Development of a Nonlethal and Minimally Invasive Protocol to Study Elasmobranch Reproduction

Author: Prohaska, Bianca K.

Source: Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science, 5(5) : 181-188

Published By: American Fisheries Society

URL: https://doi.org/10.1080/19425120.2013.788590

Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science 5:181–188, 2013

ISSN: 1942-5120 online
DOI: 10.1080/19425120.2013.788590

SPECIAL SECTION: ELASMOBRANCH LIFE HISTORY

Development of a Nonlethal and Minimally Invasive Protocol to Study Elasmobranch Reproduction

Bianca K. Prohaska*
Department of Marine Sciences, University of New England, 11 Hills Beach Road, Biddeford, Maine 04005, USA

Paul C. W. Tsang
Department of Molecular, Cellular and Biomedical Sciences, 129 Main Street, Durham, New Hampshire 03824, USA

William B. Driggers III and Eric R. Hoffmayer
National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Mississippi Laboratories, 3209 Frederick Street, Pascagoula, Mississippi 39567, USA

James A. Sulikowski
Department of Marine Sciences, University of New England, 11 Hills Beach Road, Biddeford, Maine 04005, USA

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 suggested that sex steroid hormones can be extracted from muscle tissues to identify reproductive status. Collecting for muscle biopsy is quick and minimally invasive and can be done without removing an animal from the water. Thus, the objective of the current study was to determine the efficacy of using muscle steroid hormones to assess the reproductive biology of elasmobranch fishes. The results suggest that concentrations of muscle progesterone, testosterone, and estradiol can be successfully quantified to study reproduction by radioimmunoassay. Additionally, there were significant correlations between the plasma and muscle estradiol concentrations in Spiny Dogfish *Squalus acanthias* and the progesterone, testosterone, and estradiol concentrations in Atlantic Sharpnose Sharks *Rhizoprionodon terraenovae*. The present investigation thus demonstrates that skeletal muscle is a nonlethally harvestable tissue that is well suited for studying the reproductive biology of elasmobranchs.

An essential component for assessing and managing populations of elasmobranchs (sharks, skates, and rays) is determining each species' specific reproductive biology, e.g., when sexual maturity occurs, the timing of seasonal cycles, or gestation length (Walker 2004, 2005). Despite the importance of such data to fisheries management (Walker 2004), they 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,

Subject editor: John Mandelman, New England Aquarium, Boston, Massachusetts

*Corresponding author: bprohaska@une.edu
Received October 12, 2012; accepted March 12, 2013

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, studying the reproductive biology of elasmobranchs entailed sacrificing a relatively large number of specimens and observing the 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 nonlethally study the reproductive biology of elasmobranchs is the analysis of blood steroid hormone concentrations (e.g., Perez and Callard 1993; Sulikowski et al. 2006, 2007a, 2007b; Kneebone et al. 2007; 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. 2007a, 2007b). However, obtaining blood from large species is impractical and can be problematic because of the 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 situation 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 as well (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); the 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 a chimaera, the Spotted Ratfish *Hydrolagus colliei* (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 taken with 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) whether reproductive steroid hormones can be quantified from elasmobranch skeletal muscle tissue and (2) whether there is a relationship between the muscle and plasma concentrations of these reproductive hormones.

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 Shark *Rhizoprionodon terraenovae* were selected as study organisms. Female Spiny Dogfish were primarily captured by otter trawl off the coast of Rhode Island aboard the 13.7-m bottom trawler FV *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 FV *Lady Victoria* in November 2010 in an area centered around 70.257°W and 42.917°N. Female Atlantic Sharpnose Sharks were captured by bottom longline in the Gulf of Mexico aboard the 51.8-m RV *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 the capture of an individual, 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 $\times$ *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), FL, and natural TL, which were measured to the nearest centimeter 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 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 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 millimeter 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

FIGURE 1. Centers of sampling areas for Spiny Dogfish (1 and 2) and Atlantic Sharpnose Sharks (3).

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, Massachusetts) 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, New York). Then, 500-μL aliquots of homogenate, in quadruplicate, were extracted for each hormone. Nonradiolabeled [E_2], [T], and [P_4] (Steraloids, Inc., Newport, Rhode Island) 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 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/100 μL for [T] and [P_4] and 320 pg/100 μL for [E_2]. Working stocks were utilized to cold-spike muscle homogenates for calculating sample recovery. Of the four homogenate replicates, two 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 min 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 it 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 nonspiked 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 nonspiked replicates was calculated, multiplied by a dilution factor of 8 (to convert the 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 nonradiolabeled [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] (Gordon Niswender, Colorado State, Fort Collins, Colorado), 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 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, New Jersey) 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, Ohio) was added. The radioactivity level was then measured by a Perkin-Elmer Tri-Carb 2900TR liquid scintillation analyzer (Waltham, Massachusetts). The mean intra-assay coefficients of variation were 8, 10, and 9% for [E_2], [T], and [P_4], respectively, in the Spiny Dogfish plasma assays and 7, 7, and 6% in the Atlantic Sharpnose Shark plasma assays. The mean interassay coefficients of variation were 10, 11, and 12% for [E_2], [T], and [P_4], respectively, in the Spiny Dogfish plasma assays and 8, 8, and 9% in the Atlantic Sharpnose Shark plasma assays. The mean intra-assay coefficients of variation were 8, 10, and 8% for [E_2], [T], and [P_4], respectively, in the Spiny Dogfish muscle assays and 10, 8, and 9% in the Atlantic Sharpnose Shark muscle assays. The mean interassay coefficients of variation were 10, 11, and 12% for [E_2], [T] and [P_4], respectively, in the Spiny Dogfish muscle assays. In calculating the means ± SEs for the concentrations of each steroid hormone per stage, any value that was nondetectable 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 the 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 means and SEs were graphed in Sigmaplot 12 (Systat Software, San Jose, California). All tests were considered significant at $\alpha = 0.05$.

RESULTS

A total of 31 female Spiny Dogfish (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 preovulatory females, six females 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 Atlantic Sharpnose Sharks (68–85 cm FL, 2.4–4.6 kg) were collected and divided into arbitrary discrete reproductive stages (Table 2), including one preovulatory female, 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 Spiny Dogfish and 71, 85, and 68% for Atlantic Sharpnose Sharks, respectively, while the overall mean recoveries of muscle [E_2], [T], and [P_4] extractions were 21, 23, and 20% for Spiny Dogfish and 59, 39, and 55% for Atlantic Sharpnose Sharks, respectively.

Spiny Dogfish

In preovulatory female Spiny Dogfish, [P_4] concentrations in both plasma (658 ± 186 pg/mL) and muscle (543 ± 285

TABLE 1. Mean ± SE maximum follicle diameter (MFD) and pup STL for Spiny Dogfish, by gestational stage (preovulatory [P/O], candle, early, mid, and late); *N* = sample size.

Stage	MFD (mm)	Pup STL (mm)	*N*
P/O	44.8 ± 1.5		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. Mean ± SE maximum follicle diameter (MFD) and pup STL for Atlantic Sharpnose Sharks, by gestational stage. See Table 1 for additional information.

Stage	MFD (mm)	Pup STL (mm)	*N*
P/O	15.6		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

pg/g) were similar. However, plasma [P_4] (60 ± 22 pg/mL) decreased by 10-fold during the candle stage, while muscle [P_4] (2,412 ± 420 pg/g) increased by about fivefold. Females in early gestation displayed relatively elevated plasma (1,004 ± 225 pg/mL) and muscle (1,150 ± 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 Spiny Dogfish 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 preovulatory stage (Figure 2B). Similar to [P_4], muscle [T] was not significantly related to plasma [T] in Spiny Dogfish (Kendall's tau = 0.16; $P = 0.30$).

From preovulation to mid gestation, Spiny Dogfish 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], Spiny Dogfish muscle [E_2] concentrations were significantly related to plasma [E_2] (Kendall's tau = 0.49; $P = 0.0017$).

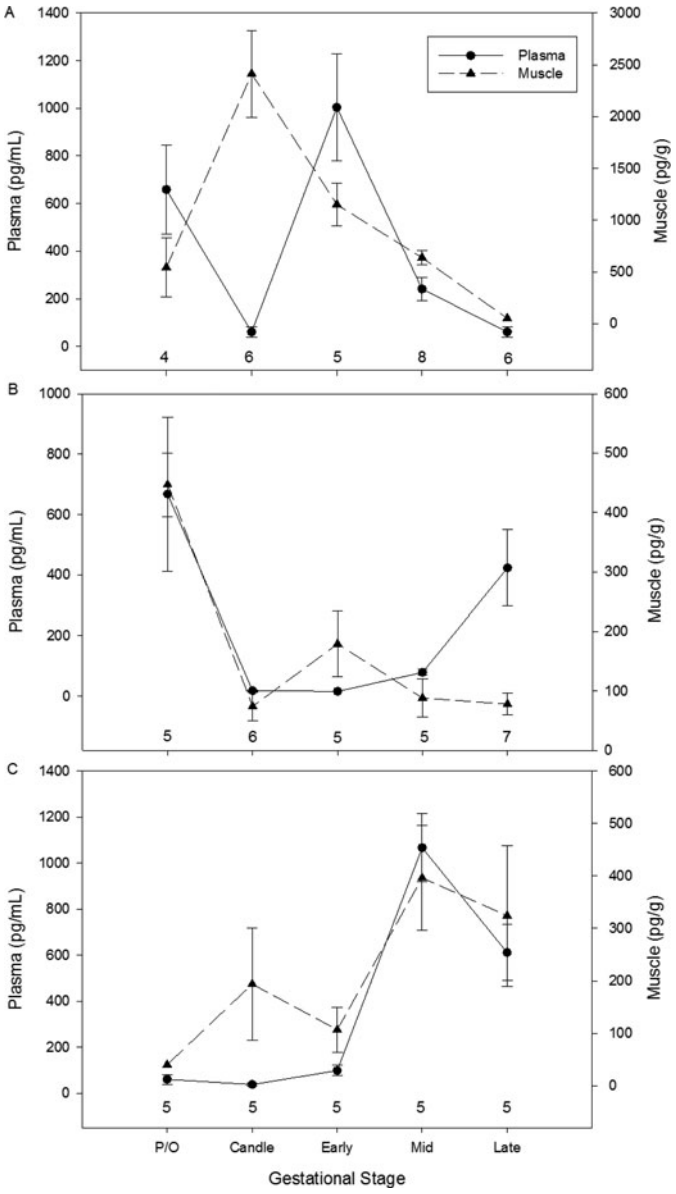

FIGURE 2. Mean concentrations of **(A)** progesterone, **(B)** testosterone, and **(C)** estradiol in plasma and muscle of Spiny Dogfish, by gestational stage (preovulatory [P/O], candle, early, mid, and late). The numbers above the *x*-axes are sample sizes; the error bars represent SEs. Note the difference in scale between the plasma and muscle.

Atlantic Sharpnose Sharks

From preovulation to early gestation, concentrations of [P_4] and [T] in Atlantic Sharpnose Sharks 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]: 3,582 ± 1,767 pg/mL; [T]: 4,840 ± 884 pg/mL) and muscle ([P_4]: 3,325 ± 1,253 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). The muscle [P_4] and [T] of Atlantic Sharpnose Sharks 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 preovulatory Atlantic Sharpnose Shark female contained relatively elevated concentrations of [E_2] in plasma (4,885 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 those of [P_4] and [T], the concentrations of Atlantic Sharpnose Shark muscle [E_2] were found to be significant predictors of plasma [E_2] (linear regression; $R^2 = 0.79$, $P = 0.00059$).

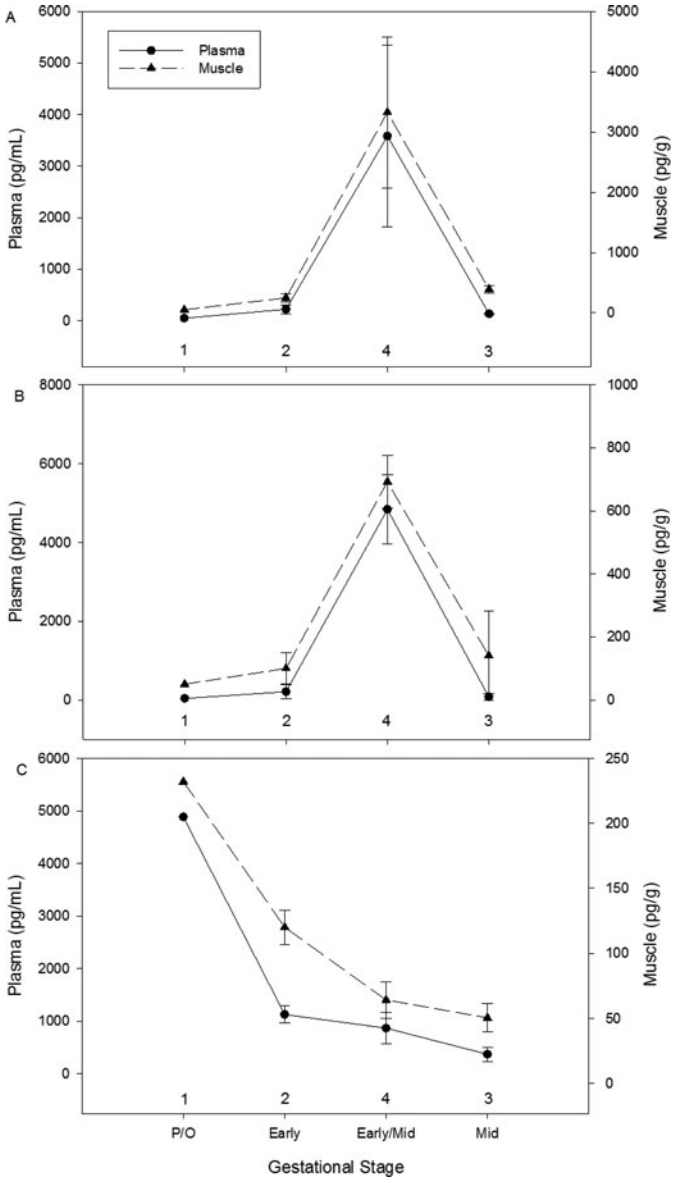

FIGURE 3. Mean concentrations of **(A)** progesterone, **(B)** testosterone, and **(C)** estradiol in plasma and muscle of Atlantic Sharpnose Sharks, by gestational stage. See Figure 2 for additional information.

DISCUSSION

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

Spiny Dogfish

In the current study, plasma and muscle [P_4] in Spiny Dogfish exhibited patterns during distinct gestational stages similar 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 decreased from mid gestation through parturition (Tsang and Callard 1987). Although not measured by Tsang and Callard (1987), the preovulatory 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 and preovulatory females (Table 1). In this species follicles 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 preovulation. 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 preovulation has yet to be studied. It is likely not related to folliculogenesis, since follicles develop concurrently with gestation in Spiny Dogfish, with theoretically little follicular growth from parturition to preovulation (Castro 2009). It is possible, however, that the peak in [T] plays 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 Spiny Dogfish remained relatively low from preovulation to mid gestation. However, substantial increases in this hormone began after mid gestation and the level 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 act as a reliable indicator of gestational stage in Spiny Dogfish. Similar to those of [P_4] and [T], preovulatory concentrations of [E_2] in Spiny Dogfish have not been previously quantified. 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).

Atlantic Sharpnose Sharks

The patterns of plasma and muscle [P_4] and [T] concentrations were similar in that they were relatively low from preovulation through early gestation before reaching a distinct peak in early–mid-gestation females. The pronounced elevation of plasma and muscle [P_4] and [T] concentrations coincided with the gestational stage at which uterine compartmentalization and implantation are suggested to arise in Atlantic Sharpnose Sharks (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 the Bonnethead *Sphyrna 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 Atlantic Sharpnose Sharks also concurrently varied, being elevated during preovulation before decreasing through mid gestation. This same pattern was also observed by Manire et al. (1995), who noted that in the Bonnethead, [E_2] peaks in the preovulatory stage during the final phase of folliculogenesis and decreases from early to mid gestation. In addition, muscle [E_2] concentrations in Atlantic Sharpnose Sharks 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 of quantifying steroid hormones in skeletal muscle and tested their efficacy, relative to plasma hormone concentrations, in assessing the reproductive status of sharks. An advantage of using skeletal muscle rather than plasma is that muscle tissue can be obtained nonlethally and with minimal invasion. The results suggest that white skeletal muscle tissue of Spiny Dogfish and Atlantic Sharpnose Sharks 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, which are coincident with gestational stage, paralleled those in plasma despite the different reproductive modes exhibited by these two sharks. Our results suggest that skeletal muscle steroid hormone content correlates well with gestational stage in yolk sac placental species. As such, future Atlantic Sharpnose Shark sampling efforts will focus on collecting individuals in the later stages of gestation to determine whether the parallel patterns observed in plasma and muscle steroid hormone concentrations are consistent throughout the reproductive cycle. While there were no significant relationships between plasma and muscle [P_4] and [T] concentrations in Spiny Dogfish, 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 that species. 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 Spiny Dogfish.

ACKNOWLEDGMENTS

We thank captains C. Brown (FV *Proud Mary*) and C. Felch (FV *Lady Victoria*) as well as the NOAA, Pascagoula, Mississippi, bottom longline survey. We thank Amy Carlson for creating our map. Thanks to the Marine Science Center (MSC) graduate program. Research was conducted as part of the requirements for a master's degree at the University of New England. We also thank the graduate and undergraduate students of the Sulikowski laboratory at the University of New England. This project was supported by a Save our Seas Foundation grant (P170) to J.A.S. This manuscript represents MSC contribution 49.

REFERENCES

Awruch, C. A., N. W. Pankhurst, S. D. Frusher, and J. D. Stevens. 2008. Endocrine and morphological correlates of reproduction in the Draughtboard Shark *Cephaloscyllium laticeps* (Elasmobranchii: Scyliorhinidae). Journal of Experimental Zoology Part A 309:184–197.

Barnett, L. A. K., R. L. Earley, D. A. Ebert, and G. M. Cailliet. 2009. Maturity, fecundity, and reproductive cycle of the Spotted Ratfish, *Hydrolagus colliei*. Marine Biology 156:301–316.

Callard, I. P., L. A. Fileti, L. E. Perez, L. A. Sorbera, G. Giannoukos, L. L. Klosterman, P. C. W. Tsang, and J. A. McCracken. 1992. Role of the corpus luteum and progesterone in the evolution of vertebrate viviparity. Integrative and Comparative Biology 32:264–275.

Castro, J. I. 2009. Observations on the reproductive cycles of some viviparous North American sharks. Aqua, International Journal of Ichthyology 15:205–222.

Castro, J. I., C. M. Woodley, and R. L. Brudek. 1999. A preliminary evaluation of the status of shark species. FAO (Food and Agriculture Organization of the United Nations) Technical Paper 380.

Castro, J. I., and J. P. Wourms. 1993. Reproduction, placentation, and embryonic development of the Atlantic Sharpnose Shark, *Rhizoprionodon terraenovae*. Journal of Morphology 218:257–280.

D'Ercole, A. J., A. D. Stiles, and L. E. Underwood. 1984. Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proceedings of the National Academy of Sciences of the USA 81:935–939.

Dulvy, N. K., Y. Sadovy, and J. D. Reynolds. 2003. Extinction vulnerability in marine populations. Fish and Fisheries 4:25–64.

Ebert, D. A. 2005. Reproductive biology of skates, *Bathyraja* (Ishiyama), along the eastern Bering Sea continental slope. Journal of Fish Biology 66:618–649.

Francis, M., C. O. Maolagain, and D. Stevens. 2001. Age, growth, and sexual maturity of two New Zealand endemic skates, *Dipturus nasutus* and *D. innominatus*. New Zealand Journal of Marine and Freshwater Research 35:831–842.

Gelsleichter, J., L. E. L. Rasmussen, C. A. Manire, J. Tyminski, B. Chang, and L. Lombardi-Carlson. 2002. Serum steroid concentrations and development

of reproductive organs during puberty in male Bonnethead Sharks, *Sphyrna tiburo*. Fish Physiology and Biochemistry 26:389–401.

Hammerschlag, N., and J. Sulikowski. 2011. Killing for conservation: the need for alternatives to lethal sampling of apex predatory sharks. Endangered Species Research 14:135–140.

Henningsen, A. D. 1998. Levels of re-circulating reproductively-related steroid hormones in female elasmobranchs. Implications for reproduction in a captive environment. Aquarium Sciences and Conservation 2:97–116.

Henningsen, A. D., F. L. Murru, L. E. L. Rasmussen, B. R. Whitaker, and G. C. Violetta. 2008. Serum levels of reproductive steroid hormones in captive Sand Tiger Sharks, *Carcharias taurus* (Rafinesque), and comments on their relation to sexual conflicts. Fish Physiology and Biochemistry 34:437–446.

Heupel, M. R., and C. A. Simpfendorfer. 2010. Science or slaughter: need for lethal sampling of sharks. Conservation Biology 24:1212–1218.

Hisaw, F. L., and A. Albert. 1947. Observations on the reproduction of the Spiny Dogfish, *Squalus acanthias*. Biological Bulletin 92:187–199.

Hoffmann, B. 1978. Use of radioimmunoassay for monitoring hormonal residues in edible animal products. Journal Association of Official Analytical Chemists 61:1263–1273.

Holt, W. V., and A. R. Pickard. 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Reviews of Reproduction 4:143–150.

IUCN (International Union for Conservation of Nature and Natural Resources). 2011. IUCN red list of threatened species, version 2011.1. Available: www.iucnredlist.org. (July 2011).

Kellar, N. M., M. L. Trego, C. I. Marks, and A. E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22:1–16.

Kneebone, J., J. A. Sulikowski, D. E. Ferguson, and P. C. W. Tsang. 2007. Endocrinological investigation into the reproductive cycles of two sympatric skate species, *Malacoraja senta* and *Amblyraja radiata*, in the western Gulf of Maine. Environmental Biology of Fishes 80:257–265.

Koob, T. J., and I. P. Callard. 1999. Reproductive endocrinology of female elasmobranchs: lessons from the Little Skate (*Raja erinacea*) and Spiny Dogfish (*Squalus acanthias*). Journal of Experimental Zoology 284:557–574.

Lasley, B. L., and J. F. Kirkpatrick. 1991. Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. Journal of Zoo and Wildlife Medicine 22:23–31.

Manire, C. A., L. E. L. Rasmussen, D. L. Hess, and H. E. Heuter. 1995. Serum steroid hormones and the reproductive cycle of the female Bonnethead Shark, *Sphyrna tiburo*. General and Comparative Endocrinology 97:366–376.

Mansour, A. A. H., D. W. McKay, J. Lien, J. C. Orr, and J. H. Banoub. 2002. Determination of pregnancy status from blubber samples in mike whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18:112–120.

Noppe, H., B. Le Bizec, K. Verheyden, and H. F. De Brabander. 2008. Novel analytical methods for the determination of steroid hormones in edible matrices. Analytica Chimica Acta 611:1–16.

Paolucci, M., and I. P. Callard. 1998. Characterization of progesterone-one-binding moieties in the Little Skate *Raja erinacea*. General and Comparative Endocrinology 109:106–118.

Payne, A. H., and D. B. Hales. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocrine Reviews 25:947–970.

Perez, L. E., and I. P. Callard. 1993. Regulation of hepatic vitellogenin synthesis in the Little Skate (*Raja erinacea*): use of a homologous enzyme-linked immunosorbent assay. Journal of Experimental Zoology 266:31–39.

Pinhal, D., O. B. F. Gadig, A. P. Wasko, C. Oliveira, E. Ron, F. Foresti, and C. Martins. 2008. Discrimination of shark species by simple PCR of 5S rDNA repeats. Genetics and Molecular Biology 31:361–365.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Available: http://www.R-project.org./ (September 2011).

Ruocco, N. L., L. O. Lucifora, J. M. Díaz de Astarloa, and O. Wöhler. 2006. Reproductive biology and abundance of the White Dotted Skate, *Bathyraja albomaculata*, in the southwest Atlantic. ICES Journal of Marine Science 63:105–116.

Sawaya, W. N., K. P. Lone, A. Husain, B. Dashti, and S. Al-Zenki. 1998. Screening for estrogenic steroids in sheep and chicken by the application of enzyme-linked immunosorbent assay and a comparison with analysis by gas chromatography-mass spectrometry. Food Chemistry 63:563–569.

Schlernitzauer, D. A., and P. W. Gilbert. 1966. Placentation and associated aspects of gestation in the Bonnethead Shark, *Sphyrna tiburo*. Journal of Morphology 120:219–231.

Skomal, G. B. 2007. Evaluating the physiological and physical consequences of capture on post-release survivorship in large pelagic fishes. Fisheries Management and Ecology 14:81–89.

Sulikowski, J. A., W. B. Driggers, G. W. Ingram, J. Kneebone, D. E. Ferguson, and P. C. W. Tsang. 2007a. Profiling plasma steroid hormones: a non-lethal approach for the study of skate reproductive biology and its potential use in conservation management. Environmental Biology of Fishes 80: 285–292.

Sulikowski, J. A., S. Elzey, J. Kneebone, J. Jurek, W. H. Howell, and P. C. W. Tsang. 2007b. The reproductive cycle of the Smooth Skate (*Malacoraja senta*) in the western Gulf of Maine. Journal of Marine and Freshwater Research 58:98–103.

Sulikowski, J. A., J. Kneebone, S. Elzey, J. Jurek, W. H. Howell, and P. C. W. Tsang. 2006. Using the composite variables of reproductive morphology, histology and steroid hormones to determine age and size at sexual maturity for the Thorny Skate *Amblyraja radiata* in the western Gulf of Maine. Journal of Fish Biology 69:1449–1465.

Sulikowski, J. A., P. C. W. Tsang, and W. H. Howell. 2004. Annual changes in steroid hormone concentrations and gonad development in the Winter Skate, *Leucoraja ocellata*. Marine Biology 144:845–853.

Tsang, P. C. W., and I. P. Callard. 1987. Morphological and endocrine correlates of the reproductive cycle of the aplacental viviparous dogfish, *Squalus acanthias*. General and Comparative Endocrinology 66:182–189.

Walker, T. I. 2004. Management measures. FAO (Food and Agriculture Organization of the United Nations) Fisheries Technical Paper 474: 285–321

Walker, T. I. 2005. Reproduction in fisheries science. Pages 81–126 *in* W. C. Hamlett and B. G. M. Jamieson, editors. Reproductive biology and phylogeny of chondrichthyes (sharks, batoids and chimaeras). Science Publishers, Enfield, New Hampshire.

Walmsley-Hart, S. A., W. H. H. Sauer, and C. D. Buxton. 1999. The biology of the skates *Raja wallacei* and *R. pullopunctata* (Batoidea: Rajidae) on the Agulhas Bank, South Africa. South African Journal of Marine Science 21:165–179.

Zeiner, S. J., and P. G. Wolf. 1993. Growth characteristics and estimates of age at maturity of two species of skates (*Raja binoculata* and *Raja rhina*) from Monterey Bay, California. NOAA Technical Report NMFS 115: 87–90.