AN ABSTRACT OF THE THESIS OF

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Title: An Assessment of the Reproductive Cycle in Black Rockfish, *Sebastes melanops* (Girard, 1856) on the Central Oregon Coast: A Blood Plasma Protein and Sex Steroid used as Potential Indicators of Sex and Stage of Gonadal Maturity

Abstract approved:

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Selina S. Heppell

Because of large declines in abundance of many Eastern Pacific rockfish populations (Genus *Sebastes*), there has been an increasing effort to improve our understanding of the role of spawning population characteristics and individual reproductive success in recruitment variability and population dynamics. Current methods for assessing sex and maturational status in rockfishes are lethal, so fishery managers would benefit from a non-lethal method to assess the reproductive potential of rockfish populations. I developed a biochemical assay to determine sex and stage of female maturity in black rockfish (*Sebastes melanops*) using a blood plasma indicator vitellogenin (VTG). The specific objectives of this research were to: 1) describe the annual hormonal and gonadal changes associated with reproduction and 2) describe the timing of reproduction in female black rockfish of different age classes.

Estradiol-17β (E_2) and VTG concentrations were compared with histologically determined ovarian stages of female maturity (i.e. pre-vitellogenic, early vitellogenic, mid-vitellogenic, post-vitellogenic, oocyte maturation, and post-spawned) from 325 female black rockfish sampled by hook-and-line from the central Oregon coast from September 2003 to January 2005. I also measured E_2 and VTG in male fish from the same time period. VTG concentrations detected in field-collected blood samples reflected the seasonal cycle reported in previous studies. Mean concentrations of VTG in mid-vitellogenic, post-vitellogenic, and females undergoing oocyte maturation can be used to distinguish between pre-vitellogenic females and early vitellogenic females during the spawning season ($P<0.05$). Females can be distinguished from males based on VTG concentrations ($P<0.0001$), with the exception of pre-vitellogenic and post-spawned females (immature fish and mature female fish in non-reproductive summer months). Mean E_2 concentrations were not as accurate for identification of sex and each individual stage of female maturity, although mid-vitellogenic and post-vitellogenic females had significantly higher concentrations of E_2 than pre-vitellogenic females ($P<0.05$). Mean concentrations of E_2 in mid-vitellogenic females, post-vitellogenic females, and females undergoing oocyte maturation were higher than in males ($P<0.0001$).

A Discriminant Function Analysis (DFA) identified VTG as the best predictor for predicting sex from plasma samples, with, 82% of fish correctly classified by sex. Of the 100 females and 30 males examined, 68% of females and 97% of males were correctly classified. The DFA choose plasma VTG, total length, and weight as the

best predictors of stage of maturity in females and some stages could be distinguished from each other with ≈65% accuracy.

The second goal of this research was to use the biochemical assay to investigate the differences in timing of reproduction in young and older females in this species. In the fall (September-November) females age 5-8 made up 43 % of early and mid-vitellogenic (stage 3 and 4) females, where as, age 9+ females made up 100% of the females that were post-vitellogenic (stage 5) and undergoing oocyte maturation (stage 6). Females' ages 6-8 captured in the winter months (December-February) made up 90% of the mid- and late vitellogenic (stage 4 and 5) and 100% of post-spawned (stages 7 and 8) fish. In the spring (March-May) 40% of post-spawned females were age 8 and 60% were age 9+. These results suggest that older females have more of a protracted spawning period than younger fish. Females from all age categories showed a similar trend of hormone and protein cycling. Concentrations of E_2 and VTG increased throughout September-December, peaked in February, decreased and remained low until the end of August. Concentrations of E_2 for early vitellogenic (stage 3) fish of the age 9+ were significantly higher than in females ages 3-5. Females' age 9+ undergoing oocyte maturation (stage 6) had higher concentrations of E_2 than females ages 6-8. Early vitellogenic (stage 3) and mid-vitellogenic (stage 4) females ages 6-8 and 9+ had higher concentrations of VTG than in females ages 3-5. Small samples sizes were prohibitive in comparing VTG and E_2 concentrations between all age groups and stages of maturity, and I did not find conclusive evidence of differences in seasonal VTG or E_2 levels among age groups.

Potentially, this newly developed vitellogenin assay may be used for other rockfish species, providing a non-lethal assessment tool to differentiate between sex and stage of maturity in females.

An Assessment of the Reproductive Cycle in Black Rockfish, *Sebastes melanops* (Girard, 1856) on the Central Oregon Coast: A Blood Plasma Protein and Sex Steroid used as Potential Indicators of Sex and Stage of Gonadal Maturity

by

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APPROVED:

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Major Professor, representing Fisheries Science

_____

Head of the Department of Fisheries and Wildlife

_____

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Brooke Martin-Mills, Author

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DEDICATION

I would like to dedicate this thesis to my padre, Jack Martin, who passed away before its completion. My Dad always reminded me to keep my faith in God, and through him I could do anything I set my mind to.

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General Introduction.

Many Pacific west coast rockfish populations are in decline. Although the exact mechanisms behind this decline are unknown, proposed reasons include overfishing and the unique life history characteristics of the *Sebastes* genus (Parker *et al.* 2000). *Sebastes* exhibit slow growth, low natural mortality, and variable recruitment, which all contribute to their vulnerability and dampen species recovery. Additionally, the lack of information pertaining to their reproductive biology makes managing rockfish populations especially difficult. Inaccurate reproductive information can lead to overly optimistic productivity estimates, and can complicate the computation of optimal harvest rates (Leaman 1991). Most of the reproductive biology for these rockfishes has been obtained through histological evaluation of the gonads (Love *et al*. 2002). However, gonad collections from summer surveys are often made during periods of reproductive inactivity, making maturity classifications difficult (Gunderson *et al.* 1980; Wyllie Echeverria 1987). There is a need to obtain correct classifications of maturity and timing of reproduction for these species.

Of the sixty-five species of rockfish on the Oregon coast, black rockfish is one of the more-extensively studied (Love *et al*. 2002). Although black rockfish have suffered recent declines, they are still commonly found in Oregon nearshore waters. Coast-wide surveys have generated substantial information on their distribution, and basic reproductive parameters such as age and length at maturity are well-documented.

These factors make black rockfish ideal subjects for further study of their reproductive biology and its management applications.

Black rockfish (*Sebastes melanops*) are widely distributed along the west coast of North America, inhabiting nearshore rocky reefs from Huntington Beach, California to Amchitka Island, Alaska (Hart 1973; Love *et al.* 2002). Like all members of their genus, black rockfish are iteroparous, moderately fecund livebearers—viviparous with internal fertilization (Moser 1967). Most rockfishes are typically long-lived and slow growing with delayed sexual maturity. Age and length at maturity in female black rockfish is variable between geographic locations (Wyllie Echeverria 1987; Wallace *et al*. 1999; Ralston and Dick 2003; Bobko and Berkeley 2004) (Table 1). Several studies have shown that the variation in age at maturity may be attributed to latitude and depth (Gunderson *et al.* 1980; Kuzis 1985).

Table. 1. Estimated age and size at 1st, 50%, and 100% maturity and maximum age of female black rockfish for Alaska, Washington, Oregon, and California. All lengths were converted to fork length for comparison given in cm.

	1st		50%		100%		
Location	yr	FL	yr	FL	yr	FL	Max Age
Alaska	—	—	10—13	39	—	—	50
Washington	6	—	7.9	42.2	—	—	20
Oregon							
2003	4	30	8	40	9	45	25
2004	—	34	7.5	39.4	—	45	17
California	5	29	7	40	11	47	—

In Alaska, the maximum age for female black rockfish has been reported at 50 years and a maximum length recorded of 648 mm (Meyer 2000). However, recent research reports the oldest black rockfish females found in nearshore waters on the central Oregon coast were no older than 17 years (Bobko 2002; Chapman 2004).

Fecundity in large female black rockfish may be as high as 1,200,000 eggs per season (Haldorson and Love 1991), although fertility is always less than fecundity due to unfertilized eggs, atresia, and/or resorption of some larvae. Bobko and Berkeley (2004) reported absolute fecundity for a post-fertilized 6-year old was only 58% of the estimated absolute fecundity for a pre-fertilized fish of the same age. Additionally, absolute fertilized fecundity estimates increased with age from 299,000 embryos for a six-year old individual to 948,000 embryos for a sixteen-year old female.

Male black rockfish are in spawning condition in July and August off Oregon (Love *et al.* 2002). Males inseminate females, and females store sperm for several weeks to months before fertilization occurs (Moser 1967; Wyllie Echeverria; Love *et al.* 2002). For one species rockfish (*Helicolenus lengenchi)*, it has been discovered that females have a specialized structure for the collection and preservation of sperm (Lisovenko 1979). Fertilization occurs in female black rockfish primarily in January and February (Bobko and Berkeley 2004). Once eggs are fertilized, gestation of developing embryos is estimated to be 37 days at 10°C (Boehlert and Yoklavich 1984). Female black rockfish exhibit matrotrophy where the mother supplies nutritional energy to her developing embryos (Boehlert and Yoklavich 1984). Females exhibit group-synchronous oocyte development and extrude one brood of

larvae per year (Moser 1967; Wyille Echeverria 1987). Females give birth to live larvae from mid-January to mid-March (Bobko and Berkeley 2004). Researchers have observed a trend of older females extruding their larvae earlier in the spawning season than younger females (Berkeley and Markle 1999; Bobko and Berkeley 2004). Berkeley *et al.* (2004a) showed that older females also produced offspring that grow faster in both length and weight, survived longer in the absence of exogenous food supply, and exhibited lower mortality rates than offspring from younger mothers. Larvae are pelagic for the first few months and are eventually transported into nearshore coastal estuaries and intertidal zones (Laroche and Richardson 1980; Bayer 1981).

Black rockfish have increased in popularity in the Oregon sport fishery over the last decade, comprising as much as one third of all landed groundfish (Love *et al.* 1996). The adult population of black rockfish off the Oregon Coast has been fished at moderately high levels to have reduced the number of age classes in the population (Wallace *et al.* 1999). The continued decline in the population of mature adults and the loss of older age classes may have severe negative ramifications for recruitment and population sustainability (Berkeley *et al.* 2004b). The basic life history parameters needed for stock assessments are age and/or size at maturity, fecundity, maximum age and rates of natural mortality. The lack of the basic reproductive parameters, particularly age and size at maturity has presented a challenge for managers to predict optimal harvest rates for many rockfish species. Hence, it is important to develop

methods for quick and accurate identification of mature spawning adult females to measure stock productivity.

Hormonal control of reproduction

The neuroendocrine system initiates and controls the process of gametogenesis and steroidogenesis primarily through activation of its hypothalamus-pituitary-gonad axis (Kah *et al.* 1993). The activation of this axis is controlled environmental and endogenous cues (Munro *et al.* 1990). Sensory structures in the brain receive these cues and activate the hypothalamus which, in turn stimulates the release of gonadotropin releasing hormone (GnRH). GnRH primarily stimulates the release of gonadotropins (GTH) from the pituitary (Yu *et al.* 1991). Gonadotropins (GTH-I and GTH-II) stimulate gametogenesis and steriodogenesis in the ovary. In some teleosts (e.g., salmonids), it is thought that GTH-I is analogous to mammalian follicle stimulating hormone (FSH), and is prevalent during ovarian follicle growth. Increased FSH (GTH-I) levels in the blood induces follicular production of estradiol-17β (E_2), where as, luteinizing hormone (LH) has been found to predominate during oocyte maturation (Redding and Patino 1993).

The commencement of hepatic vitellogenesis is initiated by ovarian E_2 production (Specker and Sullivan 1994). Vitellogenin, a large glycophospho-lipoprotein, is produced by the liver in response to E_2 stimulus, and is transported via the circulatory system to growing oocytes. Throughout oocyte growth and development, FSH stimulates the production of testosterone (T) in the follicular layer

of the ovarian follicle and the subsequent aromatization of T into E_2 occurs in the granulosa cells (Fostier *et al.* 1983). During pre-vitellogenic growth large amounts of glycoproteins are synthesized by the oocytes, lipid deposition is initiated, and the ovarian follicle increases substantially. For the duration of vitellogenesis, vitellogenin enters the ovarian follicle, is processed into yolk proteins, and is stored in yolk globules or platelets. Lipovitellin is one of the major yolk proteins made up of amino acids and lipids, which are an important nutritional source that supports embryonic development. An extensive review of ovarian follicle growth, maturation, and ovulation in teleost fish has been conducted in response to the advances made in endocrine regulation and mechanisms of ovarian follicle maturation (Patino and Sullivan 2002). Vitellogenin has been found in high concentrations in the blood stream of maturing females' weeks to months prior to spawning (Nunez-Rodriquez *et al.* 1989; Kwon *et al.* 1990). Researchers have provided convincing evidence that vitellogenin can accurately assess maturation in female fish (Johnson and Casillas 1991; Heppell and Sullivan 1999; Linares-Casenave 1993).

There is limited published work on hormone profiles in the two main genera of rockfish (*Sebastes* and *Sebasticus*). Prior to 1991, little was known about the endocrinology of the female reproductive cycle in rockfishes. As in most fishes, E_2 is the dominate hormone involved in vitellogenesis, and 17 α, 20ß-dihydroxy-4-pregnen-3-one (DHP) plays an important role in oocyte maturation and the maintenance of gestation (Nagahama *et al.* 1991; Takano *et al.* 1991). Kwon *et al*. (1999) found that E_2 concentrations increased during oocyte maturation and L-thyroxine (T_4) increased

prior to gestation suggesting that T_4 may be required for sustaining gestation. Mori *et al.* (2003) were the first to report on serum profiles of vitellogenin in relation to the production of sex steroids and gonadal development in female rockfish. For *Sebastes schlegeli*, E_2 and VTG levels increased during the primary yolk globule stage. Concentrations of E_2 and VTG remained elevated throughout the secondary and tertiary yolk globule stages. During gestation, E_2 and VTG levels decreased dramatically and DHP concentrations increased. Concentrations of DHP decreased during the month of parturition. Resting (post-spawned) females had low levels of E_2, VTG, and DHP until the onset of vitellogenesis (Mori *et al.* 2003). As of yet, the endocrinology of the reproductive cycle has not been examined for any species of west coast rockfish.

Potential applications for biochemical assays for reproduction and maturity

Researchers have successfully developed a non-lethal method for determining sex and maturity in numerous fish species including English sole (*Parophrys vetulus*), white sturgeon (*Acipenser transmontanus*), temperate basses (*Morone saxatilis*), and gag grouper (*Mycteroperca microlepis*) (Johnson and Casillas 1991; Linares-Casenave 1993; Heppell *et al.* 1999; Heppell and Sullivan 1999). The inability to detect maturity without sacrificing the fish facilitated the development of a biochemical test, an enzyme-linked immunosorbent assay (ELISA), for sex and maturity detection. The assay identifies protein (vitellogenin) in the blood. Blood plasma indicators such as VTG, E_2 and calcium (Ca^{2+}) have been used as a non-invasive method for determining

sex and status of maturation (Johnson and Casillas 1991; Heppell and Sullivan 1999; Webb *et al.* 2002).

Potentially, this method may be applied to current black rockfish studies conducted by state agencies. Oregon Department of Fish and Wildlife is conducting a long-term study to evaluate if Passive Integrated Transponder (PIT) tags can be used to effectively estimate exploitation rates of black rockfish. The data collected are used in a population estimation model to determine exploitation rates and population size each year, and additional reproductive information obtained with this non-lethal method could provide accurate size and age at maturity parameters needed in these types of models. Creating an ELISA for black rockfish is not only important to accurately access the maturity status of females and those females contributing to the spawning stock biomass, but also to create a means to measure the reproductive potential of other species of the genus *Sebastes*.

Finally, with more in depth knowledge of the reproductive physiology of rockfishes federal and state agencies could engage in artificial production of juveniles as a restorative effort to rebuild depleted stocks. In response to heavy utilization of many rockfish species institutes in Japan have engaged in artificial larvae production and juvenile release programs (Kusakari 1995 *as cited in* Mori *et al.* 2003). In order to develop efficient techniques for these programs and to monitor the reproductive potential of rockfish populations, basic information on reproductive physiology is necessary.

CHAPTER 2. A BLOOD PLASMA PROTEIN AND SEX STEROID USED AS POTENTIAL INDICATORS OF SEX AND STAGE OF GONADAL MATURITY IN BLACK ROCKFISH, *SEBASTES MELANOPS* FROM THE CENTRAL OREGON COAST

Introduction

The extreme longevity and late sexual maturity seen in rockfishes (*Sebastes* spp.) have allowed them to persist in highly variable environments. However, these same traits increase their susceptibility to over-exploitation (Parker *et al.* 2000; Heppell *et al.* 1999; Heppell *et al.* 2005). In response to the relatively recent discovery that certain species of rockfish have a greater longevity than previously thought, a re-examination of stock assessments has occurred resulting in the reclassification of several rockfish species as over-fished (PFMC 2003). Rockfish reproductive value increases with age due to increased fecundity, and they exhibit slow growth rates post-maturity. New information on offspring quality and timing of reproduction in older rockfish is leading fishery biologists and managers to reemphasize the importance of maintaining healthy age structures in their populations (Berkeley *et al.* 2004b). Reproduction provides a key to the future success of these populations and therefore understanding the reproductive biology of these species has become crucial for successful management.

While research is fairly extensive on the basic biology of rockfishes of *Sebastes* (Love *et al.* 2002), the literature pertaining to the annual reproduction and spawning cycles of the 65+ species of rockfish that inhabit the waters of the Pacific Coast of North America is fragmentary. With the current lack of reproductive

information, researchers are forced to use the best available data for each species and often pool data into a multi-species complex to predict reproductive effort (Parker *et al.* 2000; PMFC 2003). Successful prediction of status and likely trends of rockfish populations requires knowledge of the stock composition with regard to sex and maturational status.

One of the primary goals in fisheries monitoring and assessment involves identification of reproductively mature females (Parker *et al.*2000). The current method of maturation assessment (gonadal histology) requires killing the fish in order to gather reproductive information, which could be detrimental to already overfished populations (Heppell *et al.* 1999; Webb *et al.* 2002). The development of a non-lethal method to identify sex and maturity for all rockfish species of *Sebastes* could greatly enhance the ability of fisheries managers to evaluate a stocks reproductive potential. In addition, such a method would facilitate gathering basic information pertaining to the reproductive physiology of poorly known species that are lacking stock assessments. My research is directed at developing the biochemical techniques to determine sex and maturational status of black rockfish (*Sebastes melanops*), and to assess if this non-lethal methods could provide a suitable substitute for histological examination.

Materials and Methods

Sample Collection

Black rockfish (*Sebastes melanops*) were sampled by hook and line from the recreational fishery in Newport and Depoe Bay Oregon and additional fish were

sampled by spear fishing as a secondary method collection (38.855 to 44.947 N latitude, and 94.799 to 124.117 W longitude). Fish were collected during monthly sampling trips from September 2003 through January 2005 (N = 325). Paired gonads, muscle tissue, otoliths, and blood were collected from each individual fish. Blood was collected from the caudal vein with heparinized syringes (21G) immediately after capture. When possible blood was centrifuged aboard ship and all samples were stored on ice for transportation to the Hatfield Marine Science Center. Fish were measured for total length (TL) to the nearest millimeter and weighed to the nearest gram (whole fish). Sagittal otoliths were cleaned, dried and stored in coin envelopes for subsequent age determination. An otolith from each female was aged using the established break and burn method (Chilton and Beamish 1982) by Sandra Rosenfield at Washington Department of Fish and Wildlife (Olympia, Washington).

Tissue and blood collection

The gonads of each female (N = 162) and male (N = 163) were weighed (nearest 0.01 gram) and samples were preserved in 10% phosphate-buffered formalin. Gross maturity was recorded for each female specimen according to Gunderson *et al.* (1980). In order to detect if there were differences between the left and right ovary from an individual female, three cross sections of each ovary were taken (anterior, center, and posterior). Gonad tissue from females were embedded in paraffin, sectioned at 7 μm, and stained by hematoxylin and eosin (Luna 1968). Slides were examined under a compound scope (4-100x power), and the germ cells were scored

for stage of development according to the modified protocol of Shaw (1999) (See Table 2). Stage-1 (oogonia and chromatin nucleus), and stage-2 (early and late perinucleous) female fish were pooled into one group, which were both determined to be pre-vitellogenic and represent either immature fish (females not yet capable of spawning) or fish that were not reproductively active. Stage-3 through stage-6 females (early vitellogenesis through oocyte maturation) was considered maturing. Females in stage-7 (post-ovulatory follicles) and stage-8 (reabsorbing larvae) were categorized as mature (Table 2).

Table 2. Stages of ovarian development identified from gonadal biopsies modified from Shaw (1999) for female black rockfish (*Sebastes melanops*).

	Stage	Description
Pre-vitellogenic	1	Oogonia and chromatin nucleolus (8-20μ)
Pre-vitellogenic	2	Includes early and late perinucleus, and oil vacuole (25-150μ)
Early vitellogenic	3	Primary yolk stage (1° yolk): appearance of acidophilic (pink) yolk granules (165-240μ)
Mid-vitellogenic	4	Secondary yolk stage (2° yolk): yolk granules increase in number and size (200-350μ)
Post-vitellogenic	5	Migratory nucleus: nucleus moves from center of oocyte to the periphery (325-670μ)
Oocyte maturation	6	Nucleus disappears replaced by oil vacuoles and yolk globules coalesced into a yolk mass throughout the oocyte (440-700μ)
Post-ovulatory	7	Ovaries contain numerous empty post-ovulatory follicles and the next generation of oocytes
Post-spawned	8	Ovaries contain reabsorbing larvae and atretic follicles along with the next generation of oocytes

The blood plasma from these females was separated by centrifugation, snap frozen with dry ice, and stored at -80°C for later use. The steroid, estradiol-17β was extracted from the blood plasma and analyzed by radioimmunoassay (RIA). The

vitellogenin protein from blood plasma was analyzed by Enzyme-Linked Immunosorbent Assay (ELISA). Because of faulty machinery (NOVA blood analyzer), I was unable to use plasma calcium concentrations as a potential classification tool for maturity in female black rockfish. This method warrants further investigation, because Ca^{2+} concentrations are much simpler and less expensive to measure than VTG concentrations. In maturing female fish plasma VTG and plasma calcium (Ca^{2+}) concentrations increase throughout the spawning period. VTG is a carrier protein that attracts positively charged ions such as Ca^{2+}.

Steroid Radioimmunoassay and the development of an ELISA

Radioimmunoassay

The E_2 steroid was extracted from plasma following the method of Fitzpatrick *et al.* (1987) and modified by Feist *et al.* (1990) for RIA analysis. Plasma from female (N = 100) and male (N = 30) black rockfish were used in the RIA. All samples were analyzed in duplicate. On day one of the assay, 50 µL of plasma from each sample, internal steroid (high and low), and inter-assay plasma pool (N = 4) was extracted twice with 2 ml of diethyl ether. Tubes were vortexed vigorously with ether for 15 seconds, and snap frozen with liquid nitrogen for 10 seconds to remove the aqueous phase steroids. Steroid standards (250, 125, 1.25, 62.5, 31.25, 12.5, 6.25, 3.125, and 0 pg/tube) were dried in a Speed-Vac centrifuge for 15 minutes. Experimental samples (50 µL) were diluted in 150 µL PBSG, internal steroid (10 µL) was diluted in 190 µL PBSG, and 200 µL PBSG was added to total count (TC), non-specific binding (NSB),

and standards. The steroid antibody was added to all standards and experimental samples, with the exception of TC and NSB tubes. Steroid working solution was added to each tube, and tubes were covered and incubated overnight at 4°C. On day two of the assay, tubes with the steroids were resuspended in 500 µL of phosphate-buffered saline with gelatin (PBSG), vortexed for 60 seconds, and 50 µL were assayed. A charcoal solution (0.200 g charcoal and 0.3125 g dextran/ 50 ml PBSG) was used for the assay to reduce nonspecific binding. The recovery efficiency for E_2 were determined by adding tritiated steroids to tubes containing the inter-assay plasma pool (N = 4), which were extracted as described above. The average recovery efficiency for E_2 was 83%, respectively. The assay results were corrected for recovery. The intra and interassay coefficients of variation for this assay were both less than 5%, and 10%, respectively. Steroid concentrations determined by the RIA were validated by verifying that serial dilutions were parallel to standard curves.

Production of Vitellogenin (VTG) for Immunizations

Fish handling, husbandry, injection and euthanasia followed procedures approved by Oregon State University's Institutional Animal Care and Use Committee, Permit 2951. Ten immature black rockfish were captured by hook and line from the recreational fishery in Newport, Oregon, September 2003 ($N = 7$ males, and $N = 3$ females). The fish were held in live tanks aboard the fishing vessel and were transported in oxygenated coolers to the Hatfield Marine Science Center Laboratory (Newport, Oregon). Fish were allowed to acclimate to a 2500 liter flow through

seawater tank over a week's time. Rockfish were fed twice per week with live fish including bay anchovy (*Anchoa mitchilli*), shiner surfperch (*Cymatogaster aggregate)*, pile surfperch (*Damalichthys vacca*), and Pacific sandlance (*Ammodytes hexapterus*) that were caught by beach seine in Yaquina Bay (Newport, Oregon). The rockfish diet with shrimp (*Upogebia pugettensis*) and frozen fish when other prey species were unavailable.

Each of the ten rockfish was injected weekly with E_2 weekly beginning on October 1, 2003 and injections continued for five weeks. Fish were injected with 1 mg/kg^{-1} body weight of E_2 dissolved in propylene glycol at a concentration of 5 mg/ml (E_2, Sigma Chemical Company, St. Louis, Mo.). Two booster injections of E_2 were administered in weeks six and week seven. Fish were killed and the final blood collection was performed at the end of week seven. The blood from each of the ten fish was drawn with 5ml heparinized syringes fitted with 21 gauge needles syringes from the caudal vasculature. Approximately, 6-13 ml of blood was taken from each fish. Blood was transferred to 10 x 75 mm test tubes and samples were treated with the protease inhibitor aprotinin (Sigma Chemical Company, St. Louis, Mo). The tubes were maintained on ice until centrifuged at 3000 rpm for 15 minutes.

The blood plasma was transferred with heparinized pipettes to micro centrifuge tubes, snap-frozen on dry ice, and stored at -80°C for VTG purification. Additional samples of black rockfish plasma in the initial development of the ELISA and were collected from naturally vitellogenic females during vitellogenesis (October). Blood collection and processing was performed as described above.

Purification procedures for black rockfish vitellogenin

Purification of vitellogenin was preformed by Kevin J. Kroll, Biotechnology Program, University of Florida, Gainesville FL. Vitellogenin purified from blood plasma from the E_2 injected fish was performed by anion exchange chromatography (Kroll 1990; Kroll *et. al*. 1991). Briefly, two ml of plasma was loaded into a DEAE-Sephacel column equilibrated with 50mM Tris-HCl buffer containing 350mM NACl, and 0.05% NaN3 (pH 8.0). The column was eluted with a gradient of 0.0-0.3 NaCl at a flow rate of 1.69 ml/min and fractions containing vitellogenin were collected. The elution profile was monitored at and analyzed for absorbance at 280 nm. The mean protein concentration was 0.45 mg/ml (Bio-Rad standard protein assay).

Antibody production

The purified vitellogenin was sent to the monoclonal antibody facility at the University of Oregon for the production of the antiserum. Two New Zealand white rabbits (~4 kg body weight) were immunized with 500 µg purified vitellogenin. The vitellogenin was dissolved in 2.0 ml physiological saline, emulsified with 2.0 ml complete Freund's adjuvant, and injected subcutaneously. Subsequently, boosters of 100 µg vitellogenin with incomplete Freund's adjuvant were given over several weeks. Blood was collected from ear arteries 2-3 months after the primary immunization, allowed to clot overnight at 4°C, centrifuged at 4,000 x G for 30 minutes, and stored at -20°C. Laboratory methods may vary.

The antiserum was selected for the ELISA by titration experiments. A 96-well microtiter plate (Costar) was coated overnight (4° C) with 100 µl/well carbonate-bicarbonate buffer solution at a concentration of 5 µg/ml ($NaHCO_3$-Na_2CO_3) (pH 9.6) and covered with acetate microplate sealer. The antiserum was serially diluted from each of the two rabbits from 1:100 to a final concentration of 1:80,000 and standard curve samples were stored overnight (4° C) in cultured test tubes. The following day the plates were then washed in PBS-Tween (pH 7.3). Binding sites in the wells were blocked by addition of 300 µl of 2.5% NGS in carbonate buffer followed by incubation for 1 h at 37°C. The rabbit antiserum (primary antibody) was added to the wells (final volume of 50 µl/well) and incubated at 37°C for 1 hour. The goat antirabbit IgG (peroxidase conjugate secondary antibody, SIGMA) was diluted in PBS-Tween (1:2500) added to each well (300 µl/well) and incubated for 1 hour at 37°C. The plate was washed, incubated in the dark with the reactive enzyme substrate—3, 3′, 5, 5′-tetramethylbenzidine (TMB), and after approximately 11-13 minutes, peroxidase (1 M H_3PO_4) was added to stop the reaction. The absorbance was determined using a microplate reader at 450 nm.

Enzyme-linked Immunosorbent Assay

The rockfish VTG assay was developed following the published methods for gag grouper (*Mycteroperca microlepis*) (Heppell and Sullivan 1999). The development of the vitellogenin ELISA included four major steps: (1) induction of vitellogenin synthesis; (2) purification of plasma vitellogenin; (3) production of

vitellogenin-specific polyclonal antibody and (4) laboratory optimization of the assay. The assay includes 4 consecutive steps (microplate coating with antigen—primary antibody, competition reaction, incubation with secondary antibody, and enzyme substrate degradation). The optimal concentration of coating antigen (purified vitellogenin) and final antisera titer were established by titration.

Standards were made by serially diluting stock solutions of purified VTG in PBST (0.01 M NaPO4, 0.15 M NaCl, and 0.05% Tween-20, pH 7.3) containing 2.5% normal goat serum (PBST-NGS). The VTG standard concentrations ranged from 2000, 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, and 3.9 ng/ml. Plasma dilutions were also made in PBST-NGS.

Ninety-six well microplates were coated with 100 µl per well of either a 500 µg/ml solution of VTG in carbonate buffer (0.05M sodium carbonate, pH 9.6) or an equal concentration of bovine serum albumin (BSA, used to measure nonspecific binding) in the same buffer and incubated for 2 hours at 37°C. Non-specific binding sites in the wells were blocked by addition of 300 µL of 2.5% NGS in carbonate buffer followed by incubation for 1 h at 37° C. The 250 µl of diluted VTG (standard), inter-assay plasma pool (quality control), or individual plasma samples 1:2 with primary antibody solution (diluted 1:20,000 in PBST-NGS) were transferred to 12 X 75 mm glass culture tubes (VWR). The tubes were vortexed and incubated at 37° C for 1 h. From each culture tube 300 µl of solution was removed in triplicate and distributed into the wells (100 µl/well) of the microtiter plate, which were incubated at 37° C for 1 h. The plate was then washed three times with PBST in a microplate washer, and

100 µl of secondary antibody solution was added per well and incubated for 1 h at 37°C. The secondary antibody solution was diluted 1:5,000 in PBST-NGS. The plate was again washed as described above and then 100 µL of enzyme substrate—TMB was added to each well. The enzyme reaction was allowed to proceed for approximately 11-13 minutes, after which time the color development reaction was halted by the addition of 100 µL of 1 M H_3PO_4. Absorbance was then read at 450 nm on a microplate reader.

For VTG recovery analyses, samples of plasma from immature and male black rockfish or PBST-NGS were spiked with a known quantity of purified VTG. Recovery was defined as the amount of VTG measured in the plasma sample relative to the amount detected in the PBST-NGS (control) sample. The PBST-NGS sample was identical to one of the standards (125 ng VTG/mL) used in the VTG ELISA standard curve. Intra-assay variability, measured as a percentage of the coefficient of variation (100 X SD/mean), was assessed by conducting replicate measurements (N = 12) in a single ELISA (plate) of a sample of blood plasma pooled from several vitellogenic black rockfish. Inter-assay variability was evaluated in the same manner by serially diluting plasma from naturally vitellogenic females analyzed in several different assays (N = 8).

After the laboratory optimization phase, the assay was used to determine plasma VTG concentrations in 100 females and 30 males. Due to high individual variability in plasma VTG content, samples from each group of females (stages 2-8) were measured at different dilutions ranging from 1:100 to 1:20,000.

Statistical analyses

Statistical analyses were performed to determine if there were significant differences in age, weight, and total length in the correlations with response variables. Vitellogenin concentrations were correlated with E_2 concentrations, and histological stage of ovarian development in individual females. Differences in plasma concentrations of VTG and E_2 were statistically tested by one-way analysis of variance (ANOVA). The Fisher's protected least significance difference (PLSD) and Bonferroni for mean comparison were chosen to account for type I and II errors. For the Fisher's (PLSD) test, a p-value of less than 0.05 was considered to be statistically significant ($\alpha = 0.05$). A p-value less than 0.0024 were considered to be statistically significant ($\alpha = 0.05$) for the Bonferroni mean comparison test. All analyses were conducted using S-Plus for Windows, release 7.0 (Insightful Corperation, Seattle, WA).

A multivariate statistical procedure, Discriminant Function Analysis (DFA), was used to find a set of variables that predict membership of groups—sex and or stage of female maturity in black rockfish. Stepwise DFA was conducted using the variables E_2 and VTG concentrations, as well as, total length and weight to choose the best predictor(s) of sex. The variables used to choose the best predictor(s) of stage of female maturity were E_2 and VTG concentrations, as well as age, weight, and total length.

The DFA was used to predict maturity based on a range of criteria and was tested by: (1) Wilks' Lambda was used to test if the discriminate model was

significant, (2) R-squared value was the fraction of the variance in the data that was explained by a regression, (3) canonical correlation was used to measure the association between the groups, specifically the variance, and (4) F-value was used to measure the distance between individual distributions. The significance level the model was $\alpha = 0.05$. Quadratic DFA was then conducted with the variables chosen in the stepwise procedure to determine the number of observations and percent classified into the six groups of stage of maturity. Stage 1 and 2 were pooled, stages 3, 4, 5, 6, and 8 represented the six groups of stage of maturity. Stage 7 was excluded from the analysis because there was only one fish in this group.

Cross-validation was used as a method for estimating the error rates in the chosen discriminant functions (Khattree and Naik 2000). A discriminant rule is constructed from the data set as an observation is removed from the quadratic discriminant function. The constructed rule was used to classify the observation that was left out, and this procedure was repeated of all observations. The number of misclassified observations for each group was calculated and overall error rates were computed. All DFA analyses were conducted using Statistical Analysis Systems for Windows, release 6.10 (SAS Institute, Cary, North Carolina).

Results

Black rockfish were collected from the Oregon recreational fishery by hook and line from October 2003 to January 2005 (Figure 1). Fish were collected from Depoe Bay from October 2003 through September 2004. However, because of

dangerous weather conditions sampling trips out of Depoe Bay during the winter months of 2004 were canceled. The Oregon groundfish fishery was closed September 2, 2004, and sampling trips were no longer possible off of recreational charter boats. Thereafter, black rockfish were collected off of Newport only on Oregon State University research vessels. Due to inclement weather—gale-force winds the collection of samples were not feasible for the month of November 2003 and December 2004.

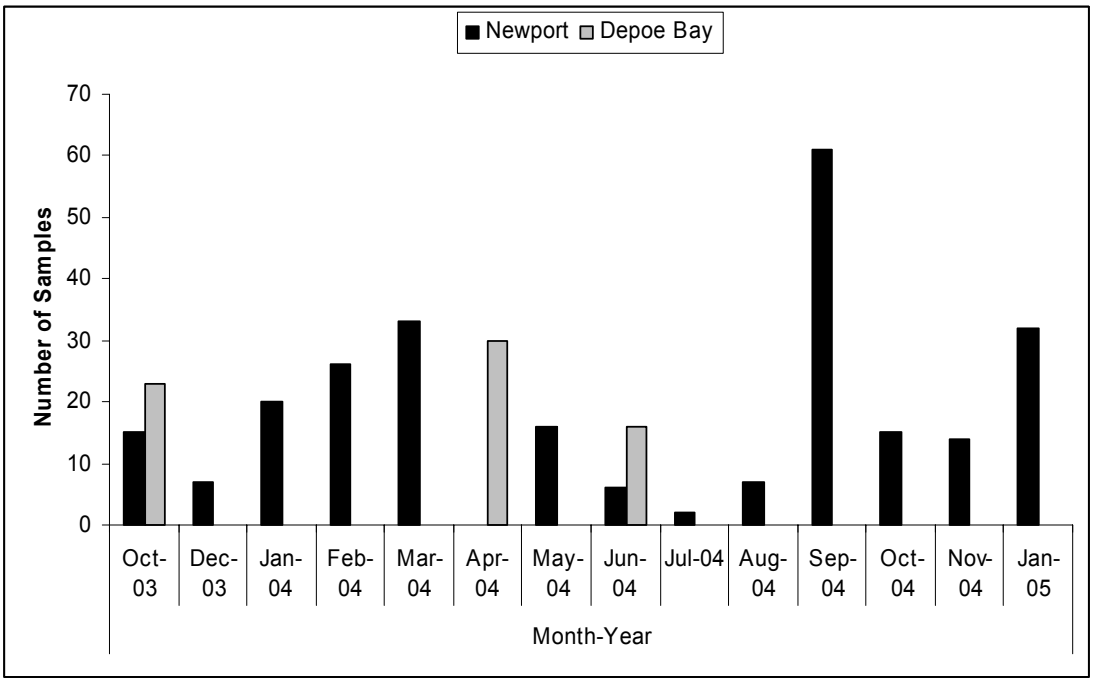

Figure 1. Distribution of sample sizes for black rockfish (*Sebastes melanops*) collected from the Oregon recreational fishery from October 2003 to January 2005. Fish were sampled from Newport and Depoe Bay, Oregon from October 2003 to September 2004. Due to the 2004 groundfish closure, fish were sampled from Newport Oregon only from October 2004 to January 2005.

Of the 325 fish examined in this study, 105 were pre-vitellogenic females, 52 were maturing (stage 3-6), 4 were mature (stage 7-8) females, and 163 were male. Stages 1 and 2 were pooled and included females that have oocytes that were pre-

vitellogenic (Figure 2a). Pre-vitellogenic females had no distinguishable yolk platlets within the oocyte. Stage-3 females were in early stage of vitellogenesis—primary yolk stage (Figure 2b). Stage-4 females were in the middle of vitellogenesis—secondary yolk stage (Figure 2c). Stage-5 females were post-vitellogenic with migrating nuclei (Figure 2d). Stage-6 females were in the stage of oocyte maturation, where the nucleus disappears and is replaced by oil vacuoles, and yolk globules coalesce (Figure 2d). Stage-7 females' ovaries contained numerous empty post-ovulatory follicles (Figure 2e). Stage-8 females' (post-spawned) ovaries contained reabsorbing larvae, atretic follicles, and next generation oocytes. (Figure 2f).

Histological examination and measurement of average ovarian follicle diameter revealed 105 stage-2 females, 19 stage-3 females, 23 stage-4 females, 6 stage-5 females, 4 stage-6 females, 1 stage-7 female, and 4 stage-8 females (Table 1). The males in this study were not classified into maturation categories. Maturing females were described as either going through vitellogenesis (stages 3 and 4), or post-vitellogenic (stage 5), or were underoing oocyte maturation. Females that were post-ovulatory (stage 7), or post-spawned (stage 8) were considered mature.

All 57 stage 3-8 females identified by histology were used for the E_2 and VTG assays. However, due to the time and cost associated with developing and running the VTG assay, not all immature females (stage 2) and males were used. Immature females (stage 2) ($N = 43$) and males ($N = 30$) were sub-sampled and utilized in both the assays.

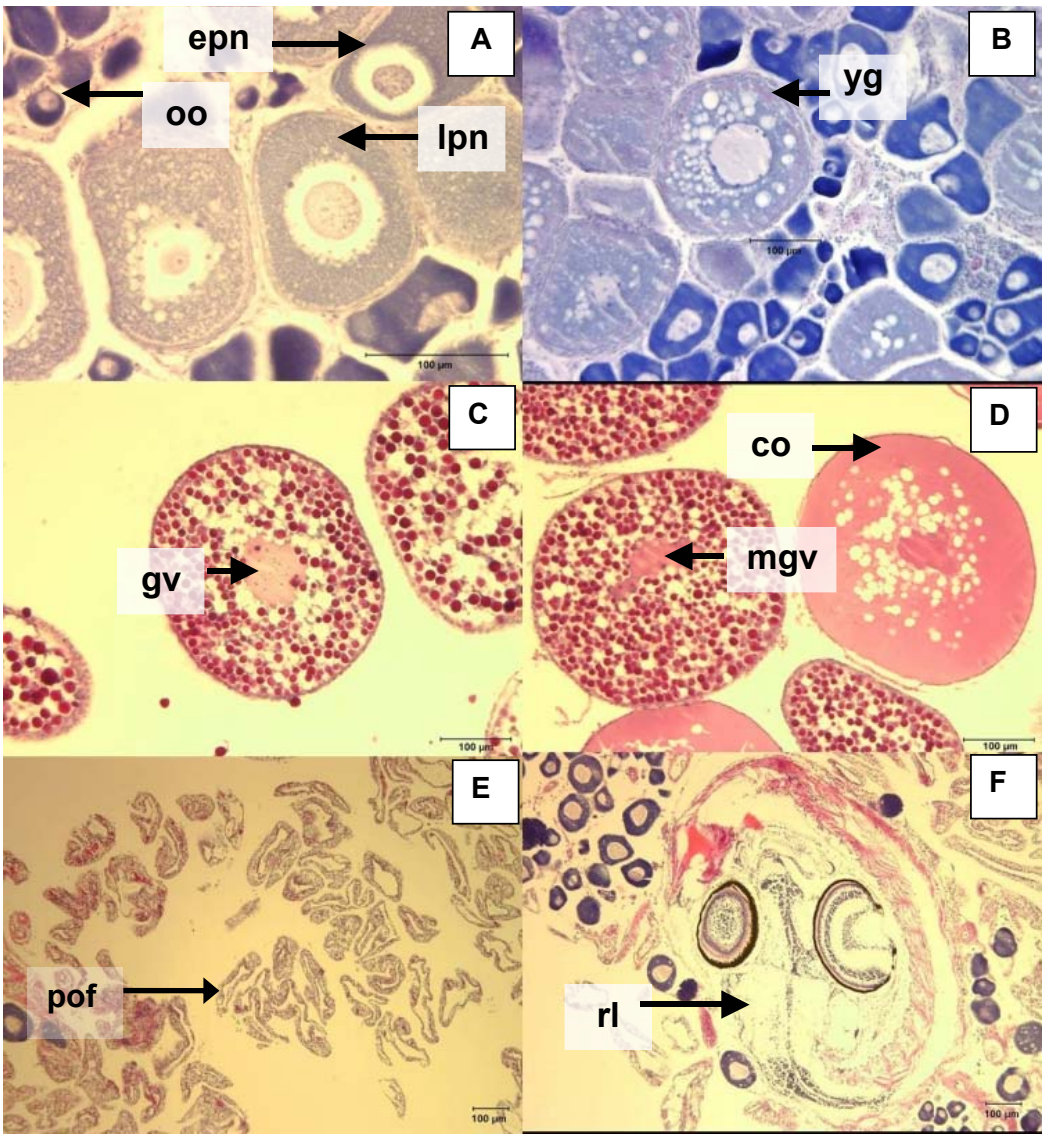

Figure 2. Histological stages of ovarian development in black rockfish. A) stages 1-2 pre-vitellogenic (magnification 400x); B) stage-3 early vitellogenesis (magnification 40x;); C) stage-4 mid-vitellogenesis (magnification 40x); D) stage-5 post vitellogenesis (left) and stage-6 oocyte maturation (right) (magnification 40x); E) stage-7 post-ovulatory follicles (magnification 4x); F) stage-8 post spawned with reabsorbing larvae (magnification 40x) (bars = 100µm) See Table 1 for detailed description of the ovarian stages. Key: co = coalesced, epn = early perinucleus—primary oocytes, lpn = late perinucleus—primary oocyte in endogenous growth, oo = oögonium, gv = germinal vesicle, mvg = migrating germinal vesicle, yg = yolk granules, rl = reabsorbing larvae.

When all fish were combined over the 18 month sampling period (females N = 162, and males N = 163) a one-way ANOVA suggested that the average size for males was greater than the average size for females but the relationship was not significant (P = 0.6106). Males averaged 70 mm longer than stages 3-8 females and 120 mm longer than stage 2 females (Figure 3). Stage 3-8 females averaged 50 mm longer than stage 2 females. Stage 7 was excluded from the analysis because there was only one fish. Maturing (stage 3-6) and mature (stage 7-8) females were on average significantly heavier than males and immature (stage 2) females (P<0.0001). Maturing females averaged 0.45 kg more than immature females and 0.43 kg more than males (Figure 4). However, there was not a significant difference in weight between immature females and males. Even though there was a similar variance seen in male and female weight, the variability in male weight was attributed to all males of all ages and maturity being pooled.

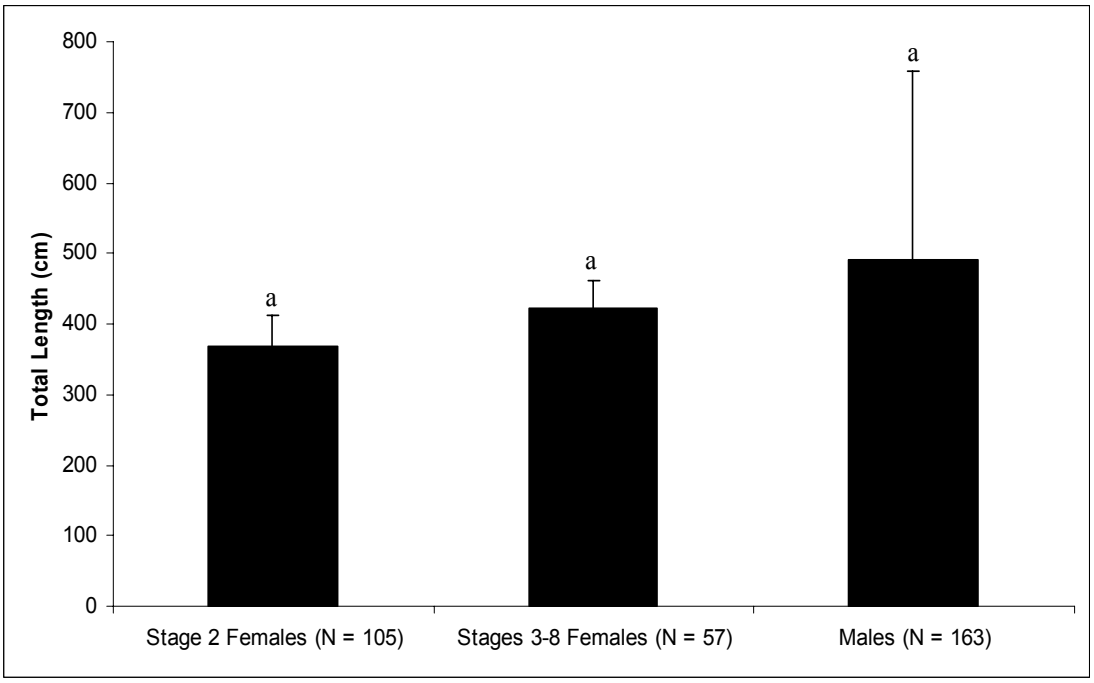

Figure 3. Mean (+ 1 SD) total length for stage 2 females, stage 3-8 females and males, collected between September 2003 and January 2005 off the central Oregon coast. The letters over each bar indicate the lack of significant differences between group means (One-way ANOVA, N = 105 for stage 2 females; N = 57 for stage 3-8; N = 163 for males).

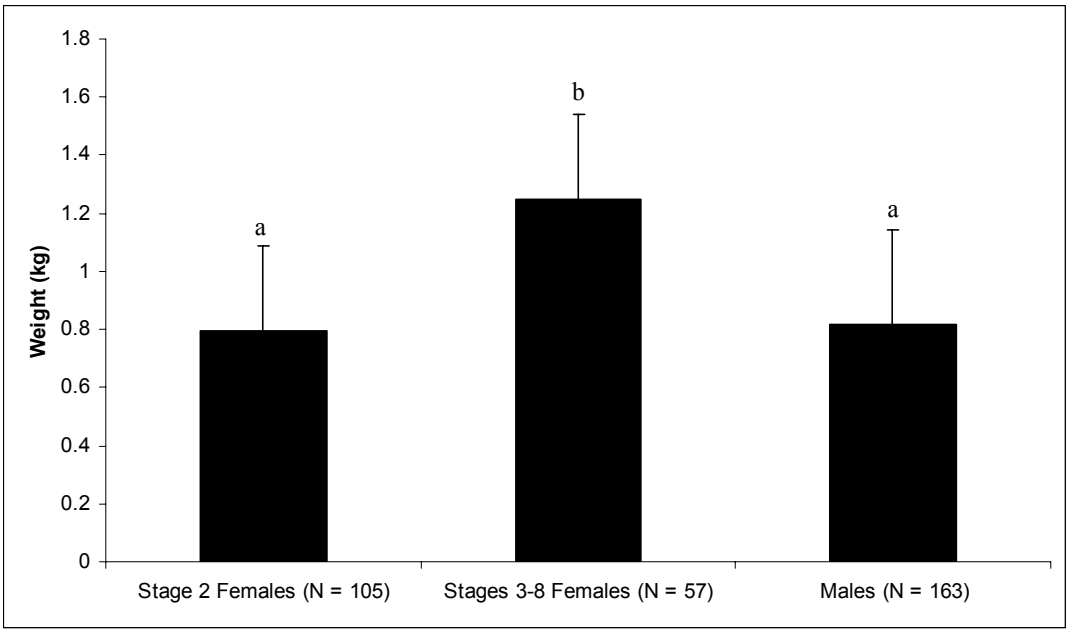

Figure 4. Mean (+ 1 SD) weight for stage 2 females, stage 3-8 females and males, collected between September 2003 and January 2005 off the central Oregon coast. The letters over each bar indicate significant differences between group means (One-way ANOVA, N = 105 for stage 2 females; N = 57 for stage 3-8; N = 163 for males).

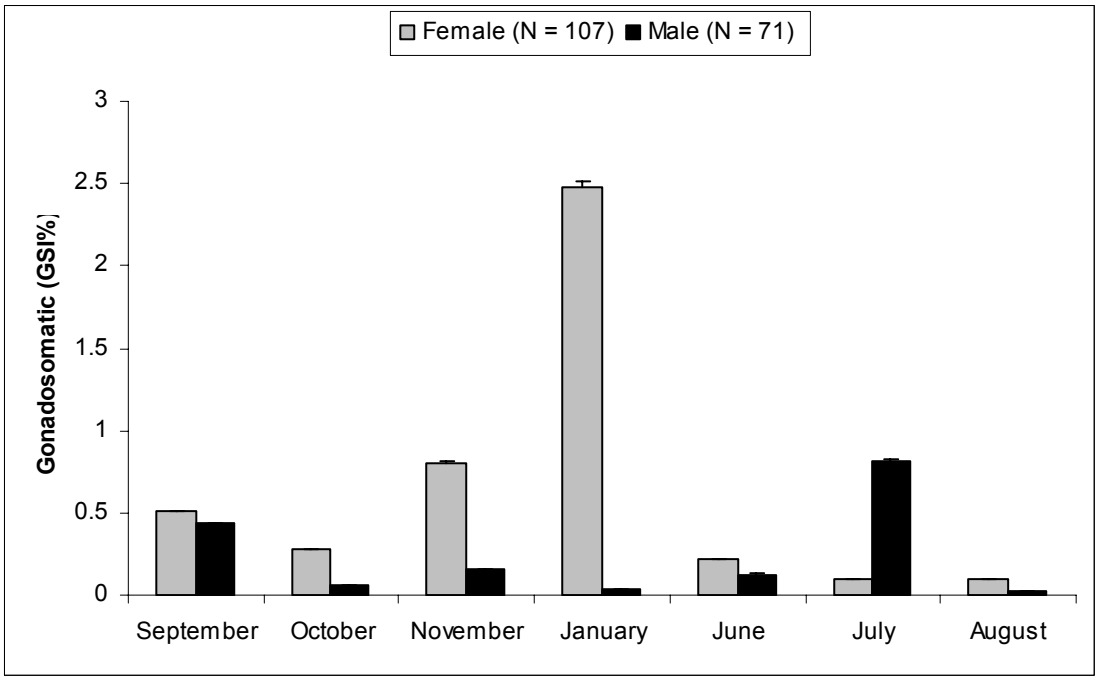

Figure 5. Monthly gonadosomatic index ((gonad weight/body weight)*100) for female and male black rockfish (females N = 107; males N = 71). Gonadal weights for both female and male samples from several months had to be discarded due to lack of standardization in weighing method.

Females had the highest gonadosomatic index (GSI) in the months of November and January, whereas males exhibited elevated GSI values in July and September (Figure 5). Gonadal weights for both female and male samples from several months had to be discarded due to lack of standardization in weighing method. Please note differences in N-values for Figure 5.

A review of monthly histological analyses revealed several interesting trends (Figure 6). As expected, the highest proportion of maturing females was found in the fall and early winter months (September-February), as reported elsewhere (Bobko and Berkeley 2004). The greatest proportion of vitellogenic females (ovarian stages 3-4) was found in October, whereas mature, post-spawned females were observed in the

spring. No reproductively active females were found in the months of June, July, or August.

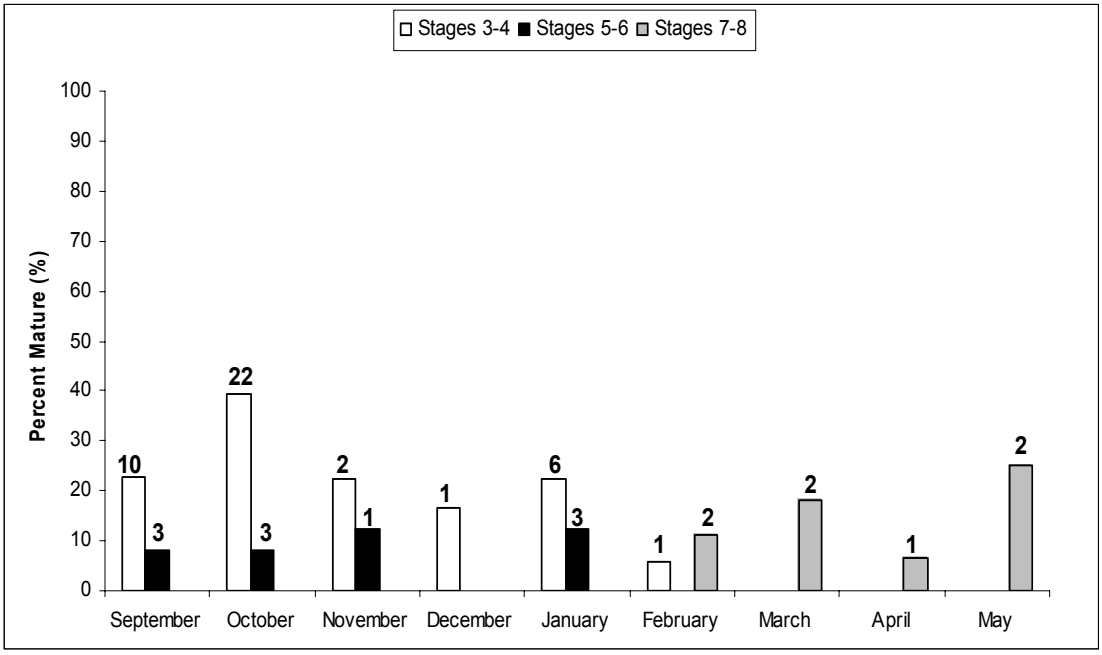

Figure 6. Percent mature (%) for female black rockfish (*Sebastes melanops*) showing stage-3 or greater ovarian development by month as shown through histology; no reproductively active females were found in the months of June, July, or August. Numerals indicate sample sizes for stages.

Mean circulating E_2 concentrations in maturing females (stage 4 and 5) sampled during the spawning season (September-February) were significantly higher than in pre-vitellogenic (stage 2) females (One-way ANOVA, $P<0.05$) (Figure 7). Pre-vitellogenic (stage 2) females had lower E_2 concentrations than females undergoing oocyte maturation (stage 6) though the relationship was suggestive, but with mariginal significance ($P = 0.087$). Early vitellogenic (stage 3) females had higher concentrations of E_2 than in pre-vitellogenic (stage 2) females though the relationship did not differ significantly ($P = 0.108$). The Fisher's protected least significance difference test showed that mid-vitellogenic (stage 4) and post-

vitellogenic (stage 5) females had higher circulating concentrations of E_2 compared to pre-vitellogenic (stage 2) ($P < 0.05$). The Bonferroni mean comparison test revealed that only mid-vitellogenic (stage 4) females E_2 concentrations were significantly different than pre-vitellogenic (stage 2) females ($P<0.0024$). Total length and weight differed significantly (one-way ANOVA, $P<0.0001$) among the six stages of female maturity. The fisher's and Bonferroni comparison test suggested that length and weight differed between pre-vitellogenic (stages 2) and (early vitellogenesis-oocyte maturation) stages 3-6 but did not differ between mid-vitellogenic (stage 4), post-vitellogenic (stage 5) and females undergoing oocyte maturation (stage 6).

Mean circulating E_2 concentrations for females that were mid-vitellogenic (stage 4), post-vitellogenic (stage 5), and undergoing oocyte maturation (stage 6) sampled throughout the year were significantly higher than in males ($P<0.0001$). Also, early vitellogenic females (stage 3) had significantly higher E_2 concentrations than in males ($P = 0.0007$). Pre-vitellogenic females (stage 2) and post spawned (stage 8) females had E_2 concentrations that were slightly higher than in males though they did not differ significantly ($P = 0.290$; $P = 0.518$). Maturing females E_2 concentrations (stage 3-6) averaged 5 ng/ml compared to 1 ng/ml for pre-vitellogenic females (stage 2) and 0.50 ng/ml for males. Both the Fisher's and Bonferroni tests suggested that females in stage 3 and stages 4-6 had significantly higher E_2 concentrations than in males ($P = 0.0007$; $P<0.0001$).

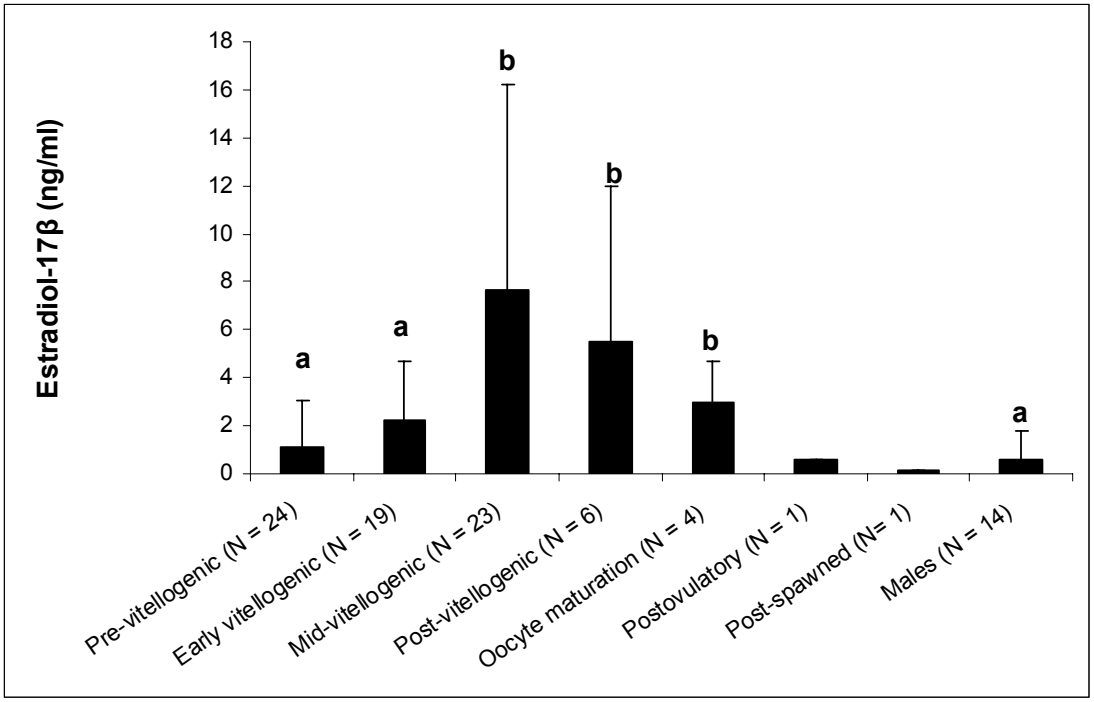

Figure 7. Mean (+ 1 SD) plasma estradiol-17β concentrations (ng/ml) in six stages of ovarian development female black rockfish for the months of September-February. The letters over each bar indicate significant differences between group means Post-ovulatory and post-spawned females were incorporated for comparison purposes.

Mean circulating concentrations of VTG for mid-vitellogenic (stage 4), post-vitellogenic (stage 5), and females undergoing oocyte maturation (stage 6) were significantly higher during the spawning season (September-February) than in immature females (stage 2) ($P<0.05$) (Figure 8). Throughout the spawning season there was not a significant difference in VTG concentrations in pre-vitellogenic females (stage 2) and early vitellogenic (stage 3) females ($P = 0.428$). However, females in stages 4, 5, and 6 had significantly higher concentrations of VTG than in early vitellogenic (stage 3) females ($P<0.05$). Mid-vitellogenic (stage 4) and post vitellogenic (stage 5) females VTG concentrations did not differ significantly (P =

0.704). However, VTG concentrations in mid-vitellogenic (stage 4) females when compared to females undergoing oocyte maturation (stage 6) were statistically significant ($P<0.05$). Both post hoc tests (Fisher's and Bonferroni) showed that females (stage 4 and 5) had higher circulating concentrations of VTG compared to immature female (stage 2) ($P<0.05$). Although VTG concentrations in pre-vitellogenic females (stage 2) were higher than in males but not significantly ($P = 0.1532$). Females in each stage of maturity (stages 3-8) had significantly higher VTG concentrations than in males ($P<0.0001$). Post-ovulatory (stage 7) and post-spawned females (stage 8) females that had recently gone through parturition and had reabsorbing larvae had low but detectable concentrations of VTG.

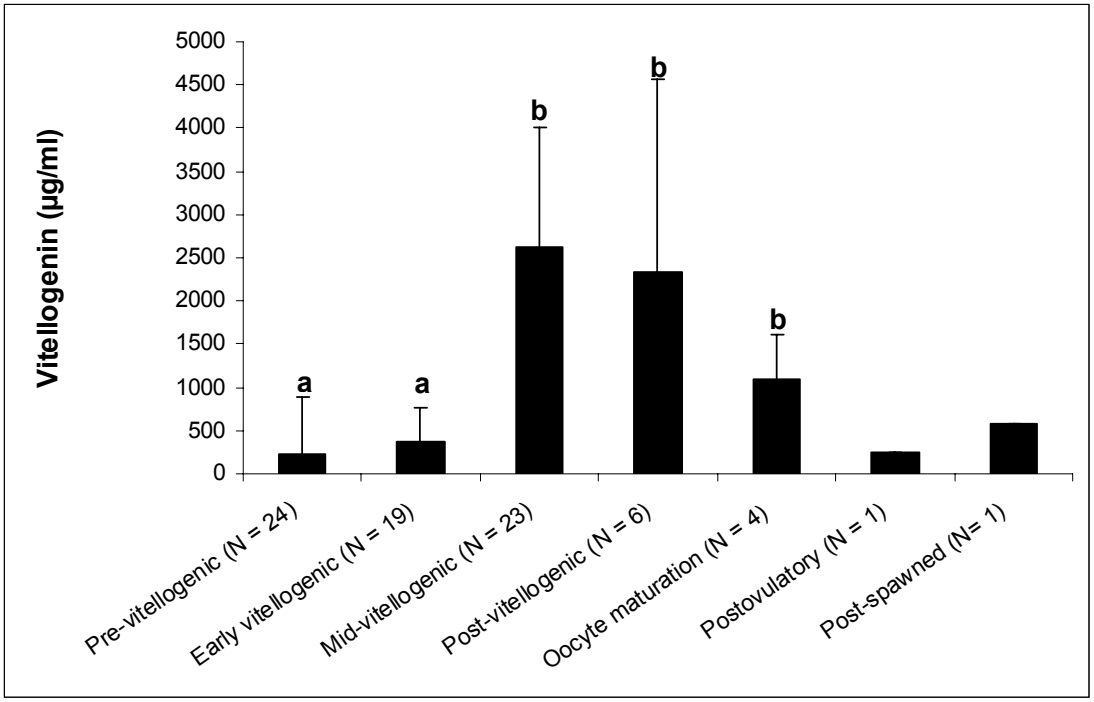

Figure 8. Mean (+ 1 SD) plasma vitellogenin concentrations (µg/ml) in six stages of ovarian development female black rockfish for the months of September-February. The letters over each bar indicate significant differences between group means. Post-spawned (stage 7 and 8) females were incorporated for comparison purposes.

Pre-vitellogenic (stage 2) concentrations of E_2 were low throughout the months of March-August (Figure 9). There was an increase in E_2 concentrations for both pre-vitellogenic females (stage 2) and maturing (stages 3-6) females in the winter months (September-November). The first vitellogenic females were observed in early September and spawning females were observed until the end of February (Figure 10). Vitellogenin concentrations increased from September to November, with a peak in February. Pre-vitellogenic females (stage 2) were observed to have low to non-detectable VTG concentrations in the months of June-August and March-May. There were no maturing females observed in the months of June-August or March-May.

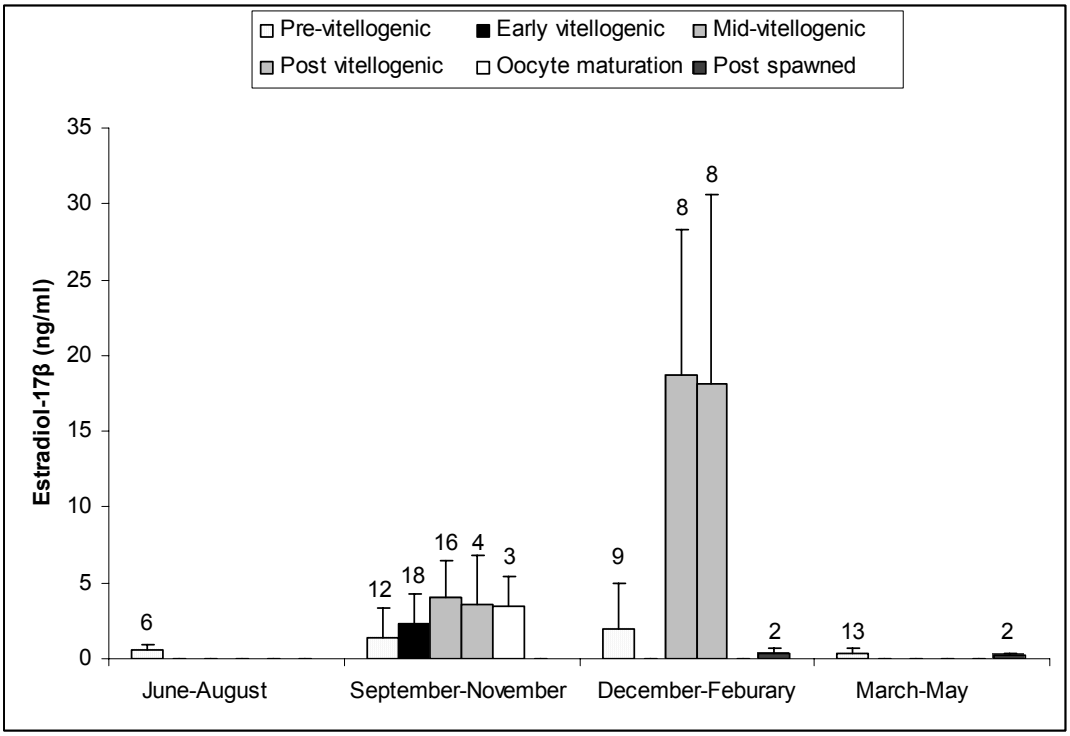

Figure 9. Quarterly mean (+ 1 SD) estradiol-17β concentrations (ng/ml) in six stages of ovarian development for female black rockfish (*Sebastes melanops)*. Numerals indicate sample sizes for stages of ovarian development.

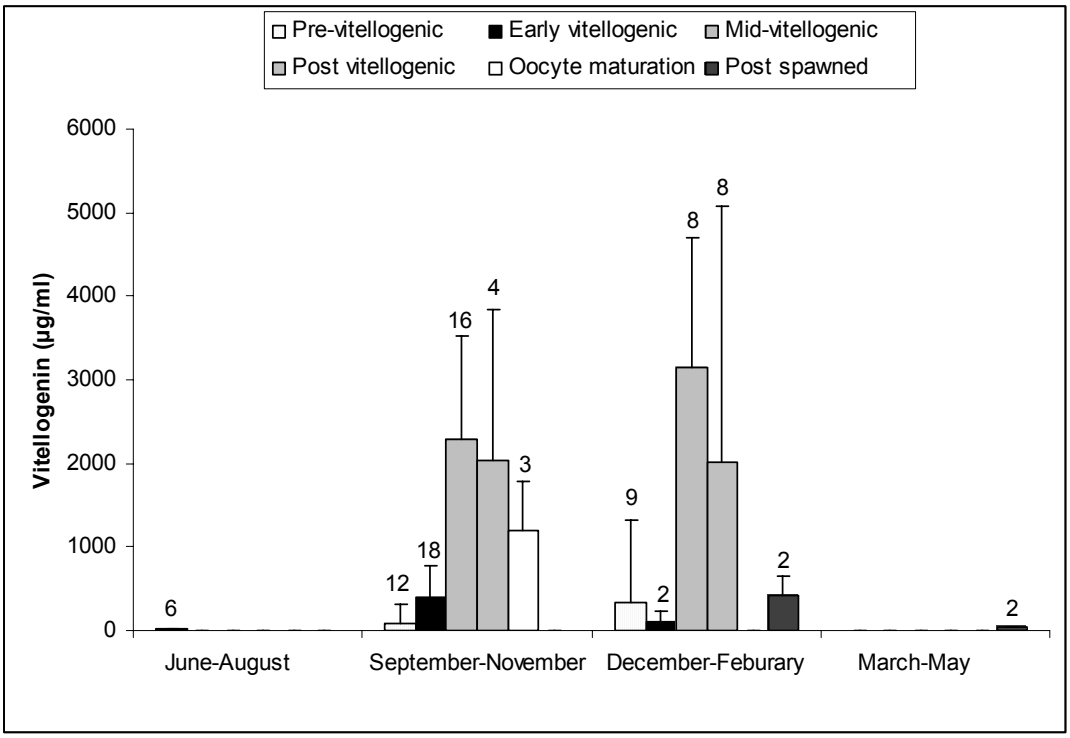

Figure 10. Quarterly mean (1 + SD) vitellogenin concentrations (µg/ml) in six stages of ovarian development female for black rockfish (*Sebastes melanops*). Numerals indicate sample sizes for stages of ovarian development.

Age-based maturity schedules were constructed to evaluate the feasibility of judging maturity of female black rockfish based on detection of VTG in their plasma (Figure 11). The proportion of mature females in all age categories detected during potential spawning months (September through February) was evaluated. An aged based maturity schedule based on VTG detection was compared to one based on histological evaluation of the stage of ovarian maturity. The two female maturity schedules were similar; however, the they differed statistically (Chi-square = 1.59, $p > 0.01$ for 10 df). Of the 57 females identified as mature based on gonadal histology, 52 were judged to be mature by detection of VTG in their plasma; the exceptions were primarily post-spawned females ($N = 4$). However, some females that did not exhibit

clear signs of maturation in their gonads had low but detectable concentrations of VTG, suggesting the VTG assay for maturity may be more sensitive than gonadal histology for early maturation detection. There were eighteen females that were not included in the maturity schedule because concentrations of VTG fell below the level of detection (100 μg/ml); of these, six were age 5, three were age 7, two were age 4 and age 10, and one individual was found for ages 3, 6, 9, 11, 14, and 17. Concentrations were detectable before yolk platelets were seen in oocytes histologically. These females were caught throughout the spawning period September-February with the exception of the two ten-year old females sampled in June, and one seventeen-year old female in October. Size-based maturity schedules were constructed using the same criteria as above (Figure 12). As with the age-based maturity schedule, there were some differences between the histological and age based two maturity schedules, but they not differ statistically (Chi-square = 0.031, $p < 0.01$ for 4 df).

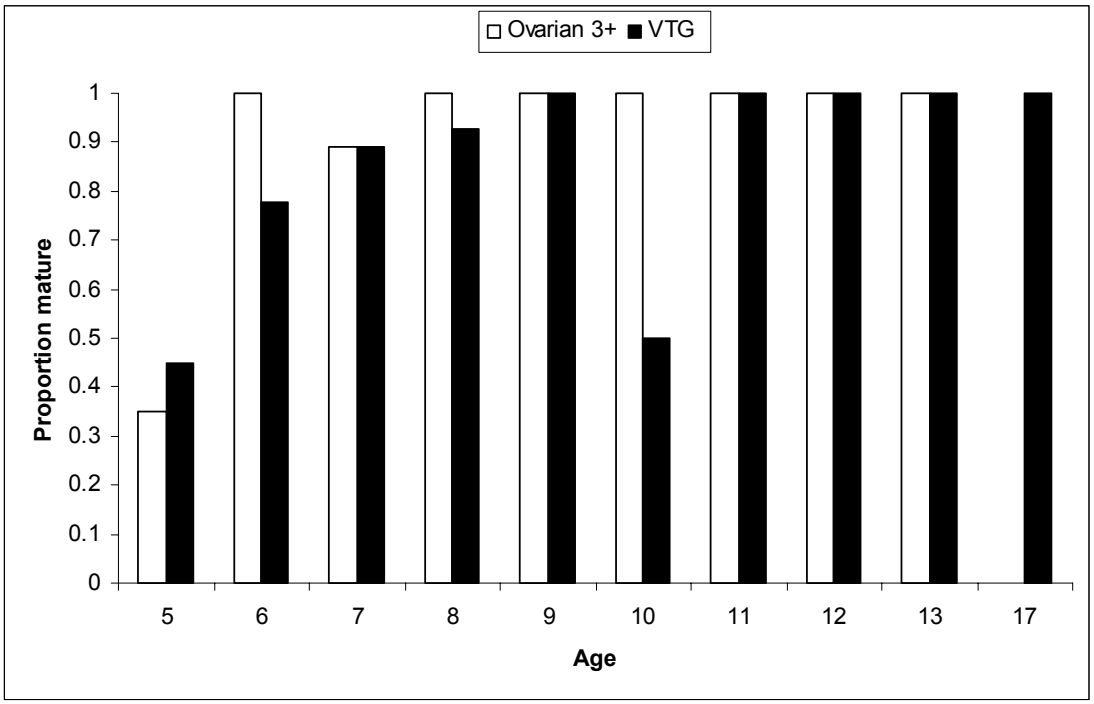

Figure 11. Maturity schedules for black rockfish predicted by the presence of vitellogenin in their blood plasma versus stage of ovarian maturity determined through gonadal histology (Table 2) during the spawning season (September-February). Females were judged to be mature if their ovary was classified as ovarian stage 3+or if they had detectable concentrations (<100µg/ml) of vitellogenin in their blood plasma (VTG). Sample sizes for the age categories were N = 20, 9, 9, 14, 7, 2, 4, 1, 1, and 1 respectively).

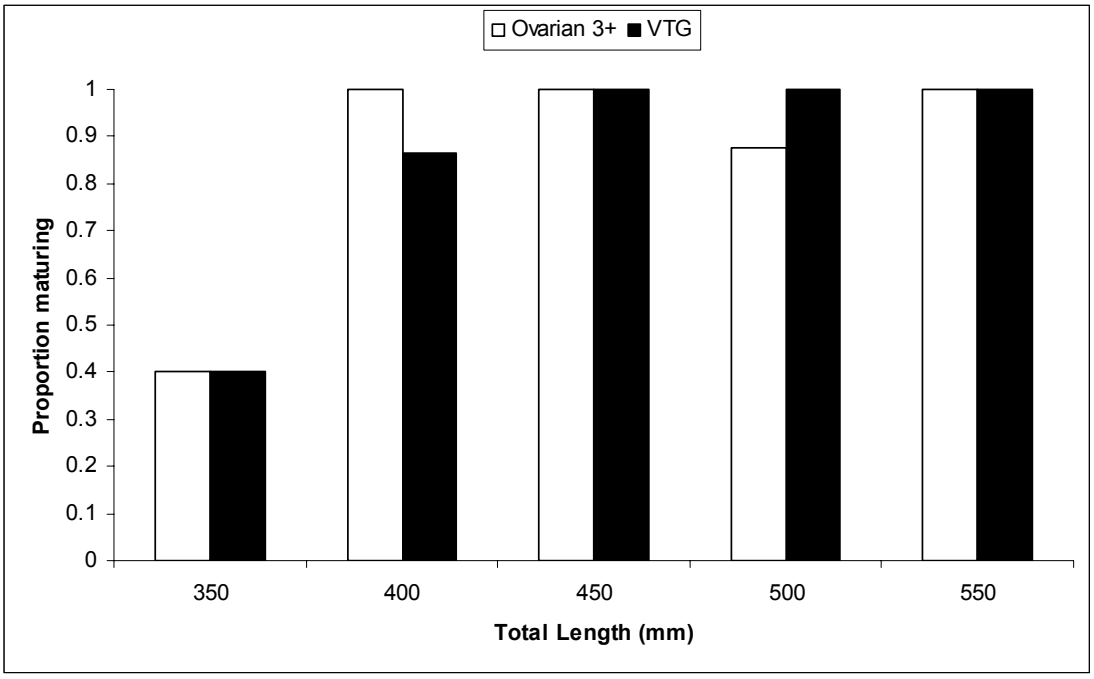

Figure 12. Maturity schedules for black rockfish predicted by the presence of vitellogenin in their blood plasma versus stage of ovarian maturity determined through gonadal histology (Figure 1). Females were judged to be mature if their ovary was classified as ovarian stage 3+ or if they had detectable concentrations (<100µg/ml) of vitellogenin in their blood plasma (VTG). Sample sizes for the six length categories were N = 5, 15, 30, 8, and 1 respectively.

A maturation "ogive" was generated from VTG maturity data to predict age at maturity. The logistic equation used to predict proportion mature at age was $p = 1/(1 + \exp(-a - bx))$ and data were fit using a power function. The predicted age at 50% maturity was 5 years of age, and 100% maturity was ~7-9 years of age (Figure 13). Average lengths for females age 5 and 7-9 were 364 and 407-438 mm respectively.

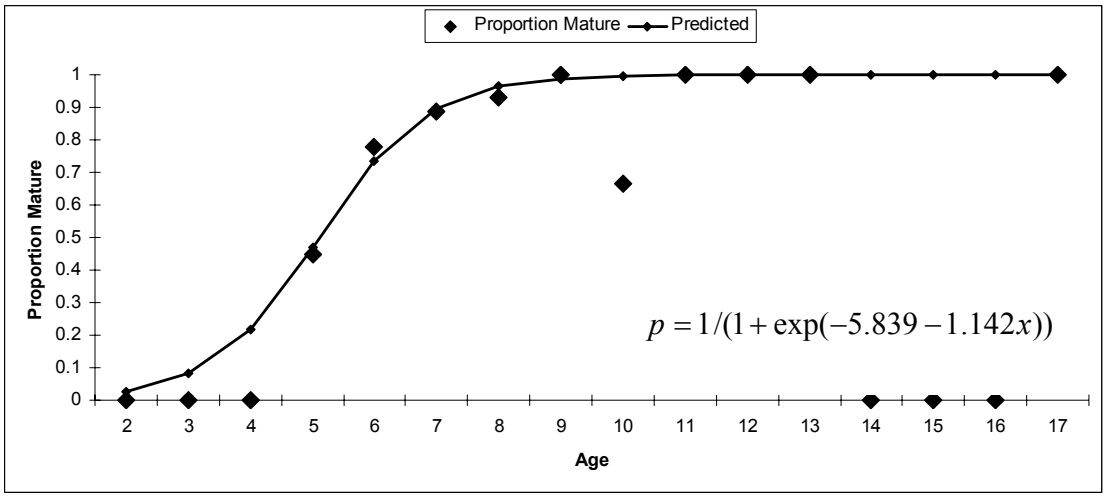

Figure 13. Age-based maturation curve for female black rockfish (*Sebastes melanops*) based on detection of vitellogenin in the bloodstream for the months of September-February.

A stepwise Discriminant Function Analysis (DFA) was used to develop a set of criteria to predict sex and maturity. The variables used to compare males and females were E_2, VTG, TL, and weight (Table 3). Males were not aged; thus, age was not included as a variable in the analysis for sex. Also, stage 7 females were excluded from the analysis because there was only one fish in this stage. Plasma VTG was chosen in the stepwise DFA as the best predictor of sex in black rockfish. Overall, 82% of the fish were correctly classified by sex. The use of these two derived

discriminant functions led to the correct classification of 68% of females and 97% of males, both exceeding the 50% probability of correctly classifying females and males by chance alone (Table 4). The cross-validation of the model revealed an error rate of 32% for females and 3% for males. Some pre-vitellogenic females (stage 2) had virtually non-detectable concentrations of VTG and were misclassified as males.

Table 3. Results from stepwise discriminant function analysis predicting sex or stage of maturity of black rockfish (*Sebastes melanops*). The blood plasma indicators used in the analysis were estradiol-17β (E_2) and vitellogenin (VTG); other variables used in the DFA were age, total length (TL) and weight (WT). In the analysis of sex, age was not used as a variable because males were not aged.

Variable	Partial R-square	F-statistic	Wilks Lambda	Average Squared Canonical Correlation
Sex; Males and Females				
VTG	0.0936	13.22	0.0906418	0.09358
Maturity; Females				
VTG	0.5762	25.28	0.42384849	0.11523
TL	0.3355	9.29	0.2816524	0.172016
WT	0.0623	1.21	0.26411768	0.180006

Table 4. Classification summary for determination of sex from the quadratic discriminant function analysis for black rockfish (*Sebastes melanops*). Values in bold are the percentages of fish correctly classified, whereas values not in bold are the percentages of misclassified fish; sample sizes {N} are in brackets.

	Classified		
Actual	**Female**	**Male**	**Total**
Female	**68**	32	100
	{68}	{32}	{100}
Male	3	**97**	100
	{1}	{29}	{30}

Plasma VTG, TL, and WT were chosen as variables in the stepwise DFA for females alone as the best predictors of stage of maturity (Table 3). Stage 7 females were excluded from the analysis because there was only one fish. When only females were used in the quadratic discriminant function using plasma VTG concentrations, TL, and WT, these variables produced correct classifications for 49% pre-vitellogenic (stage 2), 63% for early vitellogenesis (stage 3), 44% for mid-vitellogenic (stages 4), 33% for post-vitellogenic (stage 5), and 100% for oocyte maturation (stages 6) and post-spawned (stage 8) (Table 5). The cross validation of this model revealed error rates of 58% for stage 2, 53% for stage 3, 65% for stage 4, 83% for stage 5, and 100% for stages 6, and 8.

The cross validation from the quadratic discriminant function analysis revealed that some pre-vitellogenic females (48%; *N*=18) had VTG concentrations similar to early vitellogenic females (stage 3) and post-spawned females (stage 8) (7%; *N = 3*). However, there were six pre-vitellogenic (stage 2) females that were misclassified as mid-vitellogenic (7%, *N=3*) and post vitellogenic (7%, *N=3*). These six females that were misclassified were ages 10-15, and were longer and weighed more than the average pre-vitellogenic (stage 2) females. A few early vitellogenic (stage 3) had low enough concentrations of VTG that they were classified as pre-vitellogenic (stage 2) (26%; *N*=5). Mid-vitellogenic (stage 4) (35%, *N*=8) and post vitellogenic (stage 5) (48%, *N*=11) females had similar mean VTG concentrations (2000-2,600 µg/ml) and were similar in weight, which would explain why these females were misclassified into the opposing stage. Due to the variability among mid-vitellogenic (stage 4) and

post-vitellogenic (stage 5) VTG concentrations, females from each stage (50%, *N*=3) had low enough concentrations of VTG that they were classified as early vitellogenic females (stage 3). Females undergoing oocyte maturation were misclassified into stage 3 (75%, *N*=3) and stage 5 (25%, *N*=1).

Table 5. Classification summary for determination of stage of maturity in all females from the quadratic Discriminant function analysis for black rockfish (*Sebastes melanops*). Values in bold are the percentages of female fish correctly classified, whereas values not in bold are the percentages of misclassified female fish; sample sizes (N) are in the brackets. See Table 2 for the description of developmental stages.

	Classified						
Actual	Stage 2	Stage 3	Stage-4	Stage 5	Stage 6	Stage 8	Total
Stage 2	**49**	35	2	7	0	7	100
	{21}	{15}	{1}	{3}	{0}	{3}	{43}
Stage 3	16	**63**	0	11	11	0	100
	{3}	{12}	{0}	{2}	{2}	{0}	{19}
Stage 4	0	13	**44**	44	0	0	100
	{0}	{3}	{10}	{10}	{0}	{0}	{23}
Stage 5	0	50	17	**33**	0	0	100
	{0}	{3}	{1}	{2}	{0}	{0}	{4}
Stage 6	0	0	0	0	**100**	0	100
	{0}	{0}	{0}	{0}	{4}	{0}	{4}
Stage 8	0	0	0	0	0	**100**	100
	{0}	{0}	{0}	{0}	{0}	{4}	{4}

Discussion

The ELISA for VTG detection appears to be a reasonably reliable method for discriminating between male and female black rockfish as well as between maturing females (stage 4, 5, and 6) and pre-vitellogenic females (stage 2) during the spawning season (September-February) in correlation with total length and weight. Plasma VTG was chosen in the stepwise DFA as the best predictor of sex in black rockfish. Overall,

82% of the fish were correctly classified by sex when VTG was the response variable. Although VTG is a good predictor of maturity in females the variability among stages caused some females to be misclassified as reflected by the cross validation of the quadratic discriminant function analysis. Specifically, VTG concentrations cannot be used to accurately determine pre-vitellogenic females (stage 2), from either early vitellogenic (stage 3), and post-spawned (stage 8) females. Although males had lower levels of VTG than in pre-vitellogenic (stage 2) and post-spawned females (stage 8) VTG concentrations cannot be used to accurately distinguish between them. Vitellogenin concentrations for maturity detection must be at least 1, 100 µg/ml to successfully differentiate between immature (stage 2) and maturing (stage 3+) females.

Although mid-vitellogenic (stage 4) and post vitellogenic (stage 5) females had significantly higher concentrations of E_2 than pre-vitellogenic females ($P<0.0001$), there were not significant differences among all stages. Therefore, E_2 concentrations were not chosen as a variable to predict stages of female maturity. In general, mean E_2 concentrations were significantly higher in females (stages 3-6) than in males with the exception of pre-vitellogenic and post-vitellogenic females. Thus, E_2 alone was not chosen as a good predictor of sex, as has been found in other teleosts and sturgeon (Fitzpatrick 1985; Webb *et al.* 2002).

The age-based maturity schedules revealed that 68% of females were identified as mature based on gonadal histology, and 69% were by the detection of VTG in their plasma. The differences between the two maturity schedules were attributed to the

females in age classes 6, 8 and 10 that were either post-vitellogenic or were post-spawned. Also, one age 17 year old female had a high concentration of vitellogenin in the blood stream before yolk platelets were seen in oocytes histologically. The maturation curve for predicted proportion at age based revealed that the onset of maturity was 3 years, 50% maturity was age 5, and 100% maturity was achieved ~7-9 years. Females in age class 5 were determined mature based on VTG detection and not by histology which may have caused the proportion mature at to be over-estimated. Females in age classes 10, 14, 15, 16 fell below the age of predicted maturity because they were post-spawned females and had low VTG concentrations.

Several older females age 9 and older were classified as pre-vitellogenic (stage 2) due to low levels of VTG and no histological evidence of a prior spawning. Thus, these older females could not be distinguished among immature females. Although it has been proposed that older fish experience reproductive senescence has not been documented for *Sebastes* (Reznick *et al.* 2002). It is possible that some of the older females that were classified as immature did not cycle in the year of this study or may have gone atretic earlier in the season. However, complete gonadal regression was rare. Resorbtion of a few oocytes was fairly common in this species as reported for other rockfish (McDermott 1994) and occurred in most months and maturity stages.

Females ages 3-7 with elevated E_2 and VTG concentrations were observed during the spawning season (September-February) in this study. Based on these elevated concentrations these females were considered maturing. It has been reported that young female rockfish (*Sebastes*) develop an orange-yellow colored ovaries or

vitellogenic oocytes during spawning periods. Some researchers have deemed these younger females as reproductive while others have classified them as immature (Love and Westphal 1981; Nichol and Pikitch 1994; Bowers 1992; Bobko and Berkeley 2004). Black rockfish are capable of reproducing as young as three years of age (Wyllie Echeverria 1987), and according to stock assessments a small proportion of these smaller, younger females reproduce.

The reproductive cycles of numerous rockfish species have been described by histological methods (Moser 1967; Gunderson *et al.* 1980; Whyllie Echeverria 1987; Shaw 1999) but this research is the first description of the annual endocrine cycle of a Pacific coast rockfish species. Vitellogenic females detected in field-collected samples showed maturation patterns that were consistent with the histological stage of ovarian development reported in previous studies (Wyllie Echeverria 1987; Bobko and Berkeley 2004). In addition, ovarian development for black rockfish in this study was similar to the development cycles reported for other rockfish species (Moser 1967; Wyllie Echeverria 1987; Bowers 1992; Nichol and Pikitch 1994; and Shaw 1999) with the exception of seasonal timing and stage duration.

Nagahama *et al.* (1991) observed that serum E_2 concentrations for field- caught *S. taczanowskii* were 1.34 ng/ml during June-August, with a significant increase in September to a peak in February at a concentration of 6.88 ng/ml. Takano *et al.* (1991) reported that field-caught female *S. marmoratus* E_2 concentrations ranged from 1.16 to 1.87 ng/ml, where as Mori *et al.* (2003) found that captive- raised *S. schlegeli* E_2 concentrations ranged from 2.9 ng/ml to a peak of 29.6 ng/ml. Similarly, field-

caught female black rockfish from this study ranged from 0.21 ng/ml June-August, with an increase observed in September to a peak in February to 15 ng/ml. Mori *et al.* (2003) reported that the serum steroid hormone concentrations for their study were extremely high compared with those reported previously for both *S. schlegeli* and *S. taczanowskii*. Differences between E_2 concentrations for field-caught and captive-reared females vary greatly for these three species potentially due to control variables in the laboratory and possible environmental contaminants.

In this study, increased E_2 concentrations were seen in females prior to the onset of vitellogenesis, and in females undergoing oocyte maturation which coincided with the findings of Kwon *et al.* (1999) for *S. inermis*. In their study they observed resting females had lower E_2 concentrations than pre-vitellogenic females which was a trend also reflected in this study.

Bobko (2002) reported that vitellogenesis observed through gonadal histology in black rockfish began in late August and was observed through the third week in February which coincided with the vitellogenic females observed in this study. Mori *et al.* (2003) reported that mean VTG concentrations for captive-reared *S.schlegeli* mature females ranged from 1.45 to 23.9 mg/ml. In comparison, the mean VTG concentrations for mature field-caught females found in this study ranged from 0.37 mg/ml to 5.5 mg/ml. The serum VTG concentrations for female *S. schlegeli* increased from October to a peak concentration in February and remained high until April when gestation began (Mori *et al.*2003). The decrease in VTG concentrations in this study corresponded with the reported gestation and subsequent parturition dates (January-

March) for black rockfish in Oregon (Boehlert and Yoklavich 1984; Wyllie Echeverria 1987; Bobko and Berkeley 2004).

The use of the rockfish ELISA to measure VTG to determine sex is reasonably accurate, and to a lesser extent for individual stages of female maturity. However, additional research is needed to determine the natural variability in VTG and E_2 concentrations between all developmental stages in females. The variability of both VTG and E_2 in field-collected female fish indicates that the annual reproductive cycle of black rockfish needs to be monitored through repeat monthly sampling of naturally cycling fish in captivity. Under laboratory conditions, the gonadal development and serum profiles of VTG, Ca^{2+}, and sex steroids in black rockfish can be monitored for one full year. In addition, plasma 17, 20β-dihydroxy-4-pregnen-3-one (DHP) needs to be measured in female black rockfish. It has been suggested that DHP is a maturation-inducing steroid, which may be one of the endocrine factors for maintaining gestation in females.

The newly developed VTG assay should be applicable to other rockfish species, providing an assessment tool to compare reproductive cycles of maturing fish. The N-terminal amino acid sequence of VTG is highly conserved among fishes and thus, the antibody created for one species may be used on another in the same family (Heppell and Sullivan 1999). The assay would need to be validated prior to use in other rockfish species. The use of non-lethal assay may help evaluate productivity in some of the most overexploited rockfish stocks.

CHAPTER 3: DIFFERENCES IN TIMING OF REPRODUCTION AS DETERMINED BY GONADAL HISTOLOGY, PLASMA VITELLOGENIN AND ESTRADIOL-17B IN FEMALE BLACK ROCKFISH (*SEBASTES MELANOPS*).

Introduction

Black rockfish (*Sebastes melanops*) are an important resource to both commercial and sport fisheries on the Pacific west coast of the United States. Black rockfish are particularly important in the recreational fisheries of Washington, Oregon, and northern California, comprising over a third of the total recreational catch (Love 1996). The adult population in Oregon and Northern California has been fished at high levels over the past decade and has experienced a reduction in abundance and spawning biomass, as well as average age of adults (Wallace *et al.* 1999; Ralston and Dick 2003). The 2003 stock assessment for black rockfish showed through an examination of sex ratio as a function of age that the representation of females in the older age categories falls from 50% at age 10 to 10-20% by age 20. This assessment was evaluated using a variety of data sources from both recreational and commercial fisheries (Ralston and Dick 2003). Bobko and Berkeley (2004) observed a decrease in mature females age 10 and older in each year of their 4 year study, suggesting that age truncation is occurring in the nearshore Oregon black rockfish. Increased total mortality by fishing reduces the proportion of females that reach older age classes which can result in a truncated age distribution.

Berkeley *et al.* (2004a) reported that older female black rockfish produce larvae that withstand starvation longer and grow faster than the offspring of younger

females. Specifically, they found that maternal age was related to larval performance, and larval performance was related to oil globule volume at parturition. These findings suggest that, through maternal effects, the larvae of older females have an increased survivorship to the exogenous feeding stage. There may be benefits of maternal effects beyond this point of development through higher survivorship and growth, allowing larvae to pass more quickly through the most vulnerable life history stages.

Berkeley et al. (2004a,b) also found that younger female black rockfish release their larvae later in the spring than older females do, and that a disproportionate number of surviving offspring (post-settlement juveniles) have birthdates from February and March, when only older females are found giving birth. The timing of parturition of larvae may be especially important for larvae to encounter patches of zooplankton (Cushing 1969, 1975). More generally, species with protracted spawning seasons have a higher likelihood that some proportion of the spawning event will occur while environmental conditions are favorable for recruitment (Lambert 1987). Age-related differences in the timing of spawning have been observed in half beaks (*Hemiramphus brasiliensis*), and weakfish (*Cynoscion regalis*), where older females spawn first (Berkeley and Houde 1978; Shepard and Grimes 1984). In contrast, younger female American plaice (*Hippolglossoides platessoides*) generally spawn earlier than older females, although in the 1990s they spawned later than older females (Morgan 2003). Hutchings and Myers (1993) reported that younger Atlantic cod (*Gadus morhua*) have been observed to spawn before older individuals. However, the

older cod spawn over a longer period of time. Several rockfish species exhibit protracted spawning seasons. For copper (S. *caurinus*) and black rockfish (*S. melanops*), older females spawn earlier than younger individuals (Berkeley and Markle 1999; Cooper 2003; Bobko and Berkeley 2004. Larger (presumably older) yellowtail rockfish (*S. flavidus)* also spawn earlier. (Elderidge *et al.* 1991). Nichol and Pitkiach (1994) observed that younger widow rockfish spawn later than older females. Berkeley *et al*. (2004) provided evidence of protracted spawning in older females (> 9 years) but not younger females (ages 6-8). This bet-hedging reproductive strategy may reduce the impact of unpredictable and unfavorable environmental conditions, thereby, increasing reproductive success and offspring survival (Hjort 1926; Cushing 1969, 1975; Goodman 1984; Leaman and Beamish 1984).

Like many rockfish species, black rockfish are long-lived, mature late in life, and grow slowly after reaching sexual maturity. Black rockfish are viviparous (live bearing), with parturition of larvae occurring from mid-January through mid-March. Older females have been observed to extrude larvae earlier than younger females (Bobko and Berkeley 2004). However, this phenomenon requires additional study over years with variable environmental conditions. With more information on the timing of reproduction in older females and younger females, recommendations can be made to better ensure the reproductive potential of the spawning stock.

This research utilized a relatively new method for assessing the intra-annual stage of female maturity in black rockfish to determine if reproductive cycles are age-dependent. A biochemical assay was created to measure proteins in the blood.

Vitellogenin (VTG), a blood plasma protein, circulates at high concentrations and is detectable in the blood stream of vitellogenic females prior to spawning (Nunez-Rodriquez *et al.* 1989; Kwon *et al.* 1990; Lee *et al.* 1992). For several species of fishes an Enzyme Linked Immunosorbant Assay (ELISA) has been used to quantify circulating concentrations of VTG in maturing females, establish early sex determination, and detect the onset of maturation in female fish (Tao *et al.* 1993; Heppell and Sullivan 1999). The VTG ELISA was used to assess whether there were age-specific differences in the timing of vitellogenesis in female black rockfish. This technique is described in detail in Chapter 2, and may serve as a non-lethal method for additional studies of rockfish reproduction.

Methods

The annual reproductive cycle was examined in female black rockfish collected monthly (N = 4-30 per month) by hook and line from the recreational fishery in Newport and Depoe Bay Oregon from September 2003 to January 2005. Blood was taken from the caudal vein with heparinized (21G) syringes from each female. The blood was centrifuged at 3000 rpm for 15 min, and the plasma was stored at -80°C until ELISA and RIA analysis. After fish were measured for total length (mm) and body weight (kg), the ovaries were removed, weighed (g), and preserved in 10% phosphate-buffered formalin. A biopsy was taken from the middle of the right ovary, embedded in paraffin, sectioned at 7 μm, and stained by hematoxylin and eosin (Luna 1968). A biopsy was taken from the middle of the right ovary, embedded in paraffin,

sectioned at 7 μm, and stained by hematoxylin and eosin (Luna 1968). The ovaries were staged using a compound light microscope (magnification 4-100×) according to the modified protocol Shaw (1999) for rockfish (*Sebastes*) (Chapter 2; Table 2). The eight stages used in this study were 2) early and late perinucleus, 3) early vitellogenesis, 4) mid-vitellogenesis, 5) post-vitellogenic, 6) oocyte maturation, 7) post-ovulatory follicles, 8) post-spawned with reabsorbing larvae. Stage 1 (oogonia and chromatin nucleus) was combined with stage 2 (early and late perinucleus), which were both determined to be pre-vitellogenic and represent either immature fish (females not yet capable of spawning) or fish that were not reproductively active (spawning).

Otoliths collected from each fish were aged by the established break and burn method by Sandra Rosenfield at the Washington Department of Fish and Wildlife aging laboratory (Olympia, WA), following the criteria of Chilton and Beamish (1982). Vitellogenin (VTG) and Estradiol-17β (E_2) concentrations were measured in all female fish ages 3-17 for the ovarian developmental stages 3-8 (Chapter 2).

Statistical analyses

Differences in plasma VTG and E_2 concentrations were compared between female stage of maturity separated by age classes (3-5; 6-8; 9+), and were statistically tested by one-way analysis of variance (ANOVA). The age criteria used here was based on the evidence that female black rockfish enter the fishery between age 3-4, and females are 50% mature at 7.5 years, and fully mature (100%) at 9 years of age

according to recent estimates for the Oregon coast (Ralston and Dick 2003; Bobko and Berkeley 2004).

An age-length relationship and a weight-length relationship were calculated for female black rockfish in this study by fitting the data to the von Bertalanffy (1957) growth model:

$$l_t = L_\infty (1 - e^{-k(t-t_o)}) \quad (1)$$

where: l_t = length of fish at age t;

L_∞ = asymptotic length (theoretical maximum length);

k = growth coefficient;

t = current age of fish; and

t_0 = age intercept (theoretical age at zero length).

A weight-length relationship was calculated for female black rockfish in this study to obtain the allometric weight parameter by using the equation:

$$W = aL^b \quad (2)$$

Results

Of the female black rockfish collected in this study, TL increased with female age and was similar to trends found by other researchers (Figure 14). The parameters used in this model were obtained from the 1999 stock assessment—status of the black rockfish resource (Wallace *et al.* 1999). As predicted, female black rockfish weight increased with total length (cm) (Figure 15). The parameter estimates obtained from

equation 2 for the weight-length relationship were higher in this study than those obtained by other studies (Wallace *et al.* 1999; a= $1.677x10^{-5}$, b=3.00).

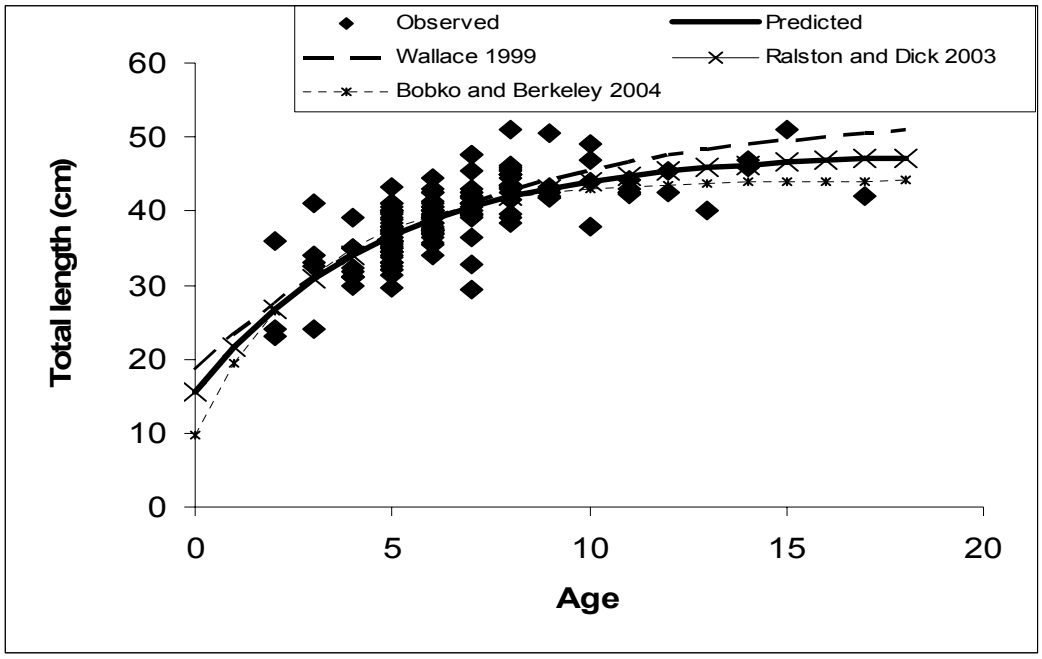

Figure 14. Relationship between total length (cm) and age in female black rockfish (*Sebastes melanops*) collected in Newport and Depoe Bay, Oregon fit to the von Bertalanffy growth model (*N*= 162).

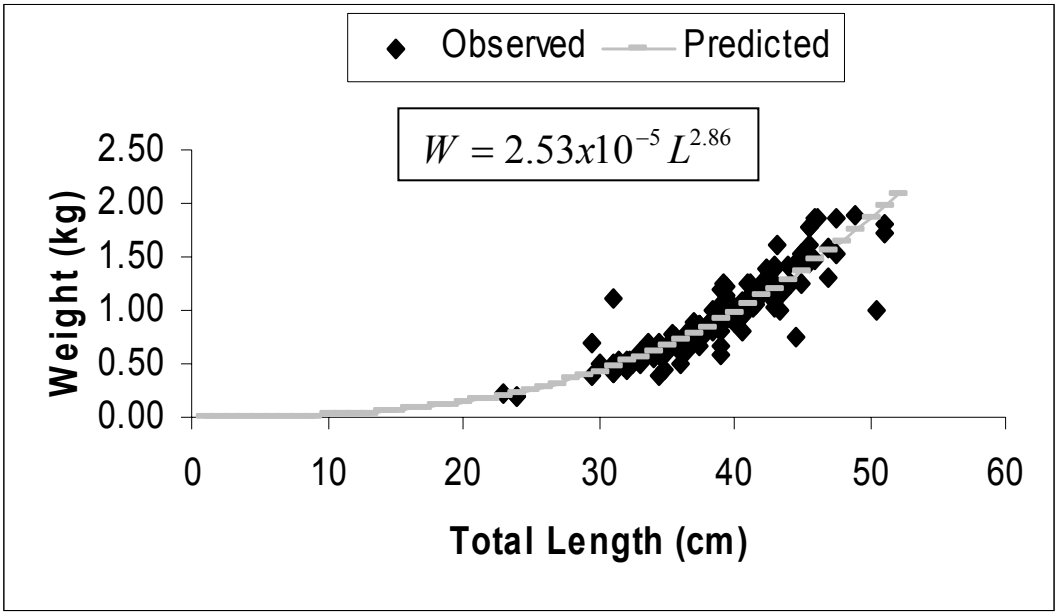

Figure 15. Relationship between total length (cm) and weight (kg) in female black rockfish (*Sebastes melanops*) collected off Newport and Depoe Bay, Oregon ($N = 162$).

In the fall (September-November) females age 5-8 made up 43 % of early and mid-vitellogenic (stage 3 and 4) females, where as, age 9+ females made up 100% of the females that were post-vitellogenic (stage 5) and undergoing oocyte maturation (stage 6) (Figure 16). Females' ages 6-8 captured in the winter months (December-February) made up 90% of the mid- and late vitellogenic (stage 4 and 5) and 100% of post-spawned (stages 7 and 8) fish (Figure 17). In the spring (March-May) 40% of post-spawned females were age 8 and 60% were age 9+ (Figure 18). Pre-vitellogenic females (stage 2) of all ages were observed throughout the months of September-May. All females sampled in the summer (June-August) were pre-vitellogenic (stage 2).

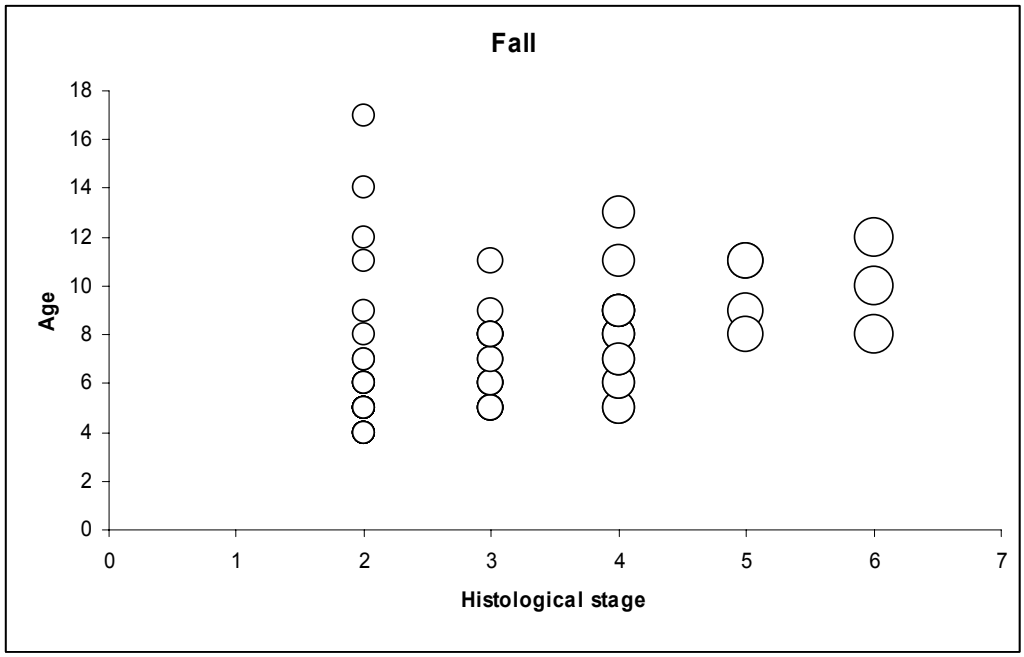

Figure 16. Maturation index for females based on histological stage and age in the fall (September – November, $N = 75$). Size of circles indicates sample sizes.

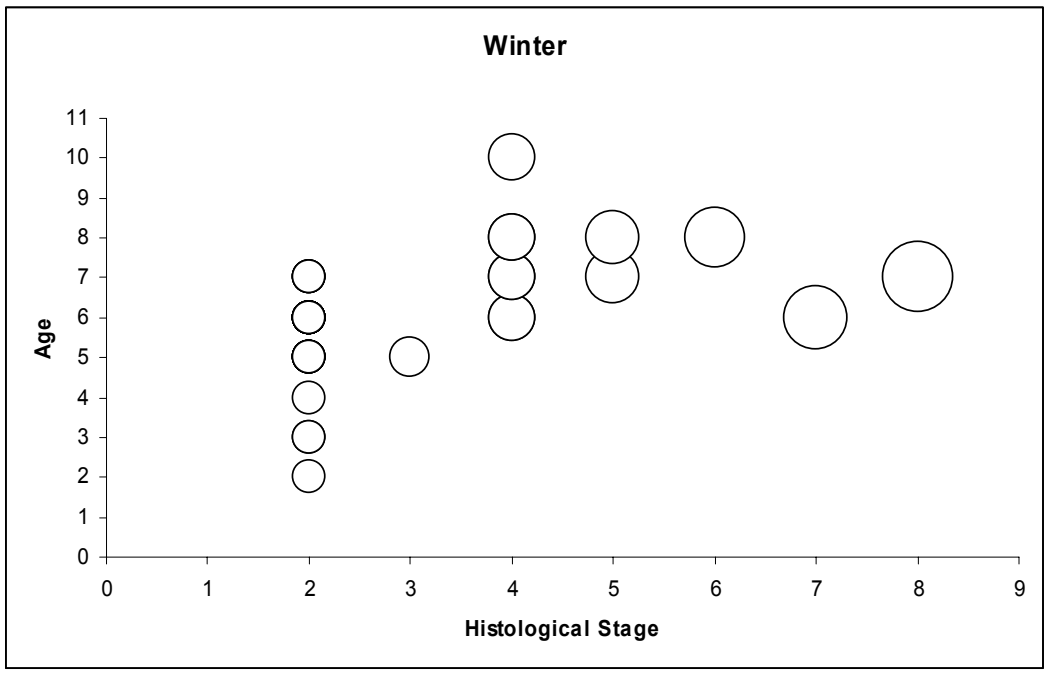

Figure 17. Maturation index for females based on histological stage and age in the winter (December – February, $N = 42$). Size of circles indicates sample sizes

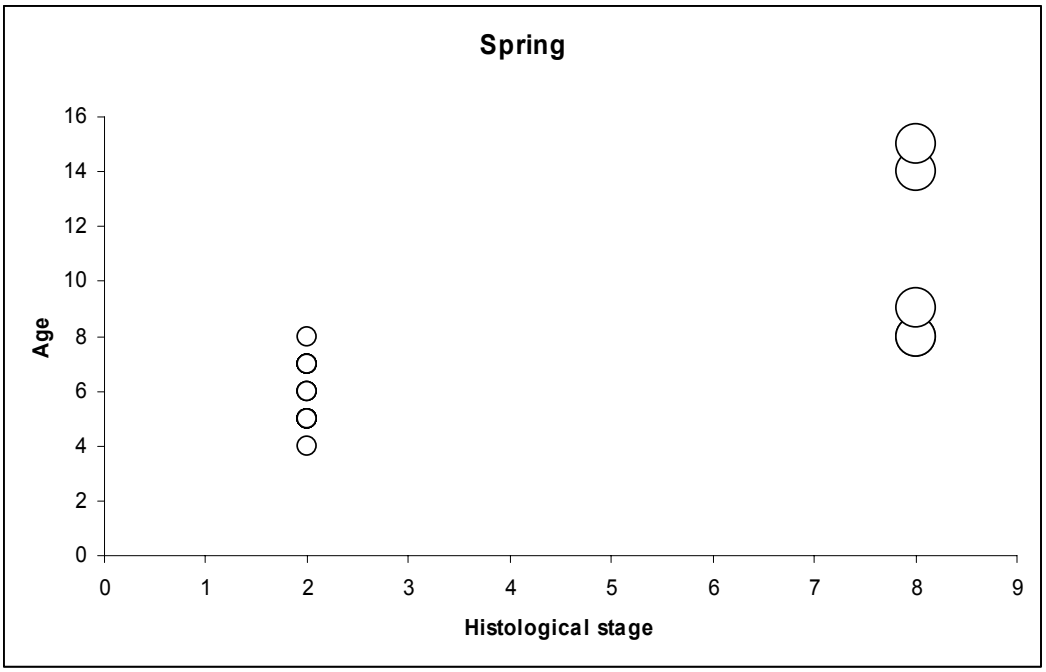

Figure 18. Maturation index for females based on histological stage and age in the spring (March – May, *N* = 29). Size of circles indicates sample sizes.

Females were separated by ovarian stages of development and E_2 concentrations were compared between age groups. A one-way ANOVA showed that early vitellogenic females in the age group 9-17 had higher concentrations of E_2 than in both age groups 3-5 and 6-8 ($P<0.05$) (Figure 19). Mean circulating concentrations of E_2 for females undergoing oocyte maturation of the age group 9-17 were greater than in females ages 6-8 of the same stage ($P<0.05$). Although, mid-vitellogenic females ages 6-8 and 9-17 had higher E_2 concentrations than in females ages 3-5 of the same stage the relationship did not differ significantly ($P = 0.32$; $P = 0.27$). Post-vitellogenic females E_2 concentrations did not differ significantly ($P = 0.97$).

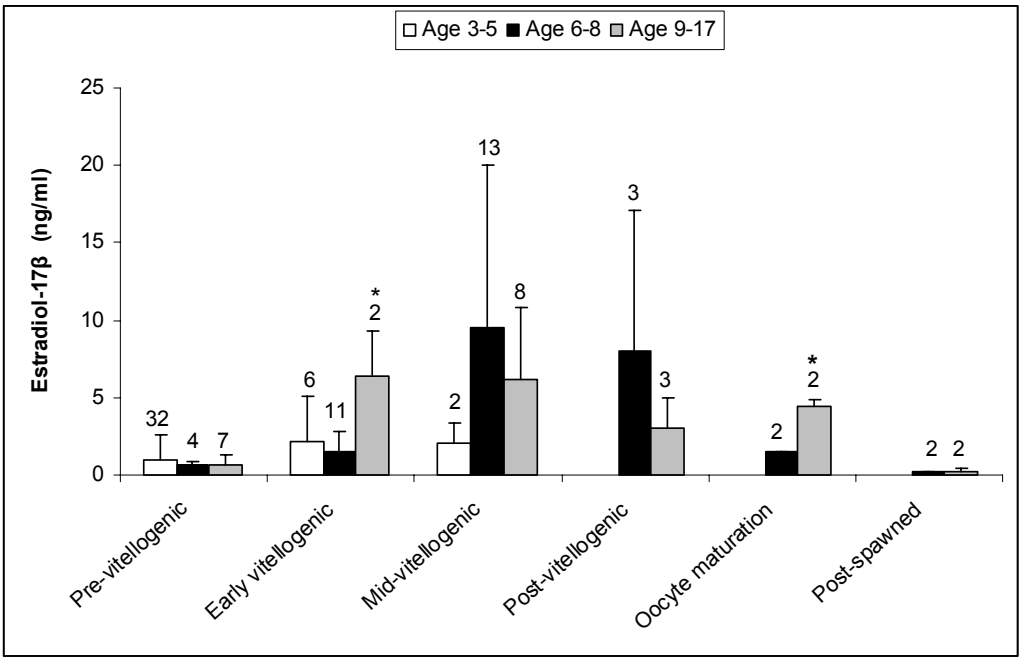

Figure 19. Mean (+ 1 SD) estradiol-17β concentrations (ng/ml) compared between ovarian stages of development and age categories for female black rockfish in the months of September-May. Numerals above each bar represent sample sizes.

Concentrations of VTG in early vitellogenic females from age groups 6-8 and 9-17 were significantly different than in females ages 3-5 of the same stage ($P<0.05$) (Figure 20). Females age 9-17 undergoing oocyte maturation had higher VTG concentrations than in females ages 6-8 ($P<0.05$). Although, mean circulating concentrations of VTG in mid-vitellogenic (ages 6-8 and 9-17) were greater than in females ages 3-5 the relationship was not statistically significant ($P = 0.55$; $P = 0.60$).

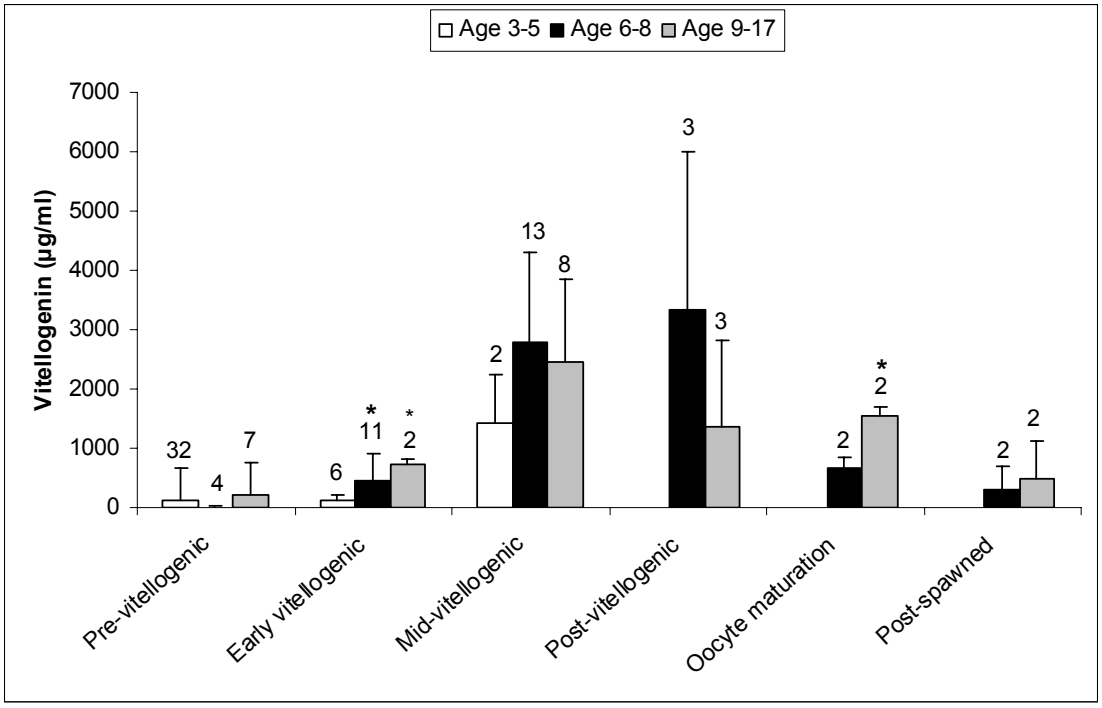

Figure 20. Mean (+ 1 SD) vitellogenin concentrations (µg/ml) compared between ovarian stages of development and age categories for female black rockfish in the months of September-May. Numerals above each bar represent sample sizes.

To further examine whether females of various ages showed different patterns in their reproductive cycles, I pooled age classes and months by season to increase sample size (Figures 21-22). Females of all age groups followed the same temporal sequence in hormone and protein production. Estradiol-17ß concentrations and VTG concentrations were low in the summer (June-August) during reproductive recrudescence. Production of E_2 began in September and increased in the months of September-November (Figure 21). There was a mean increase in E_2 concentrations for age 6-8 females between the months of September-November and December-February. There may have been a comparable increase for females age 9+ but only one female was sampled in the months of December-February. Vitellogenin

concentrations followed the same trend as E_2 concentrations (Figure 22). However, in March-May, the age 9+ females in my samples showed high vitellogenin concentrations, while both age 3-5 and 6-8 females VTG concentrations were virtually undetectable. The females age 9+ were undergoing oocyte maturation and nearing the completion of their cycle, whereas females age 6-8 were post-spawned.

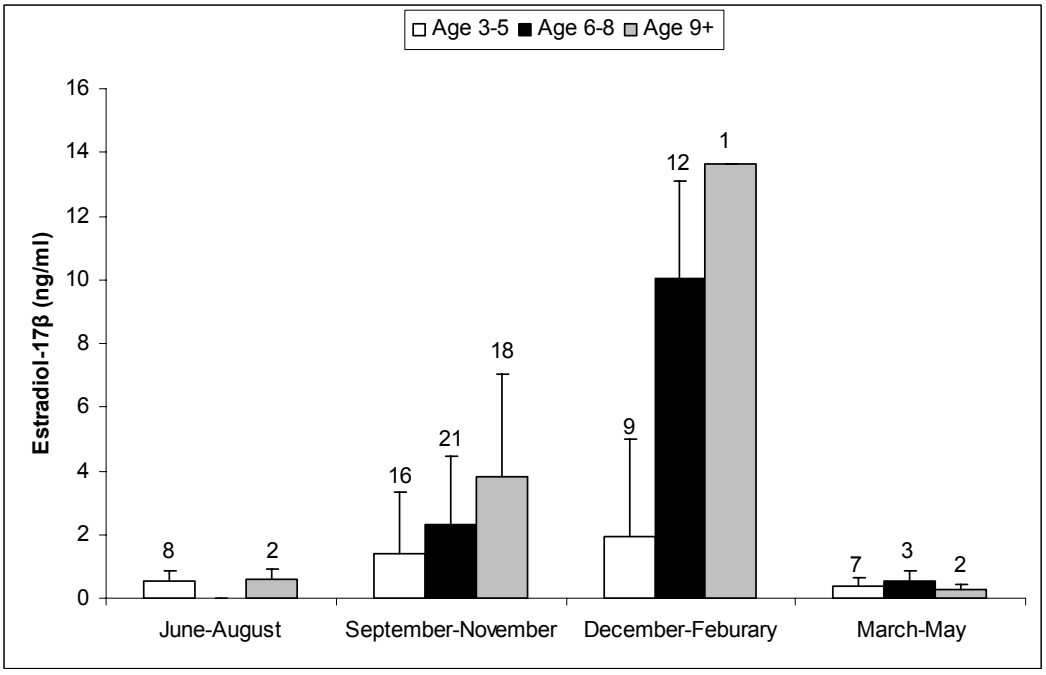

Figure 21. Mean (1+SD) quarterly estradiol-17β concentration (ng/ml) in female black rockfish. Sample numbers are for age 3-5, age 6-8, and 9+ in the months of June-August N = 8, 0, 2, September-November N = 16, 21, 18, December-February N = 9, 12, 1, and March –May N = 7, 3, 2, respectively. Numerals above each bar represent sample sizes.

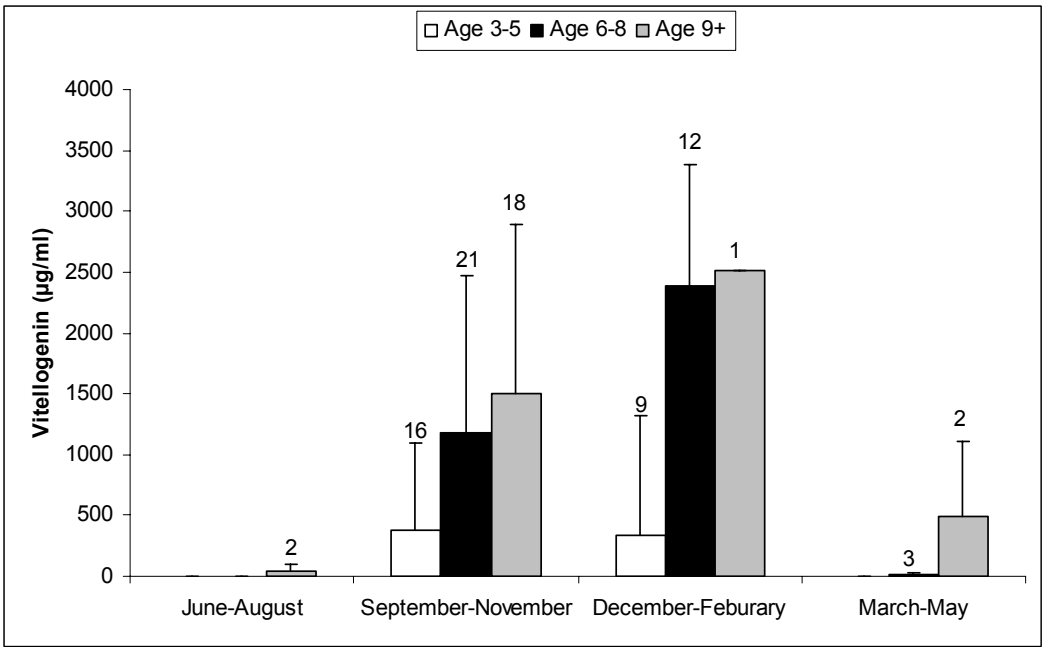

Figure 22. Mean (1+SD) quarterly vitellogenin concentration (µg/ml) in female black rockfish (*Sebastes melanops*) collected off of Newport and Depoe Bay, June-August N = 8, 0, 2, September-November N = 16, 21, 18, December-February N = 9, 12, 1, and March –May N = 7, 3, 2 respectively.

Discussion

Older females (age 9+) were observed in all stages of ovarian development throughout the spawning season (September-February). During the fall older (9+) females were post-vitellogenic or undergoing oocyte maturation where as younger females (ages 5-8) were early- to mid-vitellogenic. Younger females (5-8) sampled in the winter months made up 100% of the females that were post-spawned. However, only one female older than age 9 was sampled throughout the winter. There were no age 3-4 year old females that were undergoing vitellogenesis in the fall or winter. In the spring, 40% of the post-spawned females were age 8 years and 60% were 9+. Only pre-vitellogenic females were observed throughout the summer. These finding

coincide with the protracted spawning season previously reported for black rockfish (Bobko and Berkeley 2004).

Concentrations of E_2 for early vitellogenic (stage 3) fish of the age 9+ were significantly higher than in females ages 3-5 ($P<0.05$). Females' age 9+ undergoing oocyte maturation (stage 6) had higher concentrations of E_2 than females ages 6-8 ($P<0.05$). Early vitellogenic (stage 3) and mid-vitellogenic (stage 4) females ages 6-8 and 9+ had higher concentrations of VTG than in females ages 3-5 ($P<0.05$). Small samples sizes were prohibitive in comparing VTG and E_2 concentrations between all age groups and stages of maturity.

The three age groups (3-5, 6-8, 9+) followed a general pattern in hormone cycling. Concentrations of E_2 increased in the fall (September-November), stayed relatively high throughout the spawning period (September-February) and decreased to low concentrations by March. Several researchers have reported similar trends in hormone cycling in other rockfish species (Nagahama *et al.* 1991; Takahashi *et al.* 1991; Takano *et al.* 1991; Mori *et al.* 2003). These studies reported that E_2 concentrations increased prior to the spawning season, remained relatively high throughout vitellogenesis, and decreased prior to oocyte maturation and remained low through gestation. In this study female VTG concentrations increased in response to elevated E_2 concentrations throughout the spawning season (September-February), and decreased to low levels in March and remained low May-August. The smallest youngest females (age 3-5) during the spawning (September-November) had moderate concentrations of circulating E_2 and low but detectable concentrations of VTG. A

majority of these females had no visible evidence of yolk platelets histologically. Thus, it can be inferred that these younger females (age 3-5) were not going to spawn that year.

Age-related differences in the timing of spawning have been observed for several rockfish species.It has been observed for copper rockfish (*S. caurinus*) and black rockfish (*S. melanops*) that older females extruded larvae earlier in the spawning season (Cooper 2003; Bobko and Berkeley 2004). Eldridge *et al.* (1991) reported that larger (presumably older) yellowtail rockfish (*S. flavidus*) also extrude larvae earlier. In this study, older females were vitellogenic before younger females, suggesting that these older females were able to complete the reproductive cycle earlier than younger females. However, older females were also observed in early reproductive stages in later months, and females in younger age classes were found in post-spawning state in the winter. This suggests that there is more research needed on age and timing of larval release in this species. However, very few older female fish (9+) were sampled in this study (N = 25). The oldest female black rockfish was 17 years of age sampled in October, and was pre-vitellogenic (stage 2) but had high levels of VTG inferring that this female would have become vitellogenic that month. The reduction or removal of older larger female rockfish increases recruitment variability by reducing the length of the spawning season (Berkeley *et al.* 2004b).

It is unclear if the reason for the reduced number of older females in our collection was due to availability, or if overfishing has already reduced the abundance of older females. Ralston and Dick (2003) have estimated that only 10-20% of the

females are older than 20 years for the Oregon and northern California black rockfish stock. Kuzis (1985) showed that female black rockfish in Washington increase in age at greater depths and distance from shore. Several ripe females have been taken more than 1,000 km offshore in the Gulf of Alaska (Welch 1995). It is unknown whether Oregon female black rockfish make offshore spawning migrations.

This research has provided additional information on age-specific temporal differences in the timing of spawning in female black rockfish. Due to low samples sizes for some age groups significant differences between VTG and E_2 were not observable between all stages of maturity. I did not find conclusive evidence of differences in seasonal VTG or E_2 levels among age groups. With further studies, this methodology has the potential to give managers a better understanding of the annual endocrine cycle as it relates to the relationship between maternal effects and reproductive success.

CHAPTER 4: CONCLUSIONS AND MANAGEMENT RECOMMENDATIONS

Black rockfish (*Sebastes melanops*) are an important resource to both commercial and recreational fisheries on the Pacific West Coast. The National Marine Fishery Service has declared eight species of West Coast groundfish as overfished, seven of which are rockfish (PFMC 2003). Black rockfish have not been declared overfished but there is mounting evidence of the effects of overexploitation. Changes in black rockfish populations, such as compression in the size and age structure, have been seen over the past decade (Wallace *et al.*1999; Ralston and Dick 2003). Independent fisheries research efforts have observed fewer older females (age 9+) caught off the Oregon coast over the past decade, with the oldest female being 17 years old (Bobko and Berkeley 2004; Chapman 2004). As a result of these changes, characteristics such as age at first maturity, age at 50% maturity, and age of full maturity have been altered (Ralston and Dick 2003; Bobko and Berkeley 2004). Changes in the population age structure coupled with the loss of spawning biomass may determine recruitment success in a given year (Berkeley and Markle 1999; Berkeley *et al.* 2004b).

Management agencies from several states have recognized that a reduction in fishing mortality was needed given the reproductive constraints of rockfishes. Hence, Washington, Oregon, and California have designated management areas where fishing has been prohibited during parts of the year when particular rockfish of concern are spawning. Washington has established “no take” zones and voluntary marine reserves

in response to dramatic reductions in rockfish populations. Scuba surveys have been conducted in these reserves to measure species abundance and diversity. However, information on rockfish sex ratios and maturational status is lacking. The vitellogenin assay developed in this study could be directly applied to black rockfish within these reserves to provide information regarding sex and maturity status in females. With additional research this VTG assay may be applicable to other species of rockfish in order to evaluate the productivity of reserves. The effects of barotrauma (sudden decompression during capture that leads to injury or death) in captured rockfish have hampered our ability to collect basic biology and reproductive information on these species. However, researchers in the marine resource program (MRP) at Oregon Department of Fish and Wildlife (ODF&W) have developed a method for release that allows rockfish to reacclimatize to depth after being brought to the surface (Parker *et al.* 2006), so additional study with non-lethal sampling is now possible.

There are a number of current research projects being conducted through the MRP that could benefit from a non-invasive method for determining sex and maturity in rockfishes. In particular, the VTG ELISA created in this study could be useful for looking at sex ratios and maturity in fish that are tagged and released, such as long-term studies of fish distribution, movement, and exploitation rates that utilize Passive Integrated Transponder (PIT) tags. The data collected for the PIT tag study underway in Oregon will be used for population estimation models to determine exploitation rate and population size each year, and additional reproductive information would provide additional parameters for assessment.

The most critical research needs at this time for exploited and poorly known rockfish species are studies on physiological and biological parameters pertaining to reproduction. This information is essential to understand the effects of exploitation, population density, and environmental change on productivity and recruitment. These parameters can provide critical information for decision making by fisheries managers, and help them evaluate the overall reproductive potential of rockfish populations with appropriate measures.

This research has provided further information on the reproductive cycle in black rockfish. In this study, females were in spawning condition from September through February. Post-spawned females were observed as early as November and as late as May. Although, female black rockfish have a protracted spawning period it may be advantageous to institute temporal closures during all of or at least part of the spawning season as has been done in Washington and California.

The findings of this research have provided new information on the endocrine cycle in female black rockfish. The non-lethal method developed in this study could potentially be useful in the monitoring of the reproductive potential of black rockfish populations. With further research this method may be applicable to other rockfish species that are over-exploited or poorly understood, avoiding unnecessary mortality for research.

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