precursors into the end production of E<sub>2</sub> is limited by both the availability of a suitable substrate (Pankhurst and Riple, 2000) and by the activity of P450-arom.

In an earlier study, P-450arom activity was not affected by hCG treatment of follicles from greenback flounder (Pankhurst and Riple, 2000). This suggests that the stimulation of E<sub>2</sub> production by hCG in the present study was probably a result of an enhancement of activities of steroid converting enzymes other than P-450arom. The fact that dbcAMP also did not stimulate E<sub>2</sub> production suggests that P-450arom activity may be relatively independent of GtH in the greenback flounder. The involvement of GtH in regulating P-450 arom activity was observed in medaka follicles (Nagahama et al., 1991) but not in follicles from amago salmon (Kagawa et al., 1982), suggesting that activation of P-450 arom by GtH varies in importance among teleost species.

The high dose of dbcAMP significantly increased follicular T production in the present study, which is consistent with similar results from two salmon species, amago salmon (Kanamori and Nagahama, 1988) and Atlantic salmon (Watts et al., 2003). However, follicular E<sub>2</sub> production exhibited different patterns among these three species. DbcAMP suppressed E<sub>2</sub> production in greenback flounder follicles but stimulated E<sub>2</sub> production by amago salmon follicles (Young et al., 1983), whereas no such effect was observed in Atlantic salmon (Watts et al., 2003). Apparent P-450arom activity was suppressed by dbcAMP in flounder in a dose-dependent manner. However, concomitant accumulation of T in incubation media only occurred in the presence of a high dose of dbcAMP. In teleosts generally FSH receptors are localized in thecal cells throughout oogenesis whereas LH receptors are found on the granulosa cells only in the preovulatory stage (reviewed in Devlin and Nagahama, 2002). In brook trout (*Salvelinus fontinalis*), it was FSH but not LH that stimulated cAMP production by follicles from vitellogenic animals (Planas et al., 1997). As a consequence of its membrane permeability, dbcAMP enters all follicle cell types and increase effective intracellular cAMP levels. This may have resulted in a dual action on



steroidogenesis in follicles in the present study. On the one hand, the increase of functional cAMP in thecal cells may stimulate the *de novo* synthesis of T substrate. At the same time, the accumulation of cAMP in granulosa cells may mimic the effect of LH and attenuate P-450arom activity and E<sub>2</sub> production (reviewed in Devlin and Nagahama, 2002).

The surface epithelium of the follicle is considered as a barrier for Vtg uptake *in vitro* (Kanungo et al., 1990; Tyler et al., 1990). However, two studies in rainbow trout demonstrated that the uptake rate of Vtg into intact follicles was 1.9 to 2.8 ng mm<sup>-2</sup> follicle surface hour<sup>-1</sup> in the presence 1.0 to 2.0 mg ml<sup>-1</sup> Vtg in the medium at ≤ 15°C (Campell and Jalabert, 1979) and the rate was 20 ng mm<sup>-2</sup> follicle surface hour<sup>-1</sup> in the presence of 13 mg ml<sup>-1</sup> Vtg in the medium at 18°C (Tyler et al., 1987). These studies indicated that Vtg had the ability to pass through the epithelial layer from the surrounding medium *in vitro* even though the rate was relatively low, and that the rate of Vtg uptake *in vitro* is medium Vtg concentration-dependent as well as culture temperature-dependent. This suggests that over a finite incubation period and at a certain temperature, the amount of Vtg deposited into intact follicles (oocytes, surrounding thecal and granulosa cells, and space between different follicular cells) is in direct proportion to the concentration of Vtg in the culture medium.

*In vivo* and *in vitro* studies have documented that a highly selective Vtg receptor is located on the surface of oocytes and Vtg is sequestered into vitellogenic oocytes by receptor-mediated endocytosis (reviewed in Specker and Sullivan, 1994). *In vitro* studies by follicles of rainbow trout indicated that 85% of Vtg was deposited in the oocyte cytoplasm with 14 % in thecal cells but little in granulosa cells (Campell, 1978; Tyler et al., 1990). Vtg has a high molecular weight (300-640 kD) in teleost species (reviewed in Tyler et al., 2000) and can function as a carrier molecule, transporting steroids into oocytes during vitellogenesis in addition to its role as a yolk protein precursor (reviewed in Specker and Sullivan, 1994), which indicates it may act as an intracellular steroid sink. The distribution of Vtg in intact follicles *in vitro* and its molecular characteristics suggest that Vtg may affect steroidogenesis in follicles via multiple pathways *in vitro*.

Firstly, Vtg probably has an effect on the binding of hCG to GtH-receptors on the surface of thecal cells and this may be non-specific. The presence of high concentrations of large molecular weight proteins (e.g. Vtg) probably interfere in protein-receptor interactions of other proteins by reducing the opportunity for proteins to access receptors, or by non-specifically occupying binding site of receptors.

Secondly, as a 'steroid sink', Vtg may prevent the free diffusion of steroids between follicles and surrounding culture medium, and between different follicular cells, which may reduce the amount of steroid entering follicle cells and result in the decrease of precursor-stimulated follicular E<sub>2</sub> and/or T production. And also this may lead to an accumulation of steroid production around follicle cells and hinder the release of steroid from follicle cells into culture medium, which may increase the concentrations of steroid substrates inside follicle cells and slow the converting rate of hCG-, dbcAMP- and precursor-stimulated follicular steroid production.

Thirdly, the presence of Vtg in thecal cells may have a direct inhibitory effect on the activation of different protein kinases. This may slow the synthesis of steroid stimulated by dbcAMP and steroid precursors. And finally, as a 'large' molecule, Vtg may also direct limit the access of different steroidogenic enzymes to their substrates, and reduce steroid productions from different precursors.

In rainbow trout, where administration of vitellogenic females with a high dose of exogenous Vtg significantly decreased plasma E<sub>2</sub> concentration (Resi-Henriques et al., 1997), treatment *in vitro* with Vtg at a dose of 5.0 mg ml<sup>-1</sup> significantly reduced follicle E<sub>2</sub> production in the presence of 17P or pregnenolone (Resi-Henriques et al., 1997, 2000). These studies are in agreement with the results of the present study that *in vitro* the high concentrations of Vtg in culture medium suppress steroid production by follicles. The inhibitory effects observed in the present study indicate that Vtg may interfere in steroidogenic activity in follicles, and possibly by more than one mechanism.

A previous study (Resi-Henriques et al., 1997) showed that the elevation of plasma Vtg concentrations might act as a negative feedback loop moderating ovarian E<sub>2</sub> production, which may prevent the over-stimulation (or desensitisation) of hepatocytes by E<sub>2</sub>, and the results of the present study are consistent with this hypothesis. Generally, there is a positive correlation between plasma concentrations of E<sub>2</sub> and Vtg during the vitellogenic growth of oocytes in teleosts, e.g. sea bass (*Dicentrarchus labrax*) (Mañanós et al., 1997); common dentex (*Dentex dentex*) (Pavlidis et al., 2000); golden rabbitfish (*Siganus guttatus*) (Rahaman et al., 2000); Atlantic salmon (King and Pankhurst, 2003) and greenback flounder (Chapter 5). However, in some extended spawning species, there is often an early seasonal peak in plasma E<sub>2</sub> followed by a fall as vitellogenesis proceeds. In Atlantic halibut (*Hippoglossus hippoglossus*), a concurrent increase of plasma concentrations of Vtg and E<sub>2</sub> occurred in mid-January, after which time plasma Vtg levels continued to increase whereas plasma E<sub>2</sub> declined (Methven et al., 1992). The plasma E<sub>2</sub> concentration was high early in vitellogenesis then fell steeply to a relatively low level throughout the remainder of vitellogenesis in red gurnard (*Chelidonichthys kumu*) (Clearwater and Pankhurst, 1994). Plasma E<sub>2</sub> reached to a peak in December in Pacific herring (*Clupea pallasii*), after which it significantly decreased, but plasma Vtg remained at a relatively high concentration from January to April (Koya et al., 2003). In E<sub>2</sub>-induced vitellogenesis of black porgy (*Acanthopagrus schlegelii*), after an initial peak in October, plasma E<sub>2</sub> returned to the control level whereas plasma Vtg was maintained at significantly elevated levels until April of the next year (Lee et al., 2000). Collectively, these *in vivo* studies suggest that during vitellogenesis, the accumulation of Vtg in plasma could execute negative feedback on ovarian E<sub>2</sub> production as demonstrated *in vitro* in the present study, to prevent the over-stimulation of hepatic synthesis of Vtg. This co-regulation of E<sub>2</sub> and Vtg is possibly necessary for maintaining a normal rate of oocyte growth *in vivo*.

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## **Chapter 8**

### **General discussion**

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## 8. General discussion

Teleost vitellogenins display a high variability among species in molecular weight and form, subunit composition and degree of phosphorylation and lipidation (reviewed in Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994). Fish present various patterns of ovarian growth, and vitellogenesis is the principal event in this process which accounts in large measure for the final quality of eggs (reviewed in Tyler and Sumpter, 1996). In order to understand the reproductive strategy for any particular species, the study of characteristics of vitellogenin (Vtg) and different aspects of vitellogenesis is necessary. The first step of the present study was to isolate an intact and purified Vtg preparation and identify its molecular characteristics, and then to establish a Vtg ELISA which was sequentially used to study Vtg induction in the male by 17 $\beta$ -estradiol (E<sub>2</sub>) *in vivo* and *in vitro*, and plasma Vtg levels in relation to oocyte growth during female seasonal reproduction of females. Finally, purified Vtg was used to assess the potential feedback effect of Vtg on ovarian steroid production *in vitro*.

### 8.1. Isolation and characteristic of Vtg

Because of the diversity in characteristics of Vtgs, different isolation methods have been employed in different species to purify intact Vtg. Ion-exchange chromatography based on molecular surface charge was used in rainbow trout (*Oncorhynchus mykiss*) and sea trout (*Salmo trutta*) (Norberg and Haux, 1985), turbot (*Scophthalmus maximus*) (Silversand and Haux, 1989) and Atlantic halibut (*Hippoglossus hippoglossus*) (Norberg, 1995). Gel filtration chromatography, which separates molecules by size, was used in flounder (*Platichthys flesus*) (Emmersen and Petersen, 1976) and barfin flounder (*Verasper moseri*) (Matsubara and Sawano, 1995). In some species, such as carp (*Cyprinus carpio*) (Tyler and Sumpter, 1990), sea bass (*Dicentrarchus labrax*) (Mañanós et al., 1994), gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*) (Brion et al., 2000), a combination of these two orthogonal methods was used. Because Vtg is susceptible to proteolysis during isolation (Silversand et al., 1993), additional purification steps and frequent changes of buffers may increase the degree of

degradation. A single step of gel filtration chromatography isolated a freshwater murrel (*Channa punctatus*) Vtg as intact and purified as that obtained by two steps of chromatography (Sehgal and Goswami, 2001). In the present study, the greenback flounder Vtg was isolated from the plasma of E<sub>2</sub>-treated males by gel filtration chromatography (Chapter 2). The single symmetrical elution peak and the results of SDS-PAGE of the peak fractions (Chapter 2) demonstrated that the Vtg was intact and of high purity. The purification results also showed that the addition of a protease inhibitor (e.g. PMSF) and the reduction of storage time of plasma are important for maintaining the integrity of the Vtg molecule prior to and during purification.

The molecular weight of native greenback flounder Vtg determined by gel filtration chromatography was 540 kD (Chapter 2) and Western blotting indicated that it consisted of a single polypeptide subunit with a molecular mass of 152 kD (Chapter 3). These molecular characteristics are similar to those reported for Vtgs from other teleosts (reviewed in Specker and Sullivan, 1994; see discussion in Chapter 2). The estimation of the Vtg molecular weight can be affected by the analytical methods used (Norberg and Haux, 1985; Buerano et al., 1995), and any degradation of Vtg during separation may affect molecular weight by shifting the elution position of the Vtg peak during gel filtration chromatography (Fig. 2.6, Chapter 2). A comparison of Vtg molecular weights between different species, or within the same species in different studies should ideally use the same isolation protocols. However, even with that proviso, the characteristics of greenback flounder Vtg are generally similar to those of other teleosts and remarkably similar to those of other flatfishes (Table 2.3, Chapter 2).

## 8.2. Assessment of Vtg ELISA

For unknown reasons, the titre of the antiserum raised against greenback flounder Vtg was rather low as compared to that raised against Atlantic salmon (*Salmo salar*) when a similar immunization process was performed (Watt et al., 2003). Consequently, the concentration of antibody (1: 100) used in the present ELISA was much higher than that generally required in other assays (Watt et al., 2003;

see Table 3.1 Chapter 3). However, the antibody showed high specificity demonstrated by its only reacting with flounder Vtg but not other plasma proteins (Chapter 3) nor heterologous Vtgs (Watt et al., 2003).

The detection limit of the ELISA in the present study was  $160 \text{ ng ml}^{-1}$  which is at least 5 times higher than that of comparable competitive Vtg ELISAs (see Table 3.1 Chapter 3). However, the equivalent measurable level was  $32 \text{ } \mu\text{g ml}^{-1}$  in plasma or  $0.32 \text{ } \mu\text{g ml}^{-1}$  in medium, which was sensitive enough to measure plasma Vtg in  $\text{E}_2$ -treated male or mature female flounder (Chapters 4 and 5). The ELISA remained effective for a period of more than 3 years without the need to produce new Vtg or antibody, indicating that both assay components were stable under normal laboratory storage conditions, allowing consistent measurement of Vtg concentrations in different experiments over time.

### 8.3. Vitellogenesis in greenback flounder

In teleosts, the endocrine stimulation of vitellogenesis is mainly triggered by  $\text{E}_2$ , which is produced by the ovary under the stimulation of gonadotropin (GtH) in mature females. Afterward, Vtg is transferred to the ovary and is selectively sequestered into oocytes by receptor-mediated endocytosis, where it is converted into yolk proteins, and is responsible for the main growth phase of the oocytes (reviewed in Nagahama et al, 1995; Tyler and Sumpter, 1996). Male and juvenile fish also have Vtg genes which can be activated by treatment with exogenous  $\text{E}_2$ , or pituitary extract and various GtH preparations, which increase the titre of  $\text{E}_2$  in the plasma, resulting in the subsequent synthesis of Vtg (reviewed in Specker and Sullivan, 1994; Tyler et al., 2000). Plasma concentrations of  $\text{E}_2$  and Vtg are positively correlated during the vitellogenic growth of oocytes. However, in at least one species, the presence of high concentrations of Vtg in plasma has a negative feedback effect on ovarian steroid production (Resi-Henriques et al., 1997).

#### 8.3.1. Vtg induction in males

The induction of Vtg in plasma was achieved by the injection of fish with E<sub>2</sub> in an ethanol/saline suspension (Chapters 2 and 4), which produced a time-course of elevated plasma E<sub>2</sub> levels similar to that seen in other species treated with a single high dose of E<sub>2</sub> (Table 8.1). The dose-response relationship also was similar to that seen in other species including English sole (*Pleuronectes vetulus*) (Lomax et al., 1998), Atlantic salmon (Celius and Walther, 1998), tilapia (*Oreochromis mossambicus*) (Takemura and Kim, 2001) and largemouth bass (*Micropterus salmoides*) (Bowman et al., 2002). However, the threshold dose of E<sub>2</sub> required for Vtg induction was higher in flounder (100 µg kg<sup>-1</sup>) as compared to that required in other species e.g. 8.0 µg kg<sup>-1</sup> in English sole (Lomax et al., 1998), 10 µg kg<sup>-1</sup> in Atlantic salmon (Celius and Walther, 1998) and 1.0 µg kg<sup>-1</sup> in largemouth bass (Bowman et al., 2002). The difference in induction patterns among species may be attributed to several factors e.g. the choice of carrier may affect the uptake, release and metabolism of E<sub>2</sub> over time (Pankhurst et al., 1986), which will affect the sequential induction of hepatic vitellogenesis. The difference of maintenance temperature for each species also will have an effect. For example, a high temperature increased the expression of Vtg mRNA (Pawlowski et al., 2000) and the rate of translation and secretion of Vtg protein (Mackay and Lazier, 1993). Fish age and reproductive status also can affect Vtg induction (Bowman et al., 2002; Hotta et al., 2003). Finally, characteristics of the ELISAs used in different studies also may contribute to variable estimates of thresholds for the induction of Vtg production by estrogens.

The time-course of the induction of vitellogenesis by E<sub>2</sub> in greenback flounder indicated that the concentration of plasma Vtg reached a peak 96 h post-injection (p.i.), then rapidly declined. However, serial injections of E<sub>2</sub> could maintain a high concentration of plasma Vtg for up to 288 h p.i.. In European eels (*Anguilla anguilla*) (Luizi et al., 1997), three injections of E<sub>2</sub> resulted in a high concentration of both Vtg and E<sub>2</sub> in plasma, which was maintained for more than 30 days. These results suggested that the sustained high plasma levels of Vtg required continuous elevation of plasma E<sub>2</sub> levels. This supposition is consistent with the finding in mature female greenback flounder (Chapter 5) that high plasma concentrations of Vtg and of E<sub>2</sub> were both observed during vitellogenesis.

The concentrations of Vtg induced in plasma of greenback flounder was  $< 1.0 \text{ mg ml}^{-1}$  after a single  $E_2$  injection and  $< 2.0 \text{ mg ml}^{-1}$  after three  $E_2$  injections; both generally lower concentrations compared with those required for induction of vitellogenesis in other species. For example, the plasma Vtg concentration reached  $15.7 \text{ mg ml}^{-1}$  in sheepshead minnow (*Cyprinodon variegates*) (Bowman et al., 2000) and  $4.5 \text{ mg ml}^{-1}$  in largemouth bass (Bowman et al., 2002) after one injection of  $E_2$ , and  $40 \text{ mg ml}^{-1}$  in sheepshead minnow (Bowman et al., 2000) and  $> 9.0 \text{ mg ml}^{-1}$  in summer flounder (*Paralichthys dentatus*) (Folmar et al., 2001) after two  $E_2$  injections. However, the Vtg concentration in  $E_2$ -injected greenback flounder males was similar to that observed in the plasma of naturally vitellogenic females of the same age and generation (Chapter 5). These results suggest that absolute levels of Vtg in greenback flounder are towards the lower end of the range typically found in teleosts. No *in vitro* Vtg production was detectable in hepatocyte culture in the present study (Chapter 6) which again was probably due to the low production of Vtg in hepatocytes of flounder compared with those of other species, including other flatfishes.

Table 8.1 A comparison of time-course of Vtg induction by treatment with  $E_2$  among different species

species	age	dose & carrier	T (°C)	time-course detectable	peak	references
greenback flounder	14 months	$5.0 \text{ mg ml}^{-1}$ saline	$15 \pm 1$	48 h	96 h	this study
brown trout	immature	$1.0 \text{ mg ml}^{-1}$ saline	8-10		11 days	Sherry et al., 1999
sheepshead minnow	adult	$5.0 \text{ mg ml}^{-1}$ triethylene glycol	$25 \pm 2$	24 h	48 h	Bowman et al., 2000
rainbow trout	Juvenile	$5.0 \text{ mg ml}^{-1}$ saline	10-11	48 h	72 h	Arukwe et al., 2001
largemouth bass	24 months	$2.0 \text{ mg ml}^{-1}$ dimethyl sulfoxide	$21 \pm 2$	6 h	48 h	Bowman et al., 2002

### 8.3.2. Vitellogenesis and oocyte growth

The present study in Chapter 5 demonstrated that this species showed multiple group synchrony in oocyte development with several ovulatory events happening during a spawning season. This resulted in oocytes at several different stages of vitellogenesis being simultaneously present in the ovary. These findings are in agreement with the earlier studies of greenback flounder by Barnett and Pankhurst (1999) and Poortenaar and Pankhurst (2000a). However, there was no synchrony of spawning within the population over a four-month sampling period, in contrast to other multiple spawning flatfishes such as Atlantic halibut (*Hippoglossus hippoglossus*) (Methven et al., 1992) where several spawning peaks were observed in the population during a reproductive season. This indicates that the seasonal effect on vitellogenesis is probably not strong in greenback flounder and that this species probably exhibits scattered spawning throughout the year. This result was further proved by the fact that no seasonal variation was observed in plasma concentrations of Vtg,  $E_2$  and testosterone (T), gonadosomatic index ( $I_G$ ), and hepatosomatic index ( $I_H$ ). Some other flatfishes show an extended spawning as well; however, an evident seasonal effect was present. For example, English sole (*Parophrys vetulus*) (Johnson et al., 1991), European flounder (*Pleuronectes flesus*) (Janssen et al., 1995), and summer flounder (Merson et al., 2000) exhibited a seasonal shift of ovarian stages. Seasonal fluctuations of plasma Vtg,  $E_2$  and T were observed in plaice (*Pleuronectes platessa*) (Wingfield and Grimm, 1977) and Atlantic halibut (Methven et al., 1992). Seasonal changes of  $I_G$  and  $I_H$  were found in plaice (Wingfield and Grimm, 1977) and European flounder (Janssen et al., 1995).

The positive relationship between  $I_G$  and  $I_H$  during vitellogenesis in the present study is in agreement with the findings relating to New Zealand snapper (*Pagrus auratus*) (Scott and Pankhurst, 1992) and red gurnard (*Chelidonichthys kumu*) (Clearwater and Pankhurst, 1994). In the present study, this was accompanied by the elevation of plasma Vtg, which indicates that the increase of  $I_G$  and  $I_H$  was due to the synthesis and deposition of Vtg in hepatocytes and oocytes respectively. A proportional correlation between plasma Vtg, and  $I_G$  and  $I_H$  was also observed in

rainbow trout (Copeland et al., 1986; Tyler et al., 1990) and brook trout (*Salvelinus fontinalis*) (Schafhauser-Smith and Benfey, 2002). A further increase of  $I_G$  occurred at fish undergoing final maturation, which was similar to that seen in red gurnard (Clearwater and Pankhurst, 1994), summer flounder (Merson et al., 2000), yellowtail kingfish (*Seriola lalandi lalandi*) (Poortenaar et al., 2001) and red porgy (*Pagrus pagrus*) (Kokokiiris et al., 2000). In contrast, such an increase was not found in  $I_H$  and plasma Vtg which suggested that the increase of  $I_G$  probably resulted from oocyte hydration during final maturation as seen in Pacific herring (*Clupea pallasii*) (Gillis et al., 1990) and swordtail (*Xiphias gladius*) (Arocha, 2002).

The synchronized increase of plasma Vtg and  $E_2$  across various stages of vitellogenesis in greenback flounder indicates that, as in other species,  $E_2$  is the major steroid regulating vitellogenesis (e.g. Copeland et al., 1986; Methven et al., 1992; Roberts et al., 1999; Koya et al., 2000; Asturiano et al., 2000; Merson et al., 2000; King and Pankhurst, 2003). Levels of both Vtg and  $E_2$  peaked during advanced vitellogenesis, which is in accordance with the case of two multiple spawners i.e. gag (*Mycteroperca microlepis*) (Heppell and Sullivan, 1999) and yellowtail kingfish (Poortenaar et al., 2001). This observation indicates that high plasma  $E_2$  levels are necessary for maintaining the synthesis of Vtg in sufficient quantity. Vtg,  $E_2$  and T all showed high concentrations in fish undergoing final maturation which is consistent with the co-existence of vitellogenic and maturational oocytes in the same ovary. This is similar to the findings in multiple spawning species, such as spiny damselfish (*Acanthochromis polyacanthus*) (Pankhurst et al., 1999) and common dentex (*Dentex dentex*) (Pavlidis et al., 2000).

The positive relationship between plasma concentrations of Vtg in greenback flounder and the growth of oocytes from 200 to 400  $\mu\text{m}$  suggested that the increase of oocyte size is mainly due to the successive incorporation of Vtg into oocytes. A similar trend was observed in other species as well, e.g. rainbow trout (Copeland et al., 1986; Tyler et al., 1990), white bass (*Morone chrysops*) (Berlinsky et al., 1995), sea bass (*Dicentrarchus labrax*) (Asturiano et al., 2000),



red porgy (Kokokiiris et al., 2001) and Atlantic salmon (King and Pankhurst, 2003). The change of plasma  $E_2$  levels paralleled the increases of plasma Vtg levels and oocyte size, which is consistent with its role of  $E_2$  during gametogenesis in other teleost species (reviewed in Pankhurst and Carragher, 1991). The overall increase of plasma  $E_2$  was gradual and moderate across various oocyte sizes and reached a plateau at the end of the vitellogenic growth of oocytes. In contrast, in white bass (Berlinsky et al., 1995) and Atlantic salmon (King and Pankhurst, 2003), plasma  $E_2$  showed no sign of strong elevation at the early vitellogenic growth of oocytes but a steep increase occurred as oocytes entered late vitellogenesis.

Testosterone is converted into  $E_2$  by aromatase in the granulosa cells of vitellogenic follicles (Nagahama et al., 1995). In the present study, plasma concentrations of T started to increase at oocyte sizes of about 350  $\mu\text{m}$  (close to late vitellogenesis) and more rapidly after oocyte sizes of 400  $\mu\text{m}$  (entering final maturation). This changing trend might reflect the activity of aromatase during the process of oocyte growth, which is high during the vitellogenic stage and starts declining at the end of vitellogenesis (Chang et al., 1997). A similar finding was observed in white bass as well (Berlinsky et al., 1995). However, in species such as sea bass (Asturiano et al., 2000) and Atlantic cod (*Gadus morhua*) (Dahle et al., 2003), there was no significant change in plasma T levels during the transition of oocyte growth from vitellogenesis to maturation.

### **8.3.3. Effect of Vtg on ovarian steroid production in vitro**

The present study (refer to Chapter 7) demonstrated that the presence of high concentrations of Vtg in culture media will inhibit the synthesis of steroids by the follicles of greenback flounder *in vitro*, which is in agreement with the findings in rainbow trout (Resi-Henriques et al., 1997, 2000). Because of the disposition of Vtg in follicles *in vitro* (85% in the yolk, 14% in the thecal cells, and little in granulosa cells) (Campell, 1978; Tyler et al., 1990), and its molecular characteristic (high molecular weight) and function (steroid carrier) (Specker and Sullivan, 1994), the effect of Vtg involving in steroidogenesis *in vitro* appears to

include direct pathways, i.e. reducing the binding of FSH to its receptors on the surface of thecal cells, inhibiting the activity of the receptor-mediated adenylate cyclase-cAMP system and the activities of different converting enzymes in the cleavage pathway in thecal cells; and indirect pathways i.e. decelerating the converting rate of steroidogenic system by limiting the free diffusion of steroids between different cells and between follicles and culture medium (Fig. 8.1).

Suppression of plasma concentrations of E<sub>2</sub> was observed in vitellogenic rainbow trout treated with exogenous Vtg, with E<sub>2</sub> levels returning to normal levels after the termination of Vtg administration (Resi-Henriques et al., 1997). This indicated that high plasma Vtg might execute a negative feedback on ovarian E<sub>2</sub> production and accordingly reduce the over-stimulation (or desensitisation) of hepatocytes by E<sub>2</sub>. Circumstantial evidence for such an effect is provided by studies on seasonal reproduction in some species e.g. Atlantic halibut (Methven et al., 1992), red gurnard (Clearwater and Pankhurst, 1994) and Pacific herring (Koya et al., 2003), and in E<sub>2</sub>-induced vitellogenesis e.g. black porgy (*Acanthopagrus schlegeli*) (Lee et al., 2000). In these studies, there is an early season peak in plasma E<sub>2</sub> followed by a fall as vitellogenesis proceeds. Collectively, these results suggest that Vtg may regulate *in vivo* follicular steroidogenesis, as demonstrated *in vitro* in the present study, and thereby modulate its own production by the liver.

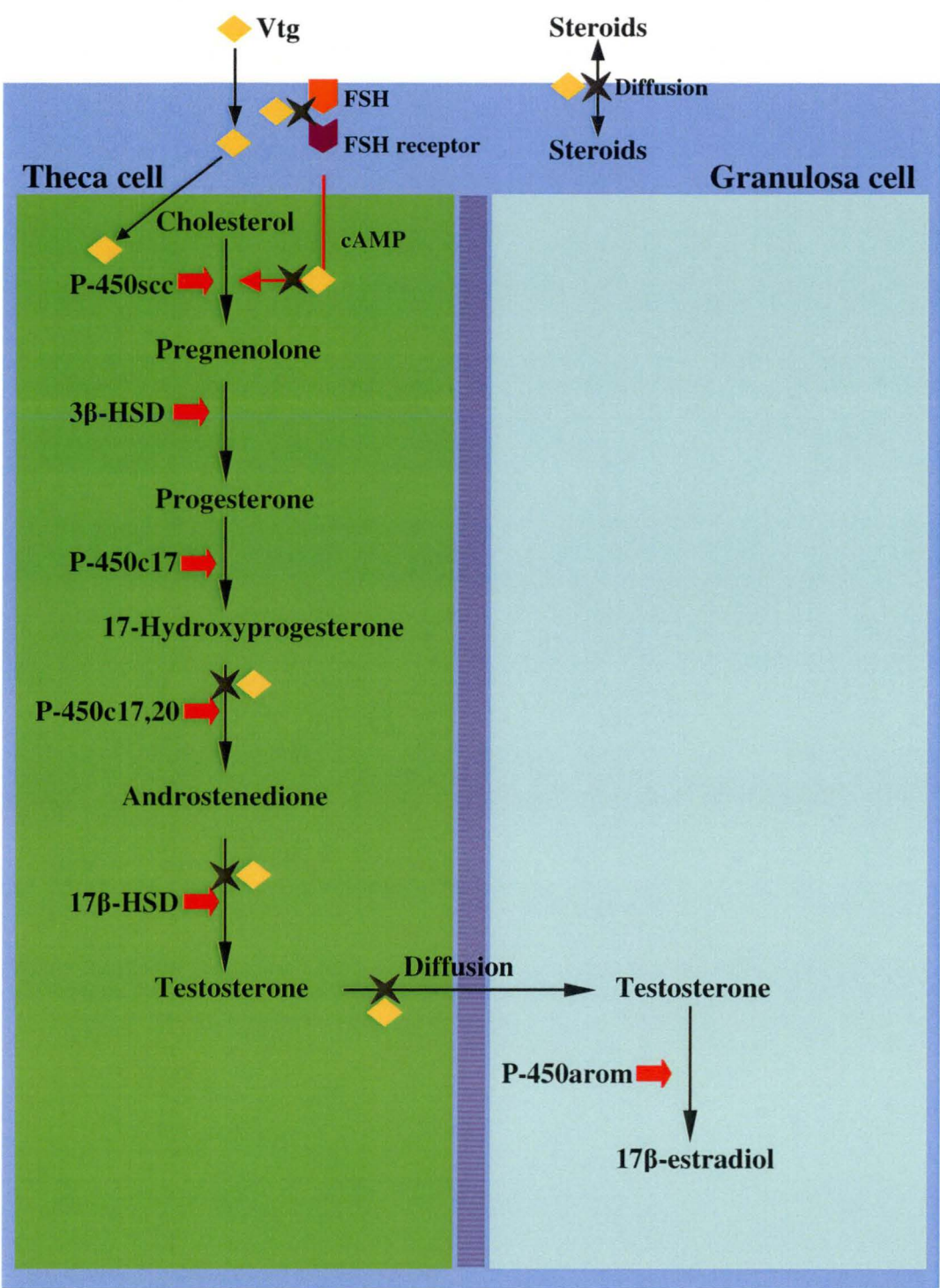


Fig. 8.1. Diagram of the possible pathways that Vtg may affect steroidogenesis *in vitro* by follicles of greenback flounder, ✕ = inhibitory effect. (Endocrine pathway modified from Kagawa 1994 and Nagahama et al., 1995)

## 8.4. Future studies

Recently, more than two forms of Vtg protein have been found in the plasma of some teleosts. Gel chromatography has identified various forms of Vtg in the same species with a large difference in molecular weight e.g. 530 kD and 320 kD in Japanese common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2003). However, as the molecular weight of different forms of Vtg more commonly have a similar value e.g. 500 kD and 550 kD in India major carp (*Cirrhinus mrigala*) (Nath and Maitra, 2001), 570 kD and 460 kD in medaka (*Oryzias latipes*) (Shimizu et al., 2002), and 532 kD, 532kD and 426 kD in perch (*Morone Americana*) (Hiramatsu et al., 2003), it is difficult to identify them only by gel chromatography. Gel chromatography isolated one Vtg (520 kD) in barfin flounder (*Verasper moseri*) (Matsubara and Sawano, 1995) but more recently two forms of Vtg with a similar molecular mass (510 kD and 540 kD) were identified in this species, and they appear to have different roles in oocyte growth (Matsubara et al., 1999). In view of these findings, the possibility of multiple forms of Vtg cannot be ruled out in greenback flounder. Further investigation of Vtg forms, the proteolytic process by which Vtgs are transformed into yolk proteins, and their respective function during oocyte growth may help us understand more about vitellogenesis in this species, and teleosts generally.

The regulation of the expression of Vtg genes by E<sub>2</sub> has been examined in rainbow trout (Vaillant et al., 1988), tilapia (*Oreochromis aureus*) (Lim et al., 1991) and white sturgeon (*Acipenser transmontanus*) (Bidwell et al., 1991), but there is little knowledge of this aspect of vitellogenesis in flatfishes. Approaches to address this dearth of knowledge could include isolation of Vtg mRNA from the liver of E<sub>2</sub>-treated males to construct cDNA probe for detecting regulation of Vtg gene expression by E<sub>2</sub> in terms of its dose-response kinetics. This approach also could lead to discovery of multiple Vtg genes and proteins in these species. In response to E<sub>2</sub> stimulation, the synthesis of Vtg mRNA appears to be more sensitive than that of the appearance of Vtg protein (Vaillant et al., 1988). The effect of environmental factors e.g. temperature (Pawlowski et al., 2000) and endocrine factors other than E<sub>2</sub> (Ding et al., 1994; Mori et al., 1998) on Vtg gene

expression also can be tested by using *in vitro* hepatocyte culture systems, although such a system (as in the present study) was incapable of producing measurable quantities of greenback flounder Vtg.

The present study (refer to Chapter 5) suggests that the seasonal effect on greenback flounder reproduction is blurred. However, in some species, temperature and photoperiod are observed to be two important environmental factors, which can be used to manipulate vitellogenesis, oocyte maturation and spawning (Thomas et al., 1994; King et al., 2003; Norberg et al., 2004). The measurement of plasma concentrations of Vtg and steroids offers a non-lethal method to monitor reproductive status, which can be used to assess the reproductive response to environmental change in greenback flounder in a future study.

*In vivo*, Vtg enters follicles via capillaries, then passes between thecal cells and granulosa cells by an intercellular route to reach the surface of oocytes where it is incorporated into oocytes by receptor-mediated endocytosis (Abraham et al., 1984). During vitellogenesis, Vtg production has a negative feedback effect on ovarian steroid production (Resi-Henriques et al., 1997, 2000; this study). The mechanism, while not fully understood, could operate at multiple levels in the endocrine cascade. Studies of *in vivo* (Abraham et al., 1984) and *in vitro* (Campbell, 1978) Vtg diffusion indicated that Vtg did not occur in granulosa cells. This may be attributed to the fact that the membrane of a granulosa cell is impermeable for a large molecule such as Vtg and, like other somatic cells, the granulosa cells apparently lack Vtg receptors, which are largely restricted to oocytes. These observations suggest an extracellular site of action of Vtg on ovarian steroidogenesis, perhaps involving non-specific interference with access of GtHs to their receptors or non-specific binding of steroid hormones and their precursors. These hypotheses need to be tested in future research.

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