MS. BIANCA PROHASKA (Orcid ID : 0000-0003-4495-073X)
MS. CAROLYN ROSE WHEELER (Orcid ID : 0000-0001-9976-8420)

Article type : Original Article

Effects of Delayed Phlebotomy on Plasma Steroid Hormone Concentrations in Two Elasmobranch Species

Running title: Reproductive analyses delayed blood draw in sharks

B. K. Prohaska[a*], P. C. W. Tsang[b], W. B. Driggers III[c], E. R. Hoffmayer[c], C. R. Wheeler[a], and J. A. Sulikowski[a]

[a]Department of Marine Sciences, University of New England, 11 Hills Beach Road, Biddeford, ME 04005, USA
[b]Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, 129 Main Street, Durham, New Hampshire 03824, USA
[c]National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Mississippi Laboratories, 3209 Frederick Street, Pascagoula, Mississippi 39567, USA

***Corresponding author**: Current address is Department of Biology, Florida State University, King Life Sciences, 319 Stadium Dr., Tallahassee, FL 32306 (313) 608-6049; E-mail: bprohaska@bio.fsu.edu

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jai.13700

Acknowledgments

We thank captains C. Brown (*F/V Proud Mary*) and C. Felch (*F/V Lady Victoria*), as well as the NOAA Fisheries, Mississippi Laboratories, Bottom Longline Survey Team. We also thank the graduate and undergraduate students of the Sulikowski Laboratory at the University of New England, and the Tsang Laboratory at the University of New Hampshire. This work was sanctioned by the UNE's Animal Care and Use Committee (IACUC protocol no. UNE-20121107SULIJ).

Summary

Measuring circulating concentrations of steroid hormones can be used as a method for determining reproductive maturity and cycles in elasmobranchs. However, it is unknown how long steroid hormones remain stable in elasmobranch blood following capture, and thus how quickly these samples should be collected for the results of subsequent steroid hormone analyses to be accurate. The objectives of this study were to determine if the sex steroid hormones progesterone, testosterone and estradiol would remain at stable concentrations in the blood of the Spiny Dogfish (*Squalus acanthias* Linnaeus, 1758) and the Atlantic Sharpnose Sharks (*Rhizoprionodon terraenovae* Richardson, 1836) that were captured, left on deck and un-refrigerated for 24 hours. Blood samples were serially drawn from five initially live sharks over a period of 24 hours. While concentrations of all three hormones did significantly fluctuate over the sampling period in both species, the resulting hormone concentrations from each sampling period still fell within the range of previously reported values for each species in their respective reproductive stage. Additionally, no significant changes in hematocrit were detected in either species over the 24-hour period. This research represents an extreme situation in which sharks were left on deck and un-refrigerated, and suggests that even when subjected to these conditions steroid hormone concentrations may fluctuate, but the resulting values may still be useful for assessing reproductive stage.

Introduction

The majority of elasmobranch fishes (sharks, skates and rays) exhibit life histories characterized by slow growth, late maturity, and low fecundity, which make these fishes particularly vulnerable to both direct and indirect fishing pressures (Dulvy et al., 2003). In general, elasmobranch life histories are similar; however, a thorough understanding of each species unique life history is essential for adequate management (Walker, 2004). Despite the importance of life history data in formulating management strategies, such data are still lacking for many elasmobranch species (Castro et al., 1999; Walker, 2004; Pinhal et al., 2008; IUCN, 2011).

Studies examining the life histories of elasmobranchs largely rely on lethal sampling methodologies (Heupel & Simpfendorfer, 2010); however, these methods should be avoided for species whose populations are threatened or endangered or when equally reliable data can be taken in a non-lethal manner. To address these concerns, researchers have begun examining the efficacy of non-lethal methods as an alternative when studying certain aspects of elasmobranch life history, such as reproductive biology (Sulikowski et al., 2007; Hammerschlag & Sulikowski, 2011). One commonly used non-lethal sampling technique is the extraction of blood from live sharks and subsequent isolation of steroid hormones, to examine physiologically-based reproductive events (e.g. Callard & Koob, 1993; Gelsleichter et al., 2002; Sulikowski et al., 2006 Kneebone et al., 2007).

Despite frequent use of steroid hormone analyses in elasmobranch research over the past 30 years, no studies have identified how rapidly a blood sample should be collected from this group of fishes before circulating plasma hormone concentrations begin to significantly fluctuate. The objective of this study was to determine if circulating concentrations of the sex steroid hormones progesterone (P_4), testosterone (T), and 17β-estradiol (E_2) were stable in sharks captured and left on deck un-refrigerated over a 24-hour period. If these steroid hormones are found to remain stable in postmortem elasmobranchs, scientists could work cooperatively with fishers by utilizing reproductive tracts and blood that would otherwise be discarded for future endocrine studies, and for the validation of non-lethal steroid hormone assays.

Materials and Methods

Five adult female Atlantic Sharpnose Sharks (*Rhizoprionodon terraenovae* Richardson, 1836) and five adult female Spiny Dogfish (*Squalus acanthias* Linnaeus, 1758) were captured and sampled using the methods described in Prohaska et al., (2013a, b). Briefly, *R. terraenovae* were captured by bottom longline in the northern 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.

All specimens were phlebotomized immediately after capture and a 5 ml aliquot of blood was collected; subsequently, all specimens were pithed. This work was sanctioned by the University of New England's Animal Care and Use Committee (IACUC protocol no. UNE-20121107SULIJ). Additional blood samples (5 ml each) were serially collected at 1 h, 3 h, 6 h, 12 h, and 24 h post-capture. Between blood collections, sharks were left on deck, shaded and un-refrigerated. Immediately after each collection, a sub sample of blood was analyzed for hematocrit (Hct) following the methods in Cicia et al., (2012). The remaining blood sample was then centrifuged at 1,242 g for 5 min. Separated plasma was removed and then stored at -20°C. After phlebotomy at time 0, fork length (FL) was recorded from each shark, which was measured to the nearest cm over a straight line along the axis of the body from the tip of the snout to the fork in the caudal fin. After sampling at time 24 h, dissections were conducted and the following data were recorded to assess reproductive stage: the five maximum follicle diameters (to the nearest mm), and pup 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.

Progesterone (P_4), testosterone (T), and 17β-estradiol (E_2) were extracted from plasma samples following the methods of Tsang and Callard (1987) and Sulikowski et al., (2004). Briefly, each plasma sample was extracted twice with 10 volumes (5 ml) 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, USA) to account for procedural loss.

Plasma steroid hormone concentrations were determined by radioimmunoassay following a protocol modified from Tsang and Callard, (1987). Non-radiolabeled P_4, T, and E_2 (Steraloids, Inc., Newport, RI, USA) were used to make stock concentrations of 80 μg ml^{-1} for P_4 and T, and 6.4 μg ml^{-1} for E_2 in absolute ethanol (ACS grade). The P_4, T and E_2 antibodies (Gordon D. Niswender, Colorado State University, Fort Collins, CO, USA) 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 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, USA) 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, USA) and the radioactivity was detected by a Perkin Elmer Tri-Carb 2900TR liquid scintillation analyzer (Waltham, MA, USA). Final concentrations were corrected for procedural loss using individual sample recoveries.

Repeated measures ANOVA were performed on plasma P_4, T, and E_2 concentrations as well as Hct by species. If a significant difference was found, a Tukey's post-hoc test was conducted. If data failed tests of normality or homogeneity of variance, the data were transformed. If transformed data still violated the assumptions, the nonparametric Friedman repeated measures analysis of variance on ranks was conducted. All data were analyzed using SigmaPlot 12.0 and were considered significant at $\alpha \leq 0.05$.

Results

153 *Rhizoprionodon terraenovae*

154 The five adult female *R. terraenovae* (mean ± SEM; 76.4 ± 2.4 cm FL) collected in this
155 study were all in the early/mid gestational stage (Prohaska et al., 2013a, b). The overall mean
156 recoveries of plasma P_4, T, and E_2 were 60, 91, and 79%, respectively. The mean intra-assay
157 coefficients of variation for *R. terraenovae* plasma P_4, T, and E_2 assays were 13, 17, and 13%,
158 respectively. Despite an initial significant decrease in P_4 between intervals 0 and 1 h,
159 concentrations of P_4 remained statistically unchanged until another significant decrease was
160 detected at hour 12. From interval 12 h to 24 h P_4 remained statistically unchanged (RM
161 ANOVA: $F_{4,29}$=17.213, $p<0.001$, β=1.000; Figure 1a). A significant fluctuation in T
162 concentrations occurred over the sampling period; however, the Tukey's post hoc test did not
163 detect a significant pairwise difference between intervals 0 h and 24 (RM ANOVA: $F_{4,29}$=5.777,
164 $p=0.002$, β=0.934; Figure 1b). Similarly, a significant fluctuation in E_2 concentrations was
165 detected over the entire sampling period; however, the Tukey's post hoc test did not detect a
166 significant pairwise difference between intervals 0 and 24 h (RM ANOVA: $F_{4,29}$=3.736, $p=0.015$,
167 β=0.701; Figure 1c). No significant difference in Hct was detected at any sampling interval
168 (Friedman: $F_{4,28}$=2.038, $p=0.119$; β=0.284; Figure 2a).

169 *Squalus acanthias*

170 The five adult female *S. acanthias* (mean ± SEM; 80.8 ± 0.8 cm FL) were all in the
171 candle gestational stage (Prohaska et al., 2013a, b). The overall mean recoveries of plasma P_4, T,
172 and E_2 were 71, 90, and 78%, respectively. The mean intra-assay coefficients of variation for *S.*
173 *acanthias* plasma P_4, T, and E_2 assays were 9, 11, and 12%, respectively. The mean inter-assay
174 coefficients of variation for *S. acanthias* plasma P_4, T, and E_2 assays were 9, 11, and 17%,
175 respectively. A significant decrease in P_4 was detected between sampling intervals 0 and 24 h,
176 with relatively little fluctuation between these intervals (RM ANOVA: $F_{3,23}$=3.324, $p=0.032$,
177 β=0.575; Figure 3a). A significant increase in T was observed at hour 6; however, no significant
178 difference was detected between interval 0 and 24 h (RM ANOVA $F_{3,23}$=9.234, $p<0.001$,
179 β=0.994; Figure 3b). A significant decrease in E_2 was evident between times 0 and 1 h; however,
180 the concentrations of E_2 remained statistically unchanged for the remainder of the sampling (RM
181 ANOVA: $F_{4,29}$=13.079, $p<0.001$, β=1.000; Figure 3c). No significant difference in Hct was
182 detected at any sampling interval (Friedman: X^2_5=7.171, $p=0.208$; Figure 2b).

183

Discussion

The aim of this study was to determine how stable steroid hormones were in sharks over a 24-hour period. This study represents a disadvantageous scenario, one in which an animal is left on deck un-refrigerated for 24 hours, and steroid hormone concentrations in both *R. terraenovae* and *S. acanthias* did significantly fluctuate over the sampling period. In *R. terraenovae*, T and E_2 were the most stable during the 24-hour sampling period; however, P_4 was only stable for one hour before it began to fluctuate. In *S. acanthias*, T and P_4 were most stable for 12 h and 24 h, respectively, while E_2 was only stable for an hour before it began to fluctuate. In instances where steroid hormone concentrations fluctuated among sampling intervals, the fluctuations were relatively minor, less than an order of magnitude and could have been influenced by small sample sizes.

Hormone concentrations in the blood are determined by both their synthesis and clearance. Once hormones are synthesized they are transported through the body by blood circulation until target tissues are reached, or catabolized by metabolic clearance typically in the liver (Norman & Litwack, 1997). When an organism dies production of hormones and blood circulation cease (Donaldson & Lamont, 2013). As the metabolic clearance rate slows, the half-life of a steroid hormone increases (Norman & Litwack, 1997). Since the sharks were initially alive when first sampled, some blood circulation likely occurred between the time 0 h and 1 h, potentially resulting in some metabolic clearance; however, the relative stability of the hormone concentrations past the first couple blood draws are likely a result of decreased or ceased hormone catabolism.

Despite significant fluctuations in hormone concentrations, particularly in regards to *R. terraenovae* P_4 and *S. acanthias* E_2, the range of values observed in this study are useful in determining gestational stage in both species. For example, using the range of mean hormone concentrations per time period alone (P_4: 1.9-6.4 ng ml^{-1}; T: 4.7-10.1 ng ml^{-1}; E_2: 660-1,049 pg ml^{-1}) *R. terraenovae* would be classified as early/mid gestation based on Prohaska et al., (2013a, b). Similarly, in *S. acanthias* mean hormone concentrations per time period alone (P_4: 80-304 pg ml^{-1}; T: 47-207 pg ml^{-1}; E_2: 44-104 pg ml^{-1}) would classify sharks as candle stage based on Prohaska et al., (2013a, b). Hormone concentrations are inherently variable; however, concentrations between stages can be many of orders of magnitude different making actual concentrations potentially not as important as relative concentrations.

While there was a trend toward decreasing Hct with time, no statistically significant changes were found in Hct in either species over the sampling period. Similarly, Hoffmayer et al., (2012, 2015) did not observed significant changes in hematocrit in serially sampled *R. terraenovae*. Despite not being statistically significant, the trend of decreasing Hct could be indicative of a hemodilution. Previous work by Frick et al., (2010), in which blood was serially drawn from Port Jackson sharks (*Heterodontus portusjacksoni* Meyer, 1793), and gummy sharks (*Mustelus antarcticus* Günther, 1870), observed an initial elevation in Hct, followed by a continuous decrease in Hct during a post-capture monitoring period, and postulated that this decrease could have been the result of repeated blood extractions, a phenomena similar to that previously observed in teleosts (Turner et al., 1983). In the current study, if hemodilution was occurring, this could account for any decreases observed in steroid hormone concentrations. To determine if hemodilution could have influenced the hormone concentrations observed in this experiment, a follow up study should be conducted in which sharks are sampled at the time of capture, subjected to the same conditions as those sharks in this study, and then sampled again at 24 h.

In addition to gaining a basic knowledge of the post-capture stability of steroid hormones, the results of this study suggest there is potential to work cooperatively with commercial fishers to use blood that would otherwise be discarded from harvested elasmobranchs for endocrine studies, and for validating non-lethal steroid hormone assays. In commercial fisheries, caudal fins are immediately cut off of harvested sharks so they can be bled; however, if researchers were working cooperatively with fishers they could request that sharks not be bled immediately. To account for any fluctuations in hormone levels post mortem, particularly like those observed in *R. terraenovae* P_4 and *S. acanthias* E_2, additional samples could be collected and analyzed to calculate a correction factor between the time post-capture that an animal is sampled and its hormone level.

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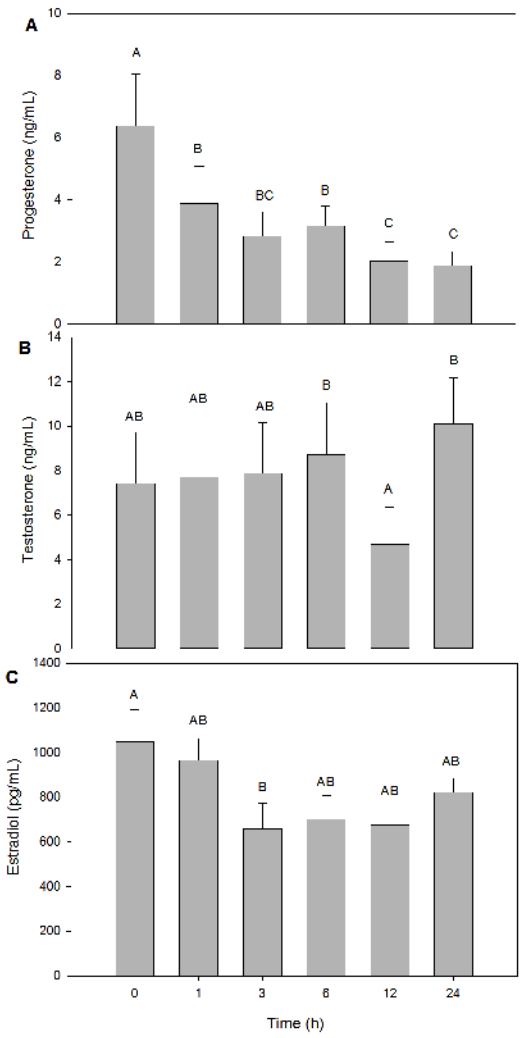
Figure Legends

Figure 1. Mean (± SEM) concentrations of Atlantic Sharpnose Sharks *Rhizoprionodon terraenovae* plasma steroid hormones progesterone (A), testosterone (B) (in nanograms per milliliter) and estradiol (C) (in picograms per milliliter) at each sampling interval. Letters above the bars denote statistically significant pairwise differences in hormone concentrations between sampling intervals ($p < 0.05$).

Figure 2. Mean percent (± SEM) hematocrit (Hct) of Atlantic Sharpnose Sharks *Rhizoprionodon terraenovae* (A) and Spiny Dogfish *Squalus acanthias* (B) blood at each sampling interval.

Figure 3. Mean (± SEM) concentrations of Spiny Dogfish *Squalus acanthias* plasma (in picograms per milliliter) steroid hormones progesterone (A), testosterone (B) and estradiol (C) at

each sampling interval. Letters above the bars denote statistically significant pairwise differences in hormone concentrations between sampling intervals ($p < 0.05$).



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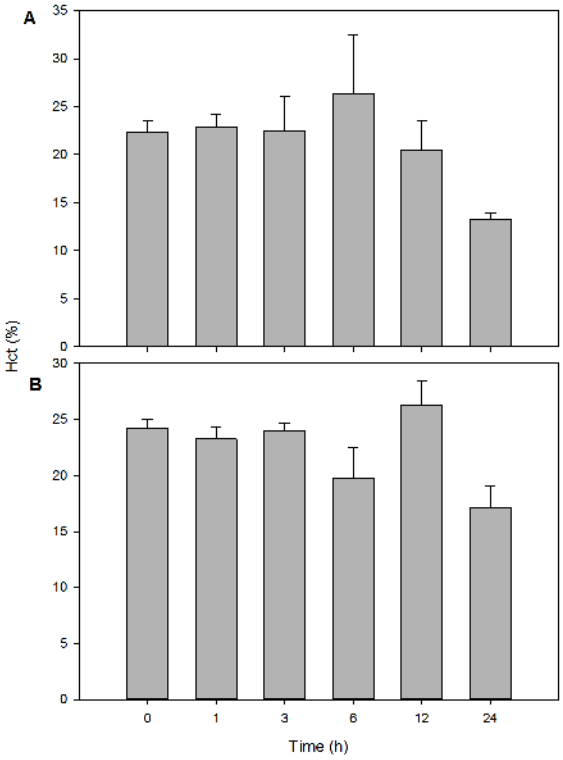

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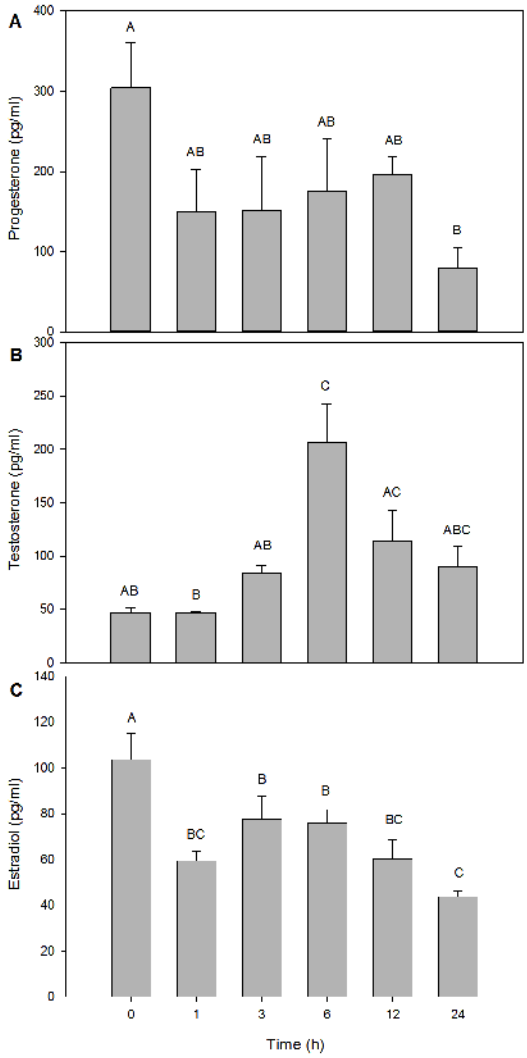

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