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Validating The Use Of Sex Steroid Hormones Extracted From The Skeletal Muscle Tissue Of The Atlantic Sharpnose Shark (Rhizoprionodon Terraenovae), Spiny Dogfish (Squalus Acanthias), And The Little Skate (Leucoraja Erinacea) To Assess Reproductive Status In Elasmobranch Fishes

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VALIDATING THE USE OF SEX STEROID HORMONES EXTRACTED FROM THE SKELETAL MUSCLE TISSUE OF THE ATLANTIC SHARPNOSE SHARK (*RHIZOPRIONODON TERRAENOVAE*), SPINY DOGFISH (*SQUALUS ACANTHIAS*), AND THE LITTLE SKATE (*LEUCORAJA ERINACEA*) TO ASSESS REPRODUCTIVE STATUS IN ELASMOBRANCH FISHES

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TABLE OF CONTENTS

ABSTRACT

VALIDATING THE USE OF SEX STEROID HORMONES EXTRACTED FROM THE SKELETAL MUSCLE TISSUE OF THE ATLANTIC SHARPNOSE SHARK (*RHIZOPRIONODON TERRAENOVAE*), SPINY DOGFISH (*SQUALUS ACANTHIAS*), AND THE LITTLE SKATE (*LEUCORAJA ERINACEA*) TO ASSESS REPRODUCTIVE STATUS IN ELASMOBRANCH FISHES

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Bianca Prohaska

University of New England, May 2013

Currently, circulating concentrations of plasma steroid hormones are used as one means to assess reproductive maturity and reproductive cycles in elasmobranchs. However, obtaining blood non-lethally from large and/or endangered species can be problematic because of difficulties involved with specimen handling. Thus, there is a need to develop new approaches and techniques to study the reproductive biology of elasmobranchs. Previous work conducted on other classes of vertebrates has demonstrated that steroid hormones can be successfully extracted from muscle tissue. The process of collecting muscle tissue samples is quick, minimally invasive, and may be conducted without removing the animal from the water, facilitating its use on larger, and/or endangered species of elasmobranchs. Thus, the objective of the current study was to develop and validate a method for extracting steroid hormones from the skeletal muscle tissue of the lecithotrophic aplacental viviparous spiny dogfish (*Squalus acanthias*), the oviparous little skate (*Leucoraja erinacea*), and the placental viviparous Atlantic sharpnose shark (*Rhizoprionodon terraenovae*). The results suggest that

concentrations of progesterone [P_4], testosterone [T], and estradiol [E_2] present in skeletal muscle of the three aforementioned species can be successfully quantified by radioimmunoassay. Additionally, there were significant correlations between plasma and muscle E_2 concentrations in *S. acanthias*, as well as plasma and muscle P_4, T and E_2 concentrations in *R. terraenovae* and *L. erinacea*. Furthermore, concentrations of muscle P_4, T and E_2 were determined to be statistically significant indicators of reproductive status. The results of the current investigation demonstrated that skeletal muscle, which can be non-lethally harvested, is a viable and practical alternative to collecting blood samples for studying the reproductive biology of elasmobranchs.

CHAPTER 1

DEVELOPMENT OF A NON-LETHAL AND MINIMALLY INVASIVE PROTOCOL TO STUDY ELASMOBRANCH REPRODUCTION

Abstract

An understanding of basic reproductive biology is essential for successful species-specific management of elasmobranch fishes (sharks, skates, and rays). Such information is often gained through gross dissection or other lethal techniques, which are not appropriate for threatened and endangered species. Previous work on other vertebrates suggests that sex steroid hormones can be extracted from muscle tissues to identify reproductive status. The process of collecting a muscle biopsy is quick, minimally invasive, and can be collected without removing an animal from the water. Thus, the objective of the current study was to determine the efficacy of muscle steroid hormones to assess the reproductive biology of elasmobranch fishes. The results from the current study suggest that concentrations of muscle progesterone, testosterone and estradiol can be successfully quantified to study reproduction by radioimmunoassay. Additionally, there were significant correlations between plasma and muscle estradiol concentrations in *Squalus acanthias*, and progesterone, testosterone and estradiol concentrations in *Rhizoprionodon terraenovae*. The results of the present investigation demonstrated that skeletal muscle is a non-lethally harvested tissue that is well suited for studying the reproductive biology of elasmobranchs.

Introduction

An essential component for assessing and managing populations of elasmobranchs (sharks, skates, and rays) is determining the species' specific reproductive biology, e.g. when sexual maturity occurs, timing of seasonal cycles, or gestation length (Walker 2004, 2005). Such information is essential for stock assessment purposes. Despite the importance of species-specific reproductive data to fisheries management (Walker 2004), these data are still unavailable for many species of elasmobranchs (Castro et al. 1999; Walker 2004; Pinhal et al. 2008), which has resulted in approximately 40% of the elasmobranch population being classified as data deficient (IUCN 2011). The lack of life history data when combined with direct and indirect fishing pressures make elasmobranchs susceptible to overexploitation worldwide, which can lead to population declines and localized extinction (Dulvy et al. 2003).

Historically, studies on the reproductive biology of elasmobranchs entailed sacrificing a relatively large number of specimens and observing changes in gross reproductive morphology over the reproductive cycle (e.g. Hisaw and Albert 1947; Zeiner and Wolf 1993; Walmsley-Hart et al. 1999; Francis et al. 2001; Ebert 2005; Ruocco et al. 2006). While this approach is still currently appropriate for elasmobranchs with sustainable population sizes (Heupel and Simpfendorfer 2010), it is not suitable for many because of their relatively low or unknown species abundance (IUCN 2011; Hammerschlag and Sulikowski 2011). Because of this limitation, alternative methods for determining size at maturity and/or reproductive cycles in elasmobranchs are being developed and validated (Sulikowski et al. 2007b).

One particular approach that has been utilized to non-lethally study the reproductive biology of elasmobranchs is the analysis of blood steroid hormone concentration (e.g. Perez and Callard 1993; Sulikowski et al. 2006; Kneebone et al. 2007; Sulikowski et al. 2007 a, b; Awruch et al. 2008; Henningsen et al. 2008). Reproductive hormones directly correlate with morphological changes within the reproductive tract, specifically at maturation (e.g. Gelsleichter et al. 2002; Sulikowski et al. 2006) and during reproductive cycles (e.g. Kneebone et al. 2007; Sulikowski et al. 2007 a, b). However, obtaining blood from large species is impractical and can be problematic because of difficulties involved with specimen handling. These restrictions also serve to protect the animals from stresses induced by handling procedures on large species (Skomal 2007). This is further compounded by the restrictions associated with sample collection from threatened and endangered species. Although plasma steroid hormone analysis is particularly useful for studying the reproductive biology of elasmobranchs, the aforementioned constraints make it necessary for new, less invasive approaches to be developed, especially for studying vulnerable species.

While steroid hormones are easily isolated from blood plasma, they are present in other body depots (Hoffmann 1978). For example, steroid hormones have been extracted from milk and/or muscle tissues from cows (Noppe et al. 2008), sheep, and chickens (Sawaya et al. 1998), urine and feces of free-ranging wildlife (Lasley and Kirkpatrick 1991; Holt and Pickard 1999), blubber from marine mammals (Mansour et al. 2002; Kellar et al. 2006) and the skeletal muscle tissue of chimaera (Barnett et al. 2009), for various reproductive analyses. Despite the successful extraction of steroid hormones from body depots other than plasma, this approach has never been conducted on an

elasmobranch. Given the lack of knowledge pertaining to the reproductive biology of elasmobranch fishes, the objectives of the present study were to determine: 1) if reproductive steroid hormones could be quantified from elasmobranch skeletal muscle tissue; and 2) if there is a relationship between muscle and plasma concentrations of these reproductive hormones.

Materials and Methods

Specimen Collection

Based on their relatively high abundance and ease of collection, the aplacental viviparous spiny dogfish (*Squalus acanthias*) and yolk sac placental viviparous Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*) were selected as study organisms. Female *S. acanthias* were primarily captured by otter trawl off the coast of Rhode Island (USA) aboard the 13.7 m bottom trawler *F/V Proud Mary* between December 2010 and February 2012, in an area centered around 71.600 °W and 41.365 °N. Additional spiny dogfish samples were captured by gill net in the Gulf of Maine aboard the 14 m bottom trawler *F/V Lady Victoria* in November 2010, in an area centered around 70.257 °W and 42.917 °N. Female *R. terraenovae* were captured by bottom longline in the Gulf of Mexico aboard the 51.8 m *R/V Oregon II*, during the 2011 NOAA Bottom Longline Survey, in an area centered around 82.628 °W and 25.822 °N (Figure 1).

Sampling

Immediately after capture, an 8 mL aliquot of blood was collected by caudal venipuncture using a heparinized needle and vacutainer. Blood samples were placed in a cooler (4°C) for up to 24 h before hematocrit analysis and centrifugation at 1,242 g for 5 min. The separated plasma was stored at -20°C in the laboratory until extraction. Gross morphological parameters were recorded including mass (kg), fork length (FL), and natural total length (TL), which were measured to the nearest cm over a straight line along the axis of the body from the tip of the snout to the posterior notch of the caudal fin, and to the posterior tip of the upper lobe of the caudal fin while in its natural position, respectively. Afterwards, all sharks were euthanized by severing the cervical vertebrae. Sharks captured in the Gulf of Maine, and off the coast of Rhode Island were kept in a shipboard cooler at (4°C) before transport, on ice, to the University of New England's Marine Science Center (MSC), and dissected approximately 24 h after capture. All sharks captured in the Gulf of Mexico were dissected approximately 1 h after capture. During dissections, the following were recorded: ovary and oviducal gland mass (to the nearest g); oviducal gland and the five maximum follicle diameters (to the nearest mm); and if present pup sex and stretch total length (STL), which was measured to the nearest mm over a straight line along the axis of the body from the tip of the snout to the posterior tip of the upper lobe of the caudal fin while fully extended along the axis of the body. In addition, a 5 g white skeletal muscle tissue sample from behind the second dorsal fin was collected and immediately stored at -20°C until extraction.

Plasma Steroid Hormone Extraction

Each plasma sample was extracted for 17β-estradiol (E_2), testosterone (T), and progesterone (P_4) following the protocols from Tsang and Callard (1987) and Sulikowski et al. (2004). A 500 μL aliquot of each plasma sample was extracted twice for each hormone with 10 volumes of ethyl ether (ACS grade), and the liquid phase was evaporated at 37 °C in a heat block under a stream of nitrogen. To account for procedural loss, each sample was spiked with 1,000 counts/min of tritiated [E_2], [T], or [P_4] (Perkin Elmer, Waltham, MA) prior to extraction. Extracts were reconstituted in phosphate buffered saline with 0.1% gelatin (PBSG).

Skeletal Muscle Tissue Steroid Hormone Extraction

Two grams of white skeletal muscle tissue from each shark were homogenized with 8 mL of cold phosphate buffered saline (PBS) for 30 s using a Kinematica PT 10-35 polytron (Bohemia, NY). Then, 500 μL aliquots of homogenate, in quadruplicate, were extracted for each hormone. Non-radiolabeled [E_2], [T], and [P_4] (Steraloids, Inc., Newport, RI) were used to make stocks (6.4 μg/mL for [E_2], and 80 μg/mL for [T] and [P_4]: all in ethanol, ACS grade) to "cold-spike" samples to calculate recoveries following extraction, and to generate the standard curves for radioimmunoassay. A 100 μL aliquot of each stock was evaporated under a stream of nitrogen, reconstituted in PBSG and serially diluted to make working stocks with final concentrations of 200 pg/100μL for [T] and [P_4], and 320 pg/100μL for [E_2]. Working stocks were utilized to cold-spike muscle

homogenates for calculating sample recovery. Of the four homogenate replicates, two replicates were cold-spiked with a 50 μL aliquot of the corresponding working stock (100 pg of [T] or [P_4]; 160 pg of [E_2]), to track sample recovery. All samples were heated in a 50 °C water bath for 15 min, briefly vortexed, and then cooled to room temperature. Samples were extracted with 10 volumes (5 mL) of 2:1 chloroform/methanol (ACS grade; Histology grade), and vortexed for 1 minute before snap freezing in a dry ice acetone bath. The liquid phase was removed by piercing the frozen homogenate layer with a pasture pipette and transferring to a new test tube. The liquid phase was then evaporated at 37 °C in a heat block under a stream of nitrogen. One mL of 70% methanol (Histology grade) was added to the dry extract, and the samples were stored at -20 °C for 24 h. Samples were centrifuged at 962 g for 10 min at 4 °C, and the liquid phase was decanted and evaporated at 37 °C in a heat block under a stream of nitrogen. Extracts were reconstituted in 200 μL of PBSG.

Individual muscle sample recoveries were calculated by subtracting the mean concentration of hormone found in the two non-spiked replicates from the mean concentration of hormone found in the two cold-spiked replicates. The resulting concentration of hormone was then divided by the concentration of hormone that was initially added to the two cold-spiked replicates (100 pg of [T] or [P_4]; 160 pg of [E_2]). To calculate the final concentration of hormone in each skeletal muscle sample (in pg/g), the mean concentration of hormone found in the two non-spiked replicates was calculated, multiplied by a dilution factor of 8 (to convert units to pg/g), and finally divided by the individual sample recovery.

Radioimmunoassay

Steroid hormone concentrations from both plasma and muscle extracts were determined following a modified radioimmunoassay procedure from Tsang and Callard (1987). Previously prepared non-radiolabeled [E_2], [T] and [P_4] stocks (6.4 µg/mL for [E_2], and 80 µg/mL for [T] and [P_4]) were utilized. Antibodies of [E_2], [T], and [P_4] (Fitzgerald Industries, Acton, MA), were diluted to final concentrations of 1:18,000, 1:10,000, and 1:2,500, respectively. Tritiated hormones, and antibodies were added to the reconstituted plasma samples (100 µL and 50 µL in duplicate) and to muscle samples (200 µL), using PBSG to bring the total volume to 400 µL. Assays were incubated at 4 °C for 24 h before separating free from bound hormone by the addition of a carbon (0.2%; Acros Organics, Fairlawn, NJ) and dextran 70 (0.02%; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) suspension followed by centrifugation at 1,242 g for 10 min at 4 °C. The resulting supernatant was then decanted into scintillation vials and 3.5 ml of Ecolume (MPO Biomedicals, Solon, OH) was added. The radioactivity level was then measured by a Perkin Elmer Tri-Carb 2900TR liquid scintillation analyzer (Waltham, MA). The mean intra-assay coefficients of variation for *S. acanthias* plasma assays were 8, 10, and 9% for [E_2], [T] and [P_4], respectively, and 7, 7, and 6% for *R. terraenovae* [E_2], [T] and [P_4] plasma assays, respectively. The mean inter-assay coefficients of variation for *S. acanthias* plasma assays were 10, 11, and 12% for [E_2], [T] and [P_4], respectively, and 8, 8, and 9% for *R. terraenovae* [E_2], [T] and [P_4] plasma assays, respectively. The mean intra-assay coefficients of variation for *S. acanthias* muscle assays were 8, 10 and 8% for [E_2], [T] and [P_4], respectively, and 10, 8, and 9% for *R.*

terraenovae [E_2], [T] and [P_4] muscle assays, respectively. The mean inter-assay coefficients of variation for *S. acanthias* muscle assays were 10, 11 and 12% for [E_2], [T] and [P_4], respectively. For calculating mean (± standard error) for concentrations of each steroid hormone per stage, any value that was non-detectable was assigned the lowest possible concentration that the assay would have been able to detect in the aliquots utilized.

Statistical Analysis

Linear regressions were performed for [E_2], [T] and [P_4] quantified from plasma and skeletal muscle. Data were transformed if variables failed tests of normality or homogeneity of variance. If transformed variables still violated the assumptions the nonparametric Kendall's tau rank correlation was conducted. All data were analyzed using R 2.13.1 (R-Core Development, 2011) and graphed using means and standard error in Sigmaplot 12 (Systat Software, San Jose, CA). All tests were considered significant at $\alpha \leq 0.05$.

Results

A total of 31 female *S. acanthias* (78-90 cm FL, 2.7-4.9 kg) were collected, and divided into discrete reproductive stages (Table 1), based on previous literature (Hisaw and Albert 1947; Tsang and Callard 1987); the group included six pre-ovulatory, six containing candles, five early gestation females containing 62-88 mm embryos, eight mid

gestation females containing 190-240 mm embryos, and six late gestation females containing 250-275 mm embryos. Ten female *R. terraenovae* (68-85 cm FL, 2.4-4.6 kg) were collected and divided into arbitrary discrete reproductive stages (Table 2), including one pre-ovulatory, two early gestation females containing 28-55 mm embryos, four early-mid gestation females containing 56-83 mm embryos, and three mid gestation females containing 85-139 mm embryos. The overall mean recoveries of plasma [E_2], [T], and [P_4] extractions were 80, 87, and 70% for *S. acanthias*, and 71, 85, 68% for *R. terraenovae,* respectively; while the overall mean recoveries of muscle [E_2], [T], and [P_4] extractions were 21, 23, and 20% for *S. acanthias,* and 59, 39, and 55% for *R. terraenovae,* respectively.

S. acanthias

In pre-ovulatory female *S. acanthias*, [P_4] concentrations in both plasma (658 ± 186 pg/mL) and muscle (543 ± 285 pg/g) were similar. However, plasma [P_4] (60 ± 22 pg/mL) decreased by 10-fold during the candle stage, while muscle [P_4] (2412 ± 420 pg/g) increased by about 5-fold. As gestation progresses, females in early gestation displayed relatively elevated plasma (1004 ± 225 pg/mL) and muscle (1150 ± 209 pg/g) [P_4] concentrations before they declined [plasma (159 ± 38 pg/mL) and muscle (386 ± 89 pg/g)] through the remainder of gestation (Figure 2a). Overall, muscle [P_4] concentrations were not significantly related to plasma [P_4] concentrations (Kendall's tau=0.16; p=0.23).

During the candle stage, [T] concentrations in *S. acanthias* plasma (23 ± 8 pg/mL) and muscle (74 ± 24 pg/g) were low, and they remained that way through mid gestation [plasma (47 ± 11 pg/mL) and muscle (134 ± 34 pg/g)]. However, during late gestation, plasma [T] increased (423 ± 127 pg/mL), while muscle [T] remained unchanged (78 ± 18 pg/g). The highest muscle (447 ± 54 pg/g) and plasma (668 ± 255 pg/mL) [T] concentrations were found in the pre-ovulatory stage (Figure 2b). Similar to [P_4], muscle [T] was not significantly related to plasma [T] in *S. acanthias* (Kendall's tau=0.16; p=0.30).

From pre-ovulation to mid gestation, *S. acanthias* had low plasma (65 ± 12 pg/mL) and muscle (113 ± 39 pg/g) [E_2] concentrations. But, concentrations of [E_2] increased in plasma (864 ± 116 pg/mL) and muscle (364 ± 73 pg/g) through the second half of gestation (Figure 2c). Unlike [P_4] and [T], *S. acanthias* muscle [E_2] concentrations were significantly related to plasma [E_2] (Kendall's tau=0.49; p=0.0017).

R. terraenovae

Throughout pre-ovulation to early gestation, concentrations of [P_4] and [T] in *R. terraenovae* were relatively low in plasma ([P_4]: 162 ± 76 pg/mL, [T]: 151 ± 119 pg/mL) and muscle ([P_4]: 182 ± 76 pg/g, [T]: 84 ± 34 pg/g). However, a marked elevation of these two hormones occurred in plasma ([P_4]: 3582 ± 1767 pg/mL, [T]: 4840 ± 884 pg/mL) and muscle ([P_4]: 3325 ± 1253 pg/g, [T]: 693 ± 84 pg/g) samples in early-mid gestation females. Concentrations of [P_4] and [T] then decreased in the plasma ([P_4]: 132 ± 9 pg/mL, [T]: 83 ± 23 pg/mL) and muscle ([P_4]: 391 ± 63 pg/g, [T]: 141 ± 91 pg/g)

through mid gestation (Figure 3 a and b). Muscle [P_4] and [T] of *R. terraenovae* were found to be significant predictors of plasma [P_4] and [T] (linear regression: [P_4] R^2=0.65, p=0.0048; [T] R^2=0.84, p=0.00022).

The pre-ovulatory *R. terraenovae* female contained relatively elevated concentrations of [E_2] in plasma (4885 pg/mL) and muscle (232 pg/g). Concentrations of [E_2] progressively decreased in both plasma (762 ± 167 pg/mL) and muscle (72 ± 12 pg/g) through early to mid gestation (Figure 3c). Similar to [P_4] and [T], concentrations of *R. terraenovae* muscle [E_2] were found to be significant predictors of plasma [E_2] (linear regression: R^2=0.79, p=0.00059).

Discussion

Concentrations of reproductive hormones were successfully detected in the muscle and plasma of female *S. acanthias* and *R. terraenovae* during specific chronological events in the reproductive cycle. In addition, significant relationships were found between plasma and skeletal muscle concentrations of [E_2] in *S. acanthias* and [E_2], [P_4], and [T] in *R. terraenovae*, suggesting that concentrations of hormones in the skeletal muscle of elasmobranchs have the potential to be an indicator of gestational stage.

S. acanthias

In the current study, plasma and muscle [P_4] in *S. acanthias* exhibited similar patterns during distinct gestational stages to those previously observed by Tsang and

Callard (1987). For example, in both studies, [P_4] concentrations were elevated in the first half of gestation, and began to decrease after mid gestation through parturition (Tsang and Callard 1987). Although not measured by Tsang and Callard (1987), the pre-ovulatory levels of [P_4] concentrations determined in the present study were relatively elevated in both plasma and muscle when compared to the other gestational stages examined. Based on the morphological data, this increase in [P_4] could inhibit further vitellogenesis (Paolucci and Callard 1998; Koob and Callard 1999), as no substantial increase in follicle diameter was noted between near parturition or pre-ovulatory females (Table 1). Follicles in this species develop concurrently with gestation, with mating and ovulation occurring shortly after birth; thus, there is presumably little follicular growth after parturition (Castro 2009). There was an observed disparity between the patterns of plasma and muscle [P_4] concentrations during the candle stage of gestation. While the reasons behind this disparity cannot be determined from the current study, the differences could be attributed to [P_4] uptake/clearance (D'Ercole et al. 1984), or metabolism (Payne and Hales 2004) in the plasma and skeletal muscle.

Testosterone concentrations in plasma and muscle displayed similar patterns, remaining low as gestation progressed from the candle stage through mid gestation. However, this hormone began to increase in the plasma near the end of gestation and peaked in both tissues during pre-ovulation. While there was no significant relationship between [T] concentrations in the plasma and muscle of this species, the patterns exhibited by plasma and skeletal muscle are consistent with the values observed by Tsang and Callard (1987). The functional significance of the peak in [T] during pre-ovulation has yet to be studied. It is likely not related to folliculogenesis, since follicles develop

concurrently with gestation in *S. acanthias*, with theoretically little follicular growth from parturition to pre-ovulation (Castro 2009). It is possible, however, that the peak in [T] may have a role in the storage and maintenance of sperm viability within the oviducal gland, which has been proposed in other elasmobranch species (Manire et al. 1995; Henningsen 1998).

Estradiol concentrations in the plasma and muscle of *S. acanthias* remained relatively low from pre-ovulation to mid gestation. However, substantial increases in this hormone began after mid gestation and remained elevated until just prior to parturition. These observed [E_2] patterns in the current study parallel those found by Tsang and Callard (1987). However, unlike [P_4] and [T] in this species, a significant relationship was found between plasma and muscle [E_2] concentrations, indicating that skeletal muscle [E_2] could potentially act as a reliable indicator of gestational stage in *S. acanthias*. Similar to [P_4] and [T], pre-ovulatory concentrations of [E_2] in *S. acanthias* have not been previously quantified. Here the present study found low concentrations in the plasma and muscle, which were expected because of the presumed minimal follicular growth after parturition (Castro 2009).

R. terraenovae

The patterns of plasma and muscle [P_4] and [T] concentrations were similar in that they were relatively low from pre-ovulation through early gestation before a distinct peak was noted in early-mid gestation females. This pronounced elevation of plasma and muscle [P_4] and [T] concentrations coincided with the gestational stage when uterine

compartmentalization and implantation are suggested to arise in *R. terraenovae* (Castro and Wourms 1993). Interestingly, similar findings were reported by Manire et al. (1995), who observed that [P_4] and [T] concentrations were heightened in *S. tiburo* during early gestation prior to implantation, which was proposed to be a result of uterine compartmentalization (Schlernitzauer and Gilbert 1966; Callard et al. 1992). Notably, muscle [P_4] and [T] concentrations were found to be significant predictors of [P_4] and [T] in the plasma, suggesting that these two hormones in the skeletal muscle tissue have the potential to be indicators of gestational stage in this species.

The patterns of plasma and muscle [E_2] concentrations in *R. terraenovae* also coordinately varied, being elevated during pre-ovulation before decreasing through mid gestation. This same pattern was also observed by Manire et al. (1995), who noted that in *S. tiburo*, [E_2] peaks in the pre-ovulatory stage during the final phase of folliculogenesis, and decreases from early to mid gestation. In addition, muscle [E_2] concentrations in *R. terraenovae* were significant predictors of plasma [E_2] concentrations, indicating that skeletal muscle [E_2], just like [P_4] and [T], has the potential to be an indicator of gestational stage in this species.

Conclusions

In the present study, we developed a method to quantify steroid hormones in skeletal muscle and tested their efficacy, when compared to plasma hormone concentrations, to assess the reproductive status of sharks. Further, an advantage of skeletal muscle over plasma is that muscle tissue can be obtained non-lethally and with

minimal invasion. The results suggest that white skeletal muscle tissue of *S. acanthias* and *R. terraenovae* is a depot for the reproductive hormones [P_4], [T] and [E_2], which can be reliably measured by radioimmunoassay. Additionally, the patterns of muscle reproductive hormones, coincident with gestational stage, paralleled those in plasma, despite the different reproductive modes exhibited by these two sharks. In the present study, our preliminary results thus far suggested that skeletal muscle steroid hormone content relates well to gestational stage in yolk sac placental species. As such, future *R. terraenovae* sampling efforts will be focused on collecting the later stages of gestation to determine if the parallel patterns observed in plasma and muscle steroid hormone concentrations are consistent throughout the reproductive cycle. On the other hand, while there were no significant relationships between plasma and muscle [P_4] and [T] concentrations in *S. acanthias*, the similar patterns exhibited by these two hormones in plasma and skeletal muscle suggest that they could still be used to broadly assess gestational stage in *S. acanthias.* Certainly, because of the stronger relationship between plasma and skeletal muscle [E_2] concentrations, this hormone may be a better indicator of gestational stage in *S. acanthias*.

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Table 1. *Squalus acanthias* morphological data. Mean (± standard error) maximum follicle diameter (MFD) in mm, and mean (± standard error) pup stretch total length (STL) in mm, which was measured to the nearest mm over a straight line along the axis of the body from the tip of the snout to the posterior tip of the upper lobe of the caudal fin while fully extended along the axis of the body, by gestational stage: pre-ovulatory (P/O), candle, early, mid, and late gestation. N represents sample size by stage.

Stage	MFD (mm)	Pup STL (mm)	N
P/O	44.8 ± 1.5	N/A	5
Candle	10.0 ± 0.4	Candle	6
Early	15.6 ± 1.2	76.8 ± 5.0	5
Mid	33.8 ± 0.6	223 ± 8.3	8
Late	44.9 ± 1.2	261 ± 5.5	6

Table 2. *Rhizoprionodon terraenovae* morphological data. Mean (± standard error) maximum follicle diameter (MFD) in mm, and mean (± standard error) pup stretch total length (STL) in mm, which was measured to the nearest mm over a straight line along the axis of the body from the tip of the snout to the posterior tip of the upper lobe of the caudal fin while fully extended along the axis of the body, by gestational stage: pre-ovulatory (P/O), early, early-mid, and mid gestation. N represents sample size by stage.

Stage	MFD (mm)	Pup STL (mm)	N
P/O	15.6	N/A	1
Early	4.80 ± 0.9	37.7 ± 9.8	2
Early-Mid	6.15 ± 1.1	66.8 ± 4.2	4
Mid	4.37 ± 0.2	119 ± 18	3

Figure Legends

Figure 1. Center of sampling areas of *S. acanthias*, points 1 and 2, and *R. terraenovae*, point 3.

Figure 2. Mean (± standard error) concentrations of plasma (pg/mL) and muscle (pg/g) steroid hormones. (A) Progesterone (B) testosterone and (C) estradiol in *Squalus acanthias*, plotted by gestational stages: pre-ovulatory (P/O), candle, early, mid, and late gestation. Numbers appearing above the x-axis represent sample size. Note the difference in scale between the plasma and muscle axes.

Figure 3. Mean (± standard error) concentrations of plasma (pg/mL) and muscle (pg/g) steroid hormones. (A) Progesterone (B) testosterone and (C) estradiol in *Rhizoprionodon terraenovae*, plotted by gestational stages: pre-ovulatory (P/O), early, early-mid, and mid gestation. Numbers appearing above the x-axis represent sample size. Note the difference in scale between the plasma and muscle axes.

Figure 1

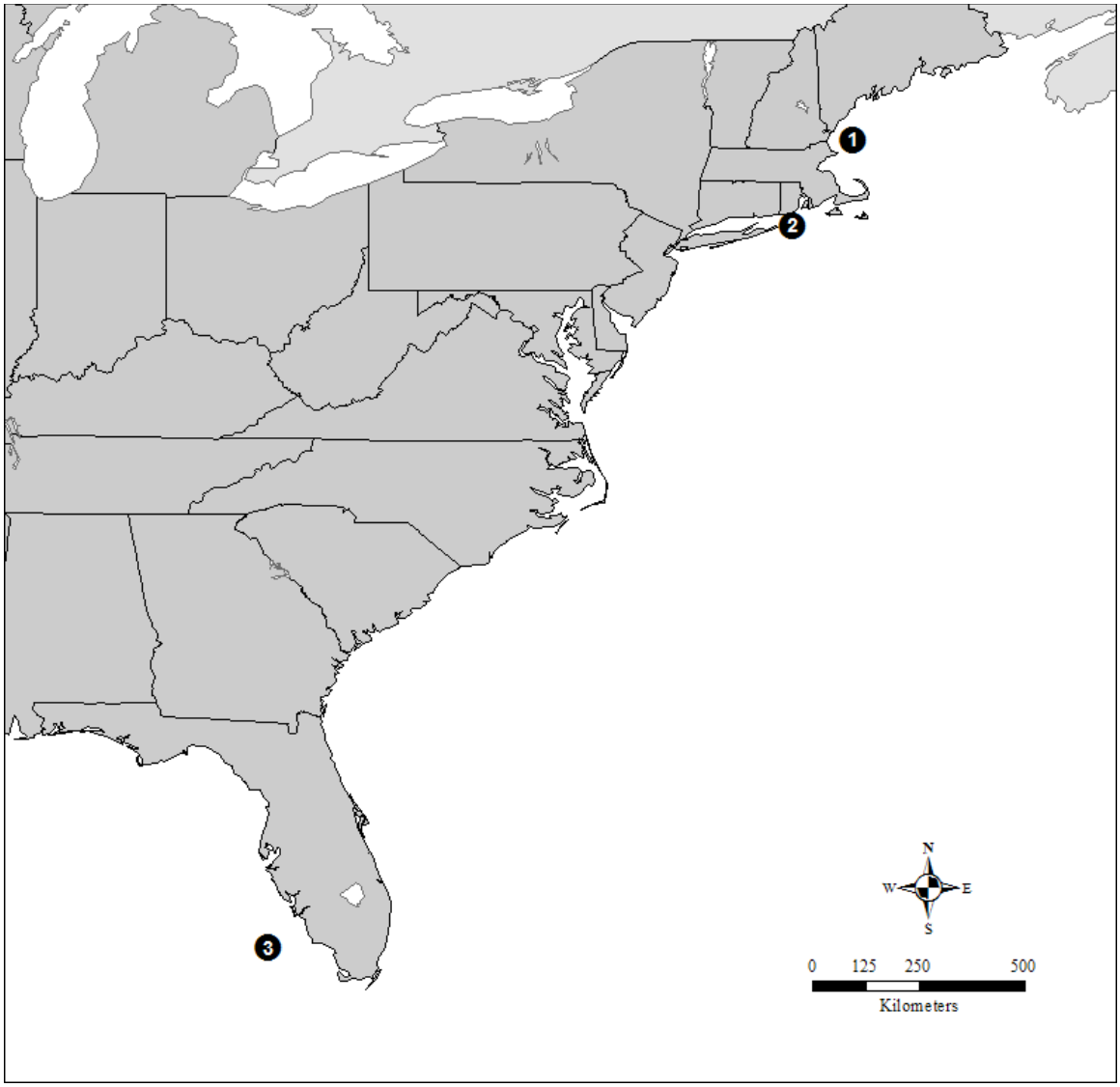


Figure 2

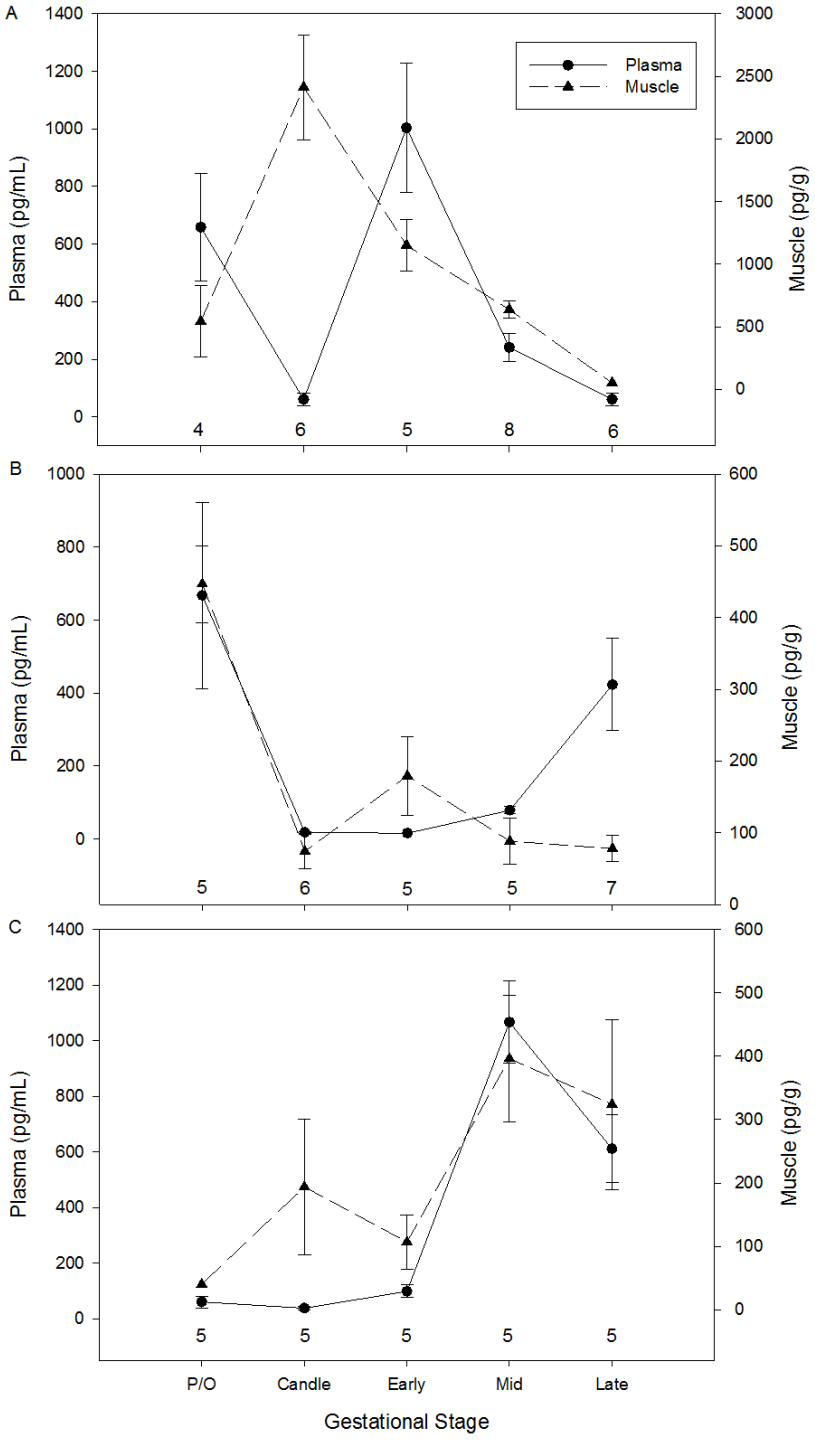
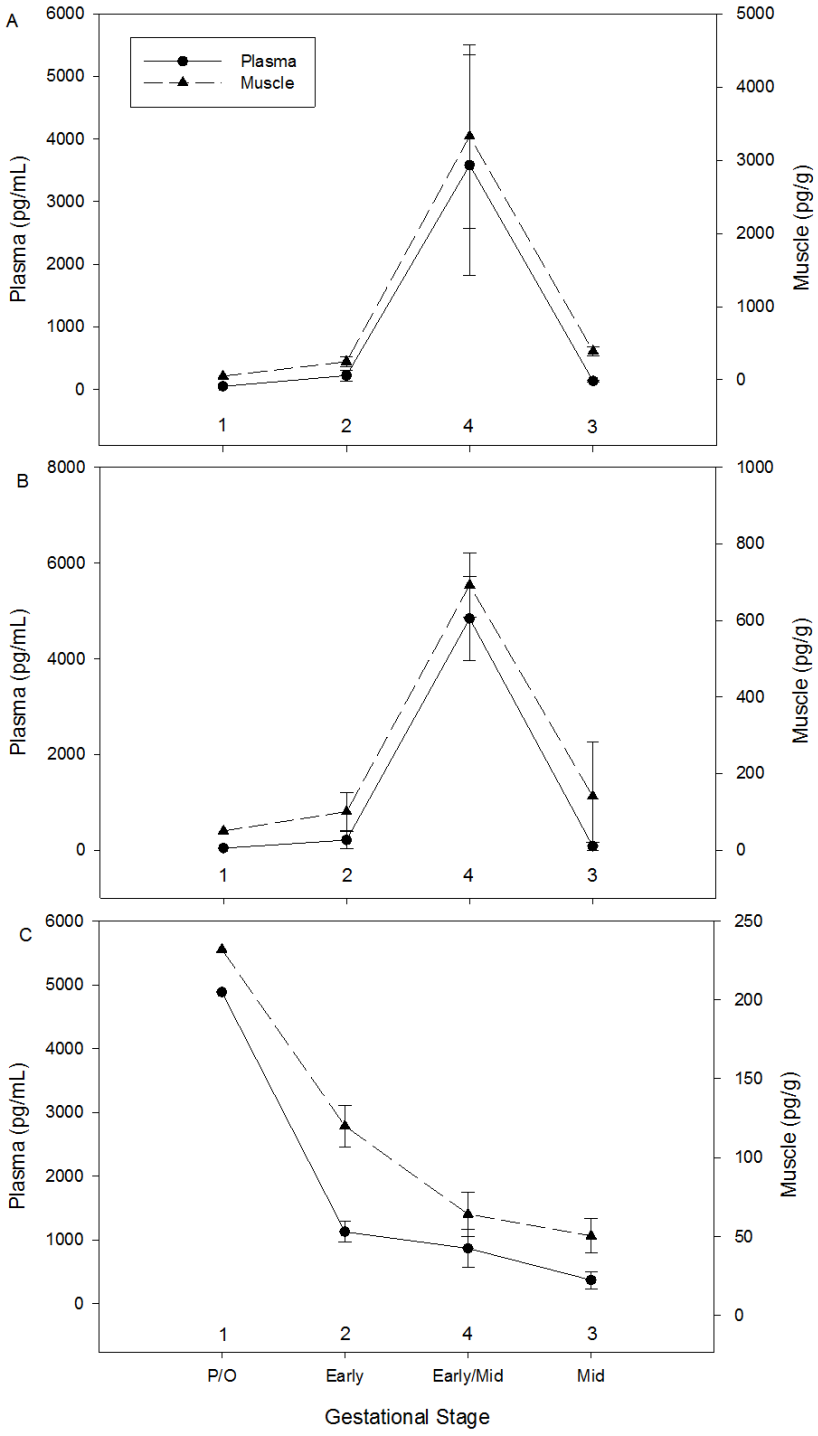

A
B
C
Plasma
Muscle
Plasma (pg/mL)
Muscle (pg/g)
4
6
5
8
6
5
6
5
5
7
5
5
5
5
5
P/O
Candle
Early
Mid
Late
Gestational Stage

Figure 3


A
B
C
Plasma
Muscle
Plasma (pg/mL)
Muscle (pg/g)
1
2
4
3
P/O
Early
Early/Mid
Mid
Gestational Stage

CHAPTER 2

ASSESSING REPRODUCTIVE STATUS IN ELASMOBRANCH FISHES USING STEROID HORMONES EXTRACTED FROM SKELETAL MUSCLE TISSUE

Abstract

Elasmobranch fishes (sharks, skates, and rays) are particularly susceptible to anthropogenic threats, making a thorough understanding of their life history characteristics essential for proper management. Historically, elasmobranch reproductive data have been collected by lethal sampling, an approach that is problematic for threatened and endangered species. However, recent studies have demonstrated that non-lethal approaches can be as effective as lethal ones in assessing reproductive status. Besides plasma, we previously reported that skeletal muscle tissue, which can be obtained non-lethally and with minimal stress, is also a depot for steroid hormones. With the diverse reproductive modes exhibited by elasmobranchs, being able to determine the efficacy of skeletal muscle steroid concentrations to assess reproductive status in these fishes will enhance our knowledge of their life histories, especially for threatened and endangered species. Thus, the objective of the current study was to determine the relationship between skeletal muscle and plasma steroid hormone concentrations, along with the associated morphological changes in the reproductive tract, during the reproductive cycles in species representing the three main reproductive modes: oviparity (*Leucoraja erinacea)*, aplacental viviparity (*Squalus acanthias*), and yolk-sac placental

viviparity (*Rhizoprionodon terraenovae*). The results show that concentrations of skeletal muscle progesterone (P_4), testosterone (T), and estradiol (E_2) were successfully quantified by radioimmunoassay in all three elasmobranch fishes. Additionally, statistically significant associations were noted between plasma and skeletal muscle P_4, T and E_2 concentrations in *R. terraenovae* and *L. erinacea*. Furthermore, concentrations of muscle P_4, T and E_2 were determined to be statistically significant indicators of reproductive status in the three aforementioned species. Overall, the results of the current study demonstrated that steroid hormones present in non-lethally harvested skeletal muscle tissue can be used as (strong) indicators of reproductive status and reproductive stage in elasmobranchs.

Introduction

The life history characteristics of elasmobranchs, such as slow growth, and maturity late in life, make these species particularly susceptible to overexploitation (Dulvy et al., 2003). As a result, anthropogenic threats, such as direct and indirect commercial fishing, and the illegal fin trade industry, have led to significant population declines in numerous elasmobranch species (Dulvy et al., 2008). In order to manage elasmobranchs effectively, a comprehensive understanding of their life history characteristics is needed. However, data on these characteristics are lacking for many species (Castro et al., 1999; Walker, 2004; Pinhal et al., 2008; IUCN, 2011). For instance, an essential life history characteristic needed for proper management is species-specific reproductive biology, such as the onset of maturity, gestation length, and seasonal

cyclicity (Walker, 2004, 2005). When this information is incorporated into fisheries models and species assessments, insight can be gained into when, where, and how often populations are reproducing, which can ultimately aid in reducing their decline through the development of management protocols that augments fishery practices (Walker, 2004, 2005). For example, the management of blacknose sharks within the United States was directly affected and improved by the inclusion of data detailing regionally distinct reproductive periodicity (NMFS, 2011).

Lethal sampling has historically been considered the most effective approach for collecting life history information from elasmobranchs, particularly because it is quick and provides a comprehensive set of data (Heupel and Simpfendorfer, 2010). More recently, the response to species declines and ethical concerns has moved elasmobranch research towards complete preservation, which has led to the development of non-lethal sampling approaches (Sulikowski et al., 2007; Heupel and Simpfendorfer, 2010; Hammerschlag and Sulikowski, 2011).

Among the non-lethal approaches to study the reproductive biology of elasmobranchs, such as intrauterine endoscopy (Carrier et al., 2003) and ultrasonography (Daly et al., 2007), analysis of plasma hormones is the most widely used (e.g Kneebone et al., 2007; Sulikowski et al., 2007; Henningsen et al., 2008). In particular, plasma steroid hormones have been found to correlate directly to reproductive events in elasmobranchs, such as the onset of maturity (Gelsleichter et al., 2002; Sulikowski et al., 2006) and reproductive activity (Sulikowski et al., 2004; Kneebone et al., 2007). Although plasma steroid hormone analysis has been conducted on many species of elasmobranchs spanning multiple reproductive modes (e.g. Gelsleichter et al., 2002;

Sulikowski et al., 2006; Awruch et al., 2008; Henningsen et al., 2008), obtaining blood can be stressful for the animal because of the significant amount of handling time and prolonged removal from the water (Skomal, 2007). A new non-lethal approach that is potentially less stressful and less invasive has been developed recently. Prohaska et al. (2013) investigated the efficacy of extracting steroid hormones from the skeletal muscle tissue of elasmobranchs representing two reproductive modes, the yolk sac placental viviparous *Rhizoprionodon terraenovae* and the aplacental viviparous *Squalus acanthias.* They found that steroid hormones can be successfully extracted and quantified from elasmobranch skeletal muscle tissue. But more importantly, fluctuations in these hormones may relate to gestational stage (Prohaska et al., 2013).

It is critical that effective non-lethal protocols for studying elasmobranch reproductive biology are thoroughly tested and validated prior to the shift towards their strict use in research, akin to what occurred for marine mammals in the United States (NMFS, 1972; Heupel and Simpfendorfer, 2010; Hammerschlag and Sulikowski, 2011). Because of the greater diversity of reproductive modes (yolk-sac placental viviparity, oviparity, and aplacental viviparity) in elasmobranchs and coupled with the promising results of Prohaska et al. (2013), the objectives of the present study were: 1) to attain a more comprehensive understanding of muscle steroid hormones in *R. terraenovae*; 2) to determine if this approach is appropriate for studying the reproductive biology of oviparous elasmobranchs; and 3) to determine whether skeletal muscle steroid hormones can be used as non-lethal indicators of reproductive status in elasmobranchs.

Methods and Materials

Specimen Collection

Female *R. terraenovae* (yolk-sac placental viviparous) and female *S. acanthias* (aplacental viviparous) were captured using the same methods, and in the same locations as previously described in Prohaska et al. (2013). Briefly, *R. terraenovae* were captured by bottom longline in the Gulf of Mexico in an area centered around 88.812 °W and 27.887 °N, while *S. acanthias* were captured by bottom trawl and gill net in the US Northwest Atlantic in an area centered around 70.115 °W and 42.471 °N. Female *L. erinacea* (oviparous) were captured by bottom trawl in November 2012 in an area centered around 70.466 °W and 42.615 °N. After capture, *L. erinacea* were maintained in an insulated 833 L live well containing ambient surface seawater. To ensure water quality was maintained, frequent water changes occurred during the approximately one and a half hour steam to the dock. All live-captured *L. erinacea* were transported from the dock in an aerated 833 L insulated live well to the University of New England's Marine Science Center (~2 h) and housed in a 3,785 L, 2.4 m diameter hexagonal tank with an open flow-through seawater system with a turnover rate of 38 L min-1. Animal husbandry of *L. erinacea* followed the protocols of Palm et al. (2011). Additionally, skates were palpated daily for up to three weeks to assess presence or absence of egg cases. Immediately prior to sampling, *L. erinacea* were euthanized by lethal pithing.

Sampling

Sampling of *R. terraenovae, S. acanthias,* and *L. erinacea* followed the same protocols as those in Prohaska et al. (2013). Briefly, at the time of sampling, an 8 mL aliquot of blood was collected and stored at 4°C for up to 24 h. Blood was analyzed for hematocrit prior to being centrifuged at 1,242 g for 5 min. Plasma was then removed and stored at -20°C until steroid hormone analysis. The following morphological parameters were recorded: mass (kg), fork length (FL; in sharks), disc width (DW; in skates) and natural total length (TL), all of which were measured to the nearest cm over a straight line along the axis of the body. Additionally, ovary and shell gland mass (g), shell gland width (mm), and follicle diameter (mm) were recorded. From sharks, pup sex and stretch total length (STL) (mm), measured to the nearest mm over a straight line along the axis of the body, were also recorded. Most importantly, a 5 g white skeletal muscle tissue sample was collected from behind the second dorsal fin from all sharks, and from the midpoint of the right wing from all skates, and immediately stored at -20°C until analysis (Prohaska et al., 2013).

Plasma Steroid Hormone Extraction

All plasma samples were extracted for progesterone (P_4) testosterone (T), and 17-β-estradiol (E_2) following the methods of Tsang and Callard (1987) and Sulikowski et al. (2004). Briefly, each plasma sample was extracted twice with 10 volumes of ethyl ether (ACS grade), and the organic phase was evaporated at 37 °C under a stream of nitrogen.

Extracts were reconstituted in phosphate buffered saline containing 0.1% gelatin (PBSG). Prior to extraction, each sample was spiked with 1,000 counts min^{-1} of tritiated P_4, T, or E_2 (Perkin Elmer, Waltham, MA) to account for procedural loss. The overall mean recoveries of P_4, T, and E_2 were 74, 91, and 78 % for *R. terraenovae*, and 51, 83, and 60% for *L. erinacea,* respectively.

Skeletal Muscle Tissue Steroid Hormone Extraction

All white skeletal muscle tissue samples were also extracted for P_4, T, and E_2 following the protocol of Prohaska et al. (2013). Briefly, for analyzing each steroid, two grams of white skeletal muscle tissue from each individual were homogenized with 8 mL of cold phosphate buffered saline (PBS) and divided in quadruplicate. All replicates were incubated at 50 °C for 15 min, prior to extraction with 10 volumes of 2:1 chloroform/methanol (ACS grade; Histology grade). The organic phase was evaporated at 37 °C under a stream of nitrogen before reconstitution in one mL of 70% methanol (Histology grade), and kept at -20° C for 24 h. Samples were then centrifuged at 962 g for 10 min at 4 °C, before decanting and evaporating the methanol phase at 37 °C under a stream of nitrogen. Dried extracts were reconstituted in PBSG. To account for procedural loss, two of the four replicates for each muscle sample were cold-spiked with the corresponding non-radiolabelled steroid hormone (Steraloids, Inc., Newport, RI) prior to extraction. The overall mean recoveries of P_4, T, and E_2 were 52, 47, 51% for *R. terraenovae*, and 61, 50, and 68% for *L. erinacea,* respectively.

Radioimmunoassay

Plasma and muscle steroid hormone concentrations were determined by radioimmunoassay, following a modified protocol from Tsang and Callard (1987). Non-radiolabeled P_4, T, and E_2 (Steraloids, Inc., Newport, RI) were used to make stock concentrations of 80 μg/mL for P_4 and T, and 6.4 μg/mL for E_2 in absolute ethanol (ACS grade). The P_4, T and E_2 antibodies (Fitzgerald Industries, Acton, MA) were diluted to final concentrations of 1:2,500 1:10,000, and 1:18,000, respectively. Tritiated hormones and antibodies were added to the reconstituted plasma and muscle samples, using PBSG to bring the total assay volume to 400 μL. After incubation at 4 °C for 24 h, free hormone was separated from bound hormone by the addition of a carbon (0.2%; Acros Organics, Fairlawn, NJ) and dextran 70 (0.02%; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) suspension, and centrifuged at 1,242 g for 10 min at 4 °C. The supernatant was combined with 3.5 mL of Ecolume (MPO Biomedicals, Solon, OH) and the radioactivity was detected by a Perkin Elmer Tri-Carb 2900TR liquid scintillation analyzer (Waltham, MA). The mean intra-assay coefficients of variation for *R. terraenovae* were 8, 7, and 5% for P_4, T, and E_2 plasma assays, respectively, and 10, 7, 4% for *L. erinacea* P_4, T, and E_2 plasma assays, respectively. The mean inter-assay coefficients of variation for *R. terraenovae* plasma assays were 12, 10, 12% for [P_4], [T], and [E_2], respectively, and 12, 12% for *L. erinacea* P_4, and E_2 plasma assays, respectively. The mean intra-assay coefficients of variation for *R. terraenovae* were 10, 8, and 7% for P_4, T, and E_2 muscle assays, respectively, and 5, 11, and 9% for *L. erinacea* P_4, T, and E_2 muscle assays, respectively. The mean inter-assay coefficients of variation for *R.*

terraenovae were 12, 13, 12% for P_4, T, and E_2 muscle assays, respectively, and 13, 12, 13% for *L. erinacea* P_4, T, and E_2 muscle assays, respectively. The mean intra-assay and inter-assay coefficients of variation for *S. acanthias* can be found in Prohaska et al. (2013). When calculating mean and standard error (± SEM) of plasma and muscle steroid hormone concentrations per stage, any value that was non-detectable was assigned the lowest possible concentration detectable by the assay for the aliquot utilized.

Statistical Analysis

Data obtained from ten *R. terraenovae* in Prohaska et al. (2013) were pooled with all *R. terraenovae* data collected in the present study for analysis. Additionally, statistical analyses were conducted on data obtained from 31 *S. acanthias* in Prohaska et al. (2013). Linear regressions were performed on plasma and skeletal muscle P_4, T, and E_2 concentrations by species. One-way ANOVAs were performed for each species on plasma and skeletal muscle P_4, T, and E_2 concentrations by gestational stage, followed by a Tukey's post-hoc test. If variables failed tests of normality or homogeneity of variance, the data were transformed. If transformed variables still violated the assumptions, the nonparametric Kendall's tau rank correlation or a Kruskal-Wallis rank sum test was performed instead of linear regression or one-way ANOVA, respectively. Multiple regression analyses were conducted by species to generate mathematical equations to model each of the following morphological parameters: maximum follicle diameter (MFD), ovary mass (OM), and shell gland mass (SGM) for both sharks and skates, and embryo STL for sharks, utilizing skeletal muscle P_4, T, and E_2 concentrations as

explanatory variables. Prior to regression analyses, all skeletal muscle P_4, T, and E_2 concentration data were log transformed to meet the assumptions of normality and homogeneity of variance. Multiple regression equations were generated by backwards selection of a fully interactive polynomial model, including all interactions between the first order hormone terms, and all first order hormone terms squared, cubed, and to the fourth power. Any term that was found to be non-significant ($P>0.05$) was removed from the model until all highest order terms were significant. If co-linearity was detected in a model, all first order terms within that model were centered and the multiple regression equation was re-generated following the same backwards selection procedure. All data were analyzed using R 2.15.2 (R-Core Development, 2012). All tests were considered significant at $\alpha \leq 0.05$.

Results

R. terraenovae

A total of 24 female *R. terraenovae* (69-92 cm FL, 1.6-7.9 kg) were sampled and assigned to the following discrete reproductive stages: five immature, four pre-ovulatory, six mid gestation (containing 100-142 mm STL embryos), and nine in late gestation (containing 314-364 mm STL embryos; see Prohaska et al. 2013 and Table 1 for gestational stage details). In addition, data from 10 female *R. terraenovae* collected in Prohaska et al. (2013) were combined with the current data set, boosting the overall sample size (n=34).

Compared to mature females (Figures 1 a, b and c), immature female *R. terraenovae* had relatively low concentrations of P_4, T, and E_2 in plasma (192 ± 46 pg/mL, n=5; 31 ± 5 pg/mL, n=5; 680 ± 140 pg/mL, n=5, respectively) and in muscle (95 ± 21 pg/g, n= 5; 50 ± 0 pg/g, n=5; 40 ± 0 pg/g, n=5, respectively).

In mature female *R. terraenovae*, from pre-ovulation to early gestation, plasma and muscle concentrations of P_4 and T were relatively low. During early-mid gestation, a significant increase in these hormones was observed, with plasma P_4 and T concentrations increasing approximately 300%, and muscle P_4 and T concentrations increasing approximately 800% (Figures 1 a and b). During mid gestation, P_4 and T concentrations significantly decreased by approximately 98% in plasma and 75% in muscle (Figures 1 a and b) (ANOVA; Plasma T: $F_{5, 28}$=19.95, P=1.88e-08; Muscle T: $F_{6, 27}$=6.343, P= 0.000296). During late gestation, plasma and muscle T and P_4 concentrations remain unchanged (Figures 1 a and b) (ANOVA; Plasma P_4: $F_{5, 28}$=13.64, P=8.72e-07; Muscle P_4: $F_{5, 28}$=14.74, P=4.12e-07). In agreement with the results of Prohaska et al. (2013), muscle P_4 and T were significantly correlated to plasma P_4 and T concentrations (P_4; linear regression: R^2=0.42, p=3.032e-05; T; Kendall's tau=0.43, p=0.001153).

During pre-ovulation, E_2 concentrations were elevated in plasma and muscle. From early to mid gestation, plasma and muscle E_2 concentrations significantly decreased by approximately 90%, while in late gestation, E_2 concentrations significantly increased by 300% in plasma and 850% in muscle (Figure 1 c) (ANOVA; Plasma E_2: $F_{5, 28}$=27.54, P=5.36e-10; Muscle E_2: $F_{5, 28}$= 62.87, P=2.38e-14). As previously reported by Prohaska

et al. (2013), *R. terraenovae* muscle E_2 concentrations were also found to be significantly correlated to plasma E_2 (linear regression: R^2=0.68, p=2.041e-09).

After performing a series of multiple regression analyses on *R. terraenovae*, muscle E_2 concentrations are significant indicators of MFD, SGM, and OM, while a combination of muscle P_4 and E_2 concentrations are significant indicators of embryo STL (Tables 2 and 3).

L. erinacea

Fourteen female *L. erinacea* (38-59 cm TL, 25-35 cm DW, 0.34-1.09 kg) were collected and divided into the following reproductive stages: five immature, two mature non-reproductively active (MFD < 12 mm), two pre-ovulatory (MFD > 19 mm), one in the process of ovulating, with a partially formed egg case, three in pre-oviposition with fully formed egg cases, and one in post-oviposition, having laid an egg case within the previous 12 h (see Table 4 for gestational stage details) (Koob et al., 1986; Koob and Callard, 1999).

Compared to mature females (Figures 2 a, b and c) , immature female *L. erinacea* had relatively low concentrations of P_4, T and E_2 in plasma (846 ± 246 pg/mL, n=5; 710 ± 204 pg/mL, n=5; 226 ± 123 pg/mL, n=5, respectively) and in muscle (387 ± 64 pg/g, n=5; 50 ± 0 pg/g, n=5; [91 ± 18 pg/g, n=5, respectively).

Plasma and muscle P_4 concentrations were elevated in mature, non-reproductively active females and decreased approximately 50% into pre-ovulation (Figure 2 a). Then, P_4 concentrations increased by 400% and 60% in plasma and muscle, respectively, during

ovulation, before a 75% decrease in plasma and 25% decrease in muscle during pre-oviposition. Plasma and muscle P_4 concentrations continued to decrease by approximately 80% during post-oviposition (Figure 2 a). Similar to P_4 in *R. terraenovae*, *L. erinacea* muscle P_4 concentrations were significantly correlated to plasma P_4 (linear regression: R^2= 0.35, p=0.02216).

In mature, non-reproductively active and pre-ovulatory females, T and E_2 concentrations were relatively low in plasma and muscle. An elevation in T and E_2 was noted during ovulation, increasing by approximately 1,500% in plasma and 500% in muscle (Figure 2 b and c). During pre-oviposition, plasma T and E_2 concentrations decreased by approximately 50%, while muscle T and E_2 increased by approximately 30%. Muscle T and E_2 concentrations continued to decrease by approximately 80% during post-ovipostion, while plasma T increased by 66%, while plasma E_2 decreased by 13% (Figure 2 b and c). Similar to P_4, *L. erinacea* muscle T and E_2 concentrations were significantly correlated to plasma T and E_2 (linear regression: [T] R^2=0.60, p=0.001063; [E_2] R^2= 0.76, p=4.665e-05).

After performing multiple regression analyses on *L. erinacea*, muscle T concentrations were significant indicators of MFD, while the combination of muscle P_4 and E_2 concentrations were significant indicators of SGM, and the combination of muscle P_4 and T concentrations were significant indicators of OM (Tables 2 and 5).

S. acanthias

A series of one-way ANOVAs were performed to investigate the relationships between P_4, T, and E_2 and gestational stage on data previously collected from 31 female *S. acanthias* (Prohaska et al., 2013). There was a significant 350% increase in muscle P_4 and a significant 90% decrease in plasma P_4 concentrations as the sharks progressed from pre-ovulation to the candle stage of gestation (Figure 3 a). Plasma P_4 concentrations significantly increased by 1,500% during the early stage of gestation, while muscle P_4 remained relatively unchanged. From mid to late gestation, plasma and muscle P_4 concentrations significantly decreased by approximately 70% (ANOVA [P_4] Plasma: $F_{4, 24}$= 17.12, P=9.2e-07; Muscle: $F_{4, 24}$=22.42, P=8.2e-08) (Figure 3 a). During pre-ovulation, muscle T concentrations were elevated, and they significantly decreased by approximately 90% in both plasma and muscle at the candle stage (Figure 3 b). Muscle T concentrations remained relatively low for the remainder of gestation, while plasma T significantly increased by 100% and 800% from mid to late gestation, respectively (ANOVA [T] Plasma: $F=_{4, 23}$=20.86, P=2.24e-07; Muscle: $F_{4, 23}$=11.11, P=3.65e-05) (Figure 3 b). Pre-ovulatory plasma and muscle E_2 concentrations were relatively low, and remained unchanged until plasma concentrations significantly increased by 1,200% and muscle concentrations increased by 200% during mid-gestation (ANOVA [E_2] Plasma: $F_{4, 19}$=33.28, P=2.43e-08; Muscle: $F_{4, 19}$=3.964, P=0.0167) (Figure 3 c).

Similar to *R. terraenovae* and *L. erinacea*, multiple regression analyses showed that a combination of muscle P_4, T, and E_2 concentrations were significant indicators of MFD, OM, SGM, and embryo STL in *S. acanthias* (Tables 2 and 6).

Discussion

In non-elasmobranch vertebrates, sex steroid hormones are successfully extracted from body depots, such as muscle, feces, and urine to study reproductive biology (e.g. Lasley and Kirkpatrick, 1991; Heppell and Sullivan, 2000; Shimizu, 2005; Barnett et al., 2009). In addition, fluctuations of the hormones present in these body depots mirror those in plasma (e.g. Hoffman and Rattenberger, 1977; Heppell and Sullivan, 2000; Shimizu, 2005; Barnett et al., 2009). For example, studies have quantified P_4 from blubber of marine mammals to assess if a female is mature and/or pregnant (Mansour et al., 2002; Kellar et al., 2006). Additionally, urinary and fecal E_2, T, and P_4 have been used to track gestation in free-ranging terrestrial vertebrates for conservation purposes (Lasley and Kirkpatrick, 1991; Shimizu, 2005). In non-elasmobranch fish, fluctuations in skeletal muscle T and E_2 concentrations are associated with maturity, sex, and reproductive cycles (Heppell and Sullivan, 2000; Barnett et al., 2009). Thus far, previous research on a few species of elasmobranchs has reported that circulating E_2, T, and P_4 concentrations are correlated with morphological changes in the reproductive tract and with specific events that occur during sexual maturation and the reproductive cycle (e.g. Koob et al., 1986; Rasmussen and Muru, 1992; Sulikowski et al., 2007). More recently, Prohaska et al. (2013) successfully extracted E_2, T, and P_4 from the skeletal muscle tissue of elasmobranchs and showed that the profiles of these hormones in the muscle are similar to those in the plasma.

Steroid hormones are essential for reproduction. Plasma E_2 concentrations are primarily linked to the growth and maturation of follicles within the ovary of elasmobranchs (e.g. Sumpter and Dodd, 1979; Manire et al., 1995; Snelson et al., 1997, Heupel et al., 1999; Tricas et al., 2000). Medium sized follicles produce the highest concentrations of E_2 (Callard et al., 1993), which is transported to the liver where binding to receptors stimulates vitellogenesis (e.g. Sumpter and Dodd, 1979; Manire et al., 1995; Snelson et al., 1997, Heupel et al., 1999; Tricas et al., 2000), and subsequent accumulation of yolk within developing follicles (Perez and Callard 1989; Koob and Callard, 1999). In addition, E_2 also plays a prominent role in the reproductive tract (Callard et al., 1989), such as enlargement of the shell gland (Koob et al., 1986) and vascularization of the uterus (Koob and Callard, 1999). Similarly, in the current study, we found that follicular growth accompanied increases in plasma and muscle E_2 concentrations. For example, *R. terraenovae* plasma and muscle E_2 significantly increased from mid to late gestation, which corresponded to a substantial increase in follicle diameter. A similar association was observed in the yolk-sac placental viviparous species, *Sphyrna tiburo,* in which serum E_2 was elevated during pre-ovulation, reduced throughout the majority of gestation, and increased again during the final stages of gestation when follicular growth was beginning (Manire et al., 1995). In *S. acanthias*, an aplacental viviparous species, plasma and muscle E_2 concentrations increased from early to mid gestation, and remained elevated for the duration of gestation, paralleling follicular growth. These results are similar to those of Tsang and Callard (1987) who previously reported that plasma E_2 increased during the second half of gestation, when cohorts of follicles with larger diameters began to develop. Interestingly, we anticipated

that the highest concentration of plasma and muscle E_2 in *L. erinacea* would have occurred at pre-ovulation during the period of peak follicular development (Koob et al., 1986), but this was not observed in the current study. This E_2 peak might not have been detected because of the high individual variability of hormone concentrations in continuously reproducing oviparous species (Williams et al., 2013). Alternatively, the skates sampled may be at the end of their follicular growth phase and/or at the beginning of ovulatory phase.

Unlike E_2, changes in P_4 are unique to reproductive mode and often linked to mode-specific reproductive events in elasmobranchs. For example, in *S. tiburo,* peaks in serum P_4 concentrations during early gestation are related to uterine compartmentalization and implantation (Schlernitzauer and Gilbert, 1966; Callard et al., 1992; Manire et al., 1995). In the current study, the peak in plasma and muscle P_4 during early-mid gestation *R. terraenovae* is also likely related to uterine compartmentalization and implantation, especially since implantation occurs when embryos are approximately 70-85 mm in length (Castro and Wourms, 1993). In *S. acanthias*, high concentrations of plasma P_4 during early gestation are linked to embryo retention (Callard et al., 1992) and vitellogenesis suppression (Paolucci and Callard, 1998; Koob and Callard, 1999). Similar to those studies, the data herein showed that *S. acanthias* plasma and muscle P_4 peaked during early gestation when little to no follicular growth was occurring, and then decreased into late gestation when follicular growth was beginning (Tsang and Callard, 1987). Despite the high individual variability in *L. erinacea* plasma P_4 concentrations (Williams et al., 2013), the patterns exhibited by plasma and muscle P_4 in the current study were similar to those in Koob et al. (1986). They noted that plasma P_4 peaks

during ovulation, and then decreases during pre and post oviposition. In addition, Koob et al. (1986) suggested that the rise in P] might be related to ovulation and the formation of egg cases, while the decrease in P_4 may be related to oviposition. Further, decreases in P_4 after ovulation, like those found in the plasma and muscle of the current study, are suggested to inhibit the early release of egg cases by hormonally controlling and tightening cervix muscles to maintain them within the uterus to undergo sclerotization and tanning (Koob and Cox, 1988; Koob and Callard, 1999).

There is a close relationship between T and E_2 in aplacental viviparous and oviparous elasmobranchs. Plasma T is primarily linked to the growth of follicles (Koob et al., 1986; Tsang and Callard, 1987), with larger follicles producing the highest concentrations, which serve as a substrate for E_2 synthesis to facilitate vitellogenesis (Tsang and Callard, 1992) and the continued accumulation of yolk by developing follicles (Perez and Callard, 1989; Koob and Callard, 1999). In the current study, the patterns of plasma and muscle T concentrations in *S. acanthias* and *L. erinacea* were similar to those reported in the aforementioned studies, i.e. increases in T are concurrent with increases in follicle diameter (Koob et al., 1986; Tsang and Callard, 1987). However, in the yolk-sac placental viviparous *R. terraenovae*, plasma and muscle T and P_4 had similar profiles, with both hormones peaking during early-mid gestation. These profiles are just like the ones exhibited in *S. tiburo* serum, in which fluctuations of T were analogous to P_4, peaking in early gestation when implantation and compartmentalization are suggested to occur (Manire et al., 1995).

Of the few studies so far that determined plasma sex steroid hormones in immature elasmobranchs, all have reported little to no detectable concentrations of these

hormones in fish classified as immature, based on length as well as the underdeveloped condition of external and internal reproductive organs (e.g. Rasmussen and Gruber, 1990; Rasmussen and Muru, 1992; Cicia et al., 2009). In the current study, we attempted to measure plasma and muscle P_4, E_2, and T concentrations in yolk-sac placental viviparous (*R. terraenovae*) and oviparous (*L. erinacea*) that were identified as immature, based on the presence of underdeveloped reproductive tracts. Similar to previous reports, concentrations of muscle and plasma P_4, E_2, and T in immature females were low relative to those that were mature. These results provided further support for the direct link between sex steroid hormones, the readiness of the reproductive tract and reproductive status in elasmobranchs (Rasmussen and Muru, 1992; Gelsleichter et al., 2002; Sulikowski et al., 2006; Sulikowski et al., 2007), as well as the potential use of the muscle hormones as a non-lethal method to assess maturity.

In addition to examining the relationships between muscle and plasma steroid hormones, multiple regression analyses were conducted in the current study to determine if skeletal muscle P_4, T, and E_2 concentrations relate to SGM, OM, and MFD in *R. terraenovae, L. erinacea,* and *S. acanthias*, as well as to embryo STL in the two species of sharks. So far, such analyses have led to the creation of several mathematical models, although the sample sizes used to generate them were low. Nonetheless, these preliminary models are a first step towards developing the use of P_4, T, and E_2 concentrations in non-lethally obtained skeletal muscle as indicators of reproductive status and gestational stage.

Conclusions

The current study reported the successful detection of steroid hormones in the muscle and plasma of female *R. terraenovae, L. erinacea* and *S. acanthias* during specific stages of their reproductive cycles. More importantly,, plasma and skeletal muscle concentrations of P_4, T, and E_2 in *R. terraenovae*, and *L. erinacea* were significantly associated, suggesting that skeletal muscle tissue is an appropriate substitute for plasma. The present results also affirm the efficacy of using the steroid hormones in non-lethally obtained skeletal muscle to assess reproductive status in elasmobranchs (Prohaska et al., 2013). The primary advantage of utilizing muscle versus blood is that obtaining muscle tissue may be less stressful and less invasive than obtaining blood, which will facilitate its use on large, as well as threatened species of elasmobranchs. Our previous work on *S. acanthias* and *R. terraenovae* suggests that white skeletal muscle tissue is a depot for P_4, T, and E_2 (Prohaska et al., 2013), the present study further suggests that skeletal muscle is also a depot for the same three hormones in the oviparous *L. erinacea*. The analysis of *L. erinacea* skeletal muscle steroid hormones, so far, suggested that they may relate well to reproductive stage. However, increasing sample size will strengthen and provide a more accurate depiction of these skeletal muscle hormones throughout the reproductive cycle of an oviparous species. On the other hand, the findings of the current study provided a more complete understanding of fluctuations in P_4, T, and E_2 during the gestation of *R. terraenovae*, reinforcing our claim that skeletal muscle steroid hormones relate well to gestation in a yolk-sac placental species.

Finally, multiple regression analyses conducted on *R. terraenovae, L. erinacea,* and *S. acanthias* suggest that skeletal muscle P_4, T, and E_2 are significantly related to changes in reproductive tract morphologies during specific stages of the reproductive cycles including gestation. The robustness of these mathematical models will be strengthened by increasing sample size before they become tools for fisheries managers.

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Table 1. *Rhizoprionodon terraenovae* morphological data. Mean (± standard error) of ovary mass (OM; g), shell gland mass (SGM; g), maximum follicle diameter (MFD; mm), and embryo stretch total length (STL; mm), by gestational stage: pre-ovulatory, early, early-mid, mid, and late gestation. N represents sample size.

Stage	OM (g)	SGM (g)	MFD (mm)	Embryo STL (mm)	N
Pre-ovulatory	24.3 ± 4.5	2.7 ± 0.4	20.0 ± 1.3	NA	5
Early			4.8 ± 0.9	37.7 ± 9.8	2
Early-Mid	5.8 ± 1.5	1.4 ± 0.3	6.9 ± 1.1	66.8 ± 4.2	4
Mid	3.6 ± 0.3	1.1 ± 0.2	4.3 ± 0.2	126 ± 6.8	9
Late	19.9 ± 3.2	2.9 ± 0.2	18.9 ± 1.0	338 ± 5.7	9

Table 2. Multiple regression analyses conducted on *Rhizoprionodon terraenovae, Squalus acanthias*, and *Leucoraja erinacea*. The relationships between steroid hormones and the response variables, maximum follicle diameter (MFD) (mm), embryo STL (mm), ovary mass (OM) (g), and shell gland mass (SGM) (g) are indicated by the R-squared (R^2) and p-value. For a more detailed account of the relationships between the hormones and the response variables, see the model definitions in Tables 3, 5, 6.

	Hormones	R^2	p-value
R. terraenovae			
MFD (mm)	E_2	0.900	1.18E-12
Embryo STL (mm)	E_2, P_4	0.843	1.52E-08
SGM (g)	E_2	0.771	1.71E-08
OM (g)	E_2	0.594	1.04E-05
L. erinacea			
SGM (g)	E_2, P_4	0.770	0.0115
OM (g)	P_4, T	0.749	0.00869
MFD (mm)	T	0.285	0.04929
S. acanthias			
MFD (mm)	E_2, P_4, T	0.902	5.03E-06
OM (g)	E_2, P_4, T	0.873	0.000381
SGM (g)	E_2, P_4, T	0.791	1.40E-06
Embryo STL (mm)	E_2, P_4, T	0.747	3.81E-05

Table 3. Multiple regression equations generated for *Rhizoprionodon terraenovae* using skeletal muscle concentrations (pg/mL) of progesterone (P_4), testosterone (T) and estradiol (E_2), as indicators of the morphological characteristics: maximum follicle diameter (MFD), embryo stretch total length (STL), ovary mass (OM), and shell gland mass (SGM). The characteristics are expressed as mean (± standard error) MFD (mm), embryo STL (mm), OM (g), and SGM (g) by gestational stage. All hormone terms within the regression equations were log transformed.

Morphological Characteristic	Morphological Predictive Regression Model	Gestational Stage				
		P/O	Early	Early/Mid	Mid	Late
MFD (mm)	MFD = 10.9025 + 8.0295 (E_2) + 0.7531 [$(E_2)^2$] - 1.1643 [$(E_2)^3$] [a]	20.0 ± 1.3	4.8 ± 0.9	6.9 ± 1.1	4.3 ± 0.2	18.9 ± 1.0
Embryo STL (mm)	Embryo STL = 138.979 + 2.4.30 (E_2) - 164.588 (P_4) - 86.555 [$(P_4)^2$] + 35.556 [$(P_4)^3$] - 142.777 [(E_2)*(P_4)] [a]	0	37.7 ± 9.8	66.8 ± 4.2	126.3 ± 6.8	337.7 ± 5.7
OM (g)	OM = - 23.398 + 6.995 (E_2)	24.3 ± 4.5	NA	5.8 ± 1.5	3.6 ± 0.3	19.9 ± 3.2
SGM (g)	SGM = - 1.64748 + 0.70060 (E_2)	2.7 ± 0.4	NA	1.4 ± 0.3	1.4 ± 0.3	2.9 ± 0.2

[a] All terms included in the model were centered.
NA- Data were not collected.

Table 4. *Leucoraja erinacea* morphological data. Mean (± standard error) of ovary mass (OM; g), maximum follicle diameter (MFD; mm), and shell gland mass (SGM; g), by reproductive stage: immature, mature (non-reproductively active), pre-ovulatory, ovulating, pre-oviposition, and post-oviposition. N represents sample size.

Stage	OM (g)	MFD (mm)	SGM (g)	N
Immature	1.5 ± 0.4	2.4 ± 1.0	0.5 ± 0.3	5
Mature	5.7 ± 1.2	9.5 ± 0.7	4.1 ± 0.3	2
Pre-ovulatory	5.9 ± 0.9	21 ± 1.6	5.5 ± 1.9	2
Ovulating	11.8	21.6	12.1	1
Pre-oviposition	8.6 ± 2.3	18 ± 0.9	6.7 ± 0.2	3
Post-oviposition	4.3	12.3	6.2	1

Table 5. Multiple regression equations generated for *L. erinacea* using skeletal muscle concentrations (pg/mL) of progesterone (P_4), testosterone (T) and estradiol (E_2), as indicators of morphological characteristics: maximum follicle diameter (MFD), ovary mass (OM), and shell gland mass (SGM). The characteristics are expressed as mean (± standard error) MFD (mm), OM (g), and SGM (g) by reproductive stage. All hormone terms within the regression equations were log transformed.

Morphological Characteristic	Morphological Predictive Regression Model	Reproductive Stage					
		Immature	Mature	Pre-ovulatory	Ovulating	Pre-Oviposition	Post-Oviposition
MFD (mm)	MFD = - 5.125 + 3.531 (T)	2.4 ± 1.0	9.5 ± 0.7	20.8 ± 1.6	21.6	18.3 ± 0.9	12.3
OM (g)	OM = 5.2419 + 2.1120 (E_2) + 7.8459 (P_4) + 0.6632 [$(P_4)^2$] - 5.9695 [$(P_4)^3$] [a]	1.5 ± 0.4	5.7 ± 1.2	5.9 ± 0.9	11.8	8.6 ± 2.3	4.3
SGM (g)	SGM = 3.7762 + 8.8563 (P_4) + 1.5244 (T) + 2.3690 [$(P_4)^2$] - 9.0439 [$(P_4)^3$] [a]	0.5 ± 0.3	4.1 ± 0.3	5.5 ± 1.9	12.1	6.7 ± 0.2	6.2

[a] All terms included in the model were centered.

Table 6. Multiple regression equations generated for *S. acanthias* using skeletal muscle concentrations (pg/mL) of progesterone (P_4), testosterone (T) and estradiol (E_2), as indicators of morphological characteristics: maximum follicle diameter (MFD), embryo stretch total length (STL), ovary mass (OM), and shell gland mass (SGM). These characteristics are expressed as mean (± standard error) hormone concentrations, MFD (mm), embryo STL (mm), OM (g), and SGM (g) by gestational stage. All hormone terms within the regression equations were log transformed and centered.

Morphological Characteristic	Morphological Predictive Regression Model	Gestational Stage				
		P/O	Candle	Early	Mid	Late
MFD (mm)	MFD = -2.649 - 2.073 (E_2) + 73.115 (P_4) - 2.883 (T) - 43.726 [$(P_4)^2$)] - 15.840 [$(P_4)^3$] + 10.372 [$(P_4)^4$] + 6.921 [$(T)^2$] + 3.018 [(T)*(P_4)] - 5.547 [(E_2)*(T)]	44.8 ± 1.5	10.0 ± 0.4	15.6 ± 1.2	33.8 ± 0.6	44.9 ± 1.2
Embryo STL (mm)	Embryo STL = 191.060 + 52.658 (E_2) - 58.531 (P_4) - 38.695 (T) - 28.323 [$(P_4)^2$] + 30.251 [(E_2)*(T)]	0	<10	76.8 ± 5.0	222.5 ± 8.1	260.7 ± 5.5
OM (g)	OM = 148.648 + 1.365 (E_2) + 74.879 (P_4) - 125.265 (T) - 58.755 [$(E_2)^2$] + 14.208[$(P_4)^2$] - 25.966 [$(P_4)^3$] - 130.792 [$(T)^2$] + 105.000 [$(T)^3$] - 52.844 [(E_2)*(T)]	110.6 ± 9.6	28.4 ± 12.9	17.0 ± 2.3	62.9 ± 7.7	128.5 ± 21.9
SGM (g)	SGM = 1.79569 - 0.26788 (E_2) - 0.32555 (P_4) + 0.15810 (T) – 0.43729 [(E_2)*(T)]	2.9 ± 0.3	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	2.4 ± 0.2

Figure Legends

Figure 1. Mean (± standard error) concentrations of plasma (pg/mL) and muscle (pg/g) steroid hormones. Progesterone (A), testosterone (B) and estradiol (C) in *Rhizoprionodon terraenovae*, plotted by gestational stage: immature, pre-ovulatory (P/O), early, early-mid, mid, and late gestation. Numbers above the x-axis represent sample size. Uppercase letters denote statistically significant pairwise differences in muscle hormone concentrations between gestational stages, while lowercase letters denote statistically significant pairwise differences in plasma hormone concentrations between gestational stages ($p<0.05$). Note the difference in scale between the plasma and muscle axes.

Figure 2. Mean (± standard error) concentrations of plasma (pg/mL) and muscle (pg/g) steroid hormones. Progesterone (A), testosterone (B) and estradiol (C) in *Leucoraja erinacea*, plotted by reproductive stage: immature, mature (non-reproductively active), pre-ovulatory, ovulating, pre-oviposition, and post-oviposition. Numbers above the x-axis represent sample size. Note the difference in scale between the plasma and muscle axes.

Figure 3. Mean (± standard error) concentrations of plasma (pg/mL) and muscle (pg/g) steroid hormones. Progesterone (A), testosterone (B) and estradiol (C) in *Squalus acanthias*, plotted by gestational stage: pre-ovulatory (P/O), candle, early, mid, and late gestation. Numbers above the x-axis represent sample size. Uppercase letters denote statistically significant pairwise differences in muscle hormone concentrations between

gestational stages, while lowercase letters denote statistically significant pairwise differences in plasma hormone concentrations between gestational stages ($p<0.05$). Note the difference in scale between the plasma and muscle axes.

Figure 1.

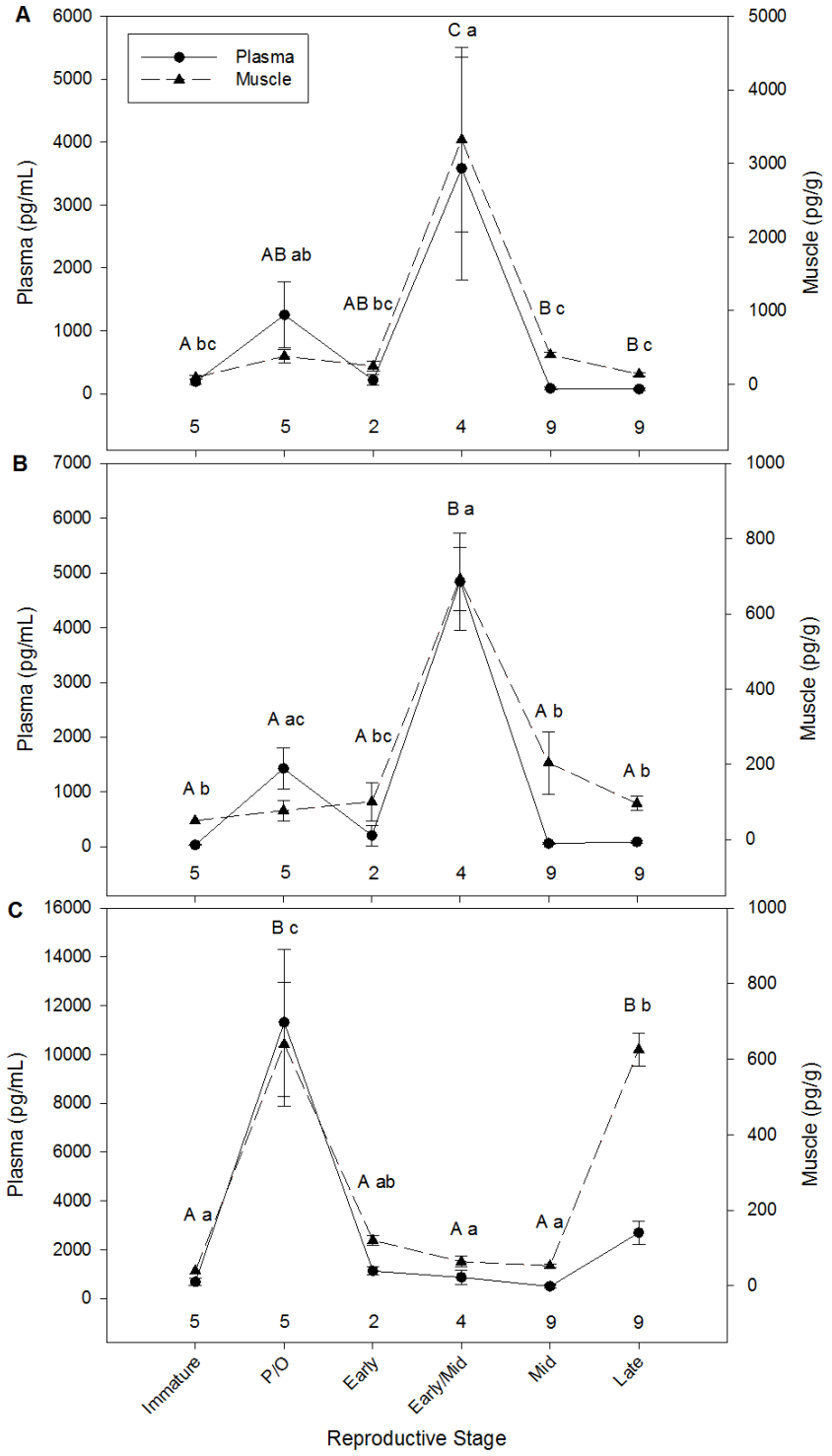

A
B
C
Plasma
Muscle
Plasma (pg/mL)
Muscle (pg/g)
A bc
AB ab
AB bc
C a
B c
B c
A b
A ac
A bc
B a
A b
A b
A a
B c
A ab
A a
A a
B b
5
5
2
4
9
9
Immature
P/O
Early
Early/Mid
Mid
Late
Reproductive Stage

Figure 2.

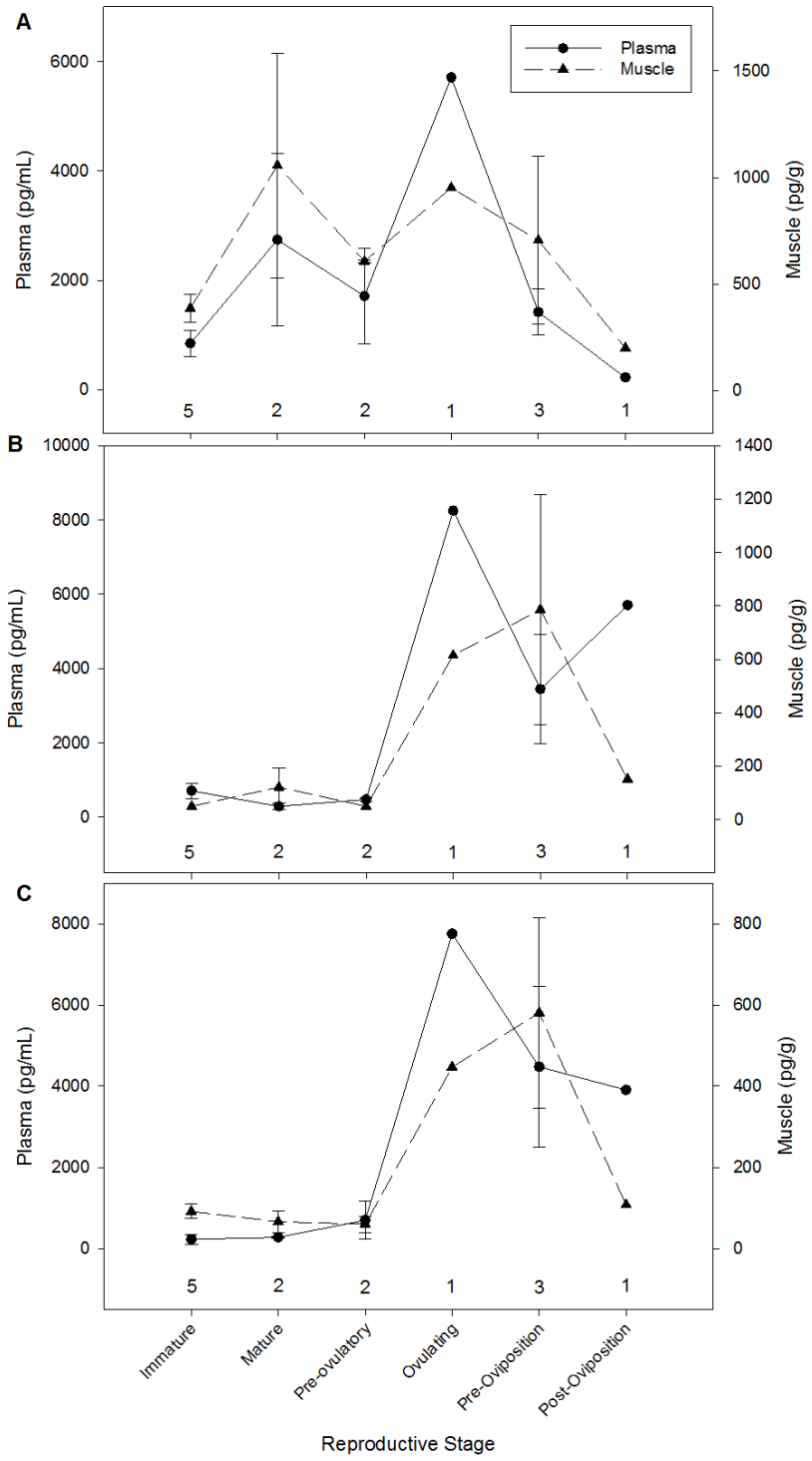
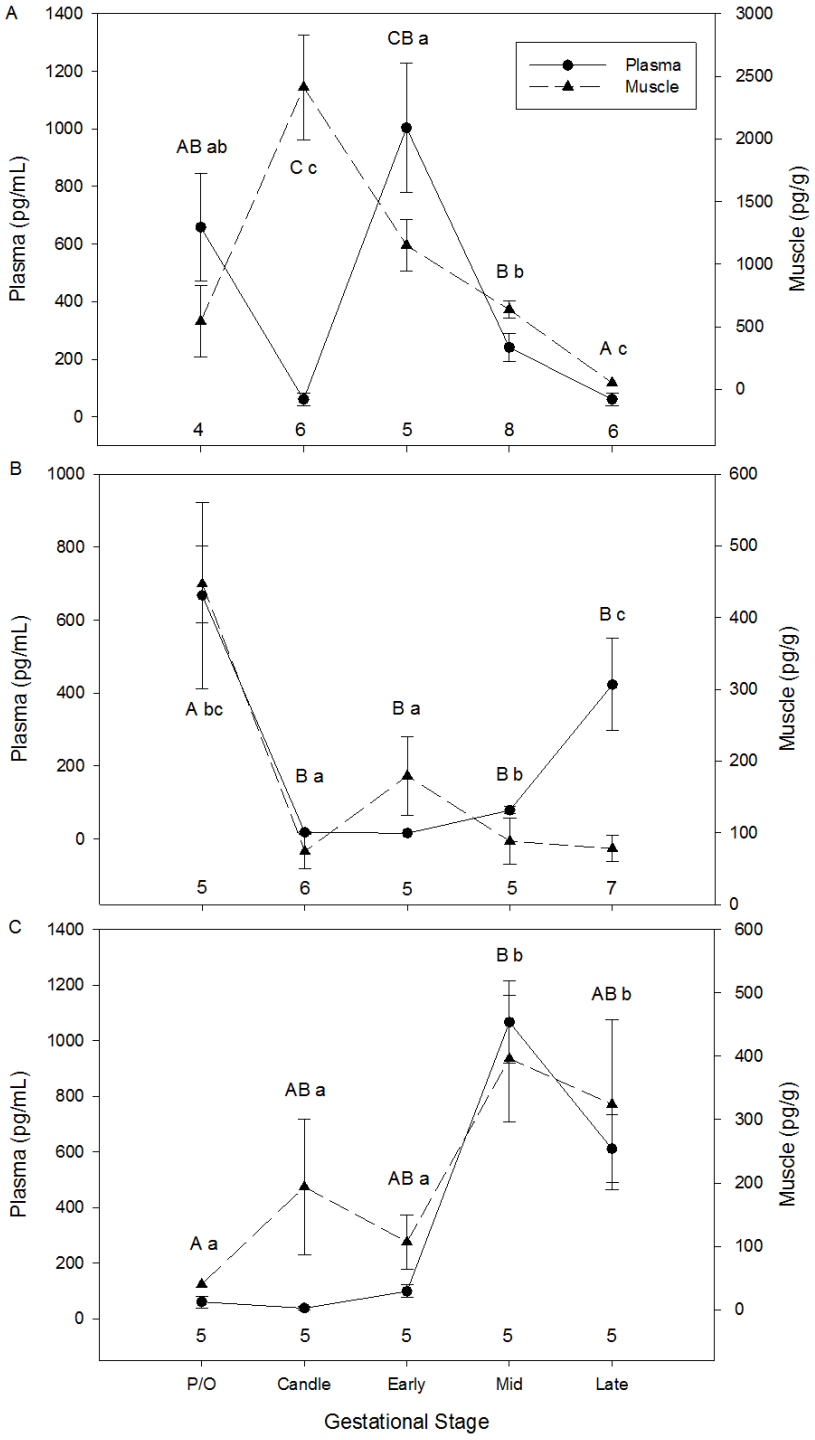

A
B
C
Plasma
Muscle
Plasma (pg/mL)
Muscle (pg/g)
0
2000
4000
6000
8000
10000
500
1000
1200
1400
5
2
2
1
3
1
Immature
Mature
Pre-ovulatory
Ovulating
Pre-Oviposition
Post-Oviposition
Reproductive Stage

Figure 3.


A
B
C
Plasma
Muscle
Plasma (pg/mL)
Muscle (pg/g)
AB ab
C c
CB a
B b
A c
B c
A bc
B a
B a
B b
A a
AB a
AB a
B b
AB b
4
6
5
8
6
5
6
5
5
7
5
5
5
5
5
P/O
Candle
Early
Mid
Late
Gestational Stage

Appendix

- Muscle extraction techniques tested. For a more detailed explanation of each technique tested please see "Bianca Prohaska Muscle Extraction Notebook #1".
    1. 6 g of white skeletal muscle tissue homogenized with 2 mL PBS
        a. 1 mL of homogenate extracted 3 times with 5 times the volume of ether
            i. Reconstitute in 500 uL
                1. Low recovery
    2. 6 g of white skeletal muscle tissue homogenized with 2 mL PBS
        a. 1 mL of homogenate extracted 3 times with 3 times the volume of ether
            i. Reconstitute in 500 uL
                1. Low recovery
    3. 6 g of white skeletal muscle tissue homogenized with 2 mL of PBS
        a. Centrifuge homogenate
            i. Homogenate would not separate
    4. 3 g of white skeletal muscle tissue homogenized with 3 mL of PBS
        a. Removed 500 uL, add 2.5 mL PBS and centrifuge
            i. Did not separate
                1. Split remaining homogenate in 2 and dry 1 under nitrogen and 1 by air
                2. Extracted both using plasma hormone extraction technique

a. No quantified hormone

5. 3 g of white skeletal muscle tissue homogenized with 3 mL of PBS
    a. Removed 500 uL, add 10 mL PBS and centrifuge
        i. Did not separate
            1. Split remaining homogenate in 2 and dry 1 under nitrogen and 1 by air
            2. Extracted both using plasma hormone extraction technique
                a. Very low concentrations of hormone
6. 2 g white skeletal muscle tissue homogenized with 8 mL of PBS
    a. 2:1 Chloroform methanol 2 rounds of extraction
        i. Homogenate did not separate during second round of extraction, turned to slush
7. 2 g white skeletal muscle tissue homogenized with 2 mL of PBS
    a. 2:1 Chloroform methanol 1 rounds of extraction
        i. Low recovery
8. 2 g white skeletal muscle tissue homogenized with 8 mL of PBS
    a. 2:1 Chloroform methanol 1 round of extraction
        i. Good recoveries for estradiol and testosterone, still fairly low for progesterone, but still able to quantify hormones from all three extracts with good replication