Updated life history and population structure assessment of spiny dogfish, Squalus acanthias, in the Gulf of Maine

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UPDATED LIFE HISTORY AND POPULATION STRUCTURE ASSESSMENT OF SPINY DOGFISH, *SQUALUS ACANTHIAS*, IN THE GULF OF MAINE

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... viii

LIST OF TABLES ..................................................................................................................... x

LIST OF FIGURES ................................................................................................................... xii

ABSTRACT ................................................................................................................................ xiv

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>I. AGE AND GROWTH WITH A COMPARISON BETWEEN AGEING STRUCTURES</td>
<td>6</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS** .................................................................................................. 8

- **Sampling Protocol** ........................................................................................................ 8
- **Preparation of Vertebrae and Spines** ................................................................................ 8
- **Sectioning and staining of vertebrae** ............................................................................... 9
- **Growth Increments in Vertebrae and Spines** .................................................................. 10
  - **First increment validation** ............................................................................................ 10
  - **Postnatal vertebrae** ..................................................................................................... 10
  - **Postnatal spines** .......................................................................................................... 11
- **Age Analysis of Vertebrae and Spines** ......................................................................... 12
  - **Precision estimates and age bias** ............................................................................... 12
  - **Marginal increment analysis** ...................................................................................... 12
Growth Determination ................................................................. 13
Longevity ................................................................................. 14
Determining the Better Structure for Age Estimation .................. 14
RESULTS .................................................................................. 15
Birthring Confirmation .............................................................. 15
Validation of Growth Increments ............................................... 16
  Bias and Precision ................................................................. 16
Wear of Dorsal Fin Spine ............................................................ 17
Differences Between Ageing Structures ....................................... 17
Growth Function Analysis .......................................................... 18
Longevity Estimates ................................................................. 19
DISCUSSION ............................................................................ 20
II. MATURITY ANALYSIS ........................................................... 36
MATERIALS AND METHODS ...................................................... 38
  Sampling Protocol ................................................................ 38
  Morphology ........................................................................... 38
  Histology ............................................................................. 39
  Physiology .......................................................................... 40
  Maturity Criteria: Morphology, Physiology, Histology ............... 41
  Parameter Characterization ..................................................... 42
  Maturity Analysis ................................................................ 43
  Statistics .............................................................................. 44
RESULTS .................................................................................. 44
ACKNOWLEDGEMENTS

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LIST OF TABLES

Table 1.1 Vertebra staining protocol ................................................................. 32
Table 1.2 Precision estimates between ageing structures .................................. 33
Table 1.3 Growth parameters by sex, structure, and growth function .................. 34
Table 1.4 Longevity estimates by sex and structure ........................................... 34
Table 1.5 Comparison of growth, longevity, and size at birth between the present study and previous studies ................................................................. 35
Table 2.1 Range of parameters used to define a maturity threshold for females ....... 60
Table 2.2 Range of parameters used to define a maturity threshold for males .......... 60
Table 2.3 Comparison of reproductive parameters between immature and mature males ........................................................................................................... 60
Table 2.4 Comparison of reproductive parameters between immature and mature females ..................................................................................................... 61
Table 2.5 Comparison of age at 50% maturity between the present study and previous studies .......................................................................................... 61
Table 3.1 Comparison of plasma steroid hormone concentrations between the present study and a previous study .............................................................. 89
Table 3.2 Comparison of fecundity by size class of females between the present study and a previous study ................................................................. 90
Table 4.1 The locations of genetic samples taken for population differentiation analyses ........................................................................................................ 108
Table 4.2 Loci characteristics and differentiation for population analysis.............108

Table 4.3 Population differentiation using pairwise $F_{ST}$ analysis.......................109
LIST OF FIGURES

Figure 1.1 Vertebrae diameter as a function of total lengths by sex .................. 26
Figure 1.2 Image of a prepared, stained, and aged vertebra .................................. 27
Figure 1.3 Standardized marginal increment analysis ........................................... 28
Figure 1.4 Bias plots between readers using age estimates obtained from vertebra and dorsal fin spines .......................................................... 29
Figure 1.5 Equation to account for lost increments on the dorsal fin spine due to wear .......................................................... 30
Figure 1.6 Two parameter von Bertalanffy growth functions by sex using age estimates obtained from vertebrae ........................................... 31
Figure 2.1 A histologically prepared and stained section of a mature testis .................. 54
Figure 2.2 Morphological and physiological reproductive parameters by age and length of males .......................................................... 55
Figure 2.3 Morphological reproductive parameters by age and length of females ....... 56
Figure 2.4 Physiological reproductive parameters by age and length of females ........ 57
Figure 2.5 Maturity ogives for males by age and size and a comparison between the present study and a previous study ........................................... 58
Figure 2.6 Maturity ogives for females by age and size and a comparison between the present study and a previous study ........................................... 59
Figure 3.1 Monthly mean values for morphological, histological and physiological parameters for mature males........................................................................................................................................84

Figure 3.2 Mean values by stage for morphological and physiological parameters of mature females.................................................................................................................................................................................................85

Figure 3.3 Largest values by month of morphological parameters for mature females...86

Figure 3.4 Mean plasma steroid hormone concentrations by stage for mature females...87

Figure 3.5 Mean plasma steroid hormone concentrations by month for mature females..88

Figure 4.1 Number of populations present using mean values for Ln P(D)..............105

Figure 4.2 Mean Q scores for population structuring based on two assumed populations present.................................................................................................................................................................................................106

Figure 4.3 Individual Q scores for population structuring based on two assumed populations present.................................................................................................................................................................................................107
ABSTRACT

UPDATED LIFE HISTORY AND POPULATION STRUCTURE ASSESSMENT OF SPINY DOGFISH, SQUALUS ACANTHIAS, IN THE GULF OF MAINE

By

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University of New Hampshire, December, 2010

Spiny dogfish (Squalus acanthias) are considered to be the most abundant shark species in the Western North Atlantic, but recently there was a decline in biomass estimates following a nearly ten-fold increase in fishery landings. Because fishing pressure has been shown to affect population dynamics in targeted species, an update of previous life history parameters is warranted to effectively manage the population. Although updated and accurate life history parameters are important, it is crucial that these parameters are applied to the correct populations, especially when there is evidence of population structuring in the Western Atlantic. This comprehensive study addressed the need for updated and accurate life history characteristics of spiny dogfish by obtaining year round samples covering the entire size range of spiny dogfish. This also enabled examination of spatial and temporal population structuring in the Western North Atlantic. While the vertebrae of spiny dogfish provided a more accurate and precise age estimate than the dorsal fin spines used in previous studies, there was no significant difference in growth prior to or following the increase in fishing pressure. Also, there was no change in the reproductive seasonality or magnitude of morphological, histological, or physiological parameters in mature individuals over this time. However, using a suite of characteristics to define maturity, there was a decrease in size and age at maturity as well
as a decrease in fecundity for female spiny dogfish in the present study compared data obtained prior to the fishing pressure. Population structuring was also observed using microsatellite regions in genomic DNA as molecular markers. With the apparent recovery of the spiny dogfish stock in the Western North Atlantic, the findings from the present study as well as a further elucidation of population structure in this region can be utilized to sustainably manage population(s) and predict the effects of fishing pressure in the future.
INTRODUCTION

The resources in the oceans were once thought to be inexhaustible, as stated by Thomas H. Huxley in 1883, then president of the Royal Society of London (Jennings et al. 2001). But, due to increases in demand and advances in technology beginning in the middle of the twentieth century, half of all global wild capture marine fisheries have since been fully exploited and a quarter are overexploited, depleted, or recovering (Hilborn and Walters 2001; FAO 2003-2010; FAO 2006). In order to maintain sustainable fisheries, given the increased fishing pressure placed on fish populations and the complex marine ecosystems which they inhabit (McCann et al. 1998), the most current, accurate, and complete data are required for effective management decisions (Hart and Reynolds 2002).

The levels of collapsed fisheries have increased over time and most recently reached 29% in 2003 (Worm et al. 2006). More worrisome is that the percentage of under or unexploited fisheries continues to decrease as these fish populations are targeted due to decline of traditional resources. For example, the collapse of the Atlantic cod, *Gadus morhua*, fishery in the United States in the 1990’s contributed to the subsequent targeting of spiny dogfish, *Squalus acanthias*, a small, temperate, coastal shark species with a circumglobal distribution (Bigelow and Schroeder 1953). As a result, this led to a greater than five-fold increase in *S. acanthias* landings from the late 1980’s to the mid 1990’s (NEFSC 1998). Due to sexual dimorphism, the large adult females were
primarily targeted (Rago et al. 1998). While, *S. acanthias* are believed to be the most abundant shark species in the North Atlantic, their life history characteristics (slow growth, late maturity, and low fecundity) make them vulnerable to heavy fishing pressure (Musick 1999; Stevens et al. 2000). Because of the selective nature of the fishery, female spawning stock biomass decreased greater than ten-fold over this time period. This prompted the Atlantic States Marine Fisheries Council (ASMFC) to declare *S. acanthias* overfished and to enact a Fishery Management Plan that essentially ended the directed fishery (ASMFC 2002). Concurrent with this decrease in spawning stock biomass was a decrease in recruitment, as record low levels of young of the year *S. acanthias* were observed for seven consecutive years according to the Northeast Fisheries Science Center (NEFSC 2003). Because fishing pressure can alter life history characteristics of fish populations (Buxton 1993; Rijnsdorp 1993; Rowell 1993; Sminkey and Musick 1995), a reassessment of *S. acanthias* life history is warranted, as all previous studies in the Western North Atlantic were conducted prior to the increased fishing pressure (Jensen 1965; Soldat 1983; Nammack et al. 1985; Tsang and Callard 1987a; Marques da Silva 1993).

Among the life history parameters, age estimates are the foundation from which other important parameters used in management, such as growth, maturity, fecundity, and mortality are built, making it one of the most influential biological variables (Campana 2001). Therefore, errors in age estimates, if not corrected, can have serious repercussions, especially when they are assimilated into fishery models to assess current and predict future stock status. Previous studies in the Western North Atlantic utilized the second dorsal fin spine as the structure for age estimates (Jensen 1965; Soldat 1983; Nammack et
al. 1985), but the results have been highly variable. Thus, by estimating age using another structure that is less prone to the variability inherent with the second dorsal fin spine, less error will be introduced in the initial age parameters, which leads to more accurate characterizations of the population.

Reproductive parameters, such as size/age at maturity and reproductive cycles, also provide essential information for fisheries managers. Previous studies have only utilized gross morphology to determine maturity and reproductive cyclicity (Jensen 1965; Nammack et al. 1985), but combining morphology with additional indicators of reproductive readiness, e.g. histological and biochemical markers, provide a more accurate assessment (Maruska et al. 1996; Sulikowski et al. 2005).

After obtaining accurate life history parameters, it is equally important that they are applied to the appropriate populations. Based on tagging studies and catch records/observations, there is evidence that S. acanthias in the Western North Atlantic may not be one panmictic population (Jensen 1965; Moore 1998; Rulifson et al. 2002; Campana et al. 2009), as previously believed. With the advent of molecular technology, a better understanding of the population structure of S. acanthias in the Western North Atlantic can be elucidated using microsatellite markers. No previous studies have examined spatial and temporal differences along the entire range of S. acanthias in the eastern United States. Using this approach, a high resolution characterization of population structure can be obtained, leading to better management of S. acanthias.

The overall goal of the current study is to provide the most complete assessment of S. acanthias life history and population structure to date, in an effort to provide
fisheries managers the data needed to ensure sustainability of populations in the Western North Atlantic.

Specifically, the hypotheses and objectives are:

Hypothesis 1: The vertebra is a more accurate and precise structure than the second dorsal fin spine for age determinations of *S. acanthisas*.

Objective 1: To develop a protocol using the vertebra as the aging structure.

Objective 2: To compare precision of age estimates between vertebrae and dorsal fin spines.

Objective 3: To fit the length at age data into growth curves for both structures.

Objective 4: To determine the better structure for age estimates based on precision, accuracy and goodness of fit to growth curves.

Hypothesis 2: Following increased fishing pressure, the age and size at maturity for *S. acanthisas* have decreased.

Objective 1: To assess age and size at maturity of *S. acanthisas*, using a combination of gross morphological, histological and endocrinological endpoints.

Hypothesis 3: The profiles of plasma steroid hormones and morphological parameters over the reproductive cycle differ between studies conducted prior to and following the increase in fishing pressure.

Objective 1: To determine plasma estradiol, progesterone and testosterone concentrations in female and male *S. acanthisas*, using radioimmunoassay.
Objective 2: To determine reproductive cycles using morphological, histological and endocrinological endpoints.

Objective 3: To assess fecundity in female S. acanthias.

Hypothesis 4: There is more than one population of S. acanthias in the Western North Atlantic.

Objective 1: To collect samples spanning three regions along the Western Atlantic and during different seasons within the Gulf of Maine.

Objective 2: To analyze samples for population differentiation using microsatellites.
CHAPTER 1

AGE AND GROWTH WITH A COMPARISON BETWEEN AGEING STRUCTURES

The spiny dogfish, *Squalus acanthias* L, is a cosmopolitan shark species found in the Atlantic and Pacific Oceans (Burgess 2002). Recently, female *S. acanthias* were heavily targeted on the east coast of the United States after the cod fishery collapse in the 1990’s (NEFSC 1998). Consequently, estimates of spawning stock biomass in the Northwest Atlantic decreased from 433,000 metric tons to 26,700 metric tons from 1988 to 1998 (NEFSC 2006). Shark populations are especially susceptible to overfishing due to their slow growth, long life span, and low fecundity (Holden 1973; Musick 1999; Stevens et al. 2000). In turn, increased fishing pressure has led to changes in population dynamics of elasmobranchs, including increased growth rate (Sminkey and Musick 1995; Stevens and West 1997; Walker 1998), a decrease in maximum size, younger age at maturity, and smaller size at maturity (Buxton 1993; Rijnsdorp 1993; Rowell 1993). This fishing pressure has had a similar effect on life history parameters of *S. acanthias*, (e.g. a decrease in maximum size and size at maturity of females (Sosebee 2005)). Changes in life history characteristics can affect management strategies, thus, updated and accurate
parameters are critical for effective management. A key component of life history studies is obtaining accurate and precise age estimates. These age estimates provide the foundation from which other important life history parameters used in management, such as growth, maturity, fecundity, and mortality are built upon, making it one of the most influential biological variables (Campana 2001). In elasmobranchs, the use of vertebral centra is the predominant means of assessing age and determining growth rates (Cailliet and Goldman 2004). In contrast, all previous ageing studies of the *S. acanthias* have utilized the second dorsal fin spine to obtain age estimates (Kaganovskaia 1933; Holden and Meadows 1962; Jensen 1965; Ketchen 1972; Ketchen, 1975; Jones and Geen 1977; Nammack et al., 1985; McFarlane and Beamish 1987). However, since the dorsal fin spines are located on the external surface of the *S. acanthias*, they are subject to environmental factors that can cause wear or breakage. Although a quantitative method is available to account for lost annuli (Ketchen 1975) and while this method has been used throughout subsequent ageing studies of the *S. acanthias*, Ketchen acknowledges that his technique may err in the direction of overestimation of age and underestimation of growth rate. Such error could result in unreliable and inaccurate age estimates, thus the need to develop alternate methods of age determination is warranted. The objectives of the present study were: 1) to develop a staining technique to visualize growth increments on vertebral centra of *S. acanthias*: 2) to compare precision of age estimates obtained between dorsal fin spines and vertebrae: 3) to fit the length at age data into growth curves using both structures: and 4) to determine which structure is more appropriate for age estimates in *S. acanthias*, based on precision, accuracy, goodness of fit of the growth curves, and observed biological data.
MATERIALS AND METHODS

Sampling Protocol

Monthly samples of male and female *S. acanthias* at 5 cm increment size classes from <35 cm to >100 cm fork length were obtained in the Gulf of Maine between July 2006 and June 2009 from commercial fishing vessels using either bottom trawl or gillnet. Additional samples were obtained from the NOAA/NMFS Spring and Autumn Bottom Trawl Surveys as well as hook and line capture during this time period.

For each *S. acanthias* collected in the field, the sex was noted, along with weight (to the nearest 0.05 kg), stretch total length (*L*<sub>ST</sub>, measured from the tip of the rostrum to the termination of the dorsal lobe of the caudal fin at its maximum extension), fork length (*L*<sub>F</sub>, measured from the tip of the rostrum to the most noticeable fork in the caudal fin), and pre-caudal length (*L*<sub>PC</sub>, measured from the tip of the rostrum to the pre-caudal pit). All length measurements were taken over the body and measured to the nearest millimeter (mm). The second dorsal fin spine and 8-12 vertebrae anterior to the first dorsal fin, including the vertebra connected to the first dorsal fin spine, were removed from each *S. acanthias* and frozen. By using the first dorsal fin spine as a landmark, it ensured that the vertebrae removed and aged were from the same region between individuals, thus reducing variability between the vertebrae used for age estimates (Officer et al. 1996).

Preparation of Vertebrae and Spines
Sectioning and staining of vertebrae

After excess tissue was removed from both structures and the individual vertebral centra were freed from the column, each respective hard part was stored frozen until further analyses commenced. Total length (cm) versus vertebral diameters was plotted to determine if vertebrae growth was proportional to fish growth. A positive relationship indicates the appropriateness of this structure for age estimations for this species. Regressions were plotted by sex and compared statistically using an analysis of covariance (ANCOVA). Sexes were pooled if no difference was found.

In order to elucidate the growth increments (which are defined as translucent and opaque band pairs in the vertebral centra), it was necessary to incorporate several procedures for each vertebra. Initially, the centra were sectioned using a Ray Tech Gem Saw with two diamond blades separated by a 0.4 mm spacer. Each centrum was sectioned through the focus along the sagittal plane, and the resulting bow-tie sections were then stained to enhance the growth increments. A variety of staining techniques were attempted, including Alizarin red (LaMarca 1966), violet blue (Johnson 1979), and silver nitrate (Schwarz 1983). In addition, a histological staining method modified based on Natanson et al. (2007) was employed and was the only method which produced visible growth increments on the vertebral centra.

Briefly, sectioned vertebrae were placed in tissue cassettes and decalcified with 100% RDO® rapid decalcifying agent (www.rdo-apex.com) for varying times from 0.5 – 1.5 hours depending on the size, with larger vertebrae having longer decalcification times. Constant movement of the solution was provided by a mixing plate and magnetic stir-bar. Afterwards, centra were rinsed with running tap water for one hour before they
were stored in 70% ethanol until staining. Sections were stained using Harris’ modified hematoxylin and brought into glycerin in preparation for mounting with Kaiser Glycerin Jelly (See Table 1.1 for procedural details). Stained sections were finally mounted on microscope slides in glycerin jelly, covered by a coverslip, and sealed with clear nail polish.

Growth Increments in Vertebrae and Spines

First increment validation

The second dorsal fin spines and vertebrae from full term pups were examined to identify the location of the growth increments, if any, that were formed in utero, which would be subtracted from the total reading of unworn spines of post natal *S. acanthias* to obtain more accurate age estimations. Full term pups, defined as pups greater than 24 cm TL with no yolk sac, were removed from uteri of female sharks in autumn, near the suspected time of parturition. Vertebrae and second dorsal fin spines were removed from each pup, cleaned, processed, and examined using the aforementioned protocol. Diameter of vertebrae and base of spines were measured to the nearest 0.1mm using vernier calipers. The number of increments, if any, on both structures were counted and the widths at the increments were measured.

Postnatal vertebrae

Upon completion of staining, each vertebra from postnatal *S. acanthias* was examined by two readers who had no prior knowledge of size, weight, sex, or month of
capture. Sections from each vertebra were viewed with a dissecting microscope using transmitted light under 30X magnification and the number of increments was counted. The vertebrae were then digitally photographed using a compound microscope with transmitted light under 40X magnification. After completing the first counts of growth increments for all vertebrae, the same readers counted growth increments on the same vertebrae a second time for precision estimates. The primary reader assigned an age using vertebrae and second dorsal fin spines for 394 and 381 S. acanthias, respectively, while the secondary reader estimated the age of 207 vertebrae and 196 dorsal spines.

*Postnatal spines*

Whole second dorsal fin spines were used for age estimations. The base of the spine at the beginning of enamel deposition was measured to the nearest 0.1 mm from anterior to posterior surfaces using vernier calipers. Spines were also examined by two readers who had no prior knowledge of size, weight, sex or month of capture of S. acanthias. Each reader counted the number of ridges and/or pigment bands on the anterior lateral surface of the spines using a dissecting microscope under 30X magnification, beginning with the first ridge at the base that did not contain a white band. Incomplete bands at the base that were in the process of forming were white and so were excluded from analysis as only complete bands were counted (Nammack et al. 1985). If external wear of the enamel on the spine surface occurred, the increment number was counted up to the point of wear and the diameter of the spine from anterior to posterior face was measured with vernier calipers. Using the method developed by Ketchen (1975), a power curve was constructed using the width at the base of unworn spines and the
number of increments observed by the readers. The width at the wear point of worn spines could then be put into the equation to account for lost increments which were then added to the count obtained up to the wear point to give an adjusted age estimate. After ageing all of the spines a first time, the readers counted the same spines a second time for precision estimates.

**Age Analysis of Vertebrae and Spines**

*Precision estimates and age bias*

Average percent error (APE) and coefficient of variation (CV) (Chang 1982) were calculated to determine the precision of the between and within reader counts for both spines and vertebrae. The equation for APE is: \[ \frac{1}{R} \sum_{i=1}^{R} \left| \frac{X_{ij} - X_j}{X_j} \right| \] and the equation for CV is: \[ \frac{\sqrt{\sum_{i=1}^{R} \left( X_{ij} - X_j \right)^2}}{\sum_{i=1}^{R} \left( X_{ij} - X_j \right)^2} \cdot \frac{R - 1}{R} \cdot \frac{1}{X_j} \] where \( X_{ij} \) is the \( i \)th age determination of the \( j \)th fish, \( X_j \) is the average age calculated for the \( j \)th fish, and \( R \) is the number of times each fish is aged. An age bias plot (Campana 2001) was used to expose any possible systematic bias in estimating age between readers.

*Marginal increment analysis*

Marginal increment analysis was used to verify the time of increment formation and provided a validation of increment periodicity. Measurements were taken from all \( S \).
acanthias with age estimates greater than one year following the protocol of Conrath et al. (2002). The margin width (\(W_M\)), which is the distance from the distal portion of the last opaque increment and the vertebra edge, as well as the previous band width (\(W_{PB}\)), between the distal portion of the next to last opaque increment and the distal portion of the last opaque increment, were recorded. The formula to determine standardized marginal increment (\(M_s\)) formation for each fish was \(M_s = (W_M) / (W_{PB})\). Mean \(M_s\) were plotted by month to examine trends in increment formation. If increments occurred annually, there will be a unimodal distribution of the marginal increments throughout the year (Manooch 1987). A multi-way ANOVA was performed to test for differences in standardized marginal increments throughout the year. A pairwise comparison was then performed to determine time of year of the band formation.

Growth Determination

Multiple growth curves were fit to length at age estimates obtained from second dorsal fin spines and stained vertebral centra from both males and females. Two versions of the von Bertalanffy growth function (VBGF) were utilized (von Bertalanffy 1938), according to the equation: 
\[ L_t = L_\infty - (L_\infty - L_0) e^{-kt}, \]
where \(L_t\) is the stretch total length at age \(t\), \(L_\infty\) is the theoretical mean maximum stretch total length, \(k\) is the growth coefficient, and \(L_0\) is the stretch total length at birth. Because size at birth was known, a two parameter version of the VBGF fixed \(L_0\), thus forcing the function to be anchored at the observed size at birth, which in the current study was 25 cm \(L_{ST}\). The three parameter version of the VBGF allowed \(L_0\) to vary. The Gompertz growth model was a third model used according to the equation: 
\[ L_t = L_0 e^{G(1-e^{-kt})}, \]
where \(L_\infty\) is equal to \(L_0 e^G\), which is the
mean maximum stretch total length \((t = \infty)\), \(k \ (= g \text{ in Ricker 1975})\) is the rate constant (per year), and \(L_0\) is the total length at birth.

**Longevity**

The maximum age estimates generated from vertebrae and second dorsal fin spines for both males and females provided a rough estimate of longevity. However, to account for a potential underestimation of maximum age using the oldest estimated ages obtained from dorsal fin spines and vertebrae due to lower frequency of fish at the extreme sizes, the method devised by Taylor (1958) to determine longevity based on VBGF parameters from observed data was utilized. The equation was later rearranged by Skomal and Natanson (2003) to solve using \(L_0\) for \(99\%\) of \(L_\infty\). The parameters were used in the following equation: 

\[
99\% \ L_\infty = \frac{L_\infty - L_0}{L_\infty (1 - 0.99)}
\]

**Determining the Better Structure for Age Estimation**

The utility of vertebrae and second dorsal fin spines as accurate ageing structures was evaluated by comparing precision estimates and growth models of each ageing structure. In order to determine which structure provided the most accurate age estimates, statistical comparisons (ANOVA) between parameter estimates generated from growth curves were calculated using the mean square of error (MSE) (Cailliet et al. 2006). Mean square error was used as an indicator of fit as opposed to the coefficient of determination \((R^2)\) because caution should be used against solely basing fit on \(R^2\) when using nonlinear models (Kvålseth 1985). In addition, \(L_\infty\) and \(L_0\) were compared to the observed maximum
total length and size at birth from individuals used in this study. While the fit of the curve is important when examining the growth functions using different ageing structures, the biological parameters such as $L_\infty$ and $L_0$ must also be taken into account based on observed data (Natanson et al. 2006) to determine if these parameters were biologically realistic. Comparisons between age estimates obtained from the two structures in the same individuals were also made to quantify differences.

**RESULTS**

Between July 2006 and June 2009, a total of 395 (147 males and 248 females) *S. acanthias* was collected, comprising 11 of the 12 months (no ageing structures from *S. acanthias* were collected in January). The length of males and females ranged from 32.5 to 84 cm $L_{ST}$ and 25 to 102 cm $L_{ST}$, respectively. There was a strong correlation ($N = 368; R^2 = 0.99$) between $L_F$ and $L_{ST}$ described by the equation: $L_F = \frac{L_{ST} - 2.1572}{1.0939}$. There was a positive logarithmic relationship between fish length and vertebrae diameter ($N = 90; R^2 = 0.93$) with no difference between sexes (ANCOVA; df = 1; $p > 0.05$) (Fig. 1.1). Because vertebrae diameter correlated with growth, it was deemed an appropriate structure for age determination, while the modified histological staining method utilized for this study also enhanced the visibility of the growth increments in the vertebrae (Fig. 1.2). Note that the decalcification step was critical, since growth increment visualization was not enhanced in vertebrae stained with hematoxylin before decalcification.

**Birthing Confirmation**
The birth ring on postnatal *S. acanthias* vertebrae was confirmed because the mean radius of the putative birth ring was not significantly different from the vertebrae radius of full term embryos, and they were both significantly smaller than the vertebrae radius at the first increment of postnatal *S. acanthias* (ANOVA; DF 2; P-value < 0.0001) (Table 1.2).

Similarly, the birth ring on postnatal *S. acanthias* second dorsal fin spine was confirmed because the mean diameter of the putative birth ring on the postnatal dorsal fin spine and the spine diameter of full term embryos are significantly smaller than the spine diameter at the first increment of postnatal *S. acanthias* (ANOVA; DF 2; P-value < 0.0001) (Table 1.2).

**Validation of Growth Increments**

Marginal increment analysis of vertebrae from all *S. acanthias* 1 year of age or older displayed a statistically significant unimodal distribution across all months (Kruskal-Wallis; DF = 10; p-value = 0.0001), suggesting that the band pairs were deposited annually with translucent bands beginning between April and July (Fig. 1.3).

**Bias and Precision**

No systematic bias was present using either the vertebrae or the dorsal fin spines to obtain age estimates (Fig. 1.4). The initial agreement on age estimates was 43.9% (90.1% within two years) using vertebrae, while the initial agreement between readers using dorsal fin spines was 30.4% (78.9% within two years). A significantly higher degree of inter- (Wilcoxon signed ranks test; n =196; p-value < 0.05) and intra-
(Wilcoxon signed ranks test; Reader 1: n = 378; p-value < 0.0001; Reader 2: n = 196; p-value < 0.001) reader precision was observed in age estimates using vertebrae than second dorsal fin spines, as indicated by lower CV and APE values (Table 1.3).

Wear of Dorsal Fin Spine

Of all the second dorsal fin spines taken for age estimates, 53% had wear to a diameter greater than the mean diameter measured for the first increment on unworn spines. The width at wear point increased with total length (ANOVA; n = 330; p-value < 0.0001), with the mean diameter of the wear point on spines = 3.13 mm. In 132 S. acanthias that were less than 60 cm LST, 6% had a wear point on the spine which would have eliminated increments, while in 249 sharks greater than 60 cm LST, that number increased to 76%. The equation to account for lost increments due to wear of the second dorsal fin spine (Ketchen 1975) was created by plotting the width of the base of 174 unworn spines against the estimated age obtained by the readers (Fig. 1.5).

Differences Between Ageing Structures

Mean vertebral derived age estimates of S. acanthias (mean = 9.2 years; range 0–24 years; STDEV 4.85) were significantly lower (ANOVA; n = 380; p-value < 0.0001) than the estimated ages derived from the second dorsal fin spines including those corrected for wear (mean = 11.2 years; range 0-28 years; STDEV 6.01). The spine age estimates were then further divided into those that were corrected for wear and those that were unworn. In individuals larger than 60 cm in which spines were corrected for wear, mean vertebral derived age estimates (13.01 years, S.D. 2.90) and mean age estimates
from dorsal spines (16.25 years, S.D. 3.46) were significantly different from each other (Wilcoxon/Kruskal-Wallis; n = 184; p < 0.001). Interestingly, mean age estimates using vertebrae (mean = 9.01 years, S.D. 2.72) and unworn spines (mean = 9.65 years, S.D. 3.11) in *S. acanthias* larger than 60 cm L<sub>ST</sub> were not significantly different (Wilcoxon/Kruskal-Wallis; n = 56; p > 0.05). This illustrates that Ketchen’s equation increases the age and thus decreases the mean size at age.

**Growth Function Analysis**

All growth curves had similar fit to length at age data generated from second dorsal spines and vertebrae of both sexes (Table 1.3) with no statistically significant difference between the fit of the models (ANOVA; N = 214; p > 0.05). Because of this, a closer examination of model parameters compared to observed data was warranted to determine which function described growth the best. The Gompertz model produced the lowest L<sub>∞</sub> values and conversely caused the highest k values for both sexes and ageing structures. This ultimately led to an overestimation of the size at birth and an underestimation of the maximum size for *S. acanthias* compared to observed values. While the fit using MSE was comparable to the VBGF’s, the Gompertz growth model did not provide realistic results for either L<sub>∞</sub> or L<sub>0</sub>, and so was excluded from further analysis.

The two and three parameter VBGF provided more realistic values of size at birth and maximum size for both ageing structures and sexes. The two parameter VBGF did not allow for comparisons between the observed size at birth and the predicted size at birth, based on L<sub>0</sub> being fixed, but the three parameter VBGF did. The three parameter
VBGF fit to vertebral derived age estimates produced values closer to the known size at birth than those obtained using dorsal fin spines. The $L_\infty$ values obtained from the two and three parameter VBGF using both ageing structures for females (range = 95.3 – 117.5 cm $L_{ST}$) and males (range = 91.5 – 105 cm $L_{ST}$) were in the appropriate range based on observed data, with the exception of the three parameter VBGF using male dorsal fin spines, which had a value that was much higher than expected. Thus, both the two and three parameter VBGF’s provided similar results, but since size at birth is known for *S. acanthias*, the two parameter VBGF was the most appropriate and provided the best biological fit for both sexes. The growth models suggest that males attain a smaller maximum size than females (Fig. 1.6). Observed size at age between sexes did not differ until age 10 (ANOVA; $N = 22; P < 0.0001$).

**Longevity Estimates**

The oldest male and female *S. acanthias* based on age estimates using vertebral centra were 17 and 24 years old, respectively. On the other hand, the oldest age estimates using dorsal fin spines for male and female *S. acanthias* were 22 and 28 years old, respectively. It should be noted that the oldest estimated age for females was a result of using Ketchen’s equation to account for an almost completely worn spine. Overall, longevity estimates were similar between sexes when using vertebral centra, but differed by 13 years, with the females obtaining an older maximum age than males when using second dorsal fin spines for age estimates. For both sexes, longevity estimates obtained using growth curves derived from vertebrae age estimates had lower values than those derived from dorsal fin spines (Table 1.4).
DISCUSSION

Previous ageing studies for *S. acanthuras* have utilized the second dorsal fin spine, including the most recent studies in the NW Atlantic (Nammack et al. 1985; Campana 2009). While this has been the traditional method, based on the predicted size at birth from the growth models, the overestimation of age while correcting for worn spines, and the better precision estimates, vertebral centra provide a more preferable means of estimating age in *S. acanthuras* than dorsal fin spines.

The first step in the use of vertebral centra as an ageing structure in *S. acanthuras* was to develop the technique to visualize the increments. It is also required that the structure be verified as an appropriate one to obtain consistent age estimates. This was accomplished based on the strong correlation between total length and vertebra diameter, the location of the birth band and first increment, and the relatively low APE and CV values showing precision of the method (Campana 2001; Jackson et al. 2007; Sharma and Borgstrom 2007). The last step in using a new ageing structure is validating the periodicity of the increment formation (Campana 2001). This was accomplished with the use of marginal increment analysis, a commonly used and accepted method to obtain validation of an ageing structure (Parsons 1993). The months during which increments formed were consistent with those reported by Nammack et al. (1985), in that the translucent band (equivalent to the faster growing region on the spines) was deposited in the spring and early summer. Ideally, each year class would be validated (Campana 2001), but the logistics of obtaining adequate animal numbers and representative sizes for each month were unrealistic.
An error component that could be eliminated by using vertebral centra compared to second dorsal fin spines relates to the anatomical location of the two ageing structures. Because the second dorsal fin spine is located externally, incidents of wear or breakage can occur. The likelihood of these incidents is positively correlated with size and thus age (Ketchen 1975) which was confirmed in the present study. Because worn or broken spines were discarded from the ageing process in initial studies of *S. acanthias*, (Kaganovskaia 1933), this led to an underestimation of age and an overestimation of growth rate due to the increased wear on spines from older fish. This discrepancy caused Ketchen (1975) to develop a power curve that compensated for the lost annuli, based on spine base width. When the power curve developed from the spine base width of unworn samples for the present study was examined, the curve fit the data differently depending on the size/age class. While the curve fit well in the region representing the smaller/younger individuals, which had a larger number of unworn spines, the larger/older individuals, who had fewer undamaged spines, were more variable and did not fit the curve as well in that region. When assessing dorsal fin spines with substantial wear from larger/older *S. acanthias*, essentially those for which the method was developed to account for lost growth increments, there was a greater difference between age estimates obtained using spines and vertebrae. Because age estimates obtained for large unworn spines were not significantly different from those obtained from the vertebrae from that same shark, while spines corrected for wear had significantly higher age estimates than those obtained from vertebrae, it suggests that there is an error in using Ketchen’s curve which overestimates the number of increments missing from worn spines (Ketchen 1975). While there is an error associated with the use of the equation
itself to account for lost increments due to wear, there is also an error associated with the interpretation and measurement of the wear point on the spine (McFarlane and King 2009). Based on their study, McFarlane and King (2009) stated these errors measuring wear point to adjust for missing annuli can cause differences anywhere from 1-19 years between readers examining the same spine. Thus, by utilizing an ageing structure such as the vertebral centrum, which is internal and not subject to environmental wear or breakage, this source of error can be eliminated which ultimately leads to increased precision and accuracy of age estimates.

Age determination is a key life history parameter as many other parameters are based on it (e.g. growth, maturity, fecundity, and mortality). By utilizing the vertebral centra with the technique described in the current study, less error will be introduced into these age dependent parameters, leading to less error propagation in fisheries models and more accurate predictions leading to sustainable management (Lai and Gunderson 1987; Rivard and Foy 1987). Previous studies have indicated that there is a large variability in age estimates among studies that utilized dorsal fin spines of *S. acanthias* as the ageing structure. For example, two studies in the Atlantic Ocean, published three years apart with both using second dorsal fin spines as the structure to estimate age, had maximum ages varying from 21 – 40 years old for females (Soldat 1982; Nammack et al.1985). The lower APE and CV obtained in the present study for vertebrae compared to dorsal spines illustrated that the growth increments were more clearly differentiated using the modified histological staining technique. Consequently, there was less ambiguity between readers which also led to better precision and accuracy of age estimates.
The two parameter VBGF using age estimates obtained from vertebral centra provided the most realistic fit to length and age data. Although the two and three parameter VBGF and Gompertz growth functions were examined in the present study, the two parameter VBGF’s using the fixed parameter L₀ was preferred because the size at birth of *S. acanthias* is known. Growth curves generated from length at age data using both ageing structures for males and females provided a good fit, based on the mean square of errors. Thus, age estimations from both ageing structures produced growth curves in which the Lᵣ parameter was realistic, but those using vertebrae were closer in approximating parameters for L₀, which was known based on the observed data obtained from the present study.

Values obtained in the present study for VBGF parameters had similarities and differences from other studies in the Western North Atlantic (Nammack et al. 1985; Campana 2009) (Table 5). Females from both the present study and those obtained by Nammack et al. (1985) have similar Lᵣ and k values, but there are large differences in these values compared those obtained by Campana et al. (2009). It must be noted though that values obtained using dorsal fin spines in the current study, as was used in previous studies, showed a slight decrease in k compared to Nammack et al. (1985). The decreased growth rate could be a result of the fishery selecting against the faster growing and thus larger fish as changes in growth rate of exploited fish populations have been observed frequently (Law 2000). The growth parameters for males on the other hand are closer between the three studies, having similar Lᵣ and k values. With the size selective nature and the magnitude of the biomass decline following fishing pressure, one would expect there to be a larger size and growth differences between females from the present study.
and that by Nammack et al. (1985) (Sminkey and Musick 1995; Stevens and West 1997; Walker et al. 1998). Sosebee (2005) provides a possible explanation, in that shortly preceding the study by Nammack et al. (1985), *S. acanthias* faced heavy commercial fishing pressure from foreign vessels, which may have already caused a decrease in size. Following the elimination of foreign fishing pressure, size and maturity estimates began to trend upward (Marques da Silva 1993) due to lowered landings of the species by U.S. commercial vessels (Rago et al. 1998). So, while Nammack et al. (1985) preceded the most recent increase in fishing pressure compared to the present study, both studies were conducted in a time of biomass increase from previous depletions.

The oldest observed ages *S. acanthias* from the three studies spans 12 years, but the range of the calculated longevities based on the VBGF parameters using the method devised by Taylor (1958) is considerably larger (65.4 years). The longevity calculation of 104 years obtained from using the VBGF parameters obtained by Campana et al. (2009) indicate that *S. acanthias* in the Canadian waters of the Western North Atlantic are slower growing and longer lived than those in the Pacific (Ketchen 1975). This seems unlikely though, considering Campana et al. (2006) validated age estimates using dorsal fin spines in the Western Atlantic with bomb radiocarbon dating and concluded that *S. acanthias* in the Pacific attain older ages than those in the Atlantic. There is not enough information to explain the cause of the large differences between the studies, as they could be due to anything from sample size or population differentiation (Campana 2009) to errors in the age estimates, but the results of the present study are relevant to management of *S. acanthias* in Atlantic waters off the United States.
In conclusion, the findings of the present study show that the vertebral centra is a more appropriate structure to estimate age in *S. acanthias* than the traditional structure, the second dorsal fin spine. The age estimates using the second dorsal fin spine introduced an increased error, which in turn would be amplified when employed in fisheries models. Conversely, the vertebra, processed with the modified histological staining technique, was validated as an appropriate ageing structure and provided better age estimates of *S. acanthias* compared to the second dorsal fin spine by having reduced precision errors and eliminating errors associated with the wear experienced by an external structure. Overall, the present study describes the use of vertebral centra to estimate age of *S. acanthias* and provided a comprehensive reassessment of *S. acanthias* age and growth parameters in the Western North Atlantic following increased fishing pressure using a structure which provided more precise and accurate age estimates that can be used for future management.
Figure 1.1. Vertebrae diameter (mm) as a function of total lengths (cm) in male (●) and female (○) *S. acanthias*. A logarithmic trend line with an $R^2 = 0.94$ was fit to the data with the equation: $y = 51.72 \ln(x) - 23.23$. 
Figure 1.2. A representative sectioned vertebra of a mature male *S. acanthias* prepared according to the modified staining method. Opaque increments of each band pair are marked (○), with the birth band indicated (○).
Figure 1.3. Mean standardized marginal increment (SMI) by month on measurements of vertebrae taken from *S. acanthias*. Error bars are two standard errors and months denoted with an asterisk (*) are significantly different (p < 0.05) from each other. Numbers above indicate sample size.
Figure 1.4. Bias plot between the primary (1) and secondary (2) readers of age estimates of *S. acanthias* made using second dorsal fin spines (A) and vertebrae (B). The primary and secondary readers read 394 and 207 second dorsal fin spines and 381 and 196 vertebrae, respectively. Error bars indicate two standard errors.
Figure 1.5. Best fit line of spine base width (mm) and estimated age (yrs) using S. acanthias unworn second dorsal fin spines (N = 174) to account for lost increments in worn spines (Ketchen 1975). The equation of the line fitted to the points was $A = 0.1808 * B_d^{2.5979}$, where $A$ is age and $B_d$ is diameter of the spine base.
Figure 1.6. *Squalus acanthias* two parameter von Bertalanffy growth functions of females (—) and males (-----) based on age estimates for females (○) and males (△) obtained from sectioned and stained vertebrae.
Table 1.1. *Squalus acanthias* vertebra staining procedure modified from Natanson et al. (2007)

<table>
<thead>
<tr>
<th>Step</th>
<th>Formula</th>
<th>Time (minutes)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% Distilled Water</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Harris Hematoxylin</td>
<td>10</td>
<td>Sections should be checked to ensure proper staining. Time can be adjusted depending on staining strength.</td>
</tr>
<tr>
<td>3</td>
<td>Water rinse</td>
<td>Until clear</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Acid Alcohol</td>
<td>2</td>
<td>Consists of 35% alcohol, 65% dH2O and 6 drops of HCL per 100 ml. Sections should be checked to ensure proper destaining. Time can be adjusted depending on staining strength.</td>
</tr>
<tr>
<td>5</td>
<td>Water rinse</td>
<td>1</td>
<td>Use agitation</td>
</tr>
<tr>
<td>6</td>
<td>running water</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Distilled water</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25% Glycerin</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>50% Glycerin</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>75% Glycerin</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>100% Glycerin</td>
<td>10</td>
<td>Tissues can be stored for longer periods at this step</td>
</tr>
</tbody>
</table>
Table 1.2. Precision estimates of within (intra) and between (inter) reader variation using Average Percent Error (APE) and Coefficient of Variation (CV). All statistical comparisons of variation are between the two ageing structures (vertebra and second dorsal fin spine). Statistical significance (p<0.05) using Kruskal Wallis signed rank test is denoted by *, **, ***; †, ††, or ‡.

<table>
<thead>
<tr>
<th>Structure</th>
<th>APE</th>
<th>SE</th>
<th>CV</th>
<th>SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-reader variation - reader 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vertebra</td>
<td>4.79*</td>
<td>0.44</td>
<td>6.77†</td>
<td>0.63</td>
<td>378</td>
</tr>
<tr>
<td>dorsal fin spine</td>
<td>7.40*</td>
<td>0.45</td>
<td>10.47†</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>vertebra</td>
<td>6.30**</td>
<td>0.56</td>
<td>8.90††</td>
<td>0.80</td>
<td>196</td>
</tr>
<tr>
<td>dorsal fin spine</td>
<td>8.75**</td>
<td>0.58</td>
<td>12.37††</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>Intra-reader variation - reader 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vertebra</td>
<td>6.23***</td>
<td>0.66</td>
<td>8.81‡</td>
<td>0.94</td>
<td>196</td>
</tr>
<tr>
<td>dorsal fin spine</td>
<td>8.82***</td>
<td>0.68</td>
<td>12.48‡</td>
<td>0.96</td>
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</tr>
</tbody>
</table>
Table 1.3. Growth parameters and goodness of fit (Mean Square of Errors (MSE)) by sex using vertebrae and second dorsal fin spines from *S. acanthias*. Two and three parameter von Bertalanffy (VB) and Gompertz growth functions were examined with the mean maximum total length ($L_\infty$), growth rate constant ($k$) and total length at birth ($L_0$). The two parameter models had a fixed $L_0$ (25 cm) at the age of birth determined in the present study.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Ageing Structure</th>
<th>Growth Function</th>
<th>$L_\infty$</th>
<th>$k$</th>
<th>$L_0$</th>
<th>MSE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Vertebra</td>
<td>VB 2 parameters</td>
<td>100.76</td>
<td>0.121</td>
<td>25</td>
<td>39</td>
<td>239</td>
</tr>
<tr>
<td>Female</td>
<td>Vertebra</td>
<td>VB 3 parameters</td>
<td>99.89</td>
<td>0.126</td>
<td>23.9</td>
<td>39</td>
<td>239</td>
</tr>
<tr>
<td>Female</td>
<td>Vertebra</td>
<td>Gompertz 2 parameters</td>
<td>95.30</td>
<td>0.202</td>
<td>25</td>
<td>37.9</td>
<td>239</td>
</tr>
<tr>
<td>Female</td>
<td>Vertebra</td>
<td>Gompertz 3 parameters</td>
<td>97.21</td>
<td>0.178</td>
<td>28.1</td>
<td>38.4</td>
<td>239</td>
</tr>
<tr>
<td>Female</td>
<td>Spine</td>
<td>VB 2 parameters</td>
<td>107.17</td>
<td>0.081</td>
<td>25</td>
<td>37.9</td>
<td>224</td>
</tr>
<tr>
<td>Female</td>
<td>Spine</td>
<td>VB 3 parameters</td>
<td>117.54</td>
<td>0.060</td>
<td>30.5</td>
<td>39.4</td>
<td>224</td>
</tr>
<tr>
<td>Female</td>
<td>Spine</td>
<td>Gompertz 2 parameters</td>
<td>96.28</td>
<td>0.153</td>
<td>25</td>
<td>39.8</td>
<td>224</td>
</tr>
<tr>
<td>Female</td>
<td>Spine</td>
<td>Gompertz 3 parameters</td>
<td>102.72</td>
<td>0.113</td>
<td>31.7</td>
<td>36.4</td>
<td>224</td>
</tr>
<tr>
<td>Male</td>
<td>Vertebra</td>
<td>VB 2 parameters</td>
<td>94.23</td>
<td>0.111</td>
<td>25</td>
<td>22.6</td>
<td>146</td>
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<tr>
<td>Male</td>
<td>Vertebra</td>
<td>VB 3 parameters</td>
<td>104.98</td>
<td>0.080</td>
<td>29.5</td>
<td>21.8</td>
<td>146</td>
</tr>
<tr>
<td>Male</td>
<td>Vertebra</td>
<td>Gompertz 2 parameters</td>
<td>84.68</td>
<td>0.202</td>
<td>25</td>
<td>21.5</td>
<td>146</td>
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<tr>
<td>Male</td>
<td>Vertebra</td>
<td>Gompertz 3 parameters</td>
<td>93.63</td>
<td>0.140</td>
<td>31.1</td>
<td>23.8</td>
<td>146</td>
</tr>
<tr>
<td>Male</td>
<td>Spine</td>
<td>VB 2 parameters</td>
<td>91.46</td>
<td>0.106</td>
<td>25</td>
<td>21.3</td>
<td>144</td>
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<tr>
<td>Male</td>
<td>Spine</td>
<td>VB 3 parameters</td>
<td>121.82</td>
<td>0.046</td>
<td>35.5</td>
<td>20.9</td>
<td>144</td>
</tr>
<tr>
<td>Male</td>
<td>Spine</td>
<td>Gompertz 2 parameters</td>
<td>83.67</td>
<td>0.184</td>
<td>25</td>
<td>21.6</td>
<td>144</td>
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<tr>
<td>Male</td>
<td>Spine</td>
<td>Gompertz 3 parameters</td>
<td>99.10</td>
<td>0.098</td>
<td>35.5</td>
<td>20.3</td>
<td>144</td>
</tr>
</tbody>
</table>

Table 1.4. Longevity estimates (in years) obtained from vertebrae and dorsal fin spines of female and male *S. acanthias*. The oldest observed values obtained by sex and structure are listed. Values for Taylor (99%) method of longevity estimates were calculated by using $L_\infty$ and $k$ values of a two parameter VBGF.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Ageing Structure</th>
<th>Oldest Observed</th>
<th>Taylor 99%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Vertebra</td>
<td>24</td>
<td>35.82504</td>
</tr>
<tr>
<td>Female</td>
<td>Spine</td>
<td>28</td>
<td>53.37201</td>
</tr>
<tr>
<td>Male</td>
<td>Vertebra</td>
<td>17</td>
<td>38.55104</td>
</tr>
<tr>
<td>Male</td>
<td>Spine</td>
<td>22</td>
<td>40.56551</td>
</tr>
</tbody>
</table>
Table 1.5. Comparison of published values of VBGF parameters and longevity with those obtained from the present study. Longevity was calculated from the VBGF parameters shown using the method devised from Taylor (1958) and modified by Skomal and Natanson (2003) to give age at 99% of $L_\infty$.

<table>
<thead>
<tr>
<th>Location</th>
<th>Study</th>
<th>Structure</th>
<th>Present</th>
<th>Nammack et al. 1985</th>
<th>Campana et al. 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE USA</td>
<td>Male</td>
<td>Vertebrae</td>
<td>94.23</td>
<td>82.49</td>
<td>88.12</td>
</tr>
<tr>
<td>NE USA</td>
<td>Male</td>
<td>Spines</td>
<td>91.46</td>
<td>0.148</td>
<td>0.099</td>
</tr>
<tr>
<td>NE USA</td>
<td>Female</td>
<td>Spines</td>
<td>100.76</td>
<td>100.5</td>
<td>133.35</td>
</tr>
<tr>
<td>Eastern Canada</td>
<td>Female</td>
<td>Spines</td>
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CHAPTER 2

MATURITY ANALYSES

Spiny dogfish (*Squalus acanthias*) is a shark species found in temperate coastal waters in the Atlantic and Pacific Oceans (Burgess 2002). Elasmobranchs, by and large, possess life history characteristics that make them vulnerable to heavy exploitation, such as long life, slow growth, late maturity, and low fecundity (Musick et al. 1999, Stevens et al. 2000). Previous studies have shown that *S. acanthias* in the Western North Atlantic also possess these characteristics (Jensen 1965; Nammack et al. 1985; Marques da Silva 1993). In the early 1990’s the cod fishery in the Western North Atlantic collapsed and fishermen were encouraged to target *S. acanthias* as an alternate species (NEFSC 1998). According to data compiled by the National Marine Fisheries Service, landings of *S. acanthias* increased from ~ 4,000 metric tons in 1989 to ~ 24,000 metric tons in 1996. Because females grow to a larger size than males, they were primarily targeted. Consequently, the increase in fishing pressure, in conjunction with the life history characteristics in *S. acanthias*, resulted in a decline in estimated spawning stock biomas of females from 433,000 metric tons to 26,700 metric tons (NEFSC 1998). This led the North Atlantic Fisheries Management Council in the USA to initiate a fisheries
management plan for *S. acanthias* in 2000, essentially ending a directed fishery (ASMFC 2002).

Central to any successful fisheries management plan is the availability of updated and accurate life history characteristics. Increased fishing pressure can alter life history characteristics, such as growth and size/age at maturity (Sminkey and Musick 1995; Buxton 1993; Rijnsdorp 1993; Rowell 1993; Sosebee 2005). Knowing the size and age that individuals reach maturity are important for management purposes, as these parameters are vital to ensure reproduction, which leads to recruitment and ultimately, sustainability or rebuilding of stocks. Typically, assessment of maturity for *S. acanthias* was determined solely by morphological parameters (Jensen 1965; Nammack et al. 1985), which do not always correlate with reproductive readiness (Maruska et al. 1996). Because of this, Sulikowski et al. (2005), in a study of the winter skate, *Leucoraja ocellata*, determined that using multiple indicators of maturity, such as plasma steroid hormone concentrations and histological analyses of reproductive tissues, in conjunction with morphological observations, provided a more accurate assessment of maturity.

A necessary component of maturity assessments is the age estimate. Even though second dorsal fin spines experience wear and breakage, leading to more variability in age estimates of *S. acanthias*, they were, nonetheless, used in previous studies (Jensen 1965; Soldat 1982; Nammack et al. 1985). Recently, a modification of a previously described histological staining technique for smooth skates (Natanson et al. 2007) determined that the vertebral centra provided a more accurate and precise means of assessing age for *S. acanthias* than second dorsal fin spines and to conclude that it is the preferred structure to estimate age (Chapter 1).
Therefore, based on the need to update life history information for *S. acanthias* of the Western North Atlantic, the present study sought to use a combination of morphological, histological and endocrinological endpoints to assess size and age at maturity. By providing an updated size and age at maturity for *S. acanthias*, a more effective management strategy for them can be implemented.

**MATERIALS AND METHODS**

**Sampling Protocol**

Monthly samples of both sexes were obtained from the Gulf of Maine between July 2006 and June 2007 from commercial fishing vessels using either bottom trawl or gillnet. Fish ranged from <35 cm to >100 cm total length (TL) and were binned into 5 cm increment size classes separated by sex. Spiny dogfish collection continued between July 2007 and June 2009 to supplement the months and size classes that were deficient in sample numbers. During this time period, additional samples were also obtained from the NOAA/NMFS Spring and Autumn Bottom Trawl Surveys as well as hook and line capture.

**Morphology**

For each *S. acanthias* collected in the field, the sex was noted, along with weight (to the nearest 0.05 kg) and stretch total length (*L*<sub>ST</sub>, measured from the tip of the rostrum to the termination of the dorsal lobe of the caudal fin at its maximum extension). The animal was euthanized by decapitation before vertebral centra were removed for age
estimates using our previously described protocol (Chapter 1). The presence of embryos, pups, or large ovarian follicles (>20mm diameter), was noted for all females. Clasper length (measured to the nearest millimeter along the inner margin of the clasper from the tip to the anterior portion of the vent) was taken for males; the presence or absence of calcification of the clasper was also noted. The gonads (ovaries or testes) were removed, patted dry, and weighed (to the nearest gram). Male and female gonadosomatic indices (GSI) were calculated as: \[ \text{gonad weight}/(\text{total body weight} - \text{gonad weight}) \times 100. \] The shell glands were removed from females, patted dry, and weighed separately (to the nearest 0.01 gram).

**Histology**

A single 2-3 mm thick segment was removed from the center of each testis and stored in 10% formalin until processing. The protocol from Sulikowski et al. (2005) for histological processing was followed. Sections (8 µm) were cut with a rotary microtome, mounted on slides, stained with hematoxylin and eosin, and sealed using a glass coverslip and cytoseal.

Each mounted section was examined with no prior knowledge of length, weight, or month of capture of the individual from which the testis was removed. The slides were viewed with a dissecting microscope using transmitted light under 40X magnification and images were digitally photographed. The mean proportion of stage VI spermatocysts (Maruska et al. 1996) was measured along a straight line distance over a representative portion of the cross section, beginning with the germinal zone (Conrath and Musick 2002).
Using a heparinized syringe and 21 gauge needle, 5 ml of blood was removed from each specimen via cardiac puncture for determination of steroid hormone concentrations. The blood was transferred to a 15 ml centrifuge tube and centrifuged at 1300 g for 5 minutes to separate the plasma from the red blood cells. The plasma was then transferred into 1.5 mL centrifuge tubes and frozen at -20°C.

The steroid hormones were extracted from mature individuals following protocol from Tsang and Callard (1987a) and Sulikowski et al. (2005). Each sample was extracted twice with 10 volumes of diethyl ether (anesthesia grade) for estradiol-17β (E₂) and testosterone (T), or petroleum ether for progesterone (P₄). The ether was then evaporated under a stream of nitrogen before the dried extracts were reconstituted in phosphate buffered saline with 0.1% gelatin (PBSG). Furthermore, approximately 1000 counts min⁻¹ (cpm) of an appropriate tritiated steroid were added into each plasma sample prior to addition of solvents to account for procedural losses during the extraction. The overall mean recoveries (i.e. how much tritiated steroid was not lost during extraction) were 81.6% for T, 69.0% for P₄, and 66.8% for E₂. The individual recoveries were used to correct the steroid concentrations for each sample. Also, the R² for standard curves/samples were calculated for T, P₄, and E₂ to assess linearity.

Plasma concentrations of E₂, P₄, and T were determined by radioimmunoassay (RIA) modified from procedures of Tsang and Callard (1987a). The tritiated T, E₂ (Amersham Biosciences, Picataway, NJ) but not P₄ (Perkin Elmer Life Sciences, Boston, MA) were purified using thin layer chromatography and a solvent system consisting of 3 parts ether:1 part hexane for E₂ (2,4,6,7,16,17⁻³H) and a solvent system consisting of 4
parts benzene:1 part acetone for T (1,2,6,7-^3^H) and P_4 (1,2,6,7-^3^H) (Sulikowski et al. 2004). Purified steroids were then extracted from the silica scraped off the plate using methanol, dried, and reconstituted in absolute ethanol to concentrations of 5 µCi ml^{-1} for T, 8 µCi ml^{-1} for E_2 and 50 µCi ml^{-1} for P_4.

Plasma concentrations of E_2, P_4, and T were determined for females while only concentrations of T were determined for males. Duplicate aliquots of the reconstituted plasma extracts received the appropriate tritiated steroid and anti-steroid in a total volume of 0.4 ml. The final anti-sera concentrations were 1:64,000 for T (#250, anti-testosterone-11-BSA; Gay and Kerlan 1978), 1:200,000 for E_2 (#244, anti-estradiol-6-BSA; Korenman et al. 1974) and 1:10,000 for P_4 (# 337, antiprogesterone-11-BSA; Gibori et al. 1997). After an overnight incubation at 4°C, free steroids were separated from bound steroids using a suspension of Norit A (0.2%) and Dextran T-70 (0.02%), followed by centrifugation at 1500 · g for 10 min at 4°C. The supernatants were then decanted into scintillation vials (USA Scientific, Ocala, FL) followed by addition of Ready Safe™ cocktail (Beckman Coulter, Somerset, N.J.). Radioactivity was determined in a Beckman LS6000IC (Fullerton, California) liquid scintillation counter. The non-labeled steroids for the standard curve were obtained from Steraloids, Inc. (Wilton, NH). The intra-assay coefficients of variance were 6.1% for T, 5.4% for P_4 and 7.2% for E_2. The interassay coefficients of variance were 8.5% for T, 10.4% for P_4 and 8.0% for E_2.

**Maturity Criteria: Morphology, Physiology, Histology**

Females were considered mature if embryos/pups or large follicles were present. However, in the absence of embryos/pups or large follicles, females were categorized
accordingly if they met morphology and physiology criteria based on values obtained from examination of mature (containing pups/embryos) and immature *S. acanthias* (Table 2.1). For example, females that did not have pups/embryos or large follicles were considered mature if they had GSI values > 0.8, combined shell gland mass > 1.0 g and plasma P₄ concentrations > 0.2 ng/ml.

Males were characterized as mature if the proportion of stage VI spermatocysts accounted for >20% of their testis cross section (Fig. 2.1). Otherwise, they were categorized as mature if they met morphology and physiology criteria based on values obtained from examination of mature (testes containing >20% stage VI spermatocysts) and immature *S. acanthias* (Table 2.2). For example, if no histological samples were taken or if the proportion of stage VI spermatocysts was <20%, they were considered mature if they had calcified claspers, a clasper length > 50 mm, GSI values > 0.5 and plasma T concentrations > 18 ng/ml. Overall, any *S. acanthias* which did not meet all maturity criteria for their sex was considered immature.

**Parameter Characterization**

The relationship between size and morphological and physiological parameters was examined by assigning female and male *S. acanthias* into 5 cm size classes. The following relationships were examined: GSI by size class for both sexes, combined shell gland mass and P₄, T, and E₂ concentrations for females, and clasper length and T concentrations for males.

The relationship between age and morphological and physiological parameters was also examined by putting female and male *S. acanthias* into age classes. The
following relationships were examined: GSI by age class for both sexes, combined shell gland and P_4 T, and E_2 concentrations for females, and clasper length and T concentrations for males.

**Maturity Analysis**

By assessing the maturity status of each *S. acanthias* (using the above criteria) and relating it to their respective L_{ST} the minimum size at maturity and minimum size at which all *S. acanthias* are mature were determined. Subsequently, a maturity ogive for size was generated for each sex, using a probit analysis to fit a logistic curve to proportion of mature individuals by size class. The size at 50% maturity (L_{50}) was calculated from this curve.

In addition, by assessing the maturity status of each *S. acanthias* (using the above criteria) and relating it to their age estimates derived from vertebral centra, the minimum age at maturity and minimum age at which all *S. acanthias* were mature could be determined. Subsequently, a maturity ogive for age (derived from vertebral centra) was generated for each sex using a probit analysis to fit a logistic curve to proportion of mature individuals by age class. The age at 50% maturity (A_{50}) was calculated from this curve. In order to compare the present findings to previous studies, maturity ogives were also generated using age estimates obtained from the second dorsal fin spine, the traditional structure used for age estimates in *S. acanthias*.

**Statistics**
Statistical comparisons were performed on the morphological and physiological parameters measured for the study. Normally distributed data were analyzed using a one-way ANOVA followed by a Tukey’s HSD to determine individual differences, while nonparametric data were analyzed using a Wilcoxon/Kruskal-Wallis signed rank test.

RESULTS

Between July 2006 and June 2009, a total of 395 (147 males and 248 females) *S. acanthias* was collected for this study, with dogfish being caught 11 of the 12 months (no *S. acanthias* were caught in January). The length of males ranged from 32.5 to 84 cm TL with a body mass of 0.045 to 2.45 kg, while females ranged from 25 to 102 cm TL with a body mass of 0.3 to 4.5 kg.

Morphological and Physiological Reproductive Parameters

*Males*

Overall, the GSI (ANOVA; DF = 17; p < 0.0001), clasper length (ANOVA; DF = 17; p < 0.0001), and plasma testosterone concentrations (ANOVA; DF = 16; p = 0.03) significantly increased as *S. acanthias* became older (Fig. 2.2 A, C, E). An increase in GSI (ANOVA; DF = 10; p < 0.0001), clasper length (ANOVA; DF = 10; p < 0.0001), and plasma testosterone concentrations (ANOVA; DF = 8; p = 0.0002) was also associated with an increase in size (Fig. 2.2 B, D, F). While these parameters varied in the degree of increase, all of them had their peak changes occur between six and nine
years old and between 55-59 and 65-69 cm TL, with significant differences occurring between age classes for GSI and clasper length and between size classes for GSI, plasma testosterone concentration and clasper length within this time frame, respectively.

**Females**

Overall, the GSI (ANOVA; DF = 21; p < 0.0001) and combined shell gland mass (ANOVA; DF = 20; p < 0.0001) significantly increased as *S. acanthias* became older (Fig. 2.3 A, C). Plasma concentrations of E2 (Wilcoxon signed rank test; DF = 18; p = 0.0005) and P4 (Wilcoxon signed rank test; DF = 18; p = 0.0003) also increased as age increased (Fig. 2.4 A, C). Further, an increase in GSI (ANOVA; DF = 14; p < 0.0001) and combined shell gland mass (ANOVA; DF = 14; p < 0.0001) was associated with an increase in size (Fig. 2.3 B, D). Similarly, plasma concentrations of E2 (Wilcoxon signed rank test; DF = 12; p = 0.0001) and P4 (Wilcoxon signed rank test; DF = 9; p = 0.0001) also increased as size increased (Fig. 2.4 B,D), but T concentrations did not (Wilcoxon signed rank test; DF =11; p = 0.33 ). While these parameters varied in the degree of increase, all of them had their peak changes occur between eight and thirteen years old, with significant differences occurring between consecutive age classes for GSI within this time frame. This also applied to size, as GSI increased between consecutive size classes, i.e. 75-79 cm and 80-84 cm.

**Maturity Status**

**Males**
Of the 147 male *S. acanthias* collected over the course of the present study, there were 70 mature and 77 immature individuals, based on the criteria described above. Using vertebral centra, age at first maturity for male *S. acanthias* occurred at 6.5 years of age, while the oldest immature dogfish was 10 years of age. Further, probit analysis yielded an A50 of 7.5 years (Fig. 2.5A). On the other hand, size at first maturity for male *S. acanthias* occurred at 61.5 cm LST, while the largest immature shark was 64.5 cm LST. The probit analysis yielded a L50 of 63.1 cm LST (Fig. 2.5B).

In addition, clasper length (Wilcoxon signed rank test; N = 145; p < 0.0001), GSI (Wilcoxon signed rank test; N = 144; p < 0.0001), and plasma T concentrations (ANOVA; N = 47; p < 0.0001) were significantly different between mature and immature individuals (Table 2.3).

**Females**

Of the 249 female *S. acanthias* caught over the course of the present study, 141 were mature and 108 were immature, based on the criteria described above. Of the 141 females classified as mature, 137 had embryos/pups or large follicles. Age at first maturity for female *S. acanthias* occurred at 6 years of age, while the oldest immature shark was 15 years of age. Further, probit analysis yielded an A50 of 9.1 years (Fig. 2.6 A). On the other hand, size at first maturity for female *S. acanthias* occurred at 74.5 cm LST, while the largest immature shark was 82.5 cm LST. Probit analysis yielded a L50 of 76.9 cm LST (Fig. 2.6 B).

In addition, the combined shell gland mass (Wilcoxon signed ranks test; N = 199; p < 0.0001), GSI (Wilcoxon signed ranks test; N = 243; p < 0.0001), and E2 (Wilcoxon
signed ranks test; \( N = 158; p < 0.0001 \) and \( P_4 \) (Wilcoxon signed ranks test; \( N = 119; p < 0.0001 \)) plasma concentrations were significantly different between mature and immature individuals (Table 2.4), while \( T \) concentrations were not (Wilcoxon signed ranks test; \( N = 114; p = 0.15 \)).

Comparison between present and previous studies

Relative to reports by Nammack et al. (1985) and Marques da Silva (1993), the findings of the present study revealed that \( L_{50} \) decreased for females and increased slightly for males. Further, for the ease of comparison with Nammack et al. (1985), data obtained using second dorsal fin spines as the ageing structure in the present study also yielded a decreased \( A_{50} \) for female \( S. acanthias \) and an increase in males (Table 2.5).

DISCUSSION

The present study, to my knowledge, is the first comprehensive report of morphological, histological, and physiological reproductive parameters of \( S. acanthias \) in the Western North Atlantic following increased fishing pressure in the 1990’s. The entire size range of \( S. acanthias \), which covers the entire age range as well, was examined to describe these reproductive parameters throughout the full life cycle. Because \( S. acanthias \) were caught year round as a part of a larger study, the possible effects of season on reproductive parameters in mature animals could confound some of the analyses (Sulikowski et al. 2005). However, this was not the case, since there were
significant differences between mature and immature individuals regardless of the reproductive stage in the adults.

Maturity Criteria

Having individual dogfish meet multiple criteria confers greater accuracy in assessing maturity status. Previously, maturity of *S. acanthias* was solely based on morphological parameters, such as clasper length and calcification for males, shell gland weight for females, and GSI for both sexes (Nammack et al. 1985). However, some parameters, such as GSI are based on the assumption that they are indicators of reproductive activity, but previous studies have found that this assumption can be violated (Teshima 1981; Parsons and Grier 1992). Therefore, this led Sulikowski et al. (2005) to use multiple parameters to assess maturity in winter skates.

Since female *S. acanthias* are an aplacental viviparous species with little time between gestations (Hisaw and Albert 1947) many of them already possess unequivocal indicators of maturity, such as the presence of pups/embryos or large follicles, as opposed to oviparous species (Sulikowski et al. 2005). Because of this, the use of multiple parameters to determine maturity in females is essentially relegated for use with immature *S. acanthias*, those becoming reproductively active for the first time, those which may have aborted due to the stress of capture, or the few that for whatever reason did not become pregnant during their last mating cycle. On the other hand, male *S. acanthias* have less distinct indicators of maturity. For this reason, a multi-faceted approach, which includes a suite of reproductive parameters whose thresholds must be met before an individual is classified as mature, provides the most robust method of
determining maturity (Maruska et al. 1996; Sulikowski et al. 2005). For each parameter, the threshold was set based on data from all mature individuals collected for the present study. While some immature individuals were above threshold levels for maturity in one or two parameters, an individual had to have been above the threshold for all parameters to be considered mature. This prevents individuals from being erroneously classified as mature. The parameters chosen to assess maturity in male and female S. acanthias have been shown to correlate with maturity in S. acanthias and other elasmobranch species (Conrath 2005). The findings of the present study showed that the chosen parameters were significantly different between mature and immature S. acanthias, thus validating their use as indicators of maturity. All in all, by relying on multiple parameters to determine maturity, it provides more confidence in assessing maturity status based on an individual’s reproductive readiness.

Size and Age at Maturity

Females

For S. acanthias in the Western North Atlantic, females attained maturity at a larger size and older age than males in the present study, consistent with earlier reports (Nammack et al. 1985, Jensen 1965, Marques da Silva 1993). However, size at maturity, compared to studies done prior to the increased fishing pressure (e.g. Nammack et al. 1985; Marques da Silva 1993), had declined for female S. acanthias. Sosebee (2005) observed a decrease in size at first maturity and L₅₀ since the onset of the fishing pressure in the early 1990’s, and the present study observed a continuation of this downward
trend, with females reaching an even smaller \( L_{50} \). Further, studies examining historical population dynamics have also identified decreased size and age at maturity as a result of overfishing (Rijnsdorp 1993; Sharpe and Hendry 2009). The relatively large decrease in their size at maturity has almost certainly been caused by the fishery targeting large female \textit{S. acanthias}.

Coinciding with the decline in \( L_{50} \), was an observed decrease in \( A_{50} \) for female \textit{S. acanthias}. The decrease in \( A_{50} \) could be attributed to the ageing technique and the structure used. For vertebral centra, the modified histological technique yielded an \( A_{50} \) of 9.1 years, while Nammack et al. (1985) and Marques da Silva (1993) reported an \( A_{50} \) of 12.1 years using second dorsal fin spines, representing an approximately 25\% decline. However, because of the different ageing structures, I also determined age using second dorsal fin spines and found an \( A_{50} \) of 10.5 years, representing an approximately 13\% decline from the earlier study. While this represents a relatively smaller decline than with the vertebral centra, there is nonetheless a downward trend in \( A_{50} \). Overall, because size and age are correlated, female \textit{S. acanthias} maturing at smaller sizes would be expected to mature at younger ages as well.

\textit{Males}

In the present study, male \textit{S. acanthias} in the Western North Atlantic reached maturity at a smaller size and younger age than females. The \( L_{50} \) found in male \textit{S. acanthias} during the present study increased slightly compared to the most recent study (\( L_{50} = 59.5 \) cm) that examined males prior to the increased fishing pressure in the Western North Atlantic (Nammack et al. 1985). Further, I found that the \( A_{50} \) using
vertebral centra and dorsal fin spines were 7.5 and 8.2 years, respectively. Interestingly, these values are greater than the $A_{50}$ (i.e. 6 years) reported by Nammack et al. (1985), and they are opposite to our findings in females, in which there was a decrease in $A_{50}$. While the differences between pre- and post-fishing pressure size and age at maturity for males is less than that for females, this could have biological relevance.

Effects of Size Selective Harvest

Biomass

A decrease in size and age at maturity in response to fishing pressure would increase biomass by allowing for a greater number of reproductive cycles over the lifespan of female $S. acanthias$. However, it is more likely that the overall productivity of the population will be decreased instead (Conover and Munch 2002). Size selective fishing pressure causes truncated size and age distributions of marine fishes (Ricker 1981; Zwanenberg 2001; Stergiou 2002). Because fecundity is correlated with size in many fish species, including $S. acanthias$ (Nammack et al. 1985; Sosebee 2005), a decrease in size and size at maturity leads to a decrease in the number of offspring per reproductive cycle. Thus, high adult mortality, as a result of the size selective increased fishing pressure on large female $S. acanthias$, leads to a greater reproductive investment of smaller and younger adults, which inherently have lower reproductive value (Festa-Bianchet 2003). This, in turn, causes a decline in population growth rate (Fenberg and Roy 2008), resulting in a decline in sustainable yield (Law and Grey 1989; Heino 1998).
Phenotypes

The changes in size and age at maturity observed in *S. acanthias* during the present study have been described in other fish species and may be attributed to size selective harvest strategies (Stokes and Law 2000). These changes could be an effect of rapid phenotypic (Reznick et al. 1990) or genetic (Law 2000) changes. The mechanism of first maturity in fish has not been definitively elucidated, but has been attributed to a variety of cues, including ratios between oxygen uptake and body size, photoperiod, and energy regulated switches (Campbell 2003; Pauly 1984; Rijnsdorp 1990; Taranger et al. 1999; Thorpe 1986; Thorpe 2007; Wright 2007). Of these factors, energy related switches could describe the sex selective changes of maturity observed in *S. acanthias* based on changes in density. Because *S. acanthias* travel in schools by sex once maturity is reached (Bigelow and Schroeder 1953), density dependent factors could play a role in energy related mechanisms and thus differential maturity changes between sexes following size selective fishing pressure (Conover and Munch 2002). Decreased numbers of female *S. acanthias* would cause less density and thus less competition for resources allowing increased energy and lipid reserves which could lead to earlier maturation. The inverse would be true for the males which were not targeted as heavily and so density of male schools may have been relatively unchanged or even increased, which could lead to the size and age at maturity remaining constant or even occurring later.

If phenotypic plasticity in these mechanisms is responsible for changes in size and age at maturity, there would be a shorter period of time to revert back to pre-fishing pressure values if fishing pressure is reduced. Genetic changes could, however, have longer lasting effects because harvest mortality imposes a much greater selective
pressure, causing rapid evolution, than the original environmental pressures which are far less severe (Darimont et al. 2009).

The idea of multiple stocks in the Western North Atlantic may have also played a role in this observation of increased size and age at maturity. Campana et al. (2009) described reproductive parameters of male *S. acanthias* caught in Canadian waters in the Western North Atlantic with the *L*_50 being comparable (63.6 cm *L*<sub>ST</sub>) and the *A*_50 being slightly higher (10 years) than those characterized in the present study. Because the majority of *S. acanthias* in the present study were caught in the Gulf of Maine, a possible mixing ground between putative Canadian and U.S. stocks (Campana et al. 2009), a change between the present study and that of Nammack et al. (1985) could be a function of sampling different stocks.

**Effects on Management**

The changes in size and age at maturity, regardless of the cause (i.e. genetic or phenotypic plasticity), are present in the *S. acanthias* population, and if they are not accounted for, will lead to flawed stock assessments (Die and Caddy 1997; Pauly 1998). Obviously, updated assessments with these new parameters should be utilized. Overall, the results of the present study contribute to the broader goal of obtaining current and accurate life history information for use in stock assessments, and thus to the successful management of the *S. acanthias* population in the Western North Atlantic.
Figure 2.1. A histological section, stained with hematoxylin and eosin, of a mature male *S. acanthias* testis at 40 X magnification. The proportion of stage VI spermatocysts was measured along a straight line representative of the entire cross section, beginning with the germinal zone.
Figure 2.2. Morphological and physiological reproductive parameters in male *S. acanthias*. Clasper length (mm ± 2 x SEM); plasma testosterone concentrations (ng/ml± 2 x SEM); and gonadosomatic index (GSI± 2 x SEM) are expressed as a function of age (years; A, C, E) and size (total length, cm; B, D, F). Significant differences (p < 0.05) between consecutive age and size classes are denoted by an asterisk (*).
Figure 2.3. Morphological reproductive parameters in female *S. acanthias*. Combined shell gland mass (g± 2 x SEM) and gonadosomatic index (GSI± 2 x SEM) are expressed as a function of age (years; A, C) and size (Total length, cm; B, D). Significant differences (p<0.05) between consecutive age and size classes are denoted by an asterisk (*).
Figure 2.4. Steroid hormone concentrations in female *S. acanthias*. Testosterone (ng/ml± 2 x SEM), estradiol (ng/ml± 2 x SEM) and progesterone (ng/ml± 2 x SEM) are expressed as a function of age (years; A, C, E) and size (total length, cm; B, D, F).
Figure 2.5. Maturity ogives for male *S. acanthias* fit to logistic curves using a probit analysis for age and size. Observed proportion mature by age (years; A) and size (total length, mm; B) classes are represented by (●). Age and size at 50% maturity are denoted by (black line), while size at 50% maturity from a previous study by Marques da Silva (1993) is denoted by (dotted line).
Figure 2.6. Maturity ogives for female *S. acanthias* fit to logistic curves using a probit analysis for age and size. Observed proportion mature by age (years; A) and size (total length, mm; B) classes are represented by (•). Age and size at 50% maturity are denoted by (black line), while size at 50% maturity from a previous study by Marques da Silva (1993) is denoted by (dotted line).
Table 2.1. The ranges of the three reproductive parameters used to define maturity of female *S. acanthias*. Mature individuals are those with the presence of embryos/pups or large follicles while immature individuals have completely undeveloped reproductive tracts. The maturity threshold is the cut-off to classify individuals as mature. Each female *S. acanthias* must be above all three thresholds to be considered mature. (Gonadosomatic index = GSI; progesterone = \(P_4\))

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<td>24</td>
<td>0.02-0.54</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>(P_4) concentration (ng/ml)</td>
<td>97</td>
<td>0.21-49.8</td>
<td>27</td>
<td>0-1.076</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. The ranges of the three reproductive parameters used to define maturity of male *S. acanthias*. Mature individuals are those with histological cross sections of testes with greater than 20% stage VI spermatocysts, while immature individuals have completely undeveloped reproductive tracts. The maturity threshold is the cut-off to classify individuals as mature. Each male *S. acanthias* must be above all three thresholds to be considered mature. (Gonadosomatic index = GSI; testosterone = T)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mature</th>
<th></th>
<th>Immature</th>
<th></th>
<th></th>
<th>Maturity Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Range</td>
<td>N</td>
<td>Range</td>
<td>Threshold</td>
<td></td>
</tr>
<tr>
<td>GSI</td>
<td>65</td>
<td>0.74-3.65</td>
<td>74</td>
<td>0-0.45</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Clasper Length (cm)</td>
<td>67</td>
<td>53-85</td>
<td>76</td>
<td>13-55</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>(T) concentration (ng/ml)</td>
<td>38</td>
<td>19.55-143.87</td>
<td>8</td>
<td>0-17.75</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Comparisons of three reproductive parameters between immature and mature male *S. acanthias*. (Testosterone = T; gonadosomatic index = GSI)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Maturity</th>
<th>N</th>
<th>Mean</th>
<th>STD ERR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T) concentration (ng/ml)</td>
<td>Immature</td>
<td>8</td>
<td>3.85</td>
<td>2.09</td>
</tr>
<tr>
<td>(T) concentration (ng/ml)</td>
<td>Mature</td>
<td>39</td>
<td>67.64</td>
<td>5.05</td>
</tr>
<tr>
<td>GSI</td>
<td>Immature</td>
<td>76</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>GSI</td>
<td>Mature</td>
<td>67</td>
<td>1.52</td>
<td>0.07</td>
</tr>
<tr>
<td>Clasper Length</td>
<td>Immature</td>
<td>76</td>
<td>30.47</td>
<td>0.98</td>
</tr>
<tr>
<td>Clasper Length</td>
<td>Mature</td>
<td>68</td>
<td>73.12</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Table 2.4. Comparisons of four reproductive parameters between immature and mature female *S. acanthias*. (Gonadosomatic index = GSI; estradiol = E₂; progesterone = P₄)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Maturity</th>
<th>N</th>
<th>Mean</th>
<th>STD</th>
<th>ERR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shell gland combined mass (g)</td>
<td>Immature</td>
<td>87</td>
<td>0.06</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Shell gland combined mass (g)</td>
<td>Mature</td>
<td>112</td>
<td>3.17</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>GSI</td>
<td>Immature</td>
<td>105</td>
<td>0.31</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>GSI</td>
<td>Mature</td>
<td>136</td>
<td>4.41</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>E₂ concentration (ng/ml)</td>
<td>Immature</td>
<td>40</td>
<td>0.08</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>E₂ concentration (ng/ml)</td>
<td>Mature</td>
<td>103</td>
<td>0.59</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>P₄ concentration (ng/ml)</td>
<td>Immature</td>
<td>27</td>
<td>0.23</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>P₄ concentration (ng/ml)</td>
<td>Mature</td>
<td>95</td>
<td>4.86</td>
<td>0.83</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5. Comparisons of age at 50% maturity (years) between the present and Nammack et al. (1985) studies. Age estimates obtained using second dorsal fin spines (Chapter 1) produced less accurate and precise results. They are only used here as a means of conveying the change in age at 50% maturity prior to and following fishing pressure without introducing the variable of ageing structure.

<table>
<thead>
<tr>
<th>Study</th>
<th>Structure</th>
<th>Sex</th>
<th>Age at 50% maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>Vertebra</td>
<td>Female</td>
<td>9.1</td>
</tr>
<tr>
<td>Present</td>
<td>Dorsal Fin Spine</td>
<td>Female</td>
<td>10.5</td>
</tr>
<tr>
<td>Nammack et al. 1985</td>
<td>Dorsal Fin Spine</td>
<td>Female</td>
<td>12.1</td>
</tr>
<tr>
<td>Present</td>
<td>Vertebra</td>
<td>Male</td>
<td>7.5</td>
</tr>
<tr>
<td>Present</td>
<td>Dorsal Fin Spine</td>
<td>Male</td>
<td>8.2</td>
</tr>
<tr>
<td>Nammack et al. 1986</td>
<td>Dorsal Fin Spine</td>
<td>Male</td>
<td>6</td>
</tr>
</tbody>
</table>
CHAPTER 3

REPRODUCTIVE ANALYSIS OF MATURE SQUALUS ACANTHIAS

The spiny dogfish, *Squalus acanthias*, is a common shark species found in temperate coastal waters of the Atlantic and Pacific Oceans (Burgess 2002). As with many elasmobranch species, due to their relatively long-life span, late age at maturity, and low fecundity, they are vulnerable to overfishing (Musick 1999, Stevens et al. 2000). In the Western North Atlantic, large female *S. acanthias* were recently harvested in a directed fishery, which was followed by a decrease in their estimated spawning stock biomass, from 433,000 metric tons to 26,700 metric tons (NEFSC 1998). Because of this decline in spawning stock biomass, *S. acanthias* was listed as overfished and a Fishery Management Plan was subsequently implemented in the Western North Atlantic (ASMFC 2002).

Large scale changes in biomass, such as those experienced by *S. acanthias* in the Western North Atlantic, can have significant effects on life history parameters of a population (Sminkey and Musick 1995; Buxton 1993; Rijnsdorp 1993; Rowell 1993; Sosebee 2005). The availability of current and comprehensive data for use in population assessments is essential to any successful management strategy. Age and size at maturity, reproductive cycles, and fecundity are among the important components of life history that assist fishery managers in making effective decisions. Age and size at maturity of *S.*
acanthias have already undergone changes following increased fishing pressure (Sosebee 2005; Chapter 2). Further, in size selective harvest strategies, such as those used with S. acanthias, changes in reproductive life history parameters lead to an overall decline in population growth rate (Fenberg and Roy 2008). Unfortunately, there have been no comprehensive studies examining reproductive life history of S. acanthias in the Western North Atlantic following the increased fishing pressure. Since changes in population dynamics affect the success of methods used to sustainably manage or rebuild overfished stocks, it is therefore imperative to update reproductive life history parameters in S. acanthias.

Previous studies in the Western North Atlantic utilized morphology or physiology to assess reproductive cycles in female S. acanthias (Jensen 1965; Nammack et al. 1985; Tsang and Callard 1987a), while only morphology was used to characterize the males (Jensen 1965; Nammack et al. 1985). When morphological, physiological, and histological parameters are combined, they provide a more accurate means of assessing reproductive state than using individual parameters (Maruska et al. 1996; Sulikowski et al. 2005). Therefore, the objectives of the present study are: 1. To determine reproductive cycles for male and female S. acanthias, using a combination of morphological, physiological, and histological parameters; 2. To determine fecundity of females; and 3. To compare findings of the current study to those from studies conducted prior to the increased fishing pressure.
MATERIALS AND METHODS

Sampling Protocol

Monthly samples of both sexes, ranging from <35 cm to >100 cm fork length and subdivided into 5 cm increment size classes, were obtained between July 2006 and June 2007 from commercial fishing vessels using either bottom trawl or gillnet. Spiny dogfish collection continued between July 2007 and June 2009 in order to complete the months and size classes that were deficient in sample numbers. At the same time, additional samples were also obtained from the NOAA/NMFS Spring and Autumn Bottom Trawl Surveys as well as from hook and line capture.

Morphology

For each spiny dogfish collected in the field, the sex, body weight (to the nearest 0.05 kg) and stretch total length (L ST, measured from the tip of the rostrum to the termination of the dorsal lobe of the caudal fin at its maximum extension) were noted before the animal was euthanized. Vertebral centra were removed for age determinations following the protocol outlined in Chapter 1. Clasper length (measured along the inner margin of the clasper from the tip to the anterior portion of the vent) was taken for males (to the nearest mm) and the presence or absence of calcification was noted. The gonad (ovary or testis) was removed, patted dry, and weighed (to the nearest 0.1 g). Male and female gonadosomatic index (GSI; defined as the gonad weight/[(total body weight – gonad weight)] x 100) was calculated. In female S. acanthias, the sex of all embryos was noted, after which they were counted, weighed (to the nearest g), and measured (fork and
stretch total lengths were taken to the nearest mm). Ovarian follicles were counted and those larger than 15 mm in diameter were measured (to the nearest mm). If there was evidence of aborted embryos, those individuals were removed from further reproductive analyses. The right and left shell glands were also removed from females, patted dry, weighed separately (to the nearest 0.01 g) and stored in 10% formalin for histological processing (to determine if *S. acanthias* females can store sperm for an extended period of time).

**Histology**

Shell glands were removed whole, while a single 2-3 mm thick segment was removed from the center of each testis before storage in 10% formalin until processing. The histological processing followed the protocol of Sulikowski et al. (2005). Sections (8 µm) were taken along a sagittal plane for shell glands and a transverse plane for testes with a rotary microtome, mounted on slides, stained with hematoxylin and eosin, and sealed using a glass coverslip and cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

Each section was examined with no prior knowledge of length, weight, or month of capture of the individual from which the shell gland or testes was removed. The slides were viewed with a dissecting microscope using transmitted light under 40X magnification and digitally photographed using SPOT basic imaging software (SPOT Imaging Solutions, Sterling Heights, MI). Serial sections of shell glands were examined for the presence of sperm, with special attention being paid to the terminal zone, as this is the region typically used for sperm storage in elasmobranchs (Pratt 1993). The proportion of stage VI spermatocysts (Maruska et al. 1996) was determined along a straight line
distance over a representative portion of the cross section, beginning with the germinal zone (Conrath and Musick 2002). After examining all the testes, the proportion of stage VI spermatocysts was determined a second time on the same cross sections for precision.

**Physiology**

Using a heparinized syringe and 21 gauge needle, 5 ml of blood was removed from each specimen via cardiac puncture for determination of steroid hormone concentrations. The blood was transferred to a 15 ml centrifuge tube and spun down at 1300 \( \cdot \) g for 5 minutes to separate the plasma from the red blood cells. The plasma was then transferred into 1.5 mL centrifuge tubes and frozen at -20°C.

The steroid hormones were extracted from mature individuals following protocol from Tsang and Callard (1987a) and Sulikowski et al. (2005). Each sample was extracted twice with 10 volumes of diethyl ether (anesthesia grade) for estradiol-17\( \beta \) (E\(_2\)) and testosterone (T), or petroleum ether for progesterone (P\(_4\)). The ether was then evaporated under a stream of nitrogen before the dried extracts were reconstituted in phosphate buffered saline with 0.1% gelatin (PBSG). Furthermore, approximately 1000 counts min\(^{-1}\) (cpm) of an appropriate tritiated steroid were added into each plasma sample prior to addition of solvents to account for procedural losses during the extraction. The overall mean recoveries (i.e. how much tritiated steroid was not lost during extraction) were 81.6% for T, 69.0% for P\(_4\), and 66.8% for E\(_2\). The individual recoveries were used to correct the steroid concentrations for each sample. Also, the R\(^2\) for standard curves/samples was calculated for T, P\(_4\), and E\(_2\) to assess linearity.
Plasma concentrations of E₂, P₄, and T were determined by radioimmunoassay (RIA) modified from procedures of Tsang and Callard (1987a). The tritiated T, E₂ (Amersham Biosciences, Picataway, NJ) but not P₄ (Perkin Elmer Life Sciences, Boston, MA) were purified using thin layer chromatography and a solvent system consisting of 3 parts ether: 1 part hexane for E₂ (2,4,6,7,16,17-³H) and a solvent system consisting of 4 parts benzene: 1 part acetone for T (1,2,6,7-³H) and P₄ (1,2,6,7-³H) (Sulikowski et al. 2004). Purified steroids were then extracted from the silica scraped off the plate using methanol, dried, and reconstituted in absolute ethanol to concentrations of 5 µCi ml⁻¹ for T, 8 µCi ml⁻¹ for E₂ and 50 µCi ml⁻¹ for P₄.

Plasma concentrations of E₂, P₄, and T were determined for females while only concentrations of T were determined for males. Duplicate aliquots of the reconstituted plasma extracts received the appropriate tritiated steroid and anti-serum in a total volume of 0.4 ml. The final anti-sera concentrations were 1:64,000 for T (#250, anti-testosterone-11-BSA; Gay and Kerlan 1978), 1:200,000 for E₂ (#244, anti-estradiol-6-BSA; Korenman et al. 1974) and 1:10,000 for P₄ (# 337, antiprogesterone-11-BSA; Gibori et al. 1997). After an overnight incubation at 4°C, free steroids were separated from bound steroids using a suspension of Norit A (0.2%) and Dextran T-70 (0.02%), followed by centrifugation at 1500 · g for 10 min at 4°C. The supernatants were then decanted into scintillation vials (USA Scientific, Ocala, FL) followed by addition of Ready Safe™ cocktail (Beckman Coulter, Somerset, N.J.). Radioactivity was determined in a Beckman LS6000IC (Fullerton, California) liquid scintillation counter. The non-labeled steroids for the standard curve were obtained from Steraloids, Inc. (Wilton, NH). The intra-assay
coefficients of variance were 6.1% for T, 5.4% for P<sub>4</sub> and 7.2% for E<sub>2</sub>. The interassay coefficients of variance were 8.5% for T, 10.4% for P<sub>4</sub> and 8.0% for E<sub>2</sub>.

Criteria for Maturity and Fecundity

Maturity was determined following criteria detailed in chapter 2 for both males and females. Briefly, each sex had to be above all threshold levels set for morphology, physiology, and histology for them to be considered mature. If any of these threshold levels was not reached, the individual <i>S. acanthias</i> was characterized as being immature.

The number of embryos and large ovarian follicles (diameter > 15 mm) were used to assess fecundity. Female <i>S. acanthias</i> fecundity was reported for all mature individuals and by 5cm size classes.

Reproductive Cycles

In order to profile patterns and trends over the reproductive cycle, mature male <i>S. acanthias</i> were separated into groups depending on their month of capture. Because female <i>S. acanthias</i> have a nearly two year gestational period, mature specimens were grouped according to a modified stage classification, based on those developed by Hisaw and Albert (1947) and used by Tsang and Callard (1987a). In the present study, females were placed into one of four stages (A through D) based on embryo/pup development, ovarian follicular development, and month of capture. Because the reproductive cycle is continuous, and because there is natural variability between individuals, an overlap between adjacent stages in embryo/pup development and ovarian follicular size was expected. Thus, to clearly differentiate between stages of the reproductive cycle, the
month the shark was sampled was also taken into account. For example, assuming a two year gestational cycle and parturition occurring in the winter, the reproductive cycle was divided into four 6-month stages beginning in February. Thus, mature female *S. acanthias* clearly in their first year of gestation (having no embryos, containing candles or embryos < 18 cm LST and having ovarian follicles < 30 mm in diameter) were characterized as being stage A if they were sampled from February to July and stage B from August to January. Mature female *S. acanthias* clearly in their second year of gestation (containing embryos > 15 mm LST with reduced or no yolk sacs and having ovarian follicles > 15 mm in diameter) were characterized as being stage C if they were sampled from February to July and stage D from August to January. In addition to dividing the reproductive cycle into four stages, dividing it into 24 months beginning with February enabled monthly comparisons to be made with higher resolution between females in their first (stages A and B) or second (C and D) year of gestation. Using this approach, the largest monthly values obtained for a number of morphological parameters were plotted to determine time of parturition and ovulation. Overall, the morphological, physiological, and histological data obtained from male and female *S. acanthias*, relative to month sampled or reproductive stage, collectively provided insight into the reproductive seasonality of both sexes.

**Statistical analysis**

Because the mean LST of male *S. acanthias* collected in October was significantly shorter than in other months (ANOVA; N = 58; p = 0.0024), the effect of size was accounted for in the monthly difference analysis by using an analysis of covariance
(ANCOVA) for any parameter that showed a significant relationship with LST. However, the LST of females were not significantly different from each other (ANOVA; N = 58; p = 0.13), so there was no need to account for the effect of size on the parameters in the analysis.

If the morphological, physiological, and histological data were distributed normally, a multi-way ANOVA was used to examine the entire year or reproductive cycle. If a significant difference was found over the entire model, Tukey’s HSD was utilized to examine differences between individual months or stages. However, if the data were not normally distributed, a nonparametric Kruskal-Wallis/Wilcoxon signed rank test was used. If this produced a significant difference, then pairwise comparisons were performed while maintaining an \( \alpha = 0.05 \). Lastly, using these methods, some of the data obtained from this part of the present study were compared to those obtained prior to increased fishing pressure (Tsang and Callard 1987a).

Stretch total length and age were regressed against total numbers of embryos and large ovarian follicles to determine if fecundity was affected by maternal size. Female *S. acanthias* were also divided into 5cm size classes and an ANOVA was run to examine differences between fecundity of size classes. Lastly, using the above methods, morphological and physiological data obtained from the present study were compared to those obtained prior to increased fishing pressure (Nammack et al. 1985; Tsang and Callard 1987a).
RESULTS

Sampling

Between July 2006 and June 2009, a total of 211 (70 males and 141 females) mature *S. acanthias* was collected for this study. For males, they had $L_{ST}$ ranging from 61.5 to 84 cm, body masses from 0.82 to 2.45 kg, and ages from 6.5 to 17 years. For females, they had $L_{ST}$ ranging from 74.5 to 102 cm, body masses from 1.50 to 4.50 kg, and ages from 9 to 22 years.

Morphology

*Males*

Clasper length ranged from 53 to 85 mm, and there was a significant positive relationship between $L_{ST}$ and clasper length ($\text{Clasper length} = 36.421288 + 0.5219327 \times L_{ST}; N = 58; p = 0.0001; R^2 = 0.29$). However, clasper length showed no significant difference between months (ANCOVA effects test; $DF = 5; p = 0.37; R^2 = 0.58$) (Fig. 3.1). In addition, GSI ranged from 0.46 to 3.79 and there was a significant positive relationship between $L_{ST}$ and GSI ($\text{GSI} = -1.135664 + 0.0355608 \times L_{ST}; N = 65; p = 0.0009; R^2 = 0.13$). Furthermore, unlike clasper length, there were significant differences in GSI between months (ANCOVA effects test; $DF = 5; p = 0.0001; R^2 = 0.58$) (Fig. 3.1), with July being higher than all other months (in which there were more than one sample collected).
Females

The combined mass of shell glands in female *S. acanthias* ranged from 0.81 to 8.95 g. There were significant differences between stages (ANOVA; N = 94; p < 0.0001), with stage B animals having the smallest shell glands (Fig. 3.2). In addition, GSI ranged from 0.73 to 14.30 and there was also a significant difference between stages and GSI (Wilcoxon signed rank tests; N = 120; p < 0.0001), with the later stages having larger GSI values (Fig. 3.2). The diameters of the largest ovarian follicle ranged from < 15 to 53 mm and they differed between stages (ANOVA; N = 95; p < 0.0001, R² = 0.10), with the later stages having greater diameters (Fig. 3.2). Lastly, the embryo sizes ranged from candled embryos to 29.5 mm, and as expected, they also differed between stages (ANOVA; N = 88; p < 0.0001) (Fig. 3.2).

Based on morphological parameters such as the largest ovarian follicle size, GSI, and combined shell gland mass by month, ovulation in female *S. acanthias* most likely occurs between January and March (Fig. 3.3). Furthermore, based on the largest embryo size within female *S. acanthias*, parturition most likely occurs between October and December (Fig. 3.3).

Histology

Males

The proportion of stage VI spermatocysts in sections from mature *S. acanthias* testes ranged from 0.20 to 0.50. While there was no relationship between L<sub>ST</sub> and proportion of stage VI spermatocysts (linear regression; N = 39; p = 0.60) (Fig. 3.1), the
p value between month and proportion of stage VI spermatocysts was less than 0.01 units from the accepted 0.05 level for statistical significance (ANOVA; N = 36; p = 0.057) with a larger trend in August, September, and October than July (Fig. 3.1).

**Females**

Of the 45 shell glands examined, most notably in the terminal zone, no spermatozoa were present. This indicated that there is no long term sperm storage in this species.

**Physiology**

**Males**

Plasma testosterone concentrations in mature male *S. acanthias* ranged from 11.54 to 143.87 ng/ml. While there was no relationship between size and plasma testosterone concentrations (linear regression; N = 38; p = 0.41), there were significant differences between month of capture (ANOVA; N = 39; p = 0.0047), with males caught in September and October having higher concentrations than those caught in other months (Fig. 3.1).

**Females**

Plasma concentrations of E2 in mature female *S. acanthias* ranged from undetectable to 4.84 ng/ml. While there was no relationship between size and plasma E2 concentrations (linear regression; N = 116; p = 0.60), there was a significant difference
between plasma E\textsubscript{2} concentrations and reproductive stage (ANOVA; N = 116; p = 0.022), with stage C females having significantly higher concentrations than stage A (Fig. 3.4). Interestingly, the magnitude and trends in E\textsubscript{2} concentrations of stage A to D dogfish throughout the reproductive cycle were similar between the present study and the one conducted prior to the increased fishing pressure by Tsang and Callard (1987a) (Table 3.1). However, when plasma E\textsubscript{2} concentrations were analyzed monthly throughout gestation, rather than by reproductive stage, there were no differences (ANOVA; N = 81; p = 0.39) (Fig. 3.5), although an increasing trend in stage C and D animals was evident.

Plasma concentrations of P\textsubscript{4} in mature female \textit{S. acanthias} ranged from 0.21 to 48.08 ng/ml. While there was no relationship between size and plasma P\textsubscript{4} concentrations (linear regression; N = 116; p = 0.60), there was a significant difference between plasma P\textsubscript{4} concentrations and reproductive stage in female \textit{S. acanthias} (ANOVA; N = 112; p < 0.0001), with stage B being significantly higher than all other stages (Fig. 3.4). As with E\textsubscript{2} concentrations, the magnitude and trends in P\textsubscript{4} concentrations of stage A to D dogfish throughout the reproductive cycle did not differ between the present study and the one conducted prior to the increased fishing pressure by Tsang and Callard (1987a) (Table 3.1). Unlike E\textsubscript{2}, plasma P\textsubscript{4} concentrations, when analyzed monthly rather than by reproductive stage, varied throughout gestation (ANOVA; N = 75; p = 0.0001), with females caught in January having elevated concentrations (Fig. 3.5).

Plasma concentrations of T in mature female \textit{S. acanthias} ranged from undetectable to 3.80 ng/ml. There was no relationship between size and plasma T concentrations (linear regression; N = 93 p = 0.23), or between plasma testosterone concentrations and reproductive stage in mature females (ANOVA; N = 93; p = 0.10)
(Fig. 3.4). Similar to E<sub>2</sub> and P<sub>4</sub> concentrations, the magnitude and trends in T concentrations of stage A to D dogfish throughout the reproductive cycle were not different between the present study and the one conducted prior to the increased fishing pressure by Tsang and Callard (1987a) (Table 3.1). When analyzed monthly rather than by reproductive stage, plasma T concentrations varied in the final year of gestation (ANOVA; N = 60; p = 0.0001), with females caught in July showing elevated concentrations (Fig. 3.5).

**Fecundity**

**Follicles**

Large ovarian follicles found in 110 female *S. acanthias* (mean L<sub>ST</sub> = 87.8) were used as an index of fecundity. The overall mean number of ovarian follicles was 5.1 (SE = 0.16) per female, per reproductive cycle. There was a significant relationship between fecundity and L<sub>ST</sub> of the mother (Large Ovarian Follicles = -10.28 + 0.18*L<sub>ST</sub> (cm); N = 109; p < 0.0001; R<sup>2</sup> = 0.37), with fecundity increasing with increased L<sub>ST</sub>. Notably, while the fecundity by size data were similar to those of Nammack et al. (1985) at the lower size classes, the data obtained for dogfish above 90 cm L<sub>ST</sub> in the present study were significantly lower than those previously reported (Table 3.2).

**Embryos**

In addition to large ovarian follicles, the number of embryos found in 93 female *S. acanthias* (mean L<sub>ST</sub> = 88.2) was used as an index of fecundity. The sex ratio of the
offspring was 1:1. The overall mean number of embryos was 4.05 (SE = 0.52) per female, per reproductive cycle. There was a significant relationship between fecundity and \( L_{ST} \) of the mother (Total number of embryos = \(-6.87 + 0.12L_{ST} \) (cm); \( N = 93; p < 0.0001; R^2 = 0.16 \), with fecundity increasing with increased \( L_{ST} \). Just as with fecundity using large ovarian follicles, the fecundity by size data using number of embryos were similar to the study by Nammack et al. (1985) at the lower size classes, but at the size classes between 90 and 100 cm \( L_{ST} \), the values obtained in the present study were significantly lower than those previously reported (Table 3.2).

**DISCUSSION**

Because reproductive life cycles of fish, including elasmobranchs vary throughout the year for many species (Bubley and Pashuk 2010; Hisaw and Albert 1947), it is important to collect data from individuals obtained year round to accurately assess seasonal life history parameters. However, previous studies examining the reproductive life history of *S. acanthias* had only been able to obtain individuals for 3-5 months of the year (Hisaw and Albert 1947; Nammack et al.1985). In the present study, female *S. acanthias* were collected for all twelve months, which provided a more complete picture of reproductive seasonality, avoiding the need to extrapolate due to gaps in the data set. In contrast, collection of male *S. acanthias* in all twelve months proved to be more difficult than for females. Males are believed to spend winters in deeper offshore waters (McMillan and Morse 1999), which made them more difficult to obtain. Nonetheless, in the present study, male *S. acanthias* were caught 6 out of 12 months. Although this is
comparable to the previous studies examining their life history (e.g. Nammack et al. 1985), caution was taken in extrapolating trends beyond the available data set. Despite the incompleteness of monthly samples for the males, my sampling of male and female *S. acanthis* in the Western North Atlantic yielded the entire size and age ranges, allowing me to relate these parameters to reproductive cycles.

Morphology has traditionally been used as the sole indicator of reproductive readiness and seasonality in many studies of elasmobranchs (Conrath 2005), including *S. acanthis* (Nammack et al. 1985), which could result in misleading or inaccurate information. However, by combining morphology with physiological and histological data, as was done in the present study, strengthens the characterization of male and female reproductive cycles.

**Males**

Data from the present study revealed that male *S. acanthis* exhibited reproductive seasonality, which has been observed in many other elasmobranch species that have distinct mating seasons (Wourms 1977; Parsons and Grier 1992). However, depending on the choice of parameter, the mating season differed by multiple months. If GSI was used as an indicator of reproductive seasonality, it peaked in July. But if testosterone concentrations and proportion of stage VI spermatocysts were used, the peak occurred in autumn. In males, using GSI as an indicator of reproductive readiness relies on the assumption that gonad size is correlated with time of mating (Stevens and Wiley 1986), but this assumption can be violated (Simpfendorfer 1992; Maruska 1996). On the other hand, reproduction is dependent on gamete maturity, which is assessed directly by
determining the proportion of mature spermatozoa and plasma concentrations of testosterone, the hormone essential for spermatogenesis (Callard 1992). Since GSI is an indirect method, and since testosterone concentrations coincided with an increase in proportion of mature spermatocysts, I concluded that mature male *S. acanthias* are reproductively active in the autumn, corroborating the findings of Nammack et al. (1985). Thus, by using a suite of reproductive parameters in the present study, discrepancies in reproductive seasonality between parameters were rectified.

**Females**

The two-year gestation period of female *S. acanthias* poses a challenge, especially when monthly values are used to examine reproductive seasonality, because individuals in their first and second years of pregnancy are sampled together each month. Previously, this issue was addressed by dividing the gestation period into four stages (A through D), based on follicle size and embryo size (Hisaw and Albert 1947). Using this approach, female *S. acanthias* exhibited different morphological and physiological trends, depending on the year of pregnancy (Hisaw and Albert 1948; Tsang and Callard 1987a). In the present study, the majority of animals caught in December and January were post partum but pre-ovulatory. Because the next reproductive cycle had not begun yet, individuals fitting these criteria were classified as stage D. This difference is a confounding factor when the present and previous studies are compared.

While classifying individuals by stage may provide more clarity for some analyses, a loss of resolution may result for others because animals for each stage were grouped in six-month blocks. Thus, in order to increase resolution in the present study,
some reproductive parameters were analyzed by month rather than by stage, using the assumption that fertilization occurs in February. Overall, classifying the reproductive cycle by stages allows for characterization of larger scale reproductive trends in *S. acanthias*, while analyses by months reveal finer scale changes that couldn’t be detected otherwise.

The time of ovulation can be determined based on morphological parameters, such as shell gland size, GSI and follicle size. The shell gland is responsible for secreting an egg membrane, as well as possibly acting as a site for sperm storage and fertilization (Carrier et al. 2004). It increases in size by two-fold just prior to and following fertilization and egg passage (Carrier et al. 2004). In the present study, the shell glands were largest in stage D and stage A female *S. acanthias*, leading up to and just following ovulation, respectively. In addition, during stage D of gestation, the increase in shell gland size is accompanied by an increase in follicle size and GSI. After ovulation, the eggs pass down the oviduct and through the shell gland, leaving the ovary devoid of large follicles. Consequently, the ovarian mass and thus GSI drastically decrease, as indicated by the dramatic difference between these parameters in stage A and D females.

When these same parameters were examined by month instead of by stage, they reached a peak in December and January, indicating that ovulation and fertilization occurred in these months. Throughout the remaining months of gestation following fertilization, shell gland mass, GSI and follicle size generally increased, as the animals went from stage A through stage D. Notably, these changes also coincided with the growth of the embryos within the uteri. As gestation progressed, the embryos reached their largest size at stage D in December, while in January, embryo size was the smallest.
This led me to conclude that parturition probably occurred before January, which was further supported by the presence of full term pups and empty uteri in animals captured in October, November, and December. Overall, while these ovulation and parturition times support those of previous studies in the Western North Atlantic (Nammack et al. 1985; Campana et al. 2009), the use of data from monthly samples in the present study provided more resolution, allowing me to pinpoint which months these events were likely to occur. These data also showed that there is little time between parturition and fertilization, with the gestation period being 22-23 months.

In females, plasma steroid hormone concentrations, which were used as an index of physiological parameters, paralleled the morphological ones. The hallmark of a healthy ovarian follicle is its ability to produce E2, which regulates vitellogenesis by the liver and in turn, provides yolk proteins for uptake by the growing follicle (Nagahama et al. 1995). In the present study, the increase in plasma concentrations of E2 throughout the reproductive cycle was mirrored by the increase in follicle size. Furthermore, the trend towards increased plasma T concentrations during the same time period may also play an important role in ovarian follicle growth. By producing T, the follicle utilizes it as substrate to produce E2. Notably, the patterns of plasma E2 and T observed in the present study were similar to those reported by Tsang and Callard (1987a), despite the greater than 20 year gap between them. Interestingly, even though S. acanthias caught for the present study had far fewer follicles to produce E2 and T, the magnitude of the plasma concentrations was similar. At this time, it is not clear whether this is due to a greater steroidogenic capacity by fewer ovarian follicles, which is more likely than the alternative that there might be other sources of E2 and T in female S. acanthias capable of
producing large amounts of these hormones (Yaron et al. 1977). While these other sources of hormones have not been identified in elasmobranchs, the brain, adipocytes, and the adrenal cortex are known sources in mammalian systems (Naftolin et al. 1975; Machinal et al. 1999; Baquendo et al. 2007).

Progesterone was a third steroid hormone examined in the present study. Unlike E$_2$ and T concentrations, that paralleled each other, P$_4$ concentrations exhibited a different pattern. The corpus luteum produces P$_4$, the hormone that maintains pregnancy (Niswender et al. 1985). In vitro experiments measuring steroid hormone synthesis by corpora lutea found those taken from stage B _S. acanthias_ produced the highest P$_4$ (Tsang and Callard 1987b), which is probably present to maintain pregnancy. An accompanying in vivo study by Tsang and Callard (1987a) also found increased plasma P$_4$ concentrations in stage A and B animals, while the present study only had a peak in stage B animals. This difference could be explained by which stages of dogfish were grouped together for the analysis. In the present study, if _S. acanthias_ that had already undergone parturition but had not yet ovulated (classified as stage D) were combined with stage A animals, the mean P$_4$ concentrations would look very similar to those reported by Tsang and Callard (1987a). While increased P$_4$ concentrations near parturition and ovulation are expected, an unexpected trend in the present study was revealed when the monthly values were examined. The expected peak in the plasma P$_4$ concentrations around December and January for the second year of gestation was observed, but there was also a second peak in December and January at the end of the first year of gestation. Previous studies on fish have determined that environmental cues
including temperature and photoperiod can affect reproductive cycles (Peter 1981; Bromage et al. 1982).

Morphological and physiological reproductive parameters have implications for management. Size frequencies in *S. acanthias* have been truncated following the increased fishing pressure in the 1990's (Sosebee and Rago 2006). Because fecundity in fish, including *S. acanthias*, has a significant relationship with size (Sosebee 2005), the decrease in the larger individuals decreases the fecundity of the population as a whole. This was evidenced by the decrease in mean size of female *S. acanthias*, compared to previous studies (Tsang and Callard 1987a; Nammack et al. 1985). Further, the decrease in size was related to the decrease in size and age at maturity of *S. acanthias* following the increased fishing pressure (see Chapter 2). As such, fecundity as a function of size (expressed as 5 cm size classes) showed no overall difference from the study of Nammack et al. (1985), except for a noticeable decrease in the 90 – 100 cm L<sub>ST</sub> size classes. While no direct causation can be attributed to differences in fecundity before and following the increased fishing pressure, the data suggest that these correlations may be related. Regardless of the cause, the decrease in fecundity could have an effect on recruitment, which was at record lows for seven years between 1997 and 2003 following the fishing pressure (Sosebee and Rago 2006). Consequently, a decrease in recruitment (be it decreased spawning stock biomass, decreased fecundity per fish, or a combination of both) could then lead to slower recovery of an over-fished population.

In summary, reproductive readiness in male and female *S. acanthias*, based on physiological and histological evidence, begins in autumn and early winter. Combined with the time of parturition and ovulation, as well as the observation that there is no
evidence for long term sperm storage in the shell gland in females, copulation occurs in late autumn and early winter in the Western North Atlantic and not throughout the year. Thus, by describing reproductive seasonality for both male and female *S. acanthias* in the Western North Atlantic, the present study provided an updated account of their life history, which could be used in future management of the population. Additionally, the decrease in fecundity attributed to the smaller size of mature females is a vital component in establishing guidelines for the *S. acanthias* fishery.
Figure 3.1. Monthly mean values for (A) clasper length, (B) GSI, (C) proportion of stage VI spermatocysts and (D) plasma T concentrations for male *S. acanthias*. Error bars represent two standard errors and numbers along the top represent sample sizes. An asterisk (*) denotes significantly different values (p< 0.05) between months.
Figure 3.2. Mean values for (A) shell gland mass, (B) GSI, (C) largest follicle diameter, and (D) $L_{ST}$ of embryos for female $S. acanthias$ by stage of gestation. Error bars represent two standard errors and numbers along the top represent sample sizes. Different letters between stages denote significantly different values ($p<0.05$).
Figure 3.3. The largest values for (A) $L_{ST}$ of embryos, (B) largest follicle diameter (C) shell gland mass, and (D) GSI for female *S. acanthias* by month.
Figure 3.4. Mean plasma (A) E₂, (B) T, and (C) P₄ concentrations for female *S. acanthias* by stage of gestation. Error bars represent two standard errors. An asterisk (*) denotes significantly different values (p < 0.05) between stages.
Figure 3.5. Mean plasma (A) T, (B) P₄, and (C) E₂ concentrations in female *S. acanthias* by month over the two year reproductive cycle. Reproductive stages A through D are denoted along the top. The year of gestation is denoted by the number after the month.
Table 3.1. Comparison of mean plasma steroid hormone concentrations (± two standard errors) by stage, between female *S. acanthias* obtained in the present study and those obtained prior to the increased fishing pressure (Tsang and Callard 1987a).

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Stage</th>
<th>Present Study</th>
<th>Tsang and Callard (1987a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Concentration (ng/ml)</td>
</tr>
<tr>
<td>Estradiol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>0.14 ± 0.06</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>0.40 ± 0.18</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
<td>0.85 ± 0.19</td>
<td>9</td>
</tr>
<tr>
<td>D</td>
<td>34</td>
<td>0.71 ± 0.28</td>
<td>5</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>11</td>
<td>0.06 ± 0.01</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>22</td>
<td>0.11 ± 0.08</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
<td>0.48 ± 0.20</td>
<td>9</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>0.33 ± 0.24</td>
<td>7</td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>0.78 ± 0.44</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>22</td>
<td>9.08 ± 2.65</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
<td>1.58 ± 0.32</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>33</td>
<td>1.77 ± 0.96</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 3.2. Comparison of mean fecundity (± 2 standard errors) by 5 cm size classes between mature female *S. acanthias* obtained in the present study and those obtained prior to the increased fishing pressure (Nammack et al. 1985), using number of large ovarian follicles and the number of embryos. The mean overall size of females from this study was 87.8 cm L<sub>ST</sub> and 96 cm L<sub>ST</sub> from Nammack et al. (1985).

<table>
<thead>
<tr>
<th>Size Class</th>
<th>Present Study 2006-2009</th>
<th>Nammack et al. 1985</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Follicles</td>
</tr>
<tr>
<td>75-79</td>
<td>9</td>
<td>3.66 ± 0.92</td>
</tr>
<tr>
<td>80-84</td>
<td>21</td>
<td>4.29 ± 0.60</td>
</tr>
<tr>
<td>85-89</td>
<td>38</td>
<td>5.11 ± 0.46</td>
</tr>
<tr>
<td>90-94</td>
<td>29</td>
<td>5.76 ± 0.52</td>
</tr>
<tr>
<td>95-99</td>
<td>11</td>
<td>6.55 ± 0.84</td>
</tr>
<tr>
<td>100-104</td>
<td>2</td>
<td>8.50 ± 1.96</td>
</tr>
<tr>
<td>105-109</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
<td>110</td>
<td>5.21 ± 0.32</td>
</tr>
</tbody>
</table>
CHAPTER 4

A SPATIAL AND SEASONAL EXAMINATION OF POPULATION STRUCTURE

The spiny dogfish (Squalus acanthias) is a cosmopolitan shark species found in the Atlantic and Pacific Oceans. Because of a nearly ten-fold increase in landings from 1987 – 1996 (NEFSC 1998) and life history parameters (e.g. slow growth, extended gestation) that are not conducive to withstand heavy fishing pressure (Holden 1973; Musick 1999; Stevens et al. 2000), the Atlantic States Marine Fisheries Commission (ASMFC) declared spiny dogfish in the Western North Atlantic to be overfished. As a result, a Fisheries Management Plan was implemented, establishing quotas that have essentially ended any directed fishery (ASMFC 2002).

Current fisheries models are based on the most recent spiny dogfish life history studies in the North West Atlantic, but these were conducted prior to the recent population decline (Jensen 1965; Nammack et al. 1985). This alone warrants a new life history assessment. However, while utilizing updated life history information in fisheries models is essential to successful management, it is equally important that these parameters are obtained from and applied to the appropriate biologically meaningful population unit. Based on several environmental variables such as temperature and prey availability, life history parameters in marine fishes can vary between populations
(Yamaguchi et al. 1998), creating the need for different strategies to sustainably manage the respective fishery.

Currently, *S. acanthias* are managed as a single panmictic stock, centering around the paradigm that the population spends the summer and fall months in more northerly waters off Canada and New England and migrate to waters south of Cape Cod, MA to spend their winter and spring months (Bigelow and Schroeder 1953). However, the unit stock theory has been challenged recently, since tagging studies (Moore 1998; Rulifson et al. 2001) and analysis of movement patterns (Sulikowski et al. 2010) suggest that there may be multiple dogfish populations in the Western Atlantic. In addition, observations suggest that a nearly year-round *S. acanthias* population exists in the Gulf of Maine (Jensen 1965). Consequently, the current unit stock theory needs to be re-evaluated for sustainable management of *S. acanthias*. In order to succeed, correctly identifying spatial and temporal population differentiation is essential to monitoring the dynamics of the individual populations and their overall contributions to production in the fishery (Hauser and Carvalho 2008).

Microsatellites are molecular markers in genomic DNA that have been used to examine population structure in a variety of marine fishes, including elasmobranchs (Keeney et al. 2005; Feldheim et al. 2001). A microsatellite is a common, simple, bi- tri- or tetra-nucleotide tandem repeat of DNA. These regions contain higher mutation rates than other regions of DNA (Weber and Wong 1993). As such, the differences in frequencies in the number of repeats of these mutations (alleles) between sampling locations can show differentiation of populations within a species (Pearse and Crandall 2004). Thus, microsatellites were recently used to identify separate populations in global
studies of *S. acanthias* (Verissimo et al. 2010). It is not entirely clear, however, whether there is one or multiple populations of *S. acanthias* along their geographic range in the Western North Atlantic. The objective of the present study was to determine the spatial and temporal population structure of *S. acanthias* in the Western North Atlantic using microsatellites. In the event that there are multiple dogfish populations in the Western North Atlantic, by characterizing the delineations between populations, present management strategies could be modified to successfully manage this fishery.

**MATERIALS AND METHODS**

**Sample Collection**

Genetic samples were obtained from a total of 305 individuals of mixed sex and unknown maturity. For spatial examination of population structure across a potential barrier of gene flow, fin clips (n = 146) were taken from *S. acanthias* at two locations south of Cape Cod, MA (North Carolina/Virginia, Delaware/New Jersey) and one location north of Cape Cod, MA (Gulf of Maine) (Table 4.1). To examine temporal population structure, fin clips (n = 159) from *S. acanthias* were collected from the Gulf of Maine during Autumn 2007, Summer 2008, Autumn 2008, in addition to those already collected in the Spring of 2007 (n = 48) for the spatial analysis component (Table 4.1). All fin clips were stored in separate vials containing 95% ethanol.

**DNA Isolation**
DNA was extracted from 15 to 25 mg of tissue using the protocol outlined in the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA). The amount and purity of extracted DNA were determined using optical density readings at 260 nm and 280 nm (Warburg and Christian 1942). The eluent was stored at -20°C.

DNA Amplification

The DNA was amplified by the polymerase chain reaction (PCR) using primers specific for 10 *S. acanthias* microsatellite loci: *U285, T289, J451, J445, H429, U273,* and *H434* (McCauley et al. 2004) and *GAI1, 3853,* and *6396* (Verissimo et al. 2010). The forward primers were labeled with fluorescent dyes. DNA was amplified in either single-locus or multiple-loci formats under the following conditions: initial denaturation for 5 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 52-61°C depending on the specific primer set, and 1 minute at 72°C, with the final extension step of 5 minutes at 72°C. The total reaction volume was 10 μL, which contained 40 ng of template DNA, 1X Amplitaq polymerase buffer (Applied Biosystems, Carlsbad, CA), 0.2 μM dNTPs, 0.2 μM each of the forward and reverse primer, and 1U Amplitaq polymerase.

DNA Genotyping

Amplified DNA from all loci were pooled by sample and sent to the Hubbard Genome Center (University of New Hampshire) for genotyping using an automated ABI 3130 genetic analyzer. Alleles were visualized using Peak Scanner 1.0 (Applied Biosystems) and scored manually based on size by comparing to an internal size
standard. A sample of known genotype was included with each 96 well plate to ensure consistency between runs.

**Statistical Analysis**

Samples collected at all locations and seasons were compared to examine temporal and spatial differentiation of populations. Multilocus genotypes were compiled and the allele frequencies were determined using Genepop 4.0 (Raymond and Rousset 1995). Genotypes were also examined for scoring errors using Microchecker 2.2.3 (Van Oosterhout et al. 2004) to detect possible stuttering, large allele dropouts, and null alleles for each locus at individual sampling location and over all locations. Null allele frequency was determined using the method devised by Dempster (1977). Deviations from Hardy-Weinberg Equilibrium (HWE) were tested for sampling locations/seasons, across all sampling using individual loci, and across all sampling locations/seasons and loci. Linkage was tested for loci within each sampling location using Genepop 4.0. The genetic variation was characterized at each locus in terms of polymorphism and heterozygosity using Genepop 4.0, and allele richness using Fstat 2.9.3.2 (Goudet 1995).

Microsatellite data were analyzed using Structure 2.3.3 (Pritchard et al. 2000) to infer population structure and numbers of populations (K) present and determine its pattern. Ten iterations using a Markov Chain Monte Carlo simulation (MCMC) admixture model were run for K-values between 1 and the total number of sampling locations/seasons (6). Unamplified loci for individuals, presumed homozygous genotypes with null alleles, were set as recessive alleles, and the model was run to account for other homozygous genotypes, which may be ambiguous due to the presence of potential null
alleles (Falush et al. 2007). Trial runs of varying lengths were examined and a burnin length of 50,000 followed by a run time of 1,000,000 steps was decided upon to minimize the effects of the starting state and have convergence of test parameters, respectively. The estimated membership coefficients for each individual, in each cluster (Q scores) were noted, i.e. if the value was evenly divided in individuals, it would indicate either no population structure or a hybrid zone. The cumulative Q scores were also examined from each sampling location/season. The probability and thus the most probable K from the results were calculated using the natural log of the estimated probability based on the K of the data [LnP (D)] chosen for the run, as indicated by the software manual following Bayes’ Theorem (Pritchard et al. 2000).

The relationship between sampling location/season relative to size and frequency of alleles was determined by employing F statistics (Fstat 2.9.3.2) and pair-wise comparisons of allele frequency differences using Fisher’s exact test [Arlequin 3.1 (Excoffier et al. 2005)]. The effects of null alleles on FST was examined using the INA method within the software Free N A (Chapuis and Estoup 2007). Allelic differentiation was examined to test the null hypothesis of a homogenous distribution of alleles across all sampling locations/seasons (Arlequin 3.1). The level of significance for p-values was adjusted using Bonferroni methods to account for multiple comparisons while maintaining a level of significance at p < 0.05.

RESULTS

Allele Characterization
The samples taken from the Atlantic Ocean along the eastern United States had 9 to 21 alleles for each locus, with a mean of 14.4, while allelic richness was moderate to high across all loci (Table 4.2). These alleles showed intermediate degrees of heterozygosity (overall = 0.643), with the lowest being 0.356 for locus T289, but all other alleles were larger than 0.5. There was no evidence of linkage disequilibrium at any loci. Of the 10 alleles analyzed by Genepop, nine were out of HWE except for locus 6396. Within these nine loci and six sampling locations/seasons, Microchecker found possible null alleles in 14 of the 60 combinations (one at H434, 3853, 639, and GA11, two at U285, three at T289, four at J445, five at U273, and seven in H429). Overall, null allele frequency was low, with nine of the ten loci having an estimated frequency less than 0.15, and the corrected FST values were similar to the uncorrected ones (Table 4.2). Thus, genotypes were not corrected for null alleles because it is unrealistic to have a single null allele state for all populations (Chapuis and Estoup 2007).

Population Structuring

By applying Bayes’ Theorem to the Ln P(D) results obtained from Structure 2.3.2, there were two populations detected (Figure 4.1). Samples taken during the Spring and Autumn of 2007 grouped together, while samples taken during the summer of 2008 showed the strongest structuring among the groups and those from Autumn of 2008 showed intermediate structuring (Fig. 4.2). Further examination of individuals from their respective sampling times revealed that the two putative populations were likely forming an admixed group in the Spring and Autumn seasons, with the most homogenous time in the Gulf of Maine being in the Summer (Fig. 4.3). The Q scores for many of the sampling
locations/seasons were near 0.5 indicating admixed populations or no structure and by examining the individual Q scores these could be differentiated. Notably, most individuals had relatively high Q scores, indicating that they are more like one population or the other, and not hybrids of the two. This showed that the populations in the Spring and Autumn were admixed. Pair-wise comparisons of $F_{ST}$ values using a Fisher’s exact test provided additional support of the Q scores, showing significant differences between $F_{ST}$ values between seasons/years sampled (Table 4.3), with Gulf of Maine S. acanthias collected during the summer of 2008 showing the largest pairwise $F_{ST}$ values. There was no significant difference between seasons and years of all samples from the Spring and Autumn in the Gulf of Maine.

DISCUSSION

Spatial Structuring

Analysis of 10 microsatellite loci from dogfish collected from 3 regions along the western Atlantic Ocean revealed that they grouped to a single population, consistent with the hypothesis of Verissimo et al. (2010), who did not sample locations north of Cape Cod, Massachusetts. However, both studies differed from conventional tagging studies performed by Rulifson et al. (2001) and Moore (1997), who proposed that there could be a population barrier near Cape Cod, Massachusetts. In the present study, we collected fin clips over a relatively short period of time (1.5 months) during the Spring of 2007 to avoid repeated sampling of the same population of S. acanthias as they migrated northward off the Atlantic coast. It was unlikely that we sampled the same population of
S. acanthias, based on our knowledge of the distance between the northern and southern most collection sites in the study and their speed of migration, as determined by pop-up satellite tagging technology (Sulikowski et al. 2010). Overall, the lack of spatial structuring among the three locations in Atlantic waters off the east coast of the United Stated and Southern Canada can be attributed to a population that moves north in the Spring and south in Autumn, as was previously hypothesized (Bigelow and Schroeder 1953; Shafer 1970; Verissimo et al. 2010).

A single population moving as a group does not explain the findings of the previous tagging studies by Rulifson et al. (2001) and Moore (1997), or accounts from the literature (Templemen 1944; Templemen 1965; Jensen 1965), commercial fishermen, and personal observations of S. acanthias being present in northern latitudes during the winter. Thus, the possibility exists that there may be multiple populations of S. acanthias in the Gulf of Maine, which could be attributed to a barrier to gene flow or finer scale population structuring. Because of their large scale movement potential and the inability to obtain S. acanthias fin clips from the northern most portion of their range in Canada and locations further offshore, temporal patterns in the Gulf of Maine were examined in an attempt to resolve this discrepancy.

Temporal Structuring

The Gulf of Maine was chosen as the location to examine temporal population structuring because it was hypothesized as a site of S. acanthias population mixing (Campana et al. 2009). Although S. acanthias are highly migratory, making distinct populations less likely, we nonetheless found significant population structuring within the
Gulf of Maine between samples obtained over 3 seasons (Spring, Summer and Autumn) and 2 years (2007, 2008). The *S. acanthias* caught in the Summer of 2008 not only showed the most population structuring, but their FST values were significantly different than any other combination of season and year within the data set. Despite the relatively small FST values for *S. acanthias* and other marine fishes, especially when compared to those for terrestrial and freshwater organisms (Waples 1998), these values, nevertheless, have significant biological relevance for *S. acanthias* living within different environments (Bekkevold et al. 2005).

The significant differences between seasons and year of sample collection revealed by pair-wise tests of FST values coincided with the results using Structure 2.3.3 and Bayes Theorem that identified a large probability of two *S. acanthias* populations present within the samples. The pairwise comparisons within the Gulf of Maine are nearing significane though and this could be attributed to the migration pattern of *S. acanthias*. Because Autumn is the deduced time of migration out of the Gulf of Maine (Sulikowski et al. 2010) and Spring is the presumed return time of that population migrating back to the region from the south along the eastern United States (Bigelow and Schroeder 1953), it is probable that two populations of *S. acanthias* in the Gulf of Maine are mixing these seasons. Thus, the samples obtained during Spring and Autumn could conceivably contain a larger percentage of one population or the other, depending on movement patterns due to transitory environmental factors occurring at various times of the year. It would follow that the summer samples would be structured more strongly than the others because this period of time is farthest away from the time when the population migrates either into or out of the Gulf of Maine. If this is the case, winter
samples (which have been collected for 1 season, with plans to collect in the upcoming one before analysis commences) would be expected to differentiate strongly as well, but for the year-round population. Indeed, Structure 2.3.3 analysis of samples collected for the present study revealed a continuum, with the Autumn 2008 samples being intermediate between the Spring/Autumn 2007 and the Summer 2008 samples. This provided evidence that the Gulf of Maine, at certain times of year, supports a heterogeneous mix of at least two populations of *S. acanthias*.

The differentiation of the summer samples could also be due to the locations where the *S. acanthias* were caught. In the present study, the summer samples were obtained closer to shore (i.e. in shallower waters) during a catch-and-release spiny dogfish tournament for families, while the samples from the other seasons were obtained farther offshore (i.e. in deeper waters) during the NMFS bottom trawl survey or aboard commercial fishing vessels. Along the west coast of the United States and Canada, groups of *Squalus suckleyii* (previously named *S. acanthias*) were found to inhabit distinct locations (McFarlane and King 2003). One group was found offshore and exhibited highly north-south migratory patterns, while there was a resident inshore group which did not migrate. Similarly, in the Atlantic, *S. acanthias* tagged offshore had much lower return rates than those tagged closer to shore at shallower depths (Templeman 1984). Thus, if there are separate near-shore and offshore populations, one would expect more fishing pressure in the shallower water, which would result in higher return rate of tags. Recently, following increased fishing pressure, Rago and Sosebee (2009) observed a shift in *S. acanthias* distribution that could be a result of one population being targeted more than another.
Besides movement patterns and capture location, the composition of the samples (i.e. segregation by gender and size) could also contribute to the observed population differentiation of *S. acanthias* in the Western North Atlantic. Although gender and maturity were not noted in collection of most of the fin clips taken for the present study, the samples collected during the NOAA bottom trawl surveys in Autumn 2007 and throughout 2008 were presumed to contain a higher percentage of immature *S. acanthias*, while those collected in the Gulf of Maine during the Spring of 2007 had a mix of genders and maturity statuses. Templeman (1984) described the increase in proportion of immature females and mature and immature males off the coast of Newfoundland, Canada during winter and early spring months. Therefore, if there is differential movement between mature and immature *S. acanthias* and/or by gender between populations, then the change in the relative proportions of these groups within the population would otherwise be hidden if only mature *S. acanthias* were sampled.

**Fishery Management Implications**

Population structure should be taken into account when determining proper management strategies, especially when more than one population is present. Currently, *S. acanthias* are managed as a single population along the Western North Atlantic (Rago and Sosebee 2009). Estimates for spawning stock biomass are determined by fishery independent sampling, which occurs mainly during the Spring and Autumn. Because these sampling times coincide with *S. acanthias* migration, the resulting biomass estimates would vary depending on when the sampling was conducted, either prior to or following the migration of one of these populations. Therefore, since there have been
increases in biomass estimates within a relatively short period of time that can not be explained by their life cycle characteristics, i.e. long lived and slow growth, the presence or absence of multiple populations most likely explains the large fluctuations in the spawning stock biomass estimates of *S. acanthias*.

Multiple populations could also play an important role in the recovery of *S. acanthias* from fishing pressure. Multiple populations provide genetic resources to respond to both natural and man-made pressures on one or more of the populations (Bonin et al. 2007). Even though there was differentiation between *S. acanthias* populations, gene flow still occurs between them, based on relatively low, though significantly different $F_{ST}$ values. Further, if increased fishing pressure targeted one population more than another, then the other population(s) could provide genetic resources to the depleted stock to maintain genetic diversity. The availability of genetic resources are especially important in a size selective fishery, such as that for *S. acanthias*, which has shown changes in age and reproduction following the increased fishing pressure (Chapters 1 - 3). In addition, the resilience and/or buffering of fishery productivity can also be enhanced by having multiple populations contributing to future catches, and ultimately lessening the impact of increased fishing pressure (Hilborn et al. 2003).

In summary, by identifying temporal differentiation of *S. acanthias* in the Gulf of Maine, it provided awareness of multiple populations that would allow fisheries management to monitor the dynamics and the overall contributions of individual populations to the fishery (Hauser and Carvalho 2010). Clearly, more work needs to be done along the eastern coast of the United States and Canada, employing higher
resolution sampling protocols to further characterize the population structure of *S. acanthias* and how it may be influenced by season, geographic location or by gender and maturity. Regardless of the nature of the population structuring in the Western North Atlantic, the current study revealed the presence of population structuring of *S. acanthias*, in an area previously believed to contain only one homogenous population.
Figure 4.1. Mean values of $\ln P(D)$, produced by ten iterations for each $K$ using an MCMC. Error bars indicate two standard errors. The most probable $K$ is denoted by an asterisk (*) with a probability $> 0.99$. 
Figure 4.2. The mean Q scores of *S. acanthias* relative to sampling location, season, and year. The two putative populations are denoted by the dark and gray bars.
Figure 4.3. The Q score for *S. acanthias* individuals grouped by sampling location, season, and year. Each vertical line indicates an individual and each color represents a putative population.
Table 4.1. The location (North Carolina/Virginia, NC/VA; Delaware/ New Jersey, DE/NJ), season/year, and number of samples (N) analyzed for population differentiation.

<table>
<thead>
<tr>
<th>Location</th>
<th>Season/year</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC/VA</td>
<td>Spring 2007</td>
<td>49</td>
</tr>
<tr>
<td>DE/NJ</td>
<td>Spring 2007</td>
<td>49</td>
</tr>
<tr>
<td>Gulf of Maine</td>
<td>Spring 2007</td>
<td>48</td>
</tr>
<tr>
<td>Gulf of Maine</td>
<td>Autumn 2007</td>
<td>54</td>
</tr>
<tr>
<td>Gulf of Maine</td>
<td>Summer 2008</td>
<td>55</td>
</tr>
<tr>
<td>Gulf of Maine</td>
<td>Autumn 2008</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 4.2. Population differentiation for *S. acanthias* in the Western North Atlantic Ocean. Ten microsatellite loci were screened. Significance was adjusted using a Bonferroni correction ($\alpha = 0.05/k$, $P \leq 0.0033$) to maintain a constant $\alpha = 0.05$ throughout all pair-wise comparisons. Significantly different values are bolded.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Alleles</th>
<th>Allelic Richness</th>
<th>$H_0$</th>
<th>Null allele frequency</th>
<th>$F_{ST}$</th>
<th>$F_{ST}$ corrected</th>
<th>Differentiation P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U285</td>
<td>18</td>
<td>11.43</td>
<td>0.681</td>
<td>0.07</td>
<td>0.045</td>
<td>0.036</td>
<td>$&gt;0.001$</td>
</tr>
<tr>
<td>T289</td>
<td>11</td>
<td>7.80</td>
<td>0.356</td>
<td>0.05</td>
<td>0</td>
<td>0.014</td>
<td>0.198</td>
</tr>
<tr>
<td>J451</td>
<td>11</td>
<td>7.67</td>
<td>0.631</td>
<td>0.08</td>
<td>0.01</td>
<td>0.011</td>
<td>$&gt;0.001$</td>
</tr>
<tr>
<td>J445</td>
<td>15</td>
<td>7.72</td>
<td>0.531</td>
<td>0.12</td>
<td>0.05</td>
<td>0.047</td>
<td>$&gt;0.001$</td>
</tr>
<tr>
<td>H429</td>
<td>13</td>
<td>9.36</td>
<td>0.546</td>
<td>0.17</td>
<td>0.02</td>
<td>0.019</td>
<td>$&gt;0.001$</td>
</tr>
<tr>
<td>U273</td>
<td>16</td>
<td>12.78</td>
<td>0.736</td>
<td>0.08</td>
<td>-0.001</td>
<td>0</td>
<td>0.023</td>
</tr>
<tr>
<td>H434</td>
<td>21</td>
<td>13.01</td>
<td>0.816</td>
<td>0.04</td>
<td>-0.001</td>
<td>0</td>
<td>0.095</td>
</tr>
<tr>
<td>GA11</td>
<td>9</td>
<td>6.43</td>
<td>0.675</td>
<td>0.05</td>
<td>0</td>
<td>0.003</td>
<td>0.194</td>
</tr>
<tr>
<td>3853</td>
<td>12</td>
<td>8.00</td>
<td>0.599</td>
<td>0.06</td>
<td>0</td>
<td>0</td>
<td>0.014</td>
</tr>
<tr>
<td>6396</td>
<td>18</td>
<td>13.98</td>
<td>0.863</td>
<td>0.02</td>
<td>0.002</td>
<td>0.002</td>
<td>0.296</td>
</tr>
<tr>
<td>Overall</td>
<td>144</td>
<td>9.90</td>
<td>0.643</td>
<td>0.06</td>
<td>0.012</td>
<td>0.013</td>
<td>$&gt;0.001$</td>
</tr>
</tbody>
</table>
Table 4.3 Population differentiation for *S. acanthias* in the Western North Atlantic Ocean based on location (North Carolina/Virginia, NC/VA; Delaware/ New Jersey, DE/NJ; Gulf of Maine, GoM), season, and year without correction for possible null alleles. Pair-wise comparisons of $F_{ST}$ values were performed using Fisher’s exact tests between all sampling locations with P-values shown in parentheses. Significance was adjusted using a Bonferroni correction ($\alpha = 0.05/\text{number of pairwise comparisons}$, $P$ is significant at $\leq 0.003$) to maintain a constant $\alpha = 0.05$ throughout all pair-wise comparisons. Significantly different sampling locations/seasons are bolded.

<table>
<thead>
<tr>
<th></th>
<th>NC/VA Spring 07</th>
<th>DE/NJ Spring 07</th>
<th>GoM Spring 07</th>
<th>GoM Autumn 07</th>
<th>GoM Summer 08</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE/NJ Spring 07</td>
<td>0.00106 (0.541)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GoM Spring 07</td>
<td>0.00017 (0.577)</td>
<td>0.00171 (0.468)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GoM Autumn 07</td>
<td><strong>0.01023 (&lt; 0.001)</strong></td>
<td><strong>0.01158 (&lt; 0.001)</strong></td>
<td>0.00709 (0.018)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GoM Summer 08</td>
<td>0.01871 (&lt; 0.001)</td>
<td>0.01967 (&lt; 0.001)</td>
<td><strong>0.01946 (&lt; 0.001)</strong></td>
<td><strong>0.03853 (&lt; 0.001)</strong></td>
<td></td>
</tr>
<tr>
<td>GoM Autumn 08</td>
<td><strong>0.01183 (&lt; 0.001)</strong></td>
<td><strong>0.00885 (&lt; 0.001)</strong></td>
<td>0.00789 (0.009)</td>
<td><strong>0.02010 (&lt; 0.001)</strong></td>
<td><strong>0.01328 (&lt; 0.001)</strong></td>
</tr>
</tbody>
</table>
CHAPTER 5

CONCLUSIONS

Following the increased fishing pressure on *S. acanthias* in the late 1990’s, updated and accurate life history information is needed for development of effective management plans. In the present study, I determined that histological staining of the vertebra provided better age estimates of *S. acanthias* than the dorsal fin spine. In addition, I also showed that a number of life history parameters have changed, including a decrease in size and age at maturity, and a decrease in fecundity. Although there is no direct evidence that these differences in life history parameters are caused by fishing pressure, the changes observed in *S. acanthias* follow a similar trend as other fish populations that have faced heavy size selective fishing pressure (Buxton 1993; Rijnsdorp 1993; Rowell 1993; Sminkey and Musick 1995).

Regardless of the cause(s) of these life history shifts, fisheries biology is an applied science, and the new parameters must be taken into account to effectively and sustainably manage the *S. acanthias* populations in the Western North Atlantic. The findings that female *S. acanthias* are smaller and younger at maturity could have both positive and negative effects to population resiliency and long-term management.
strategies. Because female *S. acanthias* reach maturity, on average, at a younger age than prior to the increased fishing pressure, they reproduce sooner and have an increased number of reproductive events throughout their lifetime. Conversely, by reaching maturity at a smaller size, combined with a decrease in growth following maturity and fecundity being correlated to size (Sosebee 2005), there is a compounded effect on lifetime fecundity, with fewer offspring being produced at each reproductive event. These changes can be due to either phenotypic plasticity or genetic changes brought about by selective fishing pressure (Reznick et al. 1990; Law 2000) which may take short or long times, respectively, to revert to previous conditions. In the future, by continuing to monitor population dynamics of *S. acanthias* in the Western North Atlantic, the mechanism(s) that contribute to shifts in life history can be elucidated, allowing fishery managers to take appropriate action for the future.

The presence of population structuring of *S. acanthias* within the Western North Atlantic also raises further research questions regarding the assumption of a single unit stock. Our pop-up satellite tag data on *S. acanthias* behavior and migration, as well as population structure data, indicate that current population sampling techniques are inadequate (Sulikowski et al. 2010), resulting in skewed estimates of population biomass. In order to better characterize and delineate *S. acanthias* populations, an intensive sampling regime that includes more sample collections from various geographic locations (spatial) in the Gulf of Maine and the northern portion of the range in Canada, and over all seasons (temporal), should be initiated. Further, when these samples are collected, the gender and maturity of individuals must be noted to determine if differential movement between these groups could be the cause of the population structuring.
In conclusion, the present study employed new and more accurate methods for assessing life history parameters of *S. acanthias* in the Western North Atlantic. The updated data will serve as guidelines for recovery efforts for *S. acanthias*. By utilizing the life history parameters characterized in the present study, by continual monitoring of population dynamics, and with further delineation of *S. acanthias* population structure in the Western North Atlantic, I hope that the fishery can be effectively and sustainably managed in the future.
LIST OF REFERENCES


Stevens, J.D. and West, G.J. 1997. Investigation of school and gummy shark nursery areas in south-eastern Australia. *Final Report to the Fisheries Research and Development Corporation for Project 93/61*.


124


APPENDICES
APPENDIX A

VERTEBRA STAINING PROTOCOL
Glycerine Jelly Preparation

- Kaiser Glycerine Jelly Recipe:
  - Water 40 mL
  - Gelatin 7 g
  - Glycerol 50 mL
  - Listerine 10 mL

- Mix water and glycerol together
- Sprinkle gelatin over the water/glycerol mixture and let sit for 5 minutes
- Melt at low heat or with a double boiler on a hot plate (gelatin melting temperature = 40° C)
- Once gelatin is in solution, remove from heat and add Listerine, stirring slowly to avoid bubbles
- Allow to cool overnight

Preparation

- Prepare acid alcohol solution with 65% distilled water, 35% ethanol, and 6 drops of hydrochloric acid per 100 mL
- Remove excess tissue from the vertebral column, making sure not to remove the neural arch
- Separate each vertebral centra

Sectioning

- Place seven spacers (enough to make 400 – 500 µm thick sections) between the two blades of the gem saw
- Section the vertebra along a transverse plane to a thickness of 400 – 500 µm
- If not continuing the process immediately, store sections in 70% ethanol

**Decalcification**

- Fill a large beaker with RDO, place a magnetic stir bar in the beaker, and place on a stir plate for constant movement
- Place sections in a separated tissue cassette and put into the RDO
- Let sections soak in the RDO for 0.5 to 1.5 hours depending on the size
  - Larger vertebrae require longer times
- Remove tissue cassettes with vertebral sections and place in a beaker under running water for 1 hour
  - Decalcified sections can be stored for future processing in 70% ethanol

**Staining**

- Label individual tissue cassettes with the sample number
- Place two biopsy foam pads within each tissue cassette and place corresponding vertebrae between them
- Begin processing according to the protocol listed below
  - Place solutions in a beaker and put on an orbital shaker to keep solutions moving
  - Place tissue cassettes in the solution for specified time
<table>
<thead>
<tr>
<th>Step</th>
<th>Formula</th>
<th>Time</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% Distilled H₂O</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Harris Hematoxylin</td>
<td>10 minutes</td>
<td>Sections should be checked to ensure proper staining</td>
</tr>
<tr>
<td>3</td>
<td>Water rinse</td>
<td>until clear</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Acid Alcohol</td>
<td>4 minutes</td>
<td>This can be adjusted depending on staining strength</td>
</tr>
<tr>
<td>5</td>
<td>Water rinse</td>
<td>1 minute</td>
<td>Use agitation</td>
</tr>
<tr>
<td>6</td>
<td>running water</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Distilled water</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25% Glycerin</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>50% Glycerin</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>75% Glycerin</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>100% Glycerin</td>
<td>10 minutes</td>
<td>Tissues can be stored for longer periods at this step</td>
</tr>
</tbody>
</table>

**Section Mounting**

- Place sectioned vertebra and a small amount of glycerin jelly on labeled slide and place on a hot plate set at temperature low enough to melt glycerin jelly in 30 to 60 seconds
  - If temperature is set too high, glycerin jelly will be too hot and cause air bubbles
- Place coverslip over the vertebra and melted glycerin jelly
  - Apply the light pressure to ensure that the glycerin jelly covers the vertebra
- Allow to cool
APPENDIX B

STEROID HORMONE RADIOIMMUNOASSAY
Solution Preparation

Phosphate Buffered Saline with Gelatin assay buffer (PBSG)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$·H$_2$O</td>
<td>5.38 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$·7H$_2$O</td>
<td>16.35 g</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>9.0 g</td>
</tr>
</tbody>
</table>

- Place gelatin in a small (40 ml) beaker with some 18 Megohm H$_2$O.
- Heat on low until dissolved completely, stirring if necessary.
- Add the rest of constituent materials to a beaker containing 800 ml 18 Megohm H$_2$O.
- When gelatin is dissolved, add slowly to the beaker.
- Adjust pH to 7.0 using NaOH or HCl and add to 1 L with 18 Megohm H$_2$O.

Charcoal suspension

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran T-70</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Prewashed Norit A charcoal</td>
<td>0.2 g</td>
</tr>
<tr>
<td>PBSG</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

- Add together and stir on ice for at least 30 minutes on a stir plate at high speed before use.
**Hormone Extraction**

1. Thaw sample

2. Label two 13 x 100 mm borosilicate tubes per sample

3. Aliquot sample into labeled borosilicate tube
   - Estradiol – 200 µL
   - Testosterone – 200 µL
   - Progesterone – 500 µL - immature females, stage C and D mature females, and all male samples
   - Progesterone – 400 µL - stage A and B mature females

4. Add 50 µL of respective tritiated steroid hormone (~1000 cpm)

5. Add 10 volumes of solvent
   - Progesterone = petroleum ether
   - Estradiol and testosterone = diethyl ether

6. Vortex for 1 minute

7. Snap freeze aqueous phase in dry ice-acetone bath

8. Decant organic phase into other labeled 13 x 100 mm borosilicate tube

9. Place tubes in 37° C H₂O bath and evaporate under a stream of nitrogen

10. Repeat steps (5) through (9)

11. Wash residue on sides of tubes with ~ 500 µL of solvent and evaporate

12. Reconstitute samples with Phosphate Buffered Saline with Gelatin (PBSG)
   - Estradiol and Testosterone – 200 µL
   - Progesterone – 250 µL – Immature females
   - Progesterone – 400 µL – Stage A and B mature females

133
- Progesterone – 500 µL – Stage C and D mature females and all males

13. Vortex and let stand at room temperature for 30 minutes

14. Remove 50 µL of reconstituted samples into labeled scintillation tubes for extraction efficiency

- Add 4 mL of Ready Safe™ cocktail (Beckman Coulter, Somerset, N.J.)

- Using scintillation counter, determine counts per minute at 5 minutes per samples

15. Cover remaining reconstituted samples with parafilm and store at 4°C

Assay Protocol (Day 1)

1. Label 12 x 75 mm borosilicate tubes

2. Add PBSG, standards (in triplicate), and two aliquot sizes of samples (in duplicate) to tubes according to:

<table>
<thead>
<tr>
<th>Standards (per 100 µL)</th>
<th>^3H-T</th>
<th>Antibody</th>
<th>PBSG</th>
<th>Standard/Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>100 µL</td>
<td></td>
<td>300 µL</td>
<td></td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>100 µL</td>
<td></td>
<td>300 µL</td>
<td></td>
</tr>
<tr>
<td>Zero</td>
<td>100 µL</td>
<td>50 µL</td>
<td>250 µL</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>25</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>50</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>100</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>200</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>400</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>800</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>1600</td>
<td>100 µL</td>
<td>50 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>Samples</td>
<td>100 µL</td>
<td>50 µL</td>
<td>*</td>
<td>varies</td>
</tr>
</tbody>
</table>

* Depends on aliquot size used for sample. Make total volume up to 400 µL

3. Cover tubes with parafilm and incubate at 4°C overnight (12 hours)
Assay Protocol (Day 2)

1. Place tubes on ice
2. Add charcoal suspension into all tubes, except totals, at the beginning of a 10 minute period. Charcoal suspension should be kept continuously stirring in an ice bath
   - Estradiol and Testosterone - 1000 µL
   - Progesterone – 750 µL
3. Add PBSG to totals tubes equivalent to volume of charcoal used in other tubes
4. Place all tubes in centrifuge carriers, place in the centrifuge and wait the remainder of the 10 minute period
5. Centrifuge for 10 minutes at 4° C at 1500 x g
6. Decant supernatant into the corresponding scintillation vials and add 4.5 mL of Ready Safe™ cocktail (Beckman Coulter, Somerset, N.J.) and vortex
7. Count samples in a Beckman liquid scintillation counter LS6000IC
APPENDIX C

GENETIC ANALYSIS PROTOCOL
DNA extraction

- Heat Dubnoff shaking water bath to 56°
- Label 3 1.5 mL eppendorf tubes and one mini spin column for each sample
- Measure 15 – 25 µL of fin clip and place in its correspondingly labeled 1.5 mL eppendorf tubes with 180 µL of buffer ATL
- Continue protocol according to Qiagen DNeasy® found at:
  http://www.qiagen.com/HB/DNeasyBloodTissueKit_EN
  o Modifications:
    ▪ Spun for 3 minutes at 12,000 rpm following addition of AW2 buffer

Polymerase Chain Reaction (PCR)

- Remove samples, dNTP’s, forward and reverse primers, and PCR buffer from freezer and allow to thaw completely
  o Keep Taq-polymerase in the freezer until use.
- Pipette 1 µL of sample into each well
  o Place all samples on same side of the well
    ▪ Can be done in advance
      • Store in freezer with cap strips on
- Make pre-mix and keep on ice until use:

<table>
<thead>
<tr>
<th></th>
<th>Per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved H₂O</td>
<td>6.15 µL</td>
</tr>
<tr>
<td>PCR Buffer</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>0.2 µL per dNTP (0.8 µL total)</td>
</tr>
<tr>
<td>Polymerase</td>
<td>0.05 µL</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.5 µL</td>
</tr>
</tbody>
</table>
- Make enough for full reaction of all samples plus some extra
  o For a 96 well plate, make enough for 105 reactions
- Using multi-channel pipette, pipette 9 µL into each well on opposite wall from where samples were pipetted
  o Total reaction volume is 10 µL
- Place cap strips on wells and keep on ice until next steps
- Use balance to equalize plate with samples and blank plate including carriers for each
  o Use water in blank plate to balance
- Spin on pulse for 30 seconds to spin solutions to the bottom of the wells
- Begin program on the thermocycler for appropriate annealing temperature
  o Program for reaction:
    1. 94° for 5 minutes (Initial denaturation)
    2. 94° for 1 minute (denaturation)
    3. ___° for 1 minute (Primer specific annealing temperature)
    4. 72° for 1 minute (Elongation)
    5. GOTO step 2, 34 times
    6. 72° for 5 minutes (Final elongation)
    7. 4° for 0 hours, 0 minutes, and 0 seconds (hold)
    8. END

- Once the thermocycler reaches 94°, place plate in and run reaction
- After completion, remove plate, label, and freeze for storage
Warning: Product will evaporate if stored for longer than a couple months

- Verification of amplification can be done via agarose gel electrophoresis
  (Only needs to be done if you are unsure if amplification is occurring)

- TAE buffer (50x solution):
  - 242 g Tris
  - 57.1 mL Acetic Acid
  - 100 mL 0.5 M EDTA (or 37.2g Na₂EDTA · 2H₂O)
  - Make to 1 L with distilled H₂O
  - pH = 8.5

- Dilute to 1x concentration for use
  - 20 mL 50x TAE buffer + 980 mL distilled H₂O

- Agarose gel. In a 250mL flask add:
  - 2 g agarose
  - 100 mL TAE buffer (1x)
  - Microwave until all agarose is dissolved (~1.5 minutes, stopping when solution begins to boil)
  - Using running water, cool so that the flask can be handled
  - Add 1 µL Ethidium bromide to solution
  - Place gel molds and combs into electrophoresis apparatus
  - Pour solution into molds (~ 50 mL per gel)
  - Allow to solidify for at least an hour before removing combs

- Once gel is solidified:
- Pour TAE (1x) into the gel box until it barely covers the gel

  o Samples
    - Remove desired amount of sample (5 mL should work) and place in another labeled eppendorf tube
    - Add equal amounts of loading dye

  o Pipette 5 µL of the size standard into the first well
  o Pipette sample + loading dye into remaining wells
  o Hook up electrodes making sure the gel is running towards the proper pole
  o Run gel at 70 volts for ~ 1 hour or until separation of bands in the dye is seen
  o Remove gel and photograph using transilluminator under UV light

Genotyping

- Combine reaction products for same sample but different primers into one well

- Complete submission form for Hubbard Genome Center

- Label plate with appropriate number given during submission and bring to Hubbard Genome Center

- Analyze results using Peak Scanner 1.0

  o Settings:
    - Size standard: GS500
    - Analysis method:
      - Adjust sizing quality
- Low quality range from 0.00 to 0.00
  - Peaks were hand scored
  - Values were binned to nearest number based on nucleotide size of the locus

**Data Formatting**

- Data were formatted for Convert:
  

  - Data could then be converted to commonly used formats for other genetics software
APPENDIX D

IACUC APPROVAL
August 2, 2006

Tsang, Paul
Animal & Nutritional Sciences
Kendall Hall
Durham, NH 03824

IACUC #: 060604
Approval Date: 07/12/2006
Review Level: B
Project: Using New Approaches to Update and Collect Fisheries Specific Data on Age Determinations of the Spiny Dogfish in the Gulf of Maine

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building,
51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564
31-Jul-2009

Tsang, Paul C
Molecular, Cellular & Biomedical Sciences, Kendall Hall
Durham, NH 03824

IACUC #: 090505
Projects: Using New Approaches to Update and Collect Fisheries Specific Data on Age Determinations of the Spiny Dogfish in the Gulf of Maine

Category: D
Approval Date: 15-Jul-2009

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction. - Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are used.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All case, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gledi Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File