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Characterization of Vitellogenesis in the Bonnethead Shark Sphyrna tiburo

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CHARACTERIZATION OF VITELLOGENESIS IN THE BONNETHEAD SHARK *SPHYRNA TIBURO*

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

Vitellogenin (Vtg) is a precursor to yolk-proteins produced in the liver of many invertebrates and non-mammalian vertebrates; its synthesis is stimulated by the hormone estradiol (E2). This study is the first to characterize vitellogenin synthesis in a placental viviparous elasmobranch, the yolk-sac placental bonnethead shark, *Sphyrna tiburo*. This study focused on determining where and when Vtg is produced, as well as what hormonal factors regulate Vtg production. The liver was confirmed as the site of Vtg production via immunohistochemistry. Immunoreactivity was also observed within granulosa cells of ovarian follicles; further experimentation is needed to determine if this is indicative of Vtg production by these cells. Using immunoblotting, the highest proportions of Vtg positive females were found in March, with Vtg production continuing into April and May. Putative Vtg production was found to begin in August for some individuals, with production continuing throughout the fall and winter months. In regards to hormonal regulation, immunohistochemical analysis identified receptors for E2 and progesterone (P4) within the liver. Comparison of the monthly E2 and Vtg cycles provides evidence that E2 stimulates Vtg production in *S. tiburo,* as high or increasing concentrations of E2 correlated with Vtg presence in the plasma. Preliminary results also suggest *in vitro* production of Vtg by liver tissue when exposed to E2. Comparison of the monthly P4 and Vtg cycles suggests P4 may inhibit Vtg synthesis, with higher levels of P4 found in the months when Vtg production declines. Additionally, the methods developed for this study were able to identify Vtg in the plasma of other elasmobranch species. Vtg detection in plasma may thus be an ideal new, nonlethal method for characterizing elasmobranch reproductive periodicity, which will aid in assessing population growth and allow for managers to possess more accurate information to make appropriate decisions for the populations.

1. INTRODUCTION

Understanding the reproductive cycles of elasmobranchs is critical for sustainable management of their populations. Many species of sharks and rays are particularly vulnerable to overexploitation due to their K-selected life history traits, including slow growth, late age at maturity, and low fecundity (Hoenig and Gruber, 1990; Stevens et al., 2000). In order to determine the status of a given elasmobranch population and decide on appropriate management strategies, scientists conduct stock assessments. These stock assessments examine the size, growth rate, and demographic make-up of a population, and also must consider information about the species' reproductive cycle, including size and/or age at maturity, mating season, gestation period, female fecundity, and breeding periodicity (Hoenig and Gruber, 1990).

Historically, understanding the reproductive cycles of elasmobranchs has generally required lethal sampling methods. A large number of males and females are typically collected throughout the year, and euthanized and dissected to examine changes in their reproductive tract morphology to determine when various reproductive events occur. However, this approach is not sustainable for many threatened elasmobranch species, and cannot be used to understand the reproductive cycles of endangered elasmobranchs. Therefore, in recent years scientists have moved to develop non-lethal methods for assessing the reproductive cycles of elasmobranchs (Awruch, 2013). These methods include ultrasonography to determine pregnancy and fecundity, as well as the assessment of plasma concentrations of reproductive hormones, including estradiol (E2), progesterone (P4), and testosterone (T), to examine maturity and reproductive stage. Tracking seasonal changes in plasma concentrations of these hormones has been found to correlate with changes in the reproductive tract, e.g. high E2 correlates with follicular development for females, while high T in males correlates with the peak time of spermatogenesis

(Awruch et al., 2008; Awruch, 2013; Hammerschlag and Sulikowski, 2011; Sulikowski et al., 2007). Plasma concentrations of these reproductive hormones are commonly assessed using either radioimmunoassays (RIA) or chemiluminescence assays (CLIA), and concentrations of these hormones in muscle samples have also been measured using RIA (Prohaska et al., 2013).

While currently available nonlethal methods for assessing elasmobranch reproduction have been found to be reliable for most species, there are a few elasmobranch species for which simply assessing reproductive hormone concentrations is not enough to provide an accurate picture of their reproductive cycle. In particular, assessing plasma hormone concentrations often does not provide accurate information about the reproductive periodicity of a species. For example, recent studies have demonstrated that the blacknose shark (*Carcharhinus acronotus*) seems to be capable of both annual and biennial reproduction in the northwest Atlantic Ocean (Driggers et al., 2004; Ford et al., unpublished data). For this species, simply monitoring E2 or P4 concentrations has proven to be less effective for assessing reproductive stage than in past studies (Gelsleichter et al., unpublished data). Lack of clarity on the reproductive periodicity of a given elasmobranch species presents issues for management of a population. Specifically, the possible presence of both annually and biennially reproducing females in a single population will greatly change the total reproductive output of the population and thus estimates of population growth. There is thus a need for a nonlethal method for determining reproductive periodicity of elasmobranch species, particularly in cases when monitoring of E2 or P4 concentrations do not effectively illustrate an individual female's reproductive stage.

One method for determining reproductive periodicity is to assess whether a female is undergoing follicular development during gestation. A potential nonlethal way to assess whether follicular development occurs concurrently with gestation would be to couple ultrasonography

with the plasma concentrations of vitellogenin, a reproductive protein that serves as a precursor to egg yolk proteins and is therefore increased during follicular development. Presence of vitellogenin in the plasma concurrent with pregnancy confirmed via ultrasonography would imply that a female is an annual reproducer, growing follicles during gestation to be prepared for mating and ovulation that same year. On the other hand, in a species with a long gestation period, lack of vitellogenin in the plasma during pregnancy would suggest that follicular development occurs separately from gestation, and that females will take a year or more off before mating and reproducing again.

Vitellogenin (Vtg) is a glycolipophosphoprotein expressed in many invertebrates, all non-mammalian vertebrates, and monotremes (Hiramatsu et al., 2006; Ho et al., 1982; Matozzo et al. 2008; Polzonetti-Magni et al., 2004; Romano et al., 2004). In all vertebrate species that have been studied to date, Vtg is synthesized in the liver of reproductively active female individuals during the period of follicular development. The synthesis of Vtg in the liver has been shown to be induced by E2 in all studied species. Several studies have also found that Vtg synthesis can be stimulated in male individuals that have been administered or exposed to E2 or synthetic estrogens, making Vtg analysis a commonly employed tool for assessing xenoestrogen exposure in toxicology studies (Denslow et al., 1999; Hiramatsu et al., 2006; Ho et al., 1982; Kleinkauf et al., 2004; Perez and Callard 1993; Yamane et al., 2013). Once Vtg has been synthesized, it is secreted into the bloodstream and taken up by growing oocytes in the ovaries via receptor-mediated endocytosis. Within the ovary, Vtg is then cleaved into the yolk proteins lipovitellin (Lv) and phosvitin (Pv), which act as nutritional sources for developing embryos (Romano et al., 2004). A large number of studies have characterized and examined Vtg in teleost fish, reptiles, amphibians, and birds, but very few studies have investigated the protein in elasmobranchs.

The first study to examine elasmobranch vitellogenesis was conducted on the lesser spotted dogfish (*Scyliorhinus canicula*, Craik, 1978). This study made estimates of plasma Vtg using two methods. Plasma phosphoprotein concentrations were first measured as a proxy for Vtg, as the yolk is the major source of phosphoproteins in animals. Plasma phosphoprotein concentrations were significantly higher for adult females when compared to adult males and immature females; it was inferred that the higher concentration in adult females was due to the production of Vtg. Estimates of plasma Vtg levels were also made using radioimmunoassays. Antiserum was generated against yolk granule proteins, and both dilutions of yolk granules and unknown plasma samples were tested in radioimmunoassay using this antiserum. A standard curve was generated using the dilutions of yolk granules, which allowed for estimates to be made of the amount of yolk granule protein likely present within unknown plasma samples. It was again observed that mature females had much higher levels of yolk granule proteins in their plasma compared to adult males and immature females. Additionally, there was a close correlation between the measurement of phosphoprotein and yolk granule protein content, indicating that the amount of phosphoprotein in the plasma did likely act as a good estimate of the amount of Vtg being produced. This was the first study to develop techniques to assess plasma concentrations of Vtg in an elasmobranch, but it did not investigate the hormonal regulation of Vtg production in elasmobranchs.

Since this initial study which developed methods for estimating plasma Vtg in elasmobranchs, more recent studies have utilized assays to definitively detect Vtg in the plasma and examine hormonal regulation of vitellogenesis in other elasmobranch species. The first study to investigate hormonal regulation of Vtg production in an elasmobranch was conducted on the viviparous spiny dogfish (*Squalus acanthias*); the roles of both E2 and P4 on Vtg production

were examined (Callard et al., 1991). When plasma concentrations of E2 were highest, Vtg was detected in the plasma of female *S. acanthias.* Similarly, concentrations of plasma Vtg were found to be highest in female *S. acanthias* when plasma concentrations of E2 were increased, and injection with E2 further increased Vtg levels during this time. It was also determined that when concentrations of P4 were high during gestation, Vtg was not present in the plasma of females. Additionally, injecting females with E2 during the time period when P4 was increased did not result in Vtg being detected in their plasma, which suggested that P4 may play a role in inhibiting Vtg production for *S. acanthias.* Concentrations of plasma Vtg were found to be highest in female *S. acanthias* when plasma concentrations of E2 were increased, and injection with E2 further increased Vtg levels during this time. This study was thus the first to confirm that E2 stimulates Vtg production in elasmobranchs, and also to find evidence that P4 may also have a role in regulating Vtg synthesis, perhaps in an antagonistic manner.

Another study conducted on the little skate (*Leucoraja erinacea*) provided further evidence that both E2 and P4 likely play a role in regulating Vtg synthesis in elasmobranchs (Perez and Callard, 1993). For this study, a direct ELISA was developed to quantify Vtg levels in normal female specimens, as well as in normal males, males treated with E2, males treated with P4, and males treated with both E2 and P4. An antibody was developed against the yolk protein lipovitellin (Lv) in *L. erinacea*, and the specificity of the antibody for Vtg was confirmed via immunoblotting. Levels of Vtg in plasma varied throughout the cycle of ovulating female *L. erinacea*, decreasing before ovulation, when P4 concentrations are known to be highest for this species, and increasing to their highest level near the time of oviposition, when E2 reaches its highest concentration. This change in Vtg levels throughout the female reproductive cycle provided evidence that E2 and P4 both may regulate Vtg synthesis in *L. erinacea*; this was

further confirmed by the tests on males injected with E2 and P4. Vtg was not detectable in control males or males treated with P4 alone. Upon injection with E2, Vtg was detectable in the plasma of males. However, when males were treated first with E2 and later injected with P4, Vtg levels were still detectable, but were significantly lower than in males treated with E2 alone. The results of this study thus suggested that E2 does stimulate Vtg synthesis in *L. erinacea*, and that P4 seems to play a role in slowing or terminating Vtg synthesis.

Further evidence of the hormonal regulation of vitellogenesis in elasmobranchs was found in a study on male and female spotted rays (*Torpedo marmorata*) (Prisco et al., 2008). Using an antibody against lizard Vtg, a protein consistent with the molecular weight of Vtg was detectable in both vitellogenic females and male specimens injected with E2. In females, E2 concentrations were also found to be highest for vitellogenic individuals. In contrast, P4 concentrations were highest in pregnant female *T. marmorata.* Vtg was not detected in the plasma or liver of these pregnant females. Vitellogenic follicles were observed within the ovary of pregnant *T. marmorata,* but their growth was arrested. This suggests that high P4 concentrations during pregnancy may act to inhibit Vtg synthesis during that time period for *T. marmorata*.

Vtg has also been investigated in two additional elasmobranch species. In the deep sea Portuguese dogfish (*Centroscymnus coelolepis*), which employs aplacental viviparity, a female-specific protein was detected in mature females. This female-specific protein reacted with an antibody against frog Vtg in an immunoblot (Tosti et al., 2006). In the cloudy catshark (*Scyliorhinus torazame*), an oviparous species, a female-specific protein was again detected and reacted with an antibody against catshark Vtg in an immunoblot. This same protein was detected in males injected with E2, suggesting E2 stimulation of Vtg synthesis in *S. torazame* as well

(Yamane et al., 2013). However, neither of these studies investigated the effect of P4 on vitellogenesis in these species, though P4 was found to vary little between immature and mature *C. coelolepis*, suggesting E2 may be more involved in regulating Vtg synthesis than P4 for this deep-sea species (Tosti et al., 2006).

In general, it has been determined that Vtg synthesis is stimulated by E2 in elasmobranchs, and there has been some evidence that P4 inhibits or terminates the protein's synthesis in both oviparous and aplacental viviparous elasmobranch species. However, no studies to date have characterized the process of vitellogenesis in a placental viviparous elasmobranch. Understanding the regulation of Vtg synthesis, and particularly the role of P4 in this process, in a placental elasmobranch may help to clarify how placentation ultimately evolved, as P4 plays a key role in maintenance of pregnancy in eutherian mammals (Brawand et al., 2008; Callard et al., 1992; Rothchild, 2003). Thus, this study focused on characterizing vitellogenesis in the yolk-sac placental bonnethead shark, *Sphyrna tiburo*. *S. tiburo* is a small coastal shark that lives in shallow coastal waters and estuaries and has been extensively studied along the eastern coast of the United States (Frazier et al., 2014). The cycles of E2, P4, and T have already been well characterized for *S. tiburo*, and the timing of various reproductive events throughout their cycle is already known (Gonzalez De Acevedo, 2014; Manire et al., 1995). It was therefore possible to correlate the determined Vtg cycle from this study with the already-known aspects of the *S. tiburo* female reproductive cycle. Additionally, since *S. tiburo* is a commonly caught shark throughout the Northwestern Atlantic and Gulf of Mexico, a sufficient number of samples from throughout the year were collected for analysis.

The primary goal of this study was to characterize vitellogenesis in *S. tiburo*. To fulfill this goal, it was first determined where Vtg is synthesized in this species. Immunohistochemistry

was conducted on *S.tiburo* liver to assess Vtg immunoreactivity in this organ. The overall seasonal cycle of Vtg in the plasma of female *S. tiburo* was also assessed. Immunoblots were conducted using anti-*S. tiburo* Vtg antibodies, testing for the presence of Vtg within the plasma of female *S. tiburo* sampled throughout the year. Finally, the effect of both E2 and P4 on Vtg synthesis was investigated. This objective was first fulfilled using immunohistochemistry to investigate the presence of E2 and P4 receptors in liver hepatocytes. Additionally, plasma concentrations of both E2 and P4 were measured; the cycles for both hormones were then compared to the determined Vtg temporal cycle for *S. tiburo.* Finally, *in vitro* liver assays were conducted. Precision-cut slices of liver were exposed *in vitro* to various concentrations of E2; the presence of Vtg within these cultures was assessed after exposure to the hormone for 72 hours. It was ultimately hypothesized that Vtg would be synthesized within the liver in *S. tiburo,* with E2 stimulating synthesis, as has been found for all other species studied to date. Based on the results of studies on other elasmobranchs, it was also hypothesized that P4 would inhibit or slow Vtg synthesis in this species. Vtg presence in plasma was expected primarily in the months of March and April, as this is the previously established time period of follicular development for *S. tiburo*, when females have many large vitellogenic ova present within their ovaries.

A secondary goal of this study was to develop an antibody against Vtg that could be used for multiple elasmobranch species. Because Vtg is a nutritional rather than regulatory protein, its amino acid sequence is not highly conserved between species (Denslow et al., 1999). Thus, investigation of Vtg in a new species often requires the development of a new antibody, which could limit the effectiveness of analyzing plasma Vtg to better understand reproduction in a broad range of elasmobranch species. For this study, three new antibodies were created against more highly conserved portions of the amino acid sequence of *S. tiburo* Vtg. All three antibodies

were tested against plasma from other elasmobranch species to determine their efficacy in analyzing Vtg in other elasmobranchs.

2. METHODS

2.1 Sample collection

Female *S. tiburo* specimens were collected from estuarine, near-shore, and offshore locations off of South Carolina, Georgia, and Florida waters in the Atlantic and Gulf of Mexico. Collections were conducted via bottom longline and gillnet fishing in both fishery-independent surveys and commercial fishing trips. Blood was collected from all captured females via caudal venipuncture and transferred to vacuum tubes containing anticoagulant and aprotinin. Anticoagulant (E-ACD) preparation followed the recipe from Walsh and Luer (2004). A solution was then prepared of 16 µg/mL aprotinin (aprotinin from bovine lung, Sigma-Aldrich, St. Louis, MO, USA) in E-ACD. For every 3 mL of blood that was collected, 350 µL of this E-ACD solution (containing 5.6 µg aprotinin) was used. The blood samples were centrifuged at 1,900 × *g* for 5 minutes; the plasma was collected and stored at -20°C until needed for analysis. Liver and ovary samples were also collected from females and fixed in 10% elasmobranch phosphate-buffered saline formalin (E-Formalin) for 48 hours, then rinsed and transferred to 70% ethanol for long-term storage at -80°C.

Liver was also collected from both male and female *S. tiburo* specimens for use in *in vitro* assays. Specimens were euthanized by anesthesia without revival before their liver was dissected. Roughly 5 grams of liver was removed and placed into a sterile sample bag containing 50 mL of phosphate buffered saline modified for elasmobranchs (E-PBS, recipe from Walsh and Luer 2004). The sample bag was then sealed and kept on ice until the liver was brought back to

the lab for thin section preparation and use in the *in vitro* assays. Before dissection, all tools used were cleaned and sterilized using 100% ethanol, and all media was filter-sterilized through a 0.22 μm filter.

2.2 Antibody development and testing

Three new antibodies were developed for use in this study against different highly conserved portions of the Vtg amino acid sequence from *S. tiburo* (Table 1), which was determined in prior studies (Gelsleichter, unpublished data). These antibodies were developed and provided by RayBiotech (Norcross, GA). All three antibodies were tested for their ability to detect Vtg via immunoblotting, indirect ELISA, and immunohistochemistry. For the second antibody, RayBiotech provided the antigen against which the antibody was developed. Dilutions of this antigen were also prepared and tested using the ELISA techniques. Ultimately, outcomes of the ELISA (see Results, Section 3.1) indicated it would be necessary to use immunoblotting rather than the indirect ELISA to detect Vtg within plasma samples.

2.3 Immunoblotting

Immunoblotting was conducted to identify the presence of Vtg in the plasma of *S. tiburo* specimens of all reproductive stages. Additionally, plasma samples from females of other elasmobranch species were tested using the same immunoblotting techniques. The other species assessed were the tiger shark (*Galeocerdo cuvier*), the spinner shark (*Carcharhinus brevipinna*), the blacktip shark (*Carcharhinus limbatus*), the great white shark (*Carcharodon carcharias*), the gulper shark (*Centrophorus granulosus*), the blacknose shark (*Carcharhinus acronotus*), the oceanic whitetip shark (*Carcharhinus longimanus*), the Atlantic stingray (*Dasyatis sabina*), and the smalltooth sawfish (*Pristis pectinata*).

Table 1. Amino acid sequences of three antibodies created against the amino acid sequence for vitellogenin in *S. tiburo*.

Antibody ID	Amino Acid Sequence
rabbit polyclonal anti-*S tiburo* Vtg antibody 1	ELAFAQLRKEDLDTIKC
rabbit polyclonal anti-*S. tiburo* Vtg antibody 2	CNLALNVLSPKLEQLGH
rabbit polyclonal anti-*S. tiburo* Vtg antibody 3	KTDSRSIRRILSKLINC

The protein concentration of each plasma sample was first measured using the Bio-Rad Protein Assay. Samples were then diluted in 2x Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 355 mM 2-mercaptethanol to a level that would ensure that 100 µg of protein was loaded into each well of a protein gel. For protein separation, 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) were used to ensure Vtg and any component proteins would be retained. The protein standard ladder used for each gel was the Precision Plus Dual Color Protein Standard (Bio-Rad). Each well was either loaded with 10 µL of the plasma sample or standard, or the appropriate volume to ensure that 100 µg of total protein were loaded into the well. All plasma samples were run in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) at 200 V until appropriate separation was visualized.

Protein transfer was performed in a Mini TransBlot Cell (Bio-Rad) onto an Immuno-Blot PVDF membrane (Bio-Rad) at 100 V for one hour. Following transfer, the PVDF membrane was incubated in a 10% blocking solution (5 grams of non-fat dry milk in 50 mL of Tris-buffered saline (TBS) pH 7.6) in order to block additional protein binding sites overnight at 4°C. The membrane was then incubated with the primary anti-Vtg antibody, which was rabbit polyclonal anti-*S. tiburo* Vtg (Table 1). For incubation with the primary antibody, first a 1% blocking solution was created by diluting the 10% solution with T-TBS (TBS with 0.05% tween-20). The primary antibody was then diluted in 1% blocking solution; the exact dilution used varied depending on which of the three anti-*S. tiburo* Vtg antibodies was used (Table 1). For use with *S. tiburo* samples, the first antibody was diluted 1:25,000, the second antibody was diluted 1:10,000, and the third antibody was diluted 1:2000. When testing the plasma of other elasmobranch species, the first antibody was diluted 1:10,000, the second antibody was diluted

1:10,000, and the third antibody was diluted 1:1000. All membranes were covered with 10 mL of the primary antibody solution and incubated overnight at 4°C in a covered container.

On the final day of immunoblotting, each membrane was incubated with the secondary antibody (anti-rabbit IgG conjugated to alkaline phosphatase, Sigma-Aldrich), diluted 1:30,000 in 1% blocking solution. Incubation with this secondary antibody solution occurred for 1 hour at room temperature, with 15 mL of the solution covering each membrane. Between all antibody incubations the membranes were rinsed five times for five minutes each in T-TBS. Finally, binding of the primary antibody to Vtg was visualized using a BCIP/NBT Alkaline Phosphatase kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Once a color reaction was visualized, the color reaction was stopped by rinsing the membranes for 5 minutes in reverse osmosis (RO) water and air-drying. Following the completion of an immunoblotting assay, all membranes were imaged using the Amersham Imager 600 (GE Healthcare, Uppsala, Sweden).

2.4 Histology and immunohistochemistry

Formalin-fixed samples of liver and ovarian tissue were dehydrated via incubation in an ascending series of graded alcohol (80% – 100%), cleared using the limonene-based solvent CitriSolv (Fisher Scientific, Hampton, NH), and embedded in paraffin. The tissues were then sectioned (5 µm) using a rotary microtome and mounted on microscope slides coated with 0.01% poly-L-lysine (Sigma-Aldrich). All ovary sections were stained using the hematoxylin and eosin (H&E) staining protocol to aid in visualization of oocyte structure and correct identification of oocyte developmental stages.

Immunohistochemistry was performed on liver sections from female *S. tiburo* using polyclonal rabbit anti-human ER-α, monoclonal mouse anti-human PR, and polyclonal rabbit

anti-*S. tiburo* Vtg (antibody 1, Table 1) as primary antibodies. Sections of ovarian tissue were also analyzed via immunohistochemistry using polyclonal rabbit anti-*S. tiburo* Vtg (antibody 3, Table 1). Tissue sections were incubated in Citrisolv for deparaffinization, rehydrated via incubation in a descending series of graded alcohols (100 – 95%), and rinsed in tap water. Sections were then incubated at 95°C in antigen retrieval solution (10 mMol sodium citrate, pH 6.0) for a period of 20 minutes. After sections were brought to room temperature, they were rinsed in water and phosphate-buffered saline (PBS), and finally blocked for nonspecific reactivity via overnight incubation with 2.5% normal goat serum in PBS at 4°C.

Following overnight blocking, all slides were rinsed twice in PBS. Endogenous peroxidase activity was quenched by incubating the slides in a 1:1 solution of 3% hydrogen peroxide and 100% methanol for 15 minutes. Sections were then rinsed twice with PBS before the appropriate primary antibody was added (Table 2). All primary antibodies were diluted in PBS containing 0.1% gelatin and 0.1% sodium azide (G-PBS). Control sections were incubated with G-PBS in place of primary antibody. The sections were incubated in primary antibody overnight at 4°C.

Following incubation with the primary antibodies, all sections were rinsed with PBS containing 0.05% Tween-20, rinsed twice with PBS, and then incubated with the appropriate secondary antibody for 1 hour at room temperature (Table 2). Antibody binding sites were then revealed using the appropriate peroxidase substrate kit. For ovarian sections, the peroxidase substrate 3’3-diaminobenzidine was used (ImmPACT DAB, Vector Laboratories). For liver sections, the ImmPACT NovaRED peroxidase substrate kit was used instead because of a high amount of endogenous melanin, which obscures the detection of DAB. Sections were incubated in the substrate mixture until there was a visible color change (~5 min), and then transferred to a

Table 2. Antibodies and dilutions used in immunohistochemistry for detection of Vtg, ER-α, and PR in formalin-fixed liver and ovary samples from *S. tiburo*.

Protein	Primary Antibody	Dilution	Manufacturer	Secondary Antibody
Vtg	Rabbit polyclonal anti-*S. tiburo* Vtg antibody 1	1/500	RayBiotech	Anti-Rabbit IgG
Vtg	rabbit polyclonal anti-*S. tiburo* Vtg antibody 3	1/200	RayBiotech	Anti-Rabbit IgG
ER-α	rabbit polyclonal anti-human ER-α	1/100	Millipore, C1355	Anti-Rabbit IgG
PR	mouse monoclonal anti-human PR	1/100	Thermo Scientific, alpha PR-22	Anti-Mouse IgG

running tap water rinse for 10 minutes. The sections were counterstained using 2% methyl green (Vector Laboratories) at 37°C for 1 hour and then rinsed in tap water for 2 minutes. Finally the sections were dehydrated in an ascending series of graded alcohols (95 – 100%), cleared in Citrisolv, and coverslips were applied using Cytoseal 60 (Electron Microscopy Sciences, Hatfield, PA).

2.5 Hormone analysis

Plasma concentrations of 17β-estradiol and progesterone were measured using AccuLite CLIA kits (Monobind, Lake Forest, CA) following the manufacturer instructions. Luminescence was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). All plasma was diluted in HAS calibrator matrix prior to conducting the assay; 1/25 for E2 and 1/10 for P4 measurements. Optimal dilution levels were determined in an earlier study (Gonzalez De Acevedo, 2014).

2.6 in vitro *liver assays*

Culture media was prepared for use with elasmobranch tissue; this culture media consisted of Leibovitz's L-15 medium supplemented with urea and salt to appropriate concentrations for elasmobranch tissue (E-L15, pH 7.2 – 7.4). The media was sterilized by filtering through a 0.22 µm filter before use. Throughout the preparation of liver slices, all liver was kept on ice. Slice preparation took place in a sterile culture hood and all materials and surfaces were sterilized with 100% ethanol prior to use.

Liver collected from female *S. tiburo* specimens was first cut into strips roughly 5 – 10 mm wide. These pieces were then cut into precision slices of 1 mm using the McIlwain Tissue Chopper (Cavey Laboratory Engineering Co. Ltd., Guildford, UK). The slices were placed into a sterile Petri dish containing E-L15 culture media until enough slices were obtained to be plated.

Test chemicals were added to the culture media (E-L15) using ethanol as the vehicle. Dilutions were made to achieve estradiol concentrations of 0 pg/mL, 3 pg/mL, 30 pg/mL, 300 pg/mL, 3000 pg/mL, and 30,000 pg/mL E2 in E-L15. Control media was created by adding an appropriate volume of 100% ethanol to the E-L15 instead, so that the control media contained a final concentration of 0.1% ethanol. Wells of sterile 24-well culture plates were then prepared by adding 1 mL of the E-L15 containing either ethanol or the appropriate estradiol concentration into each well. Four wells were dedicated to each concentration. After the plates were prepared, the liver slices were randomly placed into the wells. A total of four plates were prepared for each assay. Three of these plates were used as replicates for the Vtg detection assays. The fourth plate was reserved for histology and immunohistochemistry to be conducted at a later date on the liver slices. All plates were incubated for 72 hours at 19 – 21°C; culture media was not changed during the incubation period. After the 72 hour incubation, the liver slices and sample media were collected from each plate and stored at -80°C.

As noted, for each of the three plates dedicated to the Vtg assays, four wells were dedicated for each E2 concentration. The liver slices from these four wells were thus pooled together and homogenized using a 1:5 mass:volume ratio of tissue to homogenization buffer (PBS) in a Fast Prep 24 bead homogenizer (MP Biomedicals LLC, Solon, OH, USA). The homogenate was first centrifuged for 10 minutes at 8,000 × g; the resulting supernatant was placed in a new tube and centrifuged at 20,000 × g for 20 minutes. The final supernatant was isolated and stored at -80°C.

Protein concentrations were determined both for the culture media and liver homogenates using the Bio-Rad Protein Assay. Where protein concentrations were low, the TCA precipitation protocol was followed to precipitate proteins from samples. For liver homogenates, 100 – 200 µg

of protein was precipitated from the samples. For the culture media, 300 – 600 µg of protein was precipitated. The required volume to achieve each amount of protein was loaded into a microtube along with trichloroacetic acid (ratio 1:4 TCA to culture media or liver homogenate). The samples were centrifuged for 15 minutes at 18,407 × g and the supernatant was removed. The resulting protein pellet was washed twice with acetone; 200 µL of acetone was added to the pellet and the resulting solution was centrifuged for 5 minutes at 18,407 × g. The final pellet was dried briefly under a fume hood. Laemmli sample buffer (2x, Bio-Rad, containing 355 mM 2-mercaptethanol) was then added directly to this pellet; enough Laemmli buffer was added so the final protein content of a 10 µL load would be 100 µg for liver homogenates and 300 µg for the culture media. After the pellet had sufficiently dissolved in the sample buffer, the samples were heated at 95°C for 5 minutes to denature the proteins. Immunoblot analysis was then conducted of both the homogenates and culture media samples using the methods outlined in section 2.3. However, due to the limitations of the protein gels used, however, evidence of Vtg production was analyzed only for the liver tissue exposed to 0 pg/mL, 3 pg/mL, 300 pg/mL, and 30,000 pg/mL E2.

2.7 Data analysis

To determine relative concentrations of vitellogenin present in plasma samples, membranes were analyzed using ImageJ software following the completion of an immunoblotting assay. In order to facilitate this analysis, all immunoblots included a positive control plasma sample from a known vitellogenic female *S. tiburo*. This positive control sample was used as a baseline, and the optical density of each sample on a given membrane was compared to the optical density of the positive control.

Plasma concentrations of E2 and P4 were grouped by month of capture to examine temporal changes in concentration. Mean hormone concentrations per month were compared using a Kruskal-Wallis nonparametric test followed by Dunn-Bonferroni multiple comparisons, as the data for hormone concentrations did not pass tests for normality. A significance level of 0.05 was used for all tests.

3. RESULTS

3.1 Detection of Vtg in S. tiburo *plasma*

All three antibodies were tested against the plasma of female *S. tiburo* known to be undergoing vitellogenesis. All antibodies detected a high molecular weight protein consistent with the expected molecular weight of vitellogenin in elasmobranchs (~205 kD, Perez and Callard, 1992) in these plasma samples (Figure 1). The third antibody provided the strongest signal and was able to detect Vtg even when it was apparently present in low concentrations in the plasma; this antibody was thus used for immunoblotting. The first antibody was utilized for some of the immunohistochemistry assays, as this antibody also provided a relatively strong signal, though it did not detect Vtg when plasma concentrations appeared relatively low.

In some plasma samples, the third anti-*S. tiburo* Vtg antibody detected a second protein whose molecular weight is consistent with that of lipovitellin (Lv), one of the breakdown products of Vtg (Figure 2). The molecular weight of Lv in *L. erinacea* was determined to be either 105, 91, or 67 kD (Perez and Callard, 1992). A protein of a molecular weight around 70 kD was detected in some plasma samples, which suggests that Vtg was likely present in these samples but the protein may have degraded, resulting in the antibody detecting a component protein rather than the whole Vtg protein.

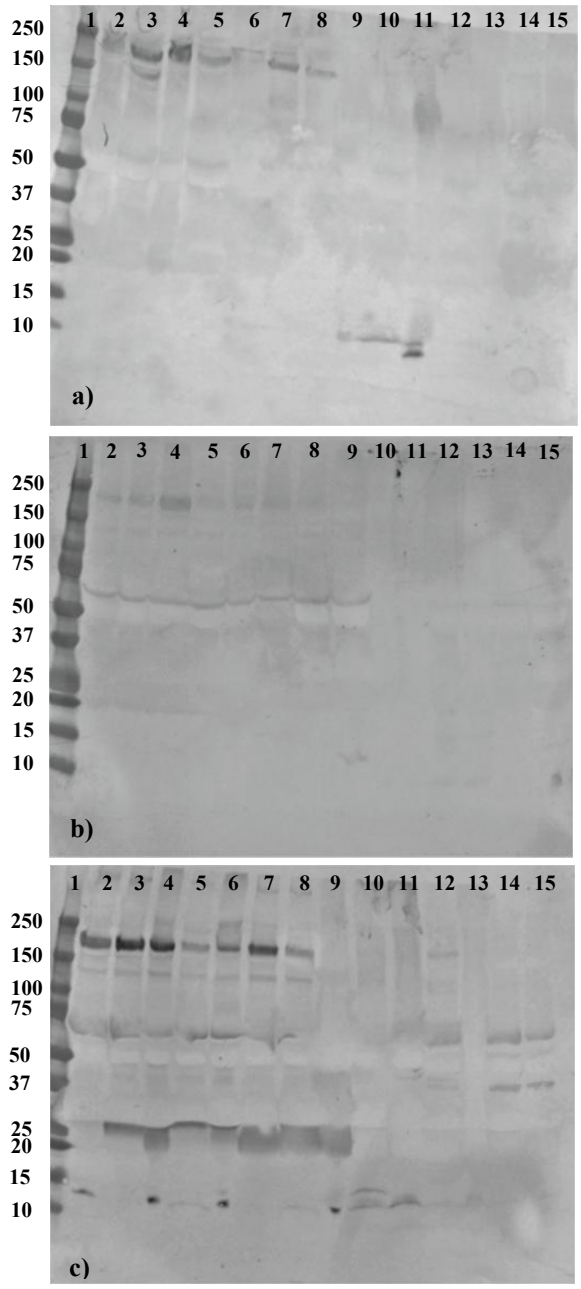

Figure 1. Results of immunoblotting testing anti-*S. tiburo* Vtg antibodies against *S. tiburo* plasma. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-8: plasma of known vitellogenic females collected in early March. Lane 9: plasma of an *S. tiburo* male. Lane 10: plasma of a mature female collected during April. Lanes 11-15: plasma of females collected during May; lanes 11-13 represent mature females, while lanes 14-15 are immature females. All antibodies were tested against the same samples: a) antibody 1 b) antibody 2 and c) antibody 3.

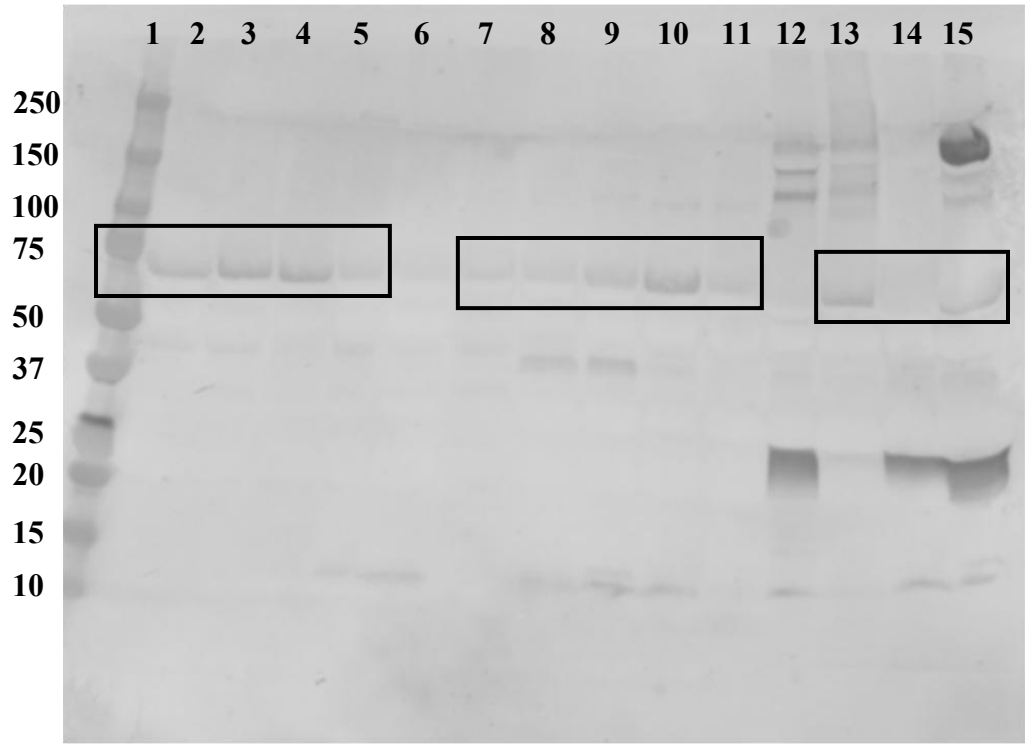

Figure 2. Immunoblot of plasma samples tested using anti-*S. tiburo* Vtg antibody 3 diluted 1:2000. Positive control of a known vitellogenic female (collected in March) is shown in lane 15. Vtg was detected for the positive control (Lane 15) and two other samples, but the antibody also detected a second protein in many samples (boxed). Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lane 2: mature postpartum female collected in October. Lane 3: mature postpartum female collected in August. Lane 4: mature female collected in November, noted to have mating scars. Lane 5: mature postpartum female collected in August. Lane 6: mature female collected in September. Lane 7: mature female collected in December with noted mating scars. Lanes 8-11: plasma from females identified as immature, collected in September (8), August (9), and October (10, 11). Lane 12: vitellogenic mature female collected in April. Lane 13: mature ovulating female collected in April. Lane 14: pregnant female collected in late September.

An attempt was made to develop an indirect ELISA to detect changes in Vtg concentrations over time. However, even when the plasma was diluted at a level of only 1/2 in the coating buffer to concentrate the protein as much as possible, the antibody detected no difference in the absorbance at 450 nm between the blank sample (coating buffer), confirmed Vtg negative, and confirmed Vtg positive plasma samples. This suggests that the antibodies were not able to detect Vtg within plasma in the indirect ELISA. However, when the dilutions were prepared of the antigen for the second antibody, differences in the amount of Vtg present were detected. This indicates that the lack of Vtg detection was not due to the techniques used in the indirect ELISA, but instead likely because the Vtg concentration in the plasma was too low for detection or other components in plasma interfered with detection.

3.2 Temporal Vtg cycle

A high molecular weight protein consistent with Vtg was detected in the plasma of female *S. tiburo* known to be undergoing vitellogenesis sampled during March. Three females sampled in April also had Vtg present within their plasma. Vtg was also detected in the plasma of one female *S. tiburo* each sampled in the months of May and October, along with two females sampled in December (Figure 3).

As noted previously, for some plasma samples the antibody did not detect Vtg, but did detect a second protein whose molecular weight is consistent with that of Lv. It is possible that Vtg was initially present in these samples, but degraded into its component parts during sample storage. When the females with this putative Lv protein in their plasma are included in the temporal Vtg cycle, there is evidence for Vtg presence in the plasma of some females in late August, with production then continuing from September through December. There was also evidence of a higher proportion of females with Vtg in their plasma in April (Figure 4).

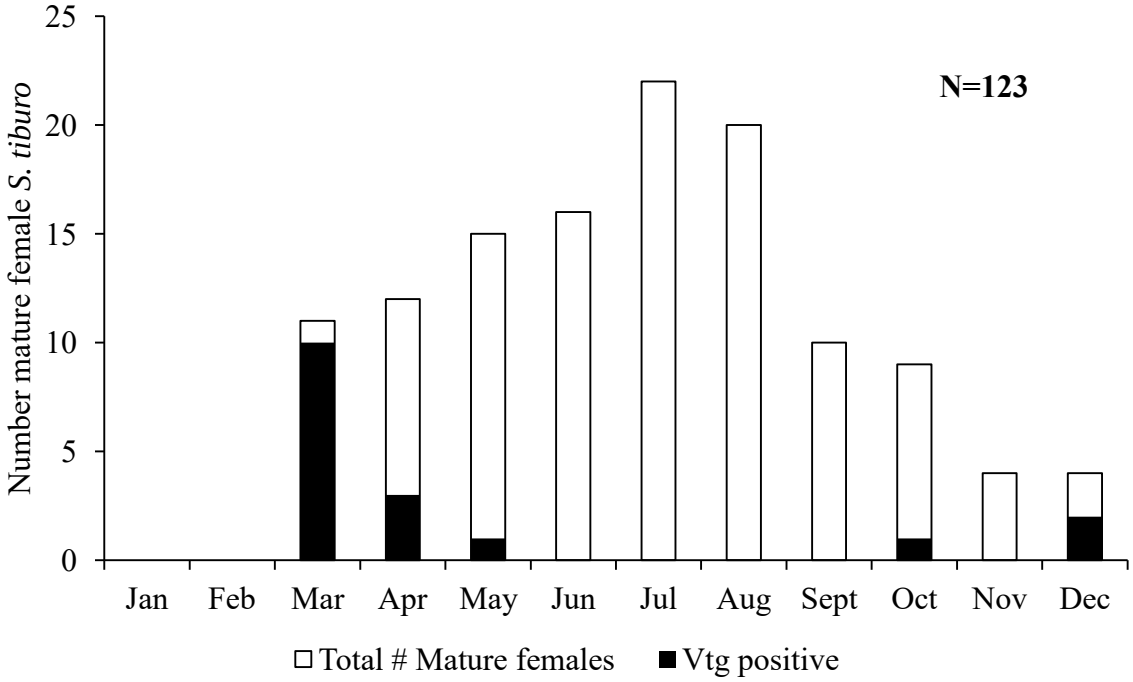

Figure 3. Proportion of mature *S.tiburo* females that were determined to have vitellogenin present within their plasma during each month.

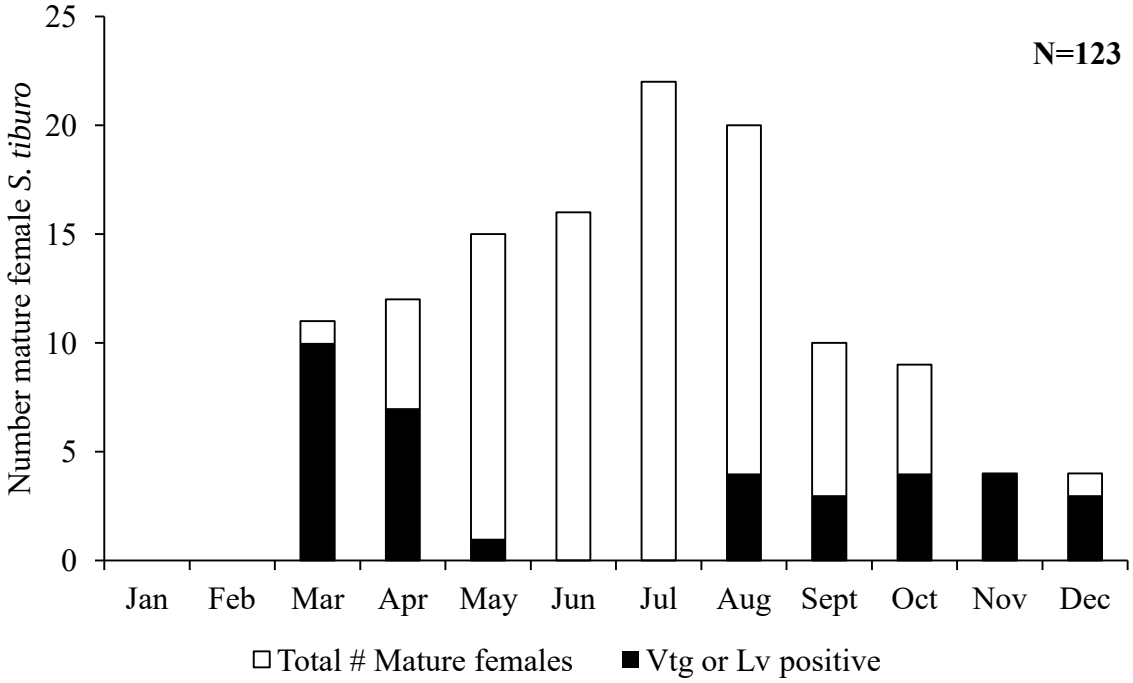

Figure 4. Proportion of mature *S.tiburo* females that were determined to have either vitellogenin or the putative lipovitellin protein present within their plasma during each month.

3.3 Temporal changes in plasma Vtg concentrations

Comparisons were first made of the amount of Vtg present in the plasma of females collected in early March and early May based on analysis of the optical density of signals on an immunoblot membrane (Figure 5a). The optical density of each sample was compared to a chosen positive control (FBay3, Lane 4 on Figure 5a). Based on this analysis, plasma concentrations of vitellogenin did vary by month. In March, the majority of females tested positive for Vtg, but the concentration of Vtg in the plasma varied between individuals. The optical density of the signals ranged from 22.2% to 129% of the optical density found for the chosen positive control sample. The concentration of Vtg present in the plasma of the female sampled in May was much lower, with an optical density 4.5% of that for the positive control plasma sample (Figure 5b).

As noted previously, three *S. tiburo* females collected in April were identified to have Vtg in their plasma, along with three additional *S. tiburo* females collected in March. One *S. tiburo* female in October had Vtg in her plasma, as did two females collected in December. An immunoblot containing all of these samples is depicted in Figure 6a, with the same positive control plasma sample (FBay3) shown in the last lane of the immunoblot. It was notable that the concentration of Vtg present in the March samples on this immunoblot appeared much lower when compared to the positive control, also collected in March. The relative optical density for the Vtg signals from the three samples on this immunoblot collected later in March ranged from 2.3 – 5.9% of the optical density of the positive control. The concentration of Vtg in the plasma samples collected in April also appeared consistently lower than that for the positive control, with optical density for the April signals ranging from 4.7% to 13.9% of the optical density for the positive control (Figure 6b).

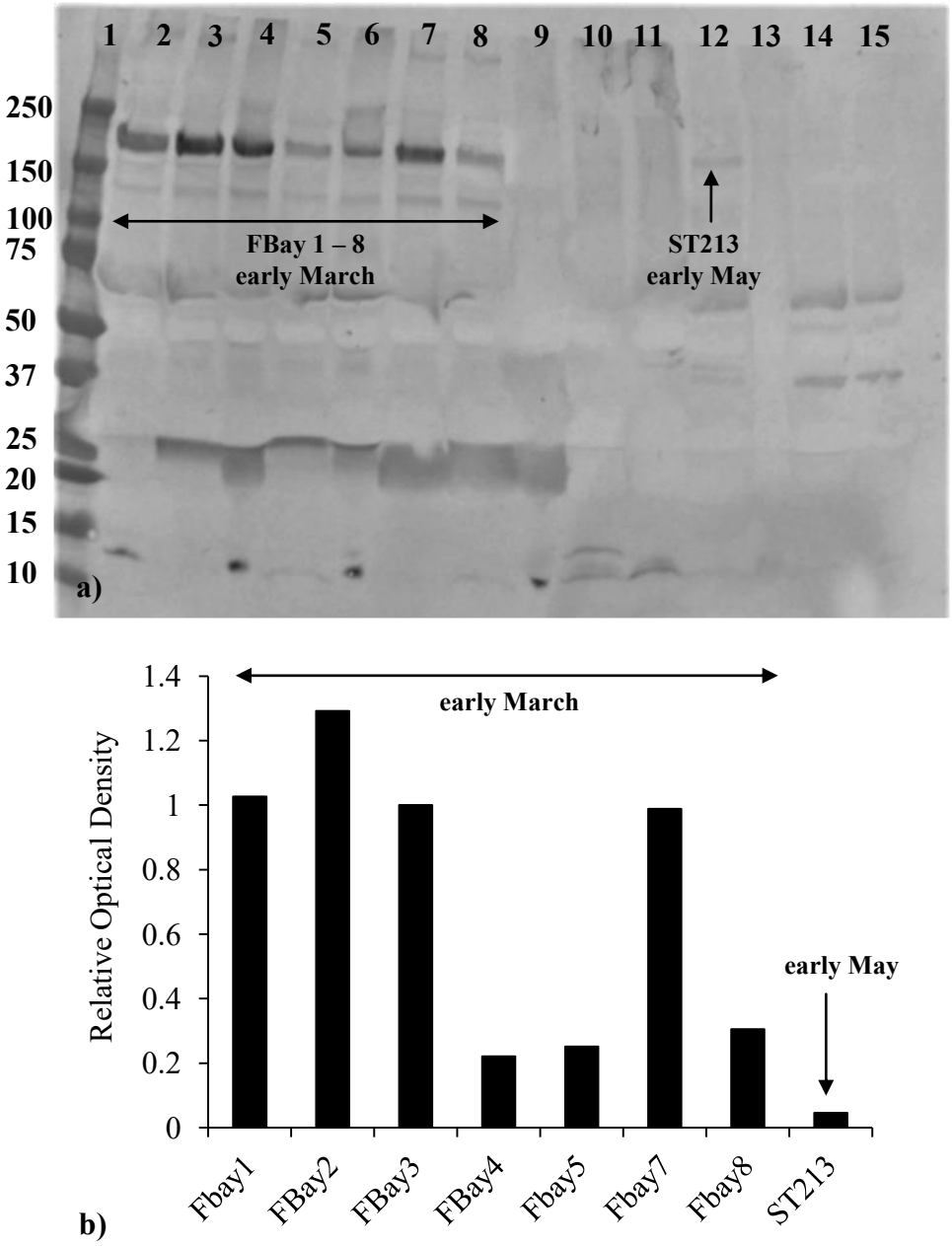

Figure 5. Analysis of relative concentrations of Vtg present in plasma samples from the months of March and May based on relative optical density. a) Immunoblot results, using the third anti-*S. tiburo* Vtg antibody. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-8: plasma samples from known vitellogenic *S. tiburo* females in early March. Individual FBay3 (lane 4) was chosen as the sample against which all other samples were compared. Lane 9: male *S. tiburo* plasma. Lane 10: plasma of a mature female from April. Lanes 11-15: plasma of females from May; lanes 11-13 represent mature females, while lanes 14-15 are immature females. Lane 12 contains sample ST213, collected in early May. b) Relative optical density of the Vtg signals from the immunoblot.

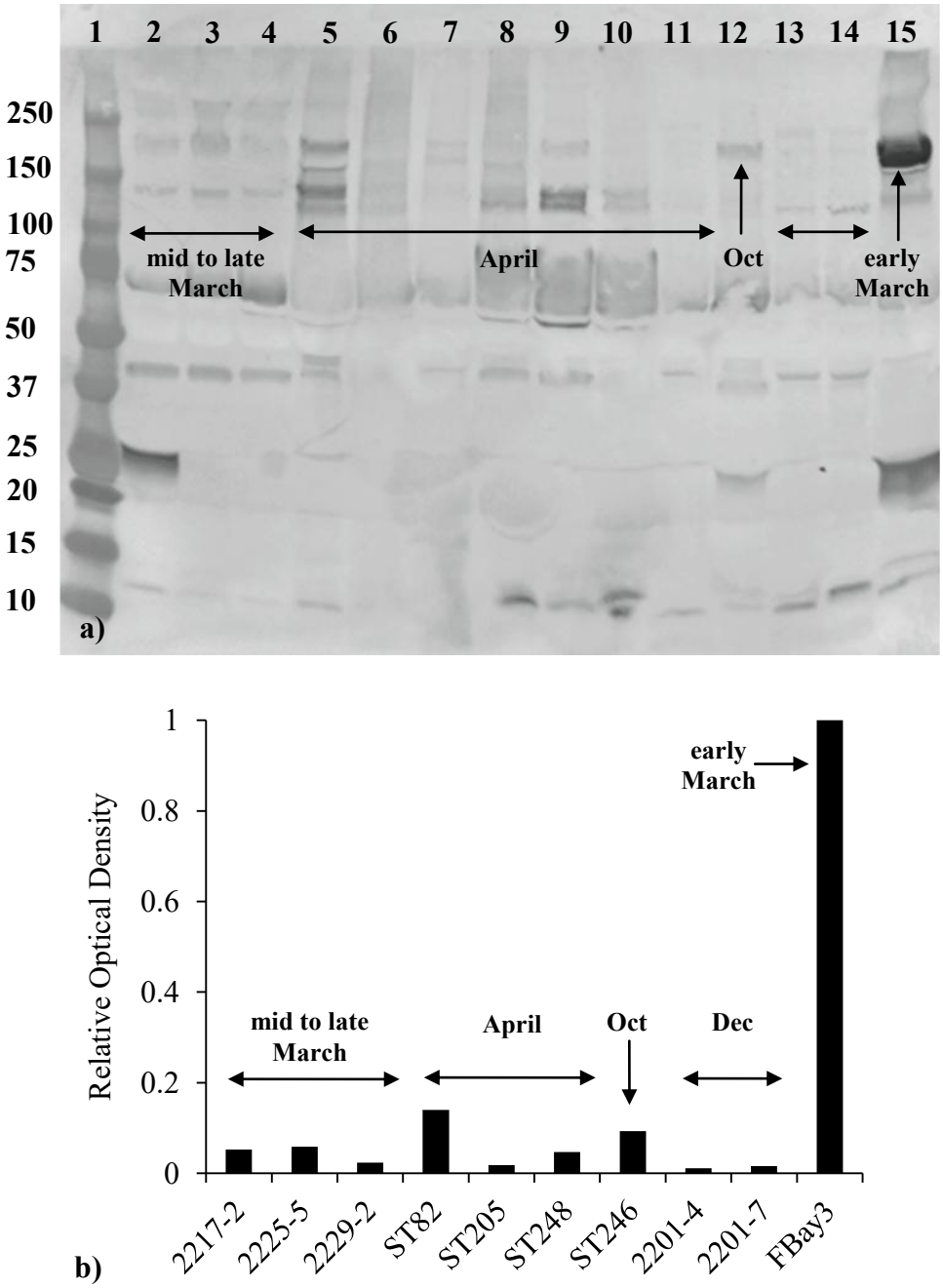

Figure 6. Analysis of relative concentrations of Vtg present in female *S. tiburo* plasma samples based on relative optical density. a) Immunoblot results, using the third anti-*S. tiburo* Vtg antibody. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-4: plasma from March (2217-2, 2225-5, 2229-2 respectively). Lanes 5-11: plasma from April (labeled in order as ST82, ST83, ST205, ST247, ST248, ST249, and 4/23/17 #1). Lane 12: plasma from October (ST246). Lanes 13-14: plasma from December (2201-4 and 2201-6 respectively). Lane 15: plasma from confirmed vitellogenic female from March (FBay3). b) Relative optical density of the Vtg signals from the immunoblot. Order of samples on graph follows order of samples on the immunoblot.

The concentration of Vtg in the plasma in October and December also appeared to be much lower than that found in March, based on comparison to the positive control. For the October sample (ST246), the optical density of the Vtg signal was 9.3% of the optical density for the positive control. Both of the December samples (2201-4 and 2201-7) showed considerably lower Vtg concentrations, with optical densities of 1.1% and 1.6% respectively when compared to the optical density of the positive control (Figure 6b).

Ultimately, it was determined that female *S. tiburo* definitively had Vtg in their plasma in the months of March, April, May, October, and December. In order to determine how the amount of Vtg varied throughout these months, the average relative optical density was calculated for each month and compared. This comparison illustrated that in general the highest amount of Vtg was present in the plasma in March, with lower amounts present in April, May, October, and December (Figure 7).

3.4 Detection of Vtg in plasma of other elasmobranch species

Of the nine other species which were tested (*G. cuvier*, *C. brevipinna*, *C. limbatus*, *C. carcharias*, *C. granulosus*, *C. acronotus*, *C. longimanus*, *D. sabina*, and *P. pectinata*), the antibodies detected proteins with molecular weights consistent with would be expected for either elasmobranch Vtg or one of its component proteins (presumably Lv) in the plasma of all species with the exception of the spinner shark (*C. brevipinna*). Clear detection of Vtg (~205 kD, Perez and Callard, 1992) occurred for four of the species: the blacktip shark *C. limbatus* (Figures 8a and 8c, antibodies 1 and 3), the great white shark *C. carcharias* (Figure 8b, antibody 2), the blacknose shark *C. acronotus* (Figure 8c, antibody 3), and the oceanic whitetip shark *C. longimanus* (Figures 8b and 8c, antibodies 2 and 3).

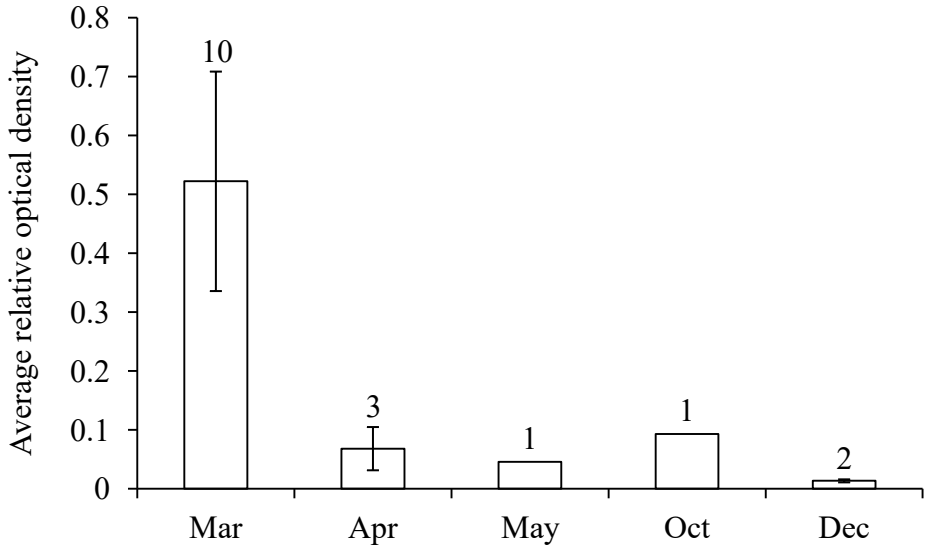

Figure 7. Average relative optical density of the Vtg signal detected in *S. tiburo* plasma from March, April, May, October, and December. Numbers above each bar indicate the sample size for each month, illustrating the number of females from that month for which the antibody clearly detected the Vtg protein. Error bars indicate standard error.

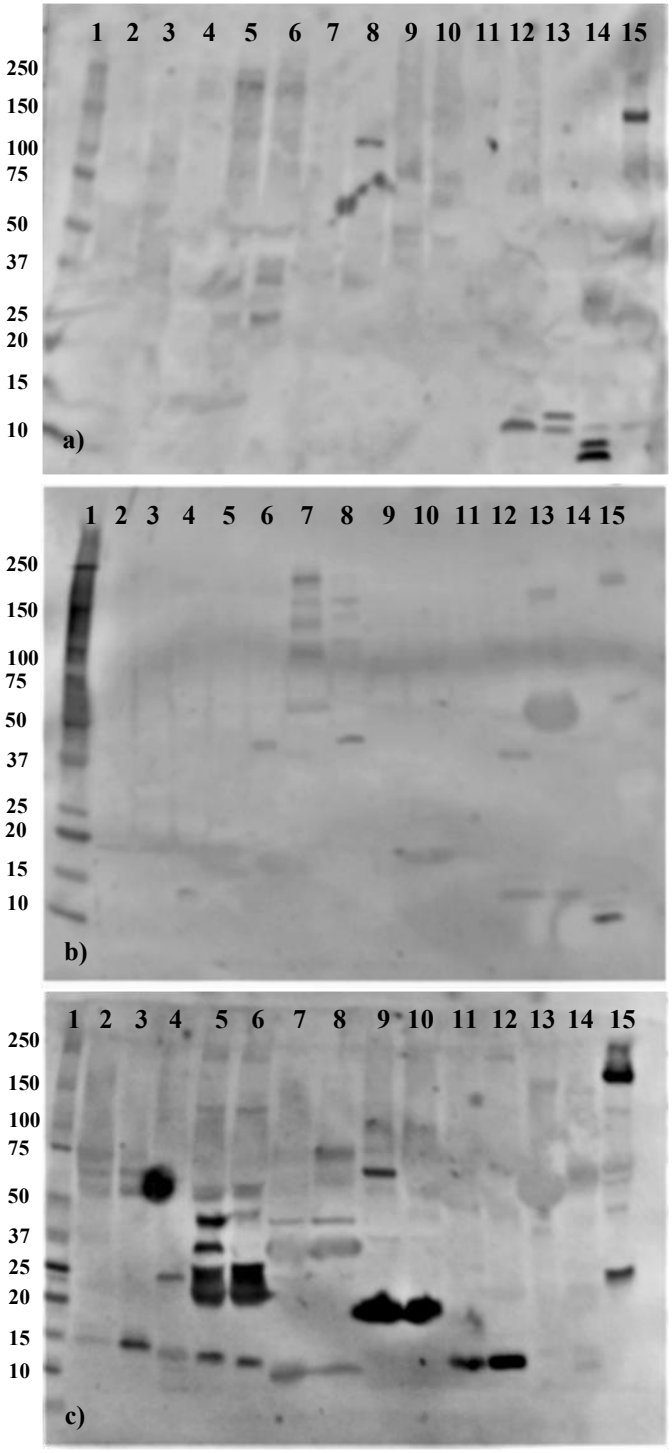

Figure 8. Immunoblots showing Vtg-like immunoreactivity with the plasma of multiple elasmobranch species. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-3: *G. cuvier*, Lane 4: *C. brevipinna*, Lanes 5-6: *C. limbatus*, Lanes 7-8: *C. carcharias*, Lanes 9-10: *C. granulosus,* Lanes 11-12: *C. acronotus*, Lanes 13-14: *C. longimanus*, Lane 15: vitellogenic *S. tiburo* (positive control). a) Antibody 1, diluted 1:10,000 b) Antibody 2, diluted 1:10,000 c) Antibody 3, diluted 1:1000.

Additionally, all three antibodies bound to other proteins present within the plasma of the elasmobranch species. However, analysis focused on proteins with molecular weights that would be expected for elasmobranch Lv. This protein is a component of Vtg, so detection of a protein with a molecular weight consistent with Lv would be indicative of putative Vtg presence in the plasma. The antibodies bound to a protein with a molecular weight around 70 kD in plasma samples from multiple species, suggesting that evidence of Vtg was being detected in the samples. The third antibody bound to a protein suggestive of the Lv component of Vtg in the plasma of the tiger shark *G. cuvier* (Figure 8c), gulper shark *C. granulosus* (Figure 8c), Atlantic stingray *D. sabina* (Figure 9, antibody 3), and smalltooth sawfish *P. pectinata* (Figure 8, antibody 3). This Lv component was also detected by the third antibody in plasma samples for which Vtg itself was clearly detected: *C. carcharias, C. longimanus,* and *S. tiburo* (Figure 8c).

All three antibodies also bound to proteins of smaller molecular weights, which likely are indicative of more extensive breakdown of Vtg and the Lv component of the protein (Figure 8c). It was not clear whether the antibodies were able to detect Vtg in the *C. brevipinna* plasma sample, but the third antibody did bind to a protein at ~25 kD, which could be indicative of Vtg, particularly if there was significant degradation of the Vtg protein within the plasma sample.

3.5 Liver immunohistochemistry

Immunohistochemistry was conducted on *S. tiburo* liver slices to detect the presence of estrogen receptors (ER-α), progesterone receptors (PR), and Vtg. Positive immunoreactivity for Vtg was observed within the hepatocytes, with strong immunoreactivity observed within the sinusoids and also around the ducts of the liver (Figure 10). Positive immunoreactivity for ER-α and PR was also observed within the hepatocytes, primarily around the ducts of the liver (Figure 11).

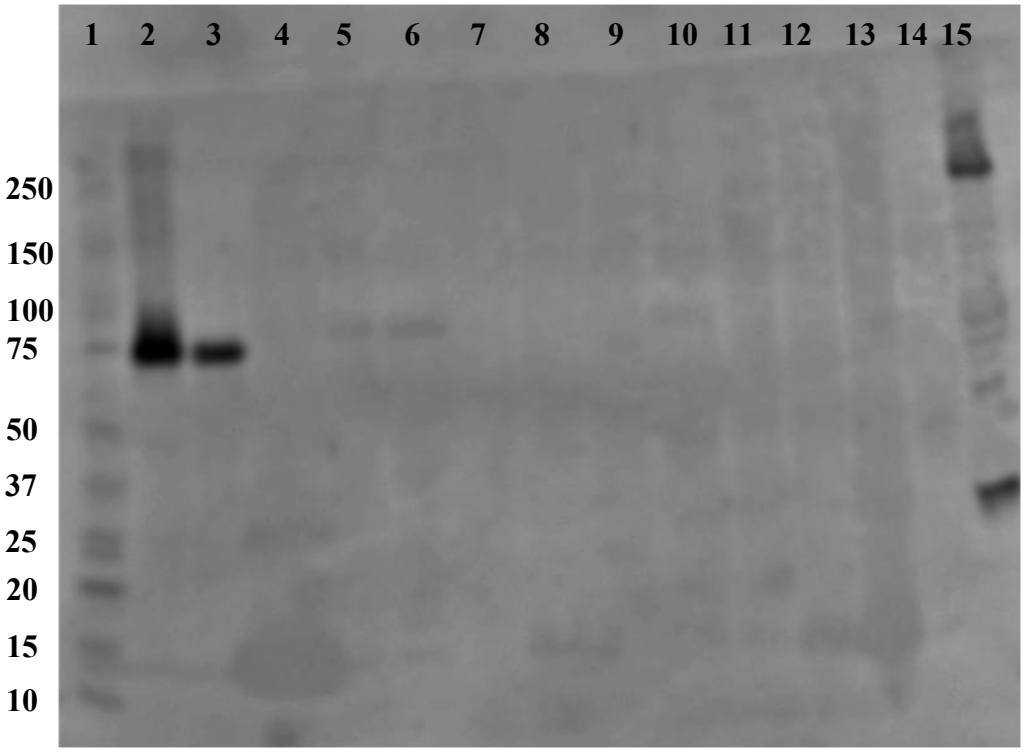

Figure 9. Immunoblot showing Vtg-like immunoreactivity with the plasma of multiple elasmobranch species. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-3: *D. sabina*, Lanes 4-14 *P. pectinata*, Lane 15: vitellogenic *S. tiburo* (positive control). Anti-*S. tiburo* Vtg Antibody 3 was used for this immunoblot, diluted 1:1000.

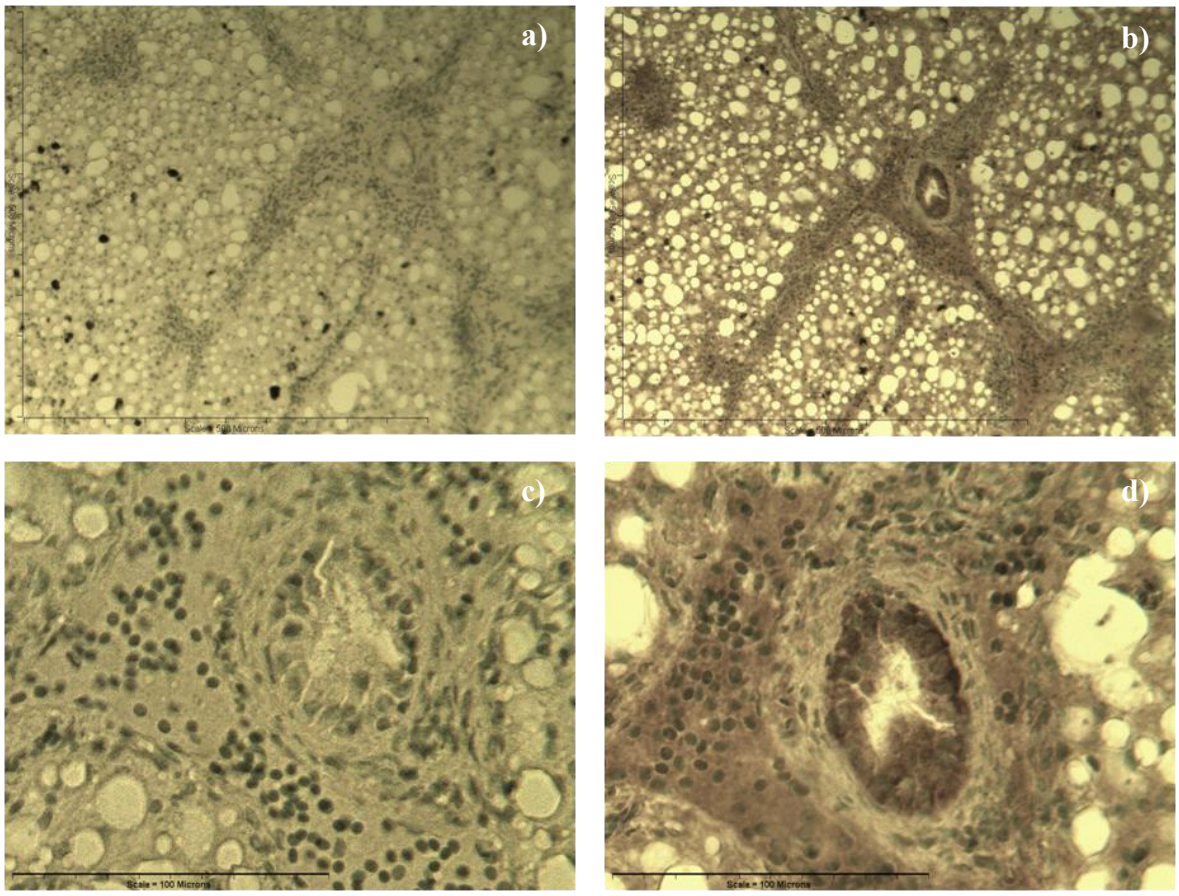

Figure 10. Immunohistochemistry of the liver of a vitellogenic *S. tiburo* female. Top row depicts magnification at 100x (scale=508 µm) of a) negative control (G-PBS) and b) reactivity with anti-*S. tiburo* Vtg antibody. Bottom row depicts magnification at 400x (scale=100 µm) of c) negative control (G-PBS) and d) reactivity with anti-*S. tiburo* Vtg antibody. Antibody 1 was used for these analyses, diluted 1:500.

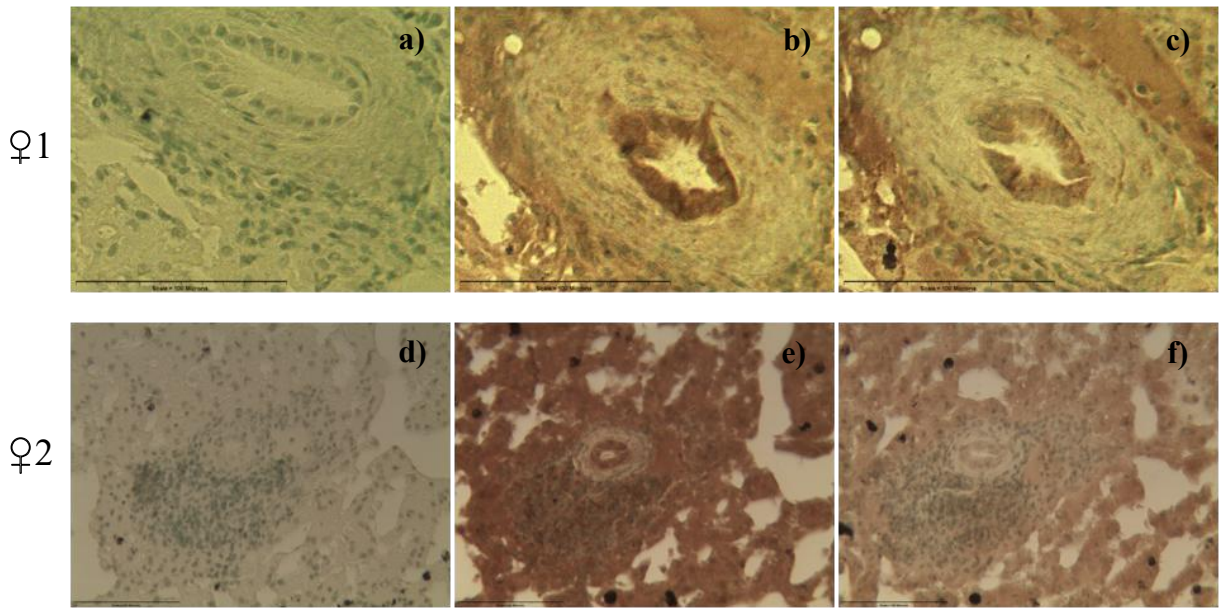

Figure 11. Immunohistochemistry of the liver of two different vitellogenic *S.tiburo* females (Identified as ♀1 and ♀2). For ♀1: a) negative control (G-PBS), b) ER-α immunoreactivity, c) PR immunoreactivity. For ♀2: d) negative control (G-PBS), e) ER-α immunoreactivity, f) PR immunoreactivity. The ER-α and PR antibodies were both diluted 1:100. All images taken at 400x magnification (scale=100 μm).

3.6 Ovary immunohistochemistry

Immunohistochemistry was conducted on sections of ovarian follicles of various stages (pre-vitellogenic through late vitellogenic) to detect the presence and localization of Vtg within the follicles. All slices were first stained using the H&E protocol to assist in visualization of structures and correct analysis of follicle developmental stage.

Pre-vitellogenic follicles were identified based on the presence of a layer of cuboidal granulosa cells and an established zona pellucida. Little positive immunoreactivity with Vtg was observed in pre-vitellogenic follicles, though potential immunoreactivity was observed within the layer of small cuboidal granulosa cells. As the follicles entered the early stages of vitellogenesis, the granulosa cells became more columnar in shape, with the overall granulosa layer increasing in thickness. The zona pellucida increased in thickness as follicles entered early vitellogenesis, but thickness decreased as follicles entered middle to late vitellogenesis. Yolk platelets began to form within the ooplasm of follicles in early vitellogenesis, and took on a clearer circular shape in middle vitellogenesis. For ovarian follicles in the early and middle stages of vitellogenesis, positive immunoreactivity was noted within the developing yolk platelets within the ooplasm, as well as in a layer just inside the zona pellucida in the ooplasm. Potential immunoreactivity was again observed within the cells of the granulosa layer, even as the cells elongated and became more columnar as vitellogenesis progressed (Figure 12).

Follicles in the late stages of vitellogenesis were identified based on the presence of many distinct cuboidal yolk platelets within the ooplasm; the zona pellucida of these follicles was also much thinner. Positive immunoreactivity with Vtg was again observed within the yolk platelets, in a thin layer inside zona pellucida in the ooplasm, and potentially within the cells of the granulosa layer (Figure 12).

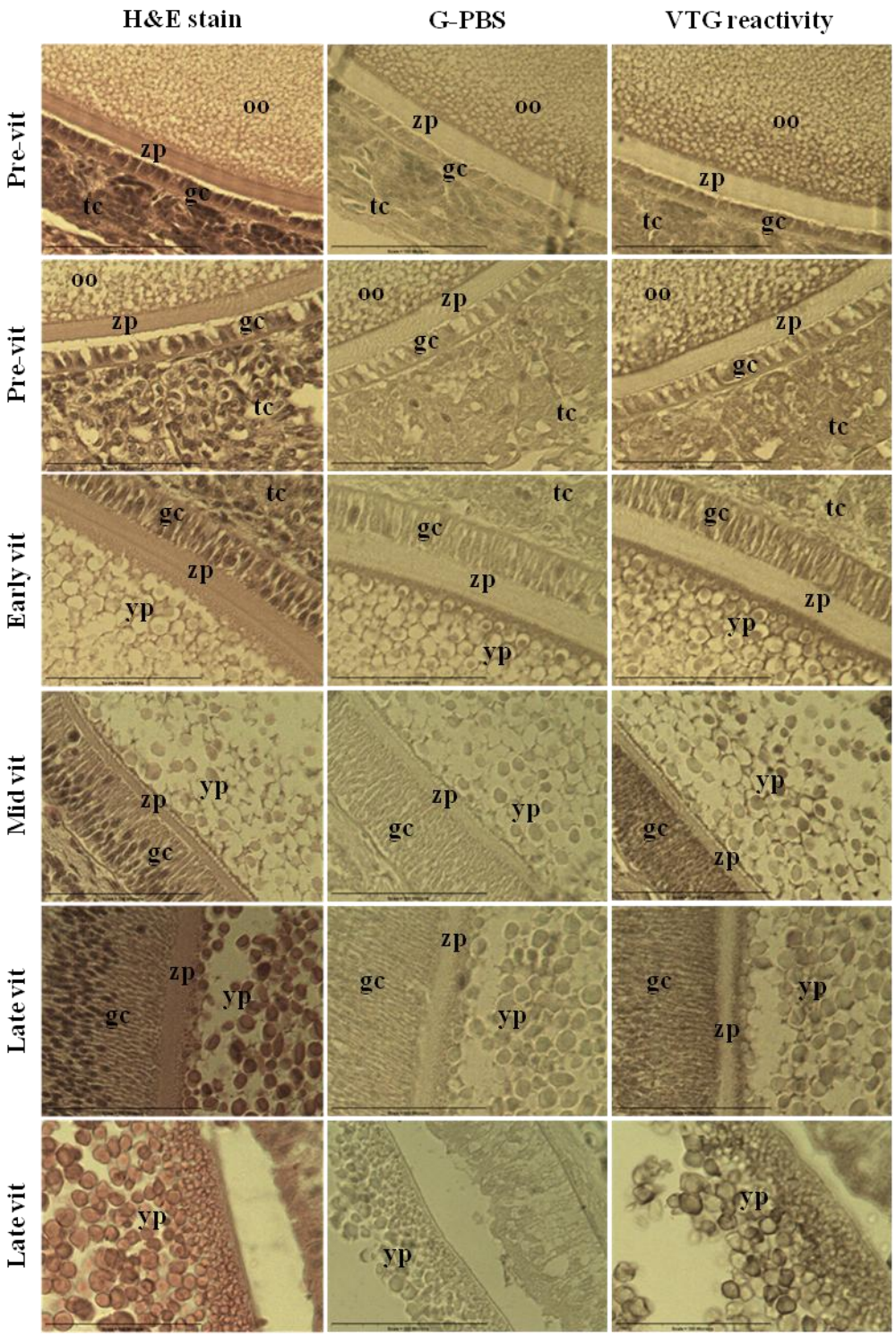

Figure 12. Immunohistochemistry of ovarian follicles throughout development, from pre-vitellogenic to late vitellogenesis. H&E stain was used to show structures; G-PBS was used as the negative control, and all follicles were stained with the third anti-*S. tiburo* antibody, diluted 1:100. Images taken at 400x magnification (scale=100 μm). Structure of follicles labeled as: oo=ooplasm, zp=zona pellucida, gc=granulosa cells, tc=theca cells, yp=yolk platelets.

3.7 Hormone cycles

Plasma concentrations of E2 were found to vary significantly by month (Kruskal-Wallis, df=8, p=0.0012). The mean plasma concentration of E2 was significantly higher in April, and significantly lower in June when compared to all other months (Kruskal-Wallis and Dunn-Bonferroni, p=0.022). In general, E2 concentrations were relatively high during the months of March and April, and then began to fall in May. Plasma concentrations of E2 remained low throughout the summer months, but began to increase as early as August and September for some females. Concentrations of E2 continued to increase throughout October, November, and December, even reaching similar concentrations as were found in March for some females.

Plasma concentrations of P4 were not found to vary significantly by month (Kruskal-Wallis, df=9, p=0.731). However, it was notable that in general, high concentrations of P4 were observed in more female *S. tiburo* captured during the months of March and May when compared to all other months, with the highest concentrations of P4 occurring during May (Figure 14).

3.8 in vitro *liver assays*

The liver homogenates were assessed first for the presence of Vtg within the tissue to determine if E2 stimulated Vtg production. Neither Vtg nor any breakdown proteins were detected within the liver homogenates for the first two liver cultures that were analyzed, from two maturing S. tiburo females (ST1929-12, Figure 15a; ST1921-1, Figure 15b). The antibody did still detect Vtg within the plasma of the positive control S. tiburo female (FBay3), indicating the lack of detection was most likely due to the lack of Vtg presence within the liver itself. Based on these initial results, further analysis of Vtg production by the liver in vitro focused on assessing Vtg presence within the culture media.

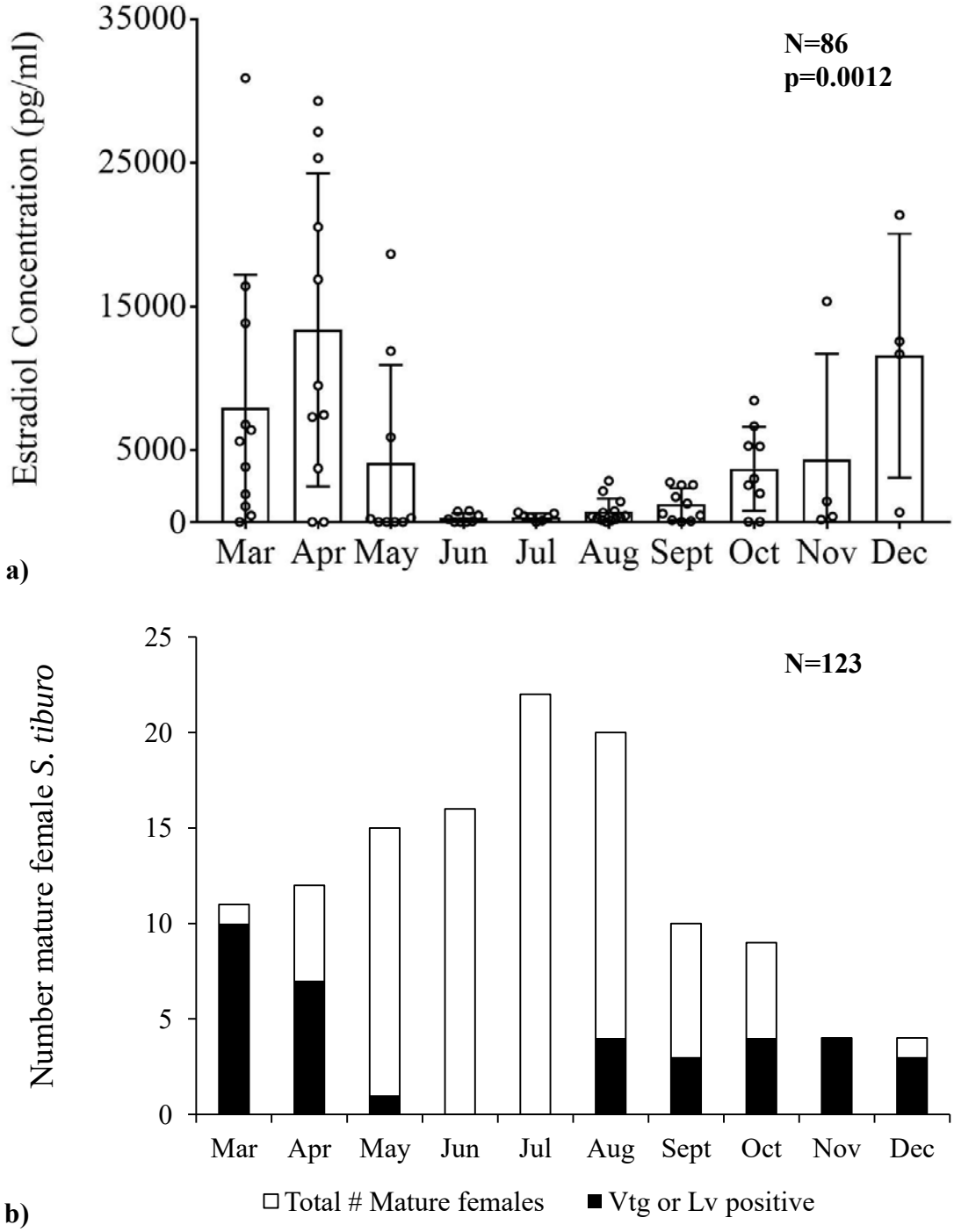

Figure 13. Comparison of monthly plasma E2 concentrations and Vtg production by female *S. tiburo.* a) Plasma concentrations of E2 in *S. tiburo* females (n=86). Values represent mean concentration, and error bars represent standard error. Significance was determined using the non-parametric Kruskal-Wallis test (p=0.0012). b) Proportion of mature *S.tiburo* females that were determined to have either vitellogenin or the putative lipovitellin protein present within their plasma during each month

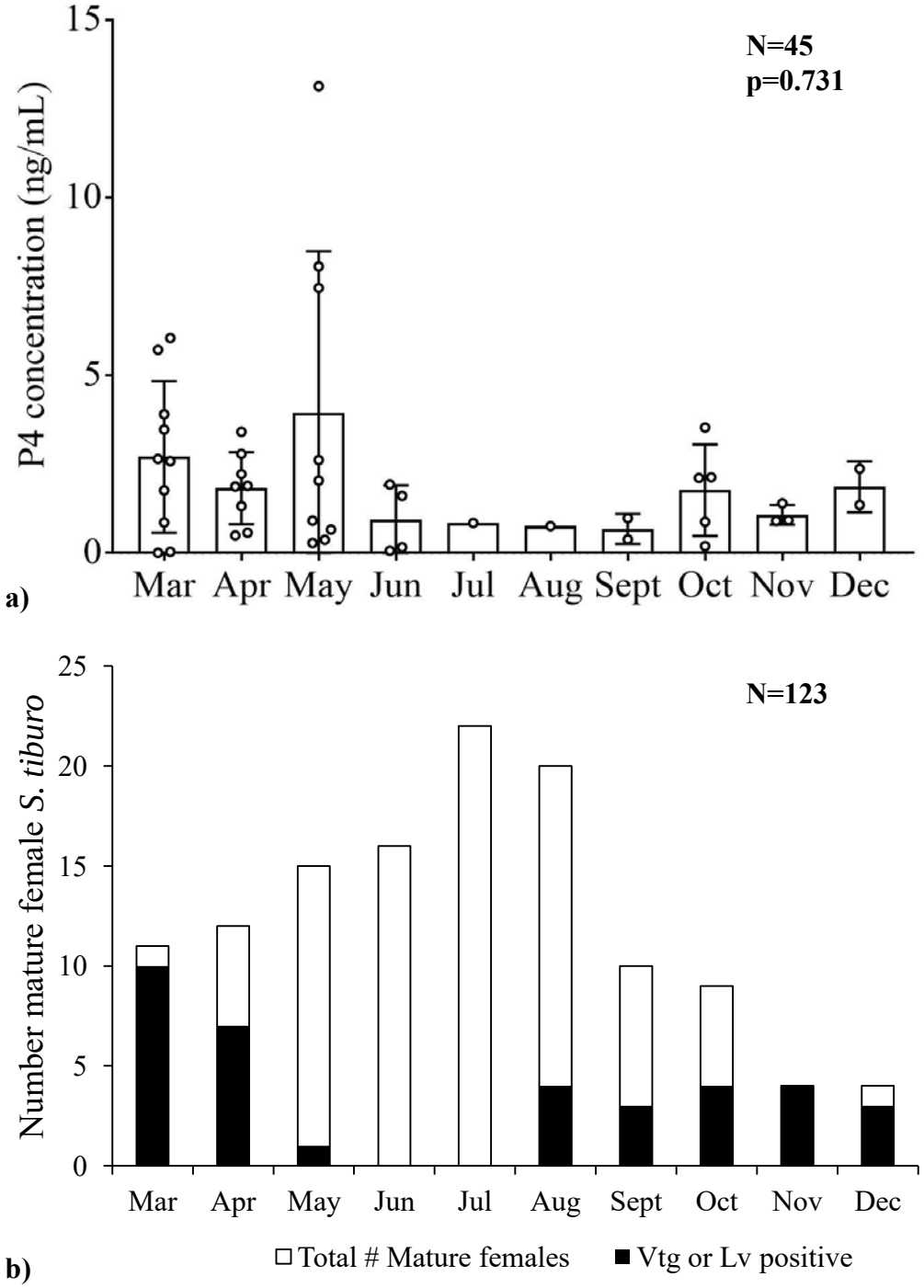

Figure 14. Comparison of monthly plasma P4 concentrations and Vtg production by female *S. tiburo.* a) Plasma concentrations of P4 in *S. tiburo* females (n=45). Values represent mean concentration, and error bars represent standard error. Significance was determined using the non-parametric Kruskal-Wallis test (p=0.731). b) Proportion of mature *S.tiburo* females that were determined to have either vitellogenin or the putative lipovitellin protein present within their plasma during each month (n=123).

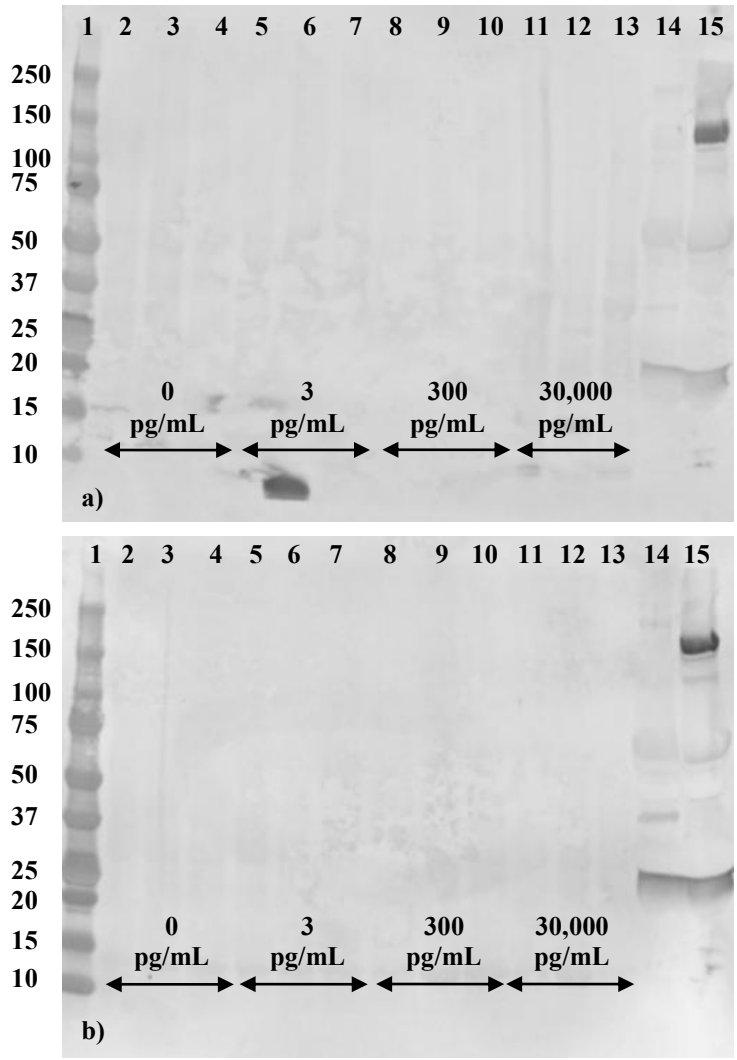

Figure 15. Immunoblots of liver homogenates from the liver cultures of two different *S. tiburo* females. a) Liver homogenates from liver culture of female ST1929-12, identified as likely maturing, captured 23 May. b) Liver homogenates from liver culture of female ST1921-1, identified as likely maturing, captured 17 May. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-13: Replicates of liver exposed to the concentrations of E2 indicated on the figure. Lane 14: Plasma sample for each of the two females, respectively. Lane 15: Positive control *S. tiburo* plasma (FBay3) from confirmed vitellogenic female.

Culture media from two mature females, three maturing females, and one immature male was assessed for Vtg presence. The two mature females (ST1933-3 and ST1943-3) were confirmed to have ovulated, and had fertilized eggs present within their uteri. All three maturing females (ST1921-1, ST1929-12, and ST1943-1) were identified as not yet reproductively active, and had no visible vitellogenic ova within their ovaries or eggs present within their uteri. However, the size of these females suggested they were likely approaching reproductive maturity, and were not truly immature females. All females were collected between 17 May and 31 May, so it was possible the liver of any females could have already been producing Vtg *in vivo*. Therefore, liver from a male *S. tiburo* was cultured in addition to the females to serve as a negative control, as males have never been identified to naturally produce Vtg *in vivo.*

The antibody detected a high molecular weight protein consistent with Vtg in only one culture media sample for one of the females identified as maturing (ST1943-1, Figure 16c). The antibody appeared to detect Vtg in one of the three control culture media samples containing 0 pg/mL E2 (Lane 2, Figure 16c). However this female also appeared to have a low concentration of Vtg present within her plasma sample (Lane 14, Figure 16c), indicating that this individual may have been producing Vtg *in vivo*, and this production continued *in vitro* by the liver tissue even when it was not exposed to E2. For this female, the antibody did detect putative Vtg component proteins in the experimental culture media samples as well. A signal around 50 kD was detected in the experimental culture media samples containing 3, 300, and 30,000 pg/mL E2. However, because there was some evidence of *in vivo* Vtg production by the liver of this female, it cannot be confirmed whether any Vtg produced in the experimental samples was the result of exposure to E2 or was simply Vtg production continuing to occur naturally *in vitro*.

The antibody did detect other proteins within the culture media samples from the four other females and one male that were assayed. Vtg itself was not detected in any of these samples, which could be due to protein degradation during the culture period or the processing of samples. The most commonly detected protein in the experimental culture media samples had a molecular weight around 50 kD; this protein could be indicative of Vtg degradation, particularly as a protein of this molecular weight had previously been detected in some *S. tiburo* plasma samples that were not stored with aprotinin (see Figure 2, section 3.1). This protein was not detected in any of the control culture media samples containing 0 pg/mL E2. The protein was also not detected consistently across individuals at all concentrations of E2 that the liver tissue was exposed to, but the protein was detected in at least one experimental replicate for all assayed liver. This protein was detected in culture media containing 3 pg/mL for two of the maturing females (ST1921-1, Figure 16a; ST1929-12, Figure 16b), one of the mature females (ST1943-3, Figure 17b), and the immature male (ST1979-1, Figure 18). This protein was also detected in culture media containing 300 pg/mL E2 for both mature females (ST1933-3, Figure 17a; ST1943-3, Figure 17b) and in culture media containing 30,000 pg/mL E2 for the immature male liver (ST1979-1, Figure 18). Though the protein was not detected consistently across all *in vitro* cultures or across all concentrations of E2, its molecular weight suggests that its presence could be indicative of Vtg production by the *S. tiburo* liver in response to E2 stimulation. The presence of this protein in experimental culture media from a male *S. tiburo* further strengthens this assumption, as the male liver was not likely producing Vtg *in vivo*, so detection of Vtg or a Vtg breakdown protein in the culture media could only be due to production that occurred *in vitro* upon exposure to E2.

The antibody also commonly detected a protein with a molecular weight just below 37 kD in multiple culture media samples from both males and females (ST1929-12, Figure 16a; ST1979-1, Figure 18). This protein was detected not only in the experimental media containing 3 – 30,000 pg/mL E2, but also within the control culture media containing 0 pg/mL E2. In females, this could be attributed to *in vivo* production of Vtg by the liver continuing once the liver was cultured. However, the protein was also detected within one replicate of control media for the male *S. tiburo*, which suggests that this protein, while it may have a sequence similar to Vtg, is not indicative of Vtg being produced by the liver. It is possible that this protein was being produced by the liver naturally *in vitro*, and that its production was unrelated to E2 stimulation.

A protein with a molecular weight between 50 – 75 kD was detected in one control culture media sample for a mature female (ST1943-3, Figure 17b, Lane 2). This female was confirmed to have ovulated and had fertilized eggs present within her uterus, but it is nonetheless possible that her liver was still producing small amounts of Vtg *in vivo*, and that this production continued *in vitro* even without exposure to E2 in the control sample. This protein was also detected within one culture media sample that contained 300 pg/mL E2 for that female (Figure 16b, Lane 9). However, within the culture samples from this female, there is still evidence of *in vitro* production of Vtg being stimulated by E2 exposure. The concentrations of putative Vtg breakdown proteins appear to be higher in the experimental culture media samples exposed to E2, based on the optical density of the signals between 37 – 50 kD detected in the experimental culture media samples, which is suggestive of Vtg production being stimulated to occur *in vitro* by exposure to E2.

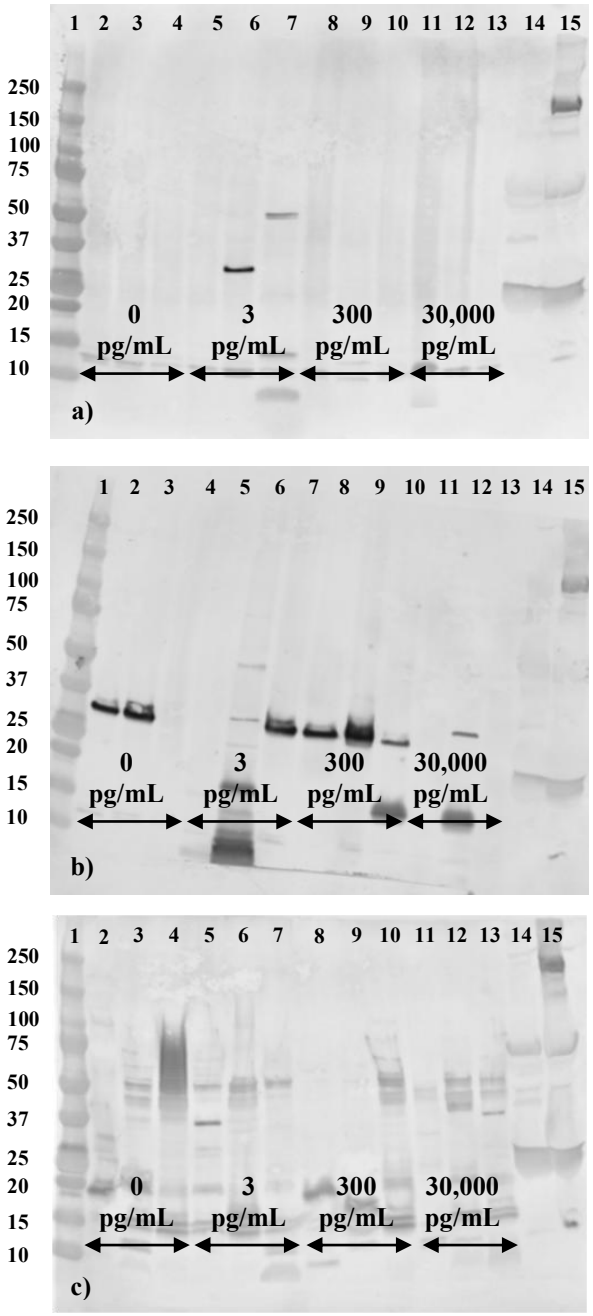

Figure 16. Immunoblots of culture media collected from the liver cultures of three different *S. tiburo* females identified as likely maturing, but not yet reproductively active. The third anti-*S. tiburo* Vtg antibody was used for these immunoblots, diluted 1:2000. a) ST1921-1, collected 17 May. b) ST1929-12, collected 23 May. c) 1943-1, collected 31 May. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-13: Replicates of culture media from liver exposed to the concentrations of E2 indicated on the figure. Lane 14: Plasma sample from the female whose liver was cultured. Lane 15: Positive control *S. tiburo* plasma (FBay3) from confirmed vitellogenic female.

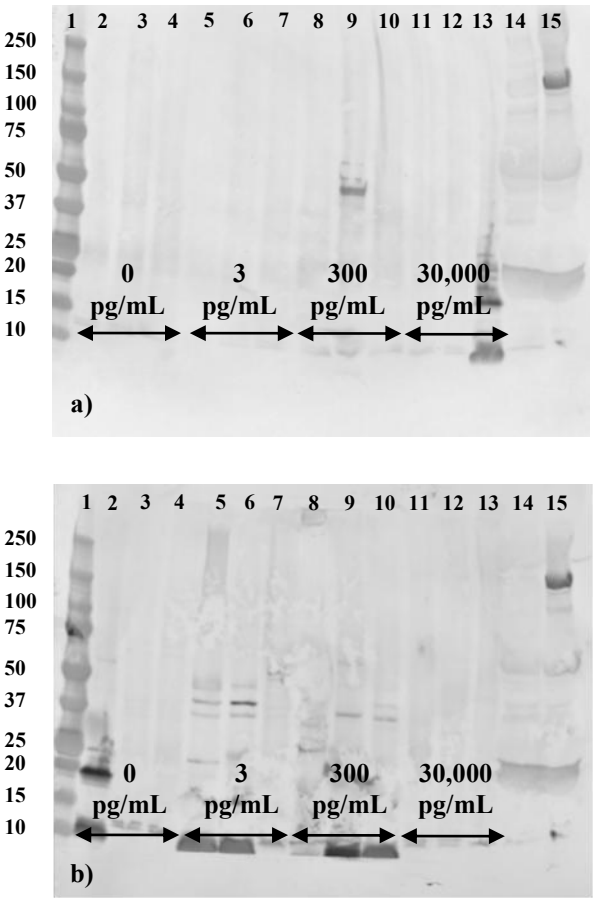

Figure 17. Immunoblots of culture media collected from the liver cultures of two different *S. tiburo* females identified as mature; both females had fertilized eggs present within their uteri. The third anti-*S. tiburo* Vtg antibody was used for these immunoblots, diluted 1:2000. a) ST1933-3, collected 24 May. b) ST1943-3, collected 31 May. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-13: Replicates of culture media from liver exposed to the concentrations of E2 indicated on the figure. Lane 14: Plasma sample from the female whose liver was cultured. Lane 15: Positive control *S. tiburo* plasma (FBay3) from confirmed vitellogenic female.

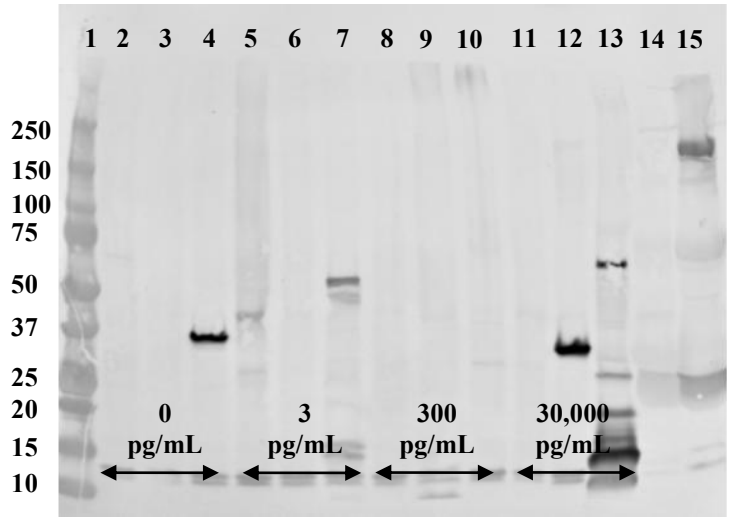

Figure 18. Immunoblot of culture media collected from the liver culture of an immature male *S. tiburo* (ST1979-1, collected 14 June), using the third anti-*S. tiburo* Vtg antibody. Lane 1: protein ladder (Precision Plus Dual Protein Color Protein Standard, Bio-Rad). Lanes 2-13: Replicates of culture media from liver exposed to the concentrations of E2 indicated on the figure. Lane 14: Plasma sample collected from ST1979-1. Lane 15: Positive control *S. tiburo* plasma (FBay3) from confirmed vitellogenic female.

4. DISCUSSION

The overall goal of this study was to characterize the process of vitellogenesis in *S. tiburo*. Characterization of the process was accomplished by determining where Vtg is produced, when the protein is produced and present within the plasma, and how the hormones E2 and P4 influence Vtg production. The liver was the expected site of Vtg production based on numerous previous studies on other organisms (Polzonetti-Magni et al., 2004; Romano et al., 2004). Immunohistochemistry using anti-*S.tiburo* Vtg antibodies was conducted on liver sections to confirm this expectation. Immunohistochemistry was also conducted on ovary sections to test for evidence of Vtg production by the granulosa cells of ovarian follicles. Temporal analysis of Vtg presence was accomplished by testing *S. tiburo* plasma samples from specimens obtained throughout the year for Vtg presence using immunoblotting. The hormonal regulation of Vtg synthesis primarily focused on the role of E2. First, immunohistochemistry was conducted to assess the presence of E2 receptors in liver hepatocytes. Plasma concentrations of E2 were then compared to Vtg presence in the plasma throughout the year. Finally, slices of liver were exposed *in vitro* to various concentrations of E2 to test for induction of Vtg synthesis. The role of P4 on Vtg synthesis was also investigated by assessing the presence of P4 receptors in liver hepatocytes and comparing P4 concentrations to Vtg presence in the plasma.

The liver was confirmed as the likely site of Vtg synthesis in *S. tiburo* using immunohistochemistry; reactivity was observed within liver hepatocytes, with the strongest reactivity observed in the sinusoids and around the ducts of the liver. These observations suggest that Vtg is likely produced within the hepatocytes and then immediately released into the bloodstream, where the protein then travels to the ovaries and is incorporated by growing oocytes. This result was expected, as it is well established that the liver is where Vtg is

synthesized in other organisms (Polzonetti-Magni et al., 2004). Additionally, the liver was confirmed as the likely primary site of Vtg synthesis in another viviparous elasmobranch, the aplacental spotted ray (*Torpedo marmorata*) using immunohistochemistry and *in situ* hybridization (Prisco et al., 2008).

It has also been proposed that the granulosa cells of ovarian follicles synthesize Vtg in *T. marmorata* (Prisco et al., 2004). Positive immunoreactivity was observed using anti-Vtg antibodies within the granulosa cells, and the authors also conducted *in situ* hybridization using a probe developed against Vtg cDNA from the zebrafish (*Danio rerio*). That probe hybridized within the granulosa cells of both previtellogenic and vitellogenic follicles; hybridization was also observed within the zona pellucida. While these results do provide evidence that the granulosa cells may produce Vtg, it should be noted that the cDNA probe used was not developed specifically against the sequence of Vtg in *T. marmorata.* The sequence of Vtg is known to vary significantly between organisms because the protein is nutritional rather than regulatory (Denslow et al., 1999). Additionally, a study that cloned Vtg cDNA from the cloudy catshark (*Scyliorhinus torazame*) found significant differences between the sequences of elasmobranch and teleost Vtg, and actually classified the cDNA sequence of *S. torazame* Vtg as more closely related to the sequences of Vtg found in amphibians and birds than sequences found in fish (Yamane et al., 2013). Thus, the binding of *D. rerio* Vtg cDNA to mRNA within the granulosa cells of *T. marmorata* may not truly indicate the presence of *T. marmorata* Vtg mRNA within these cells, but there nevertheless is evidence to support the hypothesis that granulosa cells produce Vtg.

To address this question, this study conducted immunohistochemistry on *S. tiburo* ovarian follicles of various developmental stages using an anti-*S. tiburo* Vtg antibody. Positive

immunoreactivity was observed within the granulosa cells of previtellogenic follicles and of vitellogenic follicles throughout all stages of vitellogenesis. Positive immunoreactivity was also observed inside the ooplasm in a thin layer just inside the zona pellucida in vitellogenic follicles. These results match the findings of Prisco et al. (2004) in *T. marmorata*, but do not by themselves confirm that granulosa cells produce Vtg in *S. tiburo*. After Vtg has been synthesized in the liver and travels to the ovaries, the protein must pass through the layers of the ovarian follicle, including the theca and granulosa cells, before accumulating within the ooplasm (Polzonetti-Magni et al., 2004). Thus, detection of Vtg within the granulosa cells could be explained as the protein passing through these cells before being incorporated by the oocyte. The thin layer of positive reactivity inside the zona pellucida could also be explained as Vtg entering the ooplasm before forming distinct yolk platelets. Further exploration is needed to determine if Vtg presence within granulosa cells is due to the protein being synthesized in these cells or travelling through the cells on its way to the oocyte; *in situ* hybridization using a cDNA probe against *S. tiburo* Vtg mRNA should be conducted to answer this question.

In regards to the temporal cycle of Vtg production, this study ultimately determined that Vtg is primarily produced in the month of March in *S. tiburo*, with production continuing into April and May for some individual females. The amount of Vtg produced appeared to decrease into April and May, with lower amounts of Vtg also found within the plasma of three individuals collected later in March (Figure 6). Previous studies on *S. tiburo* reproduction support this finding, with the observation of large vitellogenic ova present within the ovaries in March (Parsons, 1993). Some geographical variation has been observed in the timing of *S. tiburo* reproductive events, which explains why some females may still produce Vtg in April and even into May while other females have already ovulated and no longer produce Vtg in these months

(Manire et al., 1995; Parsons 1993). Vtg was not produced for any females during the summer gestation period of *S. tiburo* in this study, as was expected. *S. tiburo* females do not undergo gestation and follicular development concurrently, and levels of E2 remain low throughout the gestation period (Gonzalez De Acevedo, 2014; Manire et al., 1995).

A new finding of this study was the fact that Vtg was produced as early as October for one female, as well as in the month of December for two females. A putative Vtg component protein (around 70 kD) was detected in the plasma of some female *S. tiburo* in late August, suggesting that Vtg was likely present in the plasma at this time, but the protein likely degraded into its component parts during plasma storage. Overall the results of this study suggest that Vtg production may begin as early as August (after females in some populations have given birth) and continue throughout the fall, winter, and spring until ovulation occurs in March, April, or May. The lack of Vtg detection in a large number of females in August and September may again be attributed to geographical variation in *S. tiburo* reproduction, as it has been observed that females in south Florida give birth earlier in the year than females in northern Florida (Lombardi-Carlson et al., 2003). Vtg production does not appear to begin until parturition has occurred, as no Vtg was detected in gravid females and all individuals sampled in August were identified as postpartum. The onset of Vtg synthesis thus likely varies between populations depending on when parturition occurs.

Investigation of the hormonal factors regulating Vtg synthesis began by testing for the presence of receptors for E2 and P4 within liver hepatocytes. Immunohistochemistry confirmed that both ER-α and PR are present within the liver of *S. tiburo*. It was previously confirmed that Vtg is synthesized within the liver of *S. tiburo*, so the presence of ERs and PRs within the organ

leads to the hypothesis that both hormones may play a role in regulating Vtg synthesis either by stimulating, inhibiting, or terminating the process.

A comparison of the temporal Vtg cycle with the temporal E2 cycle in *S. tiburo* suggests that E2 likely stimulates Vtg production, as has been found for all other organisms studied to date. It was observed that concentrations of E2 were highest during April. The highest proportions of females were producing Vtg in March and April; notably high E2 concentrations were also observed in March. Previous studies have identified high concentrations of E2 in post-ovulatory female *S. tiburo*, which may account for the high E2 concentrations in April not coinciding with a high number of females still producing Vtg during this month (Gonzalez De Acevedo, 2014; Manire et al., 1995). Concentrations of E2 remained low throughout the summer gestation period, during which time Vtg was also not detected in the plasma of any female *S. tiburo*. However, E2 concentrations began to increase as early as August for some females, and this increase continued throughout the fall and winter, with E2 concentrations in December similar to concentrations that were found in March. Previous studies also identified high concentrations of E2 during the winter months, but attributed these higher concentrations perhaps as evidence of the hormone playing a role in sperm storage during this time period (Gonzalez De Acevedo, 2014; Manire et al., 1995). The findings of this study suggest that the increase in plasma E2 that occurs during the fall and winter also likely stimulates Vtg synthesis to begin, as Vtg was found to be present in the plasma of females in October and December, while Lv was detected in the plasma of females as early as August.

Further investigation into the role that E2 has in regulating Vtg synthesis was conducted by exposing slices of liver directly to various concentrations of E2 *in vitro*. The methods for this *in vitro* assay were based on a previous study which exposed precision-cut liver slices from the

rainbow trout (*Oncorhynchus mykiss*) to E2 and measured Vtg production. That study detected Vtg in both liver homogenates and culture media after only 24 hours, with concentrations increasing over time (Shilling and Williams, 2000). Previous studies have also exposed hepatocytes to E2 and measured Vtg production (Maitre et al., 1986; Navas and Segner, 2006; Pelissero et al., 1993; Smeets et al., 1999). However, the use of precision-cut liver slices allows for a more realistic model of what would occur *in vivo* as the structure of the whole organ, along with any cell-to-cell interactions, is maintained in the culture environment (Shilling and Williams, 2000).

For this study, precision-cut slices of *S. tiburo* liver (~1 mm) were exposed *in vitro* to E2 for a total of 72 hours; after this time both the liver and culture media were collected and analyzed. Immunoblotting was conducted to determine if Vtg was present either within homogenates of the liver or within the culture media. Vtg was not detected within the liver homogenates; this was in contrast to the aforementioned previous study, which detected Vtg within homogenates of *O. mykiss* liver after only 24 hours of exposure to E2 (Shilling and Williams, 2000). Still, the lack of Vtg detection within liver homogenates was not entirely unexpected, as Vtg is secreted by the liver and would likely then be present within the culture media. It was expected that Vtg would be present within the culture media of liver exposed to concentrations of E2 above 3 pg/mL, and that the concentration of Vtg present in the culture media would increase as the E2 concentration increased; no Vtg detection was anticipated in culture media of liver exposed to 0 pg/mL.

Ultimately, there is some evidence to suggest that exposure to E2 *in vitro* stimulated liver to produce Vtg. A protein with the molecular weight of Vtg was not detected within any of the experimental culture media samples, though Vtg did appear to be present in the control culture

media from one maturing female (ST1943-1). This female also had a low concentration of Vtg present within her plasma, so it is possible that the liver of this maturing female was still producing Vtg *in vivo* in May, and this production continued *in vitro* even without E2 exposure. For the other four females and one male whose liver was assayed, there was some evidence of Vtg production occurring when the tissues were exposed to E2 based on the presence of a putative Vtg breakdown protein. This protein, around 50 kD, was detected in experimental culture media samples from both male and female liver and was not detected within any of the control culture media samples. Presence of this 50 kD protein within the culture media thus provides some evidence that liver tissue possibly produced Vtg upon exposure to various E2 concentrations *in vitro*, though Vtg production did not occur consistently in all replicates of the experimental samples.

A protein between 50 – 75 kD was detected in one control culture media sample for the mature female identified as ST1943-3. Though this female was confirmed to have fertilized eggs within her uteri, it is still possible that her liver was producing small amounts of Vtg *in vivo* which continued *in vitro* even in the control media. For this female there was still evidence of Vtg production being stimulated by E2 exposure, as the concentrations of another putative Vtg breakdown protein (around 50 kD) appeared higher in the experimental media containing E2 based on the optical density of the signals.

The final protein detected commonly within the culture media samples had a molecular weight just below 37 kD. This protein was detected in some experimental culture media, but was also detected in control media for both female and male *S. tiburo*. Males do not naturally produce Vtg, so it could not be determined if this 37 kD protein was indicative of Vtg production, or if

the protein was produced naturally by the liver *in vitro*, unrelated to E2 exposure, and had a sequence similar enough to Vtg to allow the antibody to bind.

Ultimately, analysis of *in vitro* Vtg production focused solely on assessing when the 50 kD protein was identified within culture media, as this protein was only found in experimental culture media when the liver tissue was exposed to E2. This protein was not detected consistently but was detected in at least one replicate of media containing 3, 300, and 30,000 pg/mL E2 for all assayed liver, from both male and female specimens. Lack of consistent detection of this protein, and thus lack of consistent Vtg production by the liver *in vitro* could be due to tissue degradation or death during the culture period, or a delayed response of the liver to E2 stimulation. In initial *in vitro* assays, liver tissue was cultured for 24, 48, and 72 hours; Vtg was potentially detected after only 24 hours for one sample, but no Vtg detection occurred for any samples cultured for 48 hours. In *O. mykiss,* Vtg was detected in an ELISA after liver tissue was exposed to E2 for only 24 hours, with concentrations of Vtg increasing with increased culture time and with increased concentrations of E2 (Shilling and Williams, 2000). However, it has been noted that there are significant differences between Vtg production in bony fishes and elasmobranchs. Perez and Callard (1993) noted that concentrations of Vtg found in the plasma of *L. erinacea* were significantly lower than concentrations found in bony fishes. These lower concentrations may be due to the differing reproductive strategies between the two groups, as follicular growth occurs over a longer time period in elasmobranchs in comparison to bony fishes (Perez and Callard, 1993).

These observations lead to two possibilities. It is possible that *S. tiburo* liver was producing Vtg *in vitro*, but concentrations were too low for detection within culture media after only 72 hours. Alternately, it is possible that elasmobranch liver does not respond immediately to

E2 stimulation. Prior studies also noted complications with inducing Vtg protein synthesis in *S. tiburo* (Gelsleichter, unpublished data). In particular, those studies found no evidence of Vtg protein induction in male *S. tiburo* experimentally treated with E2 over 2-8 week periods, despite the ability to induce hepatic Vtg mRNA expression within 48 h of a single E2 injection. This suggests that there may be a significant delay in production of Vtg by the liver after E2 exposure in *S. tiburo*. This hypothesis could be further supported by the fact that E2 concentrations begin to increase in August and reach high concentrations in December. *S. tiburo* liver thus appears to be exposed to high E2 concentrations *in vivo* for a prolonged time period, but Vtg production appears to peak in March, indicating that a prolonged exposure to E2 may be necessary to induce production of Vtg. Analysis of future *in vitro* liver assays may thus instead require assessment of differing levels of Vtg mRNA expression in liver exposed to various E2 concentrations using qPCR techniques, as actual production of the protein may occur over a longer time period than liver tissue can be kept viable in culture media.

While it has been clearly established by previous studies that E2 stimulates Vtg synthesis by the liver, fewer studies have investigated the role that P4 might play in regulating Vtg synthesis. It has been proposed that P4 inhibition of vitellogenesis in viviparous organisms may have ultimately led to the evolution of placentation in eutherian mammals and the loss of vitellogenin altogether, as P4 plays a critical role in maintaining pregnancy in eutherian mammals (Callard et al., 1992; Rothchild, 2003). Several studies have found evidence of P4 slowing or terminating vitellogenesis in both oviparous and viviparous organisms. Evidence suggests that P4 injection delays Vtg synthesis in the oviparous turtle (*Chrysemys picta*), though this delay depends on the dose of P4 administered (Ho et al., 1982). In an oviparous elasmobranch, *L. erinacea*, males injected with P4 after being injected with E2 were found to

have significantly lower concentrations of Vtg present in their plasma when compared to males injected only with E2 for the same time period (Perez and Callard, 1992). In viviparous organisms, no direct experimentation has been conducted with P4 injections, but a general relationship between high plasma P4 concentrations and the termination of vitellogenesis has been noted. In general, viviparity is associated with a postovulatory rather than preovulatory increase in P4 secretion, which has previously been noted for *S. tiburo* (Callard et al., 1992; Gonzalez De Acevedo, 2014). In a viviparous snake (*Vipera aspis*), P4 concentrations rapidly increase towards the end of vitellogenesis (Bonnet et al., 2001). For viviparous elasmobranchs, including the spiny dogfish (*S. acanthias*) and spotted ray (*T. marmorata*), researchers have noted that high plasma P4 concentrations coincide with the absence or diminished presence of Vtg in the plasma (Callard et al., 1991; Prisco et al., 2008). For *T. marmorata*, it was observed that P4 concentrations increased at the time of ovulation, and continued to increase throughout gestation. It was further observed that pregnant *T. marmorata* did have vitellogenic follicles within their ovaries, but their growth was arrested. This suggests that the high P4 levels during pregnancy may inhibit Vtg synthesis by the liver (Prisco et al., 2008).

The present study identified P4 receptors within *S. tiburo* liver hepatocytes, which indicates P4 may play a role in regulating Vtg synthesis. However, plasma concentrations of P4 did not vary significantly by month in *S. tiburo*. There was a slight peak in the month of May, as well as generally higher levels of P4 in the months of March and April. These results are supported by a previous finding that P4 levels are higher in preovulatory, ovulatory, and postovulatory female *S. tiburo* compared to during gestation, with the highest concentrations detected in postovulatory females (Gonzalez De Acevedo, 2014). The highest P4 concentrations were observed in May, and it appeared that Vtg production had ceased for the majority of

females during this month; this result suggests that P4 may play a role in terminating Vtg production. Relatively high concentrations of P4 were also observed in March and April, and fewer females were observed to produce Vtg in April, which again could suggest that P4 might slow Vtg production. The high number of females with Vtg present in their plasma in March despite relatively high P4 concentrations could be explained by Vtg remaining in the plasma even after P4 has perhaps stimulated the liver to cease the protein's production. It should also be noted that the P4 concentrations in March and April were similar to what was observed in October and December, when P4 did not appear to be inhibiting Vtg production. It is possible that the effect of P4 on Vtg production varies depending on what P4 receptor is more highly expressed, as it was noted in a previous study on the painted turtle (*C. picta*) that the effect of P4 on vitellogenesis varied depending on which PR isoforms was more highly expressed (Custodia-Lora et al., 2004). Ultimately, initial evidence suggests that P4 may play a role in slowing Vtg production in *S. tiburo*, with higher P4 concentrations coinciding with an apparent break in the protein's production. However, further investigation is certainly needed into the role of P4 on Vtg production in this species. Clarification of the monthly P4 cycle in *S. tiburo* is necessary to determine correlations between P4 concentrations and Vtg presence in the plasma. Additionally, it would be beneficial to expose slices of *S. tiburo* liver to P4 *in vitro* after exposure to E2 to determine if P4 inhibits Vtg synthesis.

A secondary goal of this study was to use the antibodies and methods developed to detect Vtg in the plasma of other elasmobranch species. The antibodies detected a protein with the expected molecular weight of Vtg (~205 kD) within the plasma of four other elasmobranch species: *C. limbatus*, *C. carcharias*, *C. acronotus*, and *C. longimanus*. All three antibodies also bound to other putative Vtg component proteins present within the plasma of other elasmobranch

species, which may be indicative of Vtg presence within the plasma. These putative Vtg component proteins were detected in the plasma of *G. cuvier*, *C. granulosus*, *D. sabina*, and *P. pectinata.* The majority of these plasma samples were not stored with aprotinin, so the possibility for protein degradation existed. It is thus likely that the antibodies could detect evidence of Vtg production within the plasma of these other species, but detected component proteins rather than the large Vtg molecule due to degradation.

Nevertheless, the detection of Vtg and putative component proteins of Vtg within the plasma of other elasmobranch species suggests that the methods developed in this study could be applicable to characterizing vitellogenesis in other elasmobranch species. It may still be ideal to develop antibodies specifically against the Vtg sequence in each species in order to reduce binding to other proteins and allow for definitive detection of Vtg in the plasma. However, the methods developed for this study will certainly be applicable to other elasmobranch species, allowing for the development of a nonlethal method for determining reproductive periodicity by coupling ultrasonography with testing for Vtg presence in the plasma. Development of such a nonlethal method will be important for elasmobranch conservation and the establishment of potentially more accurate population growth models for various species, such as *C. acronotus*. Preliminary evidence suggests that the Atlantic population of *C. acronotus* may contain both annually and biennially reproducing females, but current management practices consider the population to be biennially reproducing. Accurate assessment of reproductive periodicity is needed in order to assess the reproductive output and assess population growth potential when developing management practices for *C. acronotus* and other elasmobranch species, and the methods developed through this study will allow those assessments to be conducted in a non-lethal way.

Ultimately, this study determined the temporal cycle of Vtg production in *S. tiburo*. The highest number of Vtg positive females occurred in March, with Vtg production continuing into April and May for some females likely due to individual and geographical differences in *S. tiburo* reproductive cycle. Evidence was also found of Vtg production beginning as early as August for some female *S. tiburo*, with production continuing throughout the fall and winter time period. Previous studies stated that follicular development (and thus Vtg production) occurred in the spring for *S. tiburo*, but the present results suggest Vtg production begins much earlier, likely immediately following parturition in the fall. Concentrations of Vtg were determined to be highest in March based on estimates of optical density, with lower concentrations of Vtg found in the plasma in April and May as well as during the fall and winter. The liver was confirmed to be the site of Vtg synthesis in *S. tiburo* based on immunohistochemistry. Further investigation is needed to answer the question of whether granulosa cells produce Vtg; Vtg immunoreactivity was observed but cannot conclusively be used to state that the cells produce rather than store Vtg. Correlations between high plasma E2 concentrations and Vtg presence in the plasma provide evidence that E2 likely stimulates Vtg production, but *in vitro* liver assays were unable to conclusively confirm this hypothesis. There was some evidence of Vtg production by liver tissue upon exposure to E2, but only putative Vtg component proteins were detected in experimental culture media, and these proteins were not detected consistently across replicates of the assay. Future *in vitro* assays may need to occur for longer periods of time, and assessments of mRNA expression levels in the liver may provide a more decisive answer to how E2 influences hepatic Vtg production in elasmobranchs. Preliminary evidence also suggested that P4 plays a role in inhibiting Vtg synthesis, with levels of P4 increasing towards the end of vitellogenesis in March, April, and May in individual females. However, future experimentation is needed to

confirm that P4 does slow or terminate Vtg synthesis *in vitro*. The methods developed for this study were also able to detect Vtg within the plasma of other elasmobranch species. Thus, detection of Vtg within the plasma can develop into a nonlethal method for determining when follicular development occurs. Together with ultrasonography, such a method would help clarify reproductive periodicity for some elasmobranch species, allowing for better estimates of total reproductive output and population growth when setting management decisions for elasmobranch species.

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