Androgen receptors in the bonnethead shark, Sphyrna tiburo: CDNA cloning and tissue-specific expression in the male reproductive tract

John P. Tyminski

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Androgen Receptors in the Bonnethead Shark, *Sphyrna tiburo*:
cDNA Cloning and Tissue-Specific Expression in the Male Reproductive Tract

by

John P. Tyminski

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Biology
College of Arts and Sciences
University of South Florida

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Keywords: androgen receptor, steroid hormone, polymerase chain reaction, immunocytochemistry, mRNA probe

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Dedication

To April, Matty, and the little person that is about to enter our lives
I would like to take this opportunity to convey my heartfelt gratitude to my family, friends, and colleagues for their support on this journey. First and foremost, I must thank my wife, April, for not only her unwavering love and encouragement but for her patience. She’s made a lot of sacrifices for me while I finish this up. I am indebted to Dr. Kumar Mahadevan and Mote Marine Laboratory for their support of my efforts to earn this degree. I am sincerely grateful to Bob Hueter for providing me the flexibility, the freedom, and the environment needed to get this done. I have been helped in many ways by the exceptional staff and interns at the Center for Shark Research including Cristal Ange, Stephanie Leggett, Chip Collie, Carl Luer, Charlie Manire, and Cathy Walsh. I also want to thank Dr. Gail Prins and Lynn Birch at the University of Illinois at Chicago for their generous donation of the AR21 and AR462 peptides and Dr. Elizabeth Wilson of the University of North Carolina for her donation of the AR52 antibody. I wish to also acknowledge that many of my samples were acquired through field collections from unrelated projects that were funded through agencies such as NOAA/NMFS (to the National Shark Research Consortium), the Environmental Protection Agency, and the Mote Scientific Foundation. I am indebted to my graduate committee for their time, patience, and guidance along the way. I wish to thank Dr. Jonathan Lindzey for helping to initiate this project and for indoctrinating me into the world of molecular biology. Dr. Jefferey Yoder also provided support and useful assistance. I wish to express my sincere gratitude to my wife, April, for not only her unwavering love and encouragement but for her patience. She’s made a lot of sacrifices for me while I finish this up. I am indebted to Dr. Kumar Mahadevan and Mote Marine Laboratory for their support of my efforts to earn this degree. I am sincerely grateful to Bob Hueter for providing me the flexibility, the freedom, and the environment needed to get this done. I have been helped in many ways by the exceptional staff and interns at the Center for Shark Research including Cristal Ange, Stephanie Leggett, Chip Collie, Carl Luer, Charlie Manire, and Cathy Walsh. I also want to thank Dr. Gail Prins and Lynn Birch at the University of Illinois at Chicago for their generous donation of the AR21 and AR462 peptides and Dr. Elizabeth Wilson of the University of North Carolina for her donation of the AR52 antibody. I wish to also acknowledge that many of my samples were acquired through field collections from unrelated projects that were funded through agencies such as NOAA/NMFS (to the National Shark Research Consortium), the Environmental Protection Agency, and the Mote Scientific Foundation. I am indebted to my graduate committee for their time, patience, and guidance along the way. I wish to thank Dr. Jonathan Lindzey for helping to initiate this project and for indoctrinating me into the world of molecular biology. Dr. Jefferey Yoder also provided support and useful assistance. I wish to express my sincere
thanks to Dr. Jessica Moore for her very helpful advice along the way and insightful comments to improve this manuscript. I owe a lot to Dr. Phil Motta who welcomed me into his lab when my options were few. And despite having a project that strayed from his many areas of expertise, his insight, advice, and patience have been invaluable. And finally I owe tremendous thanks to Dr. James J. Gelsleichter. Jimmy G, you’ve managed to balance being a skilled advisor while at the same time being a great friend. That has not been an easy task at times I’m sure. I am very grateful to have had the opportunity to work so closely with you.
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Androge Receptors in the Bonnethead Shark (*Sphyrna tiburo*): cDNA Cloning and Tissue-specific Expression in the Male Reproductive Tract

John P. Tyminski

**ABSTRACT**

Androgens and the androgen receptor (AR) play important roles in virilization, spermatogenesis, and sexual behavior in vertebrates. An understanding of the distribution and levels of expression of the ARs on the cellular and tissue level demonstrates the pattern of responsiveness to the androgenic hormones in a given organism. In this study, a fragment of the AR gene was cloned and sequenced from the bonnethead shark, *Sphyrna tiburo*, an elasmobranch species with a well-defined annual reproductive cycle. Acquiring this gene sequence facilitated the construction of species-specific AR polymerase chain reaction (PCR) primers and species-specific AR mRNA probes that were used to screen reproductive tissues for evidence of AR gene expression using reverse transcription (RT)-PCR and *in situ* hybridization (ISH), respectively. The RT-PCR screens demonstrated AR gene expression in the testes, epididymides, seminal vesicles, and claspers of male sharks. The use of relative PCR revealed that these organs have variable levels of AR gene expression that significantly differ with the stage of the shark’s seasonal reproductive cycle. ISH results localized the AR RNA in the interstitial cells, Sertoli cells, and developing sperm of the testes, and mature spermatozoa within the seminal vesicles and the epididymides. Immunocytochemical methods used to detect the
AR protein using a rabbit polyclonal antibody, PG-21, produced comparable results in the shark testes but did not yield positive results in the seminal vesicles or the epididymides. However, the Leydig gland, whose secretions contribute to the seminal fluid, demonstrated consistent AR immunoreactivity. Results of ICC in male and female embryos of *S. tiburo* revealed AR protein in the developing kidney but not in the embryonic reproductive structures. By characterizing AR distribution in the reproductive tract of male *S. tiburo*, this study provides the basis for future research on the direct and indirect effects of androgenic hormones in this species.
Introduction

Steroids Hormones

Steroid hormones act by coordinating the suite of complex events involved in differentiation, reproductive development, and the physiological response to diverse stimuli (Evans, 1988). The lipophilicity of these molecules enables them to directly pass across cell plasma membranes and enter the cytoplasm and nucleus (Whitfield et al., 1999). Once in the target cell, these hormonal ligands can bind to high-affinity receptors that are expressed in a tissue specific manner (Whitfield et al., 1999). This binding of the hormone elicits a structural alteration (or transformation) of the receptor, which in turn enables the hormone-receptor complex to bind to specific sites on the DNA and directly regulate gene expression (Evans, 1988). The three major classes of steroid hormones include the adrenal steroids, vitamin D₃, and the sex steroids (Evans, 1988). Sex steroids, including progesterone, estrogen, and androgens, control reproduction and sexual development in vertebrates through binding with their cognate receptors.

Androgenic Hormones

Androgens are male sex steroids secreted by the gonads that regulate virilization, spermatogenesis, and sexual behavior (Steinberger, 1971; Borg, 1994). Testosterone (T) is the main androgen in most vertebrates and is produced largely by Leydig cells which lie between the testicular spermatocysts. In some tissues, T is converted to another biologically active form of androgen, dihydrotestosterone (DHT), by the action of 5α-
reductase (Hadley, 2000). Of the 11-oxygenated androgens, 11-ketotestosterone (11KT) appears to be the most important androgen in teleost fishes (Borg, 1994).

**Androgen Receptors**

As with all steroid hormones, the physiological action of androgenic hormones is mediated through their cognate receptors. The androgen receptor (AR) is a member of the subfamily of steroid receptors (SR) which belongs to the broader superfamily of nuclear receptors (Hadley, 2000). It has been generally considered that SRs evolved in the chordate lineage about 400 to 500 million years ago from an ancestral estrogen receptor (ER) gene via gene duplication (Baker, 1997; Baker 2003). However, the isolation of an ER ortholog from the mollusk *Aplysia californica* would indicate that SRs evolved from a primordial gene that pre-dates the origin of bilaterally symmetrical animals (Thornton *et al.*, 2003). As with all the members of the nuclear receptor superfamily, ARs have four major functional domains: (1) a hypervariable transcriptional activation domain (TAD); (2) a highly conserved DNA-binding domain (DBD); (3) a variable hinge region; and (4) a moderately conserved ligand (hormone) binding domain (LBD) (Evans, 1988; Beato, 1989; Heinlein and Chang, 2006) (Figure 1). The AR protein functions as a steroid-activated transcription factor (Rundlett *et al.*, 1990). After binding to the androgenic hormone, the AR dissociates from its coregulatory proteins, translocates to the nucleus and binds as a homodimer to a specific nucleotide sequence of DNA and positively or negatively regulates transcription of its target gene (Betka and Callard, 1998, Heinlein and Chang, 2006). Although the molecular targets of androgens...
remain largely unknown, there is evidence in certain cell types for the downstream effects of these steroid hormones. For example, growth factors such as myostatin and insulin-

Figure 1. Structure of the typical androgen receptor. The major functional domains include the transcriptional activation domain (TAD), the DNA-binding domain (DBD), a hinge region, and the ligand binding domain (LBD).
like growth factor-I appear to be direct targets of AR in mammalian satellite cells (Chen et al., 2005).

Since ARs are essential for androgens to have a physiological effect, the presence and distribution of ARs determines the pattern of cellular responsiveness to the hormonal ligand within an organism. One of the tools that has developed in recent years to facilitate this area of study has been cDNA cloning. The highly conserved nature of the AR DBD and LBD has facilitated its cDNA cloning in a number of vertebrate species including mammals (Tan et al., 1988), birds (Nastiuk and Clayton, 1994), amphibians (GenBank accession no. X58955), reptiles (Young et al., 1995) and teleost fishes (Touhata et al., 1999; Takeo and Yamashita, 1999; Todo et al., 1999; Blásquez and Piferrer, 2005). These sequences have not only provided insight into steroid receptor evolution, but also facilitated the development of species-specific molecular probes to examine the expression of these receptors on a cellular level. Significant work has been devoted to evaluating the tissue-specific expression of the AR gene and/or the presence of the AR protein in reproductive tissues as a means of understanding the role of androgenic hormones in a given organism. In non-mammalian vertebrate species, ARs have been found in testis, ovary, brain, liver, spleen, kidney, and muscle cells (Table 1). In mammals, ARs have been demonstrated in a number of male reproductive organs including the testis, epididymis, seminal vesicle, phallus, as well as spermatozoa (Table 2).
Table 1. Examples of vertebrate non-mammalian studies demonstrating presence of ARs (SBA=steroid binding assay; ICC=immunocytochemistry; ISH=in situ hybridization; AA=androgen antagonists; TA=transactivation assays; Q-PCR=quantitative PCR; WB=western blot; CS=cloning/sequencing).

<table>
<thead>
<tr>
<th>Species</th>
<th>Reprod. Stage</th>
<th>Detection Methods</th>
<th>AR Sensitive Organ/Tissue</th>
<th>Cell Types</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalus acanthias</td>
<td>Adult</td>
<td>SBA</td>
<td>Testis</td>
<td>Nuclear and cytosolic extracts</td>
<td>Cuevas and Callard 1992</td>
</tr>
<tr>
<td>Micropogonias Undulates</td>
<td>Adult</td>
<td>SBA</td>
<td>Ovary, brain, liver, drumming muscle</td>
<td>Primarily the membrane fraction, less cytosolic/nuclear binding</td>
<td>Braun and Thomas 2004</td>
</tr>
<tr>
<td>Gambusia affinis</td>
<td>Adult</td>
<td>ICC, ISH, SA</td>
<td>Distal region of anal fin rays</td>
<td>Mesenchyme, epithelial cells</td>
<td>Ogino et al. 2004</td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>Adult</td>
<td>ICC</td>
<td>Brain</td>
<td>Nuclei of neurons, some neural cytoplasm</td>
<td>Gelinas and Callard 1997</td>
</tr>
<tr>
<td>Anguilla japonica</td>
<td>Adult, Juv.</td>
<td>TA, Q-PCR</td>
<td>Spleen, liver, testis</td>
<td>N/A</td>
<td>Todo et al. 1999</td>
</tr>
<tr>
<td>Oncorhynchus Mykiss</td>
<td>Adult</td>
<td>TA</td>
<td>Testis</td>
<td>N/A</td>
<td>Takeo and Yamashita 1999</td>
</tr>
<tr>
<td>Dicentrarchus Labrax</td>
<td>Adult</td>
<td>Q-PCR</td>
<td>Testis, ovaries, brain, head, kidney, liver, spleen</td>
<td>N/A</td>
<td>Blázquez and Piferrer 2005</td>
</tr>
<tr>
<td>Rana catesbeiana</td>
<td>Adult</td>
<td>ICC</td>
<td>Laryngeal muscle (both sexes)</td>
<td>Primarily muscle fiber nuclei, some muscle fiber cytoplasm</td>
<td>Boyd et al. 1999</td>
</tr>
<tr>
<td>Triturus marmoratus</td>
<td>Adult</td>
<td>ICC, WB</td>
<td>Testis</td>
<td>Primord. germ, 1° &amp; 2° spermatogonia, spermatocytes, interstitial cells</td>
<td>Arenas et al. 2001</td>
</tr>
<tr>
<td>marmoratus</td>
<td>Adult</td>
<td>ICC</td>
<td>Brain</td>
<td>Nuclei of telencephalon, diencephalon, and mesencephalon</td>
<td>Belle and Lea 2001</td>
</tr>
<tr>
<td>Streptopelia Risoria</td>
<td>Adult</td>
<td>ICC</td>
<td>Brain</td>
<td>N/A</td>
<td>Young et al. 1995</td>
</tr>
<tr>
<td>Cnemiophorus Uniparens</td>
<td>Adult</td>
<td>CS</td>
<td>Kidney</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Examples of vertebrate mammalian studies demonstrating presence of ARs (ICC=immunocytochemistry; ISH=in situ hybridization; AA=androgen antagonists; TA=transactivation assays; Q-PCR=quantitative PCR; WB=western blot; NB=northern blotting; ImmB=immunoblotting; ImmF=immunofluorescence; SAR=steroid autoradiography).

<table>
<thead>
<tr>
<th>Species</th>
<th>Reprod. Stage</th>
<th>Detection Methods</th>
<th>AR Sensitive Organ/Tissue</th>
<th>Cell Types</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rattus norvegicus</td>
<td>Adult</td>
<td>ICC, NB, PCR</td>
<td>Testis</td>
<td>Sertoli cells</td>
<td>Hill et al. 2004</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>Adult</td>
<td>ICC, AA</td>
<td>Testis, epididymis</td>
<td>Sertoli, Leydig, myoid, epithelial, stromal</td>
<td>Zhu et al. 2000</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>Adult</td>
<td>WB, ICC, ImmB</td>
<td>Testis</td>
<td>Leydig, muscle of vessels, myoid nuclei, Sertoli, spermatids</td>
<td>Vornberger et al. 1994</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>Post-natal</td>
<td>ICC</td>
<td>Prostate</td>
<td>Mesenchymal, epithelial, smooth muscle</td>
<td>Prins and Birch 1995</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>Adult</td>
<td>ICC, ISH</td>
<td>Pituit., testis, prostate, seminal vesicle</td>
<td>Sertoli, myoid, Leydig, epithel., stromal, endothelial</td>
<td>Pelletier et al. 2000</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>Adult</td>
<td>ICC, WB</td>
<td>Testis, efferent ductules, vas deferens</td>
<td>Leydig, Sertoli, peritubular, epithelial, stromal, connective</td>
<td>Zhou et al. 2002</td>
</tr>
<tr>
<td>M. musculus</td>
<td>Embryo-Post-natal</td>
<td>SAR</td>
<td>Efferent ductules, urogenital sinus, Wolffian ducts, epididymites, ductus deferens, seminal vesicles</td>
<td>Mesenchymal/stromal cells</td>
<td>Cooke et al. 1991</td>
</tr>
<tr>
<td>Macropus eugenii</td>
<td>Embryo, Juv.</td>
<td>ICC</td>
<td>Testis, brain, urogenital sinus, phallus, Wolffian d., epidid.</td>
<td>Epithelial, mesenchyme, stroma, Sertoli, interstitial</td>
<td>Butler et al. 1998</td>
</tr>
<tr>
<td>Capra hircus</td>
<td>Adult</td>
<td>ICC</td>
<td>Efferent ductules, epididymis, and ductus deferens</td>
<td>Epithelial, connective tissue, and peritubular smooth muscle</td>
<td>Goyal et al. 1998</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Embryo</td>
<td>ICC</td>
<td>Urogenital sinus, Müllerian and Wolffian ducts</td>
<td>Epithelial, mesenchyme, Leydig precursors</td>
<td>Sajjad et al. 2004</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>Embryo</td>
<td>ICC</td>
<td>Urogenital tracts, phallus</td>
<td>Epithelial, mesenchyme</td>
<td>Sajjad et al. B 2004</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>Adult</td>
<td>ICC</td>
<td>Testis</td>
<td>Sertoli, Leydig, peritubular myoid</td>
<td>Suárez-Quian et al. 1999</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>Adult</td>
<td>ImmF, ImmB</td>
<td>Sperm</td>
<td>Midpiece at site of the mitochon.</td>
<td>Solakidi et al. 2005</td>
</tr>
</tbody>
</table>
**Elasmobranchs**

The male reproductive tract of elasmobranchs is similar to that of amphibians and amniotes in its basic organization and embryologic origins (Gilbert, 1973). The testes of the male shark are paired, symmetrical structures positioned at the anterior end of the peritoneal cavity and are the site of spermatogenesis. The testes are typically attached to the dorsal surface of this cavity by the mesorchia (Wourms, 1977). Each testis is in close association with the epigonal organ and is often embedded in the anterior portion of this immune functioning structure (Carrier *et al.*, 2004). The testes of mature sharks vary greatly in size and are often enlarged during the breeding season in species with a seasonal reproductive cycle (Parsons and Grier, 1992).

The spermatocyst (also known as a follicle or ampullae) is the functional and structural unit of the elasmobranch testis (Stanley, 1966). It is a spherical structure comprised of many spermatoblasts. A spermatoblast is defined as a single Sertoli cell and its cohort of germ cells (Parsons and Grier, 1992). Leydig-like cells are found in the interstitium of the shark testis (Pudney and Callard, 1984b). All the cells within a spermatocyst are essentially in the same stage of maturation. New cysts are formed continuously in the adult male shark from the fixed germinal sites on the lateral (or dorsolateral) aspect of the testis (Callard, 1988). In carcharhinid and sphyrid sharks, development of the spermatocysts proceeds from the germinal sites on one wall across the diameter of the testis to the opposite wall (Pratt, 1988). Once spermatogenesis is complete, a connection is made to the terminal branch of the collecting ducts that enables spermatozoa to enter the efferent ducts (Callard, 1988). In contrast, the testis of the lamnid and alopiid sharks
is divided into lobes. In these families, the germinal sites are located near the center of
each lobe and the development of the maturing spermatocysts proceeds radially from this
central zone to efferent ducts at the circumference of the lobe (Pratt, 1988).

All the ducts of the male shark are derived embryologically from the opisthonephric
(Wolffian) duct (Callard, 1988). Spermatozoa are released from the testis through
efferent ducts or *vasa efferentia*. The number of ducts in the testis of the male shark
ranges from 2 to 6 (Wourms, 1977). The efferent ducts (or *ductus efferens*) join the
epididymis which typically forms a coiled tubule and serves as storage organs for
spermatozoa. The epididymis then gives rise to the *ductus deferens* (or ampulla
epididymis) which further functions for sperm storage (Jones and Jones, 1982). The
posterior portion of the *ductus deferens* becomes a thick-walled straight tube called the
seminal vesicle (or the *ampulla ductus deferens*) (Carrier et al., 2004). The seminal
vesicle functions primarily as a repository for sperm. Typically, the posterior end of the
seminal vesicles terminates with a sphincter muscle that leads into sperm sacs which can
be highly variable in size and location (Carrier et al., 2004). Mature spermatozoa pass
from the sperm sacs into the urogenital sinus and through the urogenital papilla where it
enters the cloaca (Wourms, 1977).

Internal fertilization is accomplished in sharks by copulatory organs known as claspers.
Each clasper is formed by a cartilaginous element that supports the medial margin of the
pelvic fin and extends beyond the posterior margin as a rod (Wourms, 1977). In
immature sharks, the claspers are small and flexible (uncalcified). With the onset of
maturity, the claspers calcify, become unbendable, and form articulations with the pelvic fin base (Carrier et al., 2004). During copulation, sperm is passed from the urogenital papilla into the clasper groove where it is flushed into the oviduct by seawater expelled from the siphon sac. The siphon sacs of the male shark lie just beneath the skin on the ventral side. The sacs are filled with seawater by repeated flexing of the clasper prior to copulation (Wourms, 1977).

**Steroids/Androgens in Elasmobranchs**

In most vertebrates, gonadal steroids are produced by Leydig cells that lie between spermatocysts. However, in male elasmobranchs the majority of these hormones are synthesized by Sertoli cells within the testicular spermatocysts (Simpson and Wardle, 1967; Holstein, 1969; Pudney and Callard, 1984a; DuBois et al., 1989). There is also evidence that Leydig-like cells (Pudney and Callard, 1984a, 1984b) and true Leydig cells (Marina et al., 2002) contribute to gonadal steroidogenesis in the early stages of spermatogenesis in some elasmobranch species. However, the relative contribution of these cell types to steroidogenesis is believed to be supplemental compared to that of Sertoli cells (Gelsleichter, 2004).

Androgen-dependent targets have not been systematically studied in male elasmobranchs and only a few of the 600 species of Squaliformes and Rajiformes have been evaluated for the tissue-specific roles of steroid hormones (Callard, 1988). However, associations have been demonstrated between androgen levels and seasonal stages that suggest T and/or DHT are involved in reproduction in elasmobranchs. For example, Manire and
Rasmussen (1997) found that peak T and DHT levels in August coincided with maximum testicular development in the bonnethead shark (*Sphyrna tiburo*) but then dropped off significantly during the mating period (October-November). Studies of steroid hormone production in the Atlantic stingray, *Dasyatis sabina*, revealed that serum androgen levels in the male increased during the onset of spermatocyte development (August-October), decreased after maximum testis growth (November-December), then increased again during the peak of mating activity (January-March) (Snelson *et al.*, 1997; Tricas *et al.*, 2000). In the epaulette shark (*Hemiscyllium ocellatum*), a species with a protracted mating period, male androgen levels measured through radioimmunoassay revealed a single broad peak (July-October) that also appeared to correlate with reproductive activity (Heupel *et al.*, 1999). Cuevas and Callard (1992), through the use of steroid binding analyses on cytosolic and nuclear extracts, found direct evidence that ARs in the testis of the spiny dogfish (*Squalus acanthias*) were primarily localized in early stage (pre-meiotic and meiotic) spermatocysts. The finding that androgens are involved in early spermatogenesis was supported by a similar study using semi-quantitative RT-PCR to measure AR mRNA in the same species (Betka and Callard, 1998). The elevated levels of circulating androgens in sharks during mid and late stages of spermatogenesis may further indicate a role in development of gonoducts and/or the maturation and viability of spermatozoa. However, direct evidence of this relationship is presently missing. Piercy *et al.* (2003) found evidence that the genital ducts of *D. sabina* go through morphological and histological changes at different stages of the seasonal reproductive cycle and suggested that these changes could be directly or indirectly androgen mediated. Although the rate of clasper growth increases sharply with the onset
of testicular development (Collenot, 1969), a direct role of androgens in clasper development has not yet been demonstrated. Changes in circulating androgen levels were not found to be correlated with the rate of clasper growth in captive *S. tiburo* (Gelsleichter *et al.*, 2002).

The bonnethead shark, *Sphyrna tiburo*, represents an abundant inshore species with a well-defined seasonal reproductive cycle. Males become functionally mature and exhibit fully calcified claspers at about 2 years of age (Parsons, 1993; Gelsleichter *et al.*, 2002). Gonadal development begins in the late spring and a peak gonadosomatic index (GSI) occurs in late summer, about 2 months prior to mating (Parsons and Grier, 1992; Manire and Rasmussen, 1997). Testicular regression begins prior to mating and continues until the following spring when gonadal recrudescence begins (Parsons and Grier, 1992; Manire and Rasmussen, 1997). The steroidal cycles of this species have been well characterized (Manire *et al.*, 1995; Manire and Rasmussen, 1997; Manire *et al.*, 1999), however, the functional roles of steroids in *S. tiburo* reproduction is unknown. In the case of the androgens, it is not known how T and DHT contribute to the cellular remodeling that takes place in the reproductive structures during the course of the annual cycle.

**Objectives of this Study**

The goal of this study was to examine the functional role of androgens in the reproductive tissues of the male bonnethead shark. This was accomplished by characterizing the presence and pattern of ARs, which are essential for these sex steroid
hormones to function. The AR was primarily localized in two forms: 1) the AR mRNA; and 2) AR protein. To detect AR mRNA, a molecular probe was developed to identify tissues and/or cells expressing AR mRNA through *in situ* hybridization (ISH) and northern blotting techniques. Relative or semi-quantitative PCR was used to estimate the levels of AR mRNA expression. The presence of the AR protein in male reproductive tissues was investigated by immunocytochemistry (ICC) using an antibody specific to a portion of the AR protein from humans. A further goal of this study was to initiate a preliminary examination of the role of androgens in the embryos of *S. tiburo* using immunocytochemical methods.
Materials and Methods

Sample Collection

*Sphyrna tiburo* specimens were collected from 4 sites along the southwest Florida coast using gill nets (11.4 cm stretch mesh size). The study sites included Yankeetown (28° 59’ N, 82° 49’ W), Tampa Bay (27° 41’ N, 82° 38’W), Charlotte Harbor (26° 31’ N, 82° 08’ W), and Long Key (24° 50’N, 80 °49’ W) (Figure 2). Mature male specimens were collected throughout the year during all stages of the reproductive cycle. Immature males and embryos from pregnant females were also collected for study. Embryos were pre-implantation and ranged from 4-8 cm total length. All free-swimming specimens were measured, weighed and sexed at time of capture. Reproductive tissues (testes, epididymides, and seminal vesicles) were dissected from euthanatized or recently dead sharks and flash-frozen in liquid nitrogen and stored at -80°C until use. Additional tissues, such as clasper cartilage and heart muscle, were also collected for AR screening. To avoid RNase contamination, all instruments coming in contact with the dissected tissues were cleaned with RNaseZap® decontamination solution prior to use (Ambion, Austin, TX). All animal collection and handling procedures followed the specifications outlined in IACUC protocols for this study from the University of South Florida (#2556 under P. Motta) and Mote Marine Laboratory (06-10-JT1 under J. Tyminski).
Figure 2. Map of Florida demonstrating the collection sites used in this study.
Cloning AR cDNA

**RNA Extraction and cDNA Synthesis**

Total RNA was prepared from approximately 100 mg of mature male *S. tiburo* testis. The frozen tissue was placed in liquid nitrogen and finely ground using a pre-chilled mortar and pestle. The powdered tissue was homogenized in Trizol\textsuperscript{®} reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions with a ratio of 1.0 ml Trizol per 50-100 mg of tissue. Samples were vortexed and incubated at room temperature (RT) for 5 min. Water-saturated phenol-chloroform-isoamyl alcohol (Ambion) was added at 0.2 ml per ml of Trizol and shaken vigorously by hand for 15 seconds before a 2-3 min incubation. The mixture was then centrifuged at 12,000 g (4°C) for 15 min and the RNA-containing aqueous phase removed and precipitated in 100% isopropanol (0.5ml/ ml Trizol solution). After 10-min incubation, the sample was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed and the resultant RNA pellet was resuspended with 1.0 ml of 75% ethanol at RT. The sample was vortexed and centrifuged at 7,500 g for 5 min at 4°C. The resulting RNA pellet was briefly air-dried and resuspended in 50 μl of water treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, MO). The isolated RNA was quantified using spectrophotometry and its integrity evaluated by visual inspection after running a 2-4 μl sub-sample on a 1% agarose gel with ethidium bromide. The extracted RNA was then reverse transcribed producing complementary DNA (cDNA) using random hexamer primers and murine leukemia virus (MuLV) reverse transcriptase using the GeneAmp\textsuperscript{®} RNA PCR Core Kit (Applied Biosystems, Foster City, CA).
**PCR Primer Development**

Since no complete AR gene sequences were available for an elasmobranch at the initiation of this project, degenerate primer sequences for polymerase chain reaction (PCR) amplification of *S. tiburo* AR cDNA were designed from a comparison of GenBank-published AR sequences from 10 species (Appendix I) using Block Maker software (Henikiff *et al.*, 1995). This program aligned the sequences and identified the most conserved regions. The results of these comparisons were analyzed using CODEHOP software (Rose *et al.*, 1998) to select the most appropriate sequences for degenerate DNA primer construction. Two sets of AR oligonucleotide primers were commercially produced (Integrated DNA Technologies, Coralville, IO) using standard de-salting conditions. The primer sequences were as follows: AR3-Fw; 5′-CGGCTCCTGCAAGGTGTT(C/T)TT(C/T)AA(A/G)(A/C)G-3′; AR3-Rv; 5′-GAGATGATCTCGGCATCAT(C/T)TC(deoxy-inosine)GG-3′; ARn-Fw; 5′-(A/C)G(A/C/G/T)(A/C)G(A/C/G/T)AA(A/G)AA(C/T)TG(C/T)CC-3′; ARn-Rv; 5′-(C/T)TG(A/C/G/T)C(G/T)(C/T)TC(A/C/G/T)CC(A/C/G/T)A(A/G)(C/T)TC-3′. Efforts were also made to clone β-actin with the intention of utilizing this constitutively expressed gene as a standard and/or a positive control during downstream applications. Non-degenerate primers were constructed using sequences successfully used to amplify a portion of this gene in the houndshark (*Triakis scyllium*) (GenBank Accession No. AB084472). The actin primers used were BA-Fw; 5′-GGATGATGAAATTGCAGC-3′ and BA-Rv; 5′-CGTTGTAAGAAATGTCGAGC-3′ (C. Harutu, personal communication, July 31, 2002).
**PCR Conditions**

To amplify the AR cDNA fragment, a nested PCR strategy was employed in which two sets of AR primers were used in two successive reactions. The product of the first reaction (AR3 primers) was used as the cDNA template for re-amplification in a second reaction using the ARn primers that anneal to internal sites of that initial product. All PCR reactions included 10 pmol of the respective degenerate primers in a 25 μl reaction run on a Techgene compact thermocycler (Techne, Cambridge, UK). The PCR conditions for both reactions were identical: 2 min at 94°C, 20 cycles of 30 s at 95°C, 30 s at 60°C with decreasing temperature proportionately each cycle to 51°C, 1 min at 72°C followed by 15 additional cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C then 7 min at 72°C. Standard PCR was used to amplify β-actin cDNA utilizing non-degenerate primers and a program of 95°C for 2 min followed by 33 cycles of 45 s at 95°, 45 s at 50°, and 45 s at 72° then 7 min at 72°. All PCR products were analyzed on 1.2% agarose gels stained with ethidium bromide.

**Cloning and Sequencing**

PCR products corresponding to the predicted AR 324 bp fragment and the 275 bp β-actin gene fragment were recovered from agarose gels and purified using the Qiaex II Gel Extraction Kit (Qiagen, Germantown, MD). The putative AR and β-actin fragments were ligated into pCRII plasmid vectors (TA Cloning® Kit; Invitrogen) and transformed into *E. coli* competent cells (One Shot®, Invitrogen) following the Invitrogen protocol. Positive (white) colonies were selected and cultured in Luria broth containing 100 μg/ml of ampicillin. Plasmids were isolated using the PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen). A restriction digest of the purified plasmid DNA using
EcoRI verified the presence of an insert of the appropriate size. The cloned gene fragments were sequenced by an independent laboratory (Macrogen, Inc., Seoul, Korea) using a 3730xl DNA Analyzer and the results submitted to BLASTX for comparison to known sequences (Altschul et al., 1997). The sequence was aligned against other known AR sequences using CLUSTAL W (Thompson et al., 1994) and BOXSHADE 3.21.

**PCR for Evaluating AR**

*Isolation of Total RNA and Reverse Transcription*

Total RNA was isolated using the RNeasy® Mini kit (Qiagen) following the manufacturer’s protocol. The extracted RNA was quantified by spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE) and examined for quality by running a 2 μl aliquot on a 1% agarose gel with ethidium bromide. The RNA was then reverse transcribed using the Advantage® RT-for-PCR Kit (Clontech, Mountain View, CA) with 0.5 μg of total RNA and random hexamer primers. The cDNA from samples of *S. tiburo* testes, epididymides, and seminal vesicles were aliquoted and stored at -80°C.

*Bonnethead Shark Specific Primers and AR Screening*

*S. tiburo*-specific non-degenerate AR primers were designed from the sequence data obtained through cloning (AR-BH1-Fw; 5′-ATGCCGTCTGAGAAAGTGCT-3′; AR-BH1-Rv; 5′-AAATTGCTGCATCCTCGGTA-3′). These primers were tested in a PCR reaction using cDNA from *S. tiburo* testis and resulted in the amplification of the expected 174 bp fragment. These primers were then utilized to screen total RNA extracted from reproductive tissues for AR expression using the Titanium™ One Step
RT-PCR Kit (BD Biosciences, Palo Alto, CA). Similarly, *S. tiburo* specific primers were generated for \( \beta\text{-actin} \) for potential use as a positive control for PCR (BH-actin2-Fw; 5′-GAATTGCAGCGCTTGTCATA-3′; BH-actin2-Rv; 5′-TCTCCATGTCATCCCATCCAGTTG-3′).

**Relative PCR to Quantify AR Gene Expression**

Quantification of AR gene transcripts was achieved through the use of multiplex PCR reactions using *S. tiburo*-specific AR primers and Competimer™/18s rRNA oligonucleotides (Ambion). These universal oligonucleotide primers are a more effective standard than the more commonly used \( \beta\text{-actin} \) since rRNA levels do not vary from tissue to tissue. However, since rRNA is such a major component of total RNA, the addition of the Competimers is necessary to modulate the efficiency of the PCR template while not affecting the performance of the AR target within the multiplex PCR. To evaluate the log-linear range of AR product formation, an individual mature male testis cDNA sample was used in a series of identical PCR reactions using the AR primers. Individual reaction tubes were sequentially removed from the thermocycler at two cycle intervals starting with the 21st and ending with the 39th cycle. Subsequent gel electrophoresis and image analysis of these PCR products indicated that the mid-point of the detectable log-linear range occurred after 28 cycles of amplification. An annealing temperature of 68°C was found to be optimal for the *S. tiburo*-specific AR primers. Since the 18s primers function efficiently with annealing temperatures from 55-68 C°, it was determined that a 68°C annealing temperature could be used for all multiplex relative PCR reactions. Hence the relative PCR conditions were: 94°C for 3 min followed by 28 cycles of 94°C for 45 s, 68°C for 1 min, and 72°C for 1 min with a final extension of 72°C for 5 min. Once the appropriate
annealing temperature and ideal number of cycles were determined, another series of identical multiplex reactions were conducted to assess the appropriate Competimer to 18s primer ratio. Increasing concentrations of 18s Competimers were added to a series of reactions containing both the 18s and AR primers and run using the aforementioned PCR conditions. A 9:1 ratio of Competimer to 18s primer was found to be optimal to produce an 18s band of the same relative intensity as that of the amplified AR product. Samples of cDNA from the 3 reproductive structures were amplified using this established method and electrophoretically separated on 1.5% agarose gels with ethidium bromide and digitally photographed under UV light. Individual bands from the images were identified and measured for maximum optical density (MOD) using Gel-Pro Express 4.0 software (Media Cybernetics Inc., Bethesda, MD). For each individual RNA sample evaluated, the MOD of the AR band was divided by the MOD of the 18s band to produce a relative measure of AR gene expression. Samples were grouped by reproductive stage using criteria established in previous studies (Gelsleichter et al., 2003). The stages were defined as resting/quiescent (December-April), spermatogenesis (May-August), and mating (September-November). Mean AR to 18s ratios by stage were analyzed for significance using a one-way analysis of variance (ANOVA) followed by the Tukey’s Honestly Significant Differences (HSD) test (InStat 3, GraphPad Software Inc., San Diego, CA). Data sets that failed tests of normality and/or equal variance were log-transformed prior to analysis.
**In Situ Hybridization**

**Probe Labeling**

AR antisense and sense cRNA probes were constructed by *in vitro* transcription using the MEGAscript® High Yield Transcription Kit (Ambion). Linearized plasmid containing the AR fragment was used as the DNA template for a digoxigenin (DIG)-labeled probe with DIG-labeled UTP (Roche Applied Science, Indianapolis, IN). Both RNA probes were evaluated for their labeling efficiency using the direct detection method as described in the DIG Northern Starter Kit (Roche).

**Preparation of Tissue Sections**

Tissue sections for *in situ* hybridization (ISH) were fixed for 48 h in 4% paraformaldehyde prepared using phosphate buffered saline modified for use with elasmobranch tissues (E-PBS) (Appendix II) then transferred to 100% ethanol for storage. Tissues were removed from the ethanol and incubated overnight in a 30% sucrose solution made with water treated with 0.1% dimethyl pyrocarbonate (DMPC) (Sigma). The ISH protocol followed the procedures outlined by Dijkman *et al.* (1995) with some modifications. After embedding in Tissue-Tek® O.C.T. compound (Sakura Finetek U.S.A., Inc., Torrance, CA), 5 μm frozen sections were cut on a Minotome Plus™ cryostat, (Triangle Biomedical Sciences, Durham, NC), mounted directly on Superfrost Plus slides (A. Daigger and Company, Wheeling, IL), and incubated overnight at 40°C to fix the RNA in the tissue. Tissue sections were then used immediately for ISH or stored for later use at -80°C.
**Pretreatment of Slides**

Frozen slides were incubated for 2 h at 40°C and each tissue was then circled using a PAP pen (Electron Microscopy Sciences, Hatfield, PA). Tissue sections were incubated in phosphate buffered saline (PBS) containing 4% paraformaldehyde for 5 min at RT. The sections were then washed once with PBS for 3 min then twice for 5 min with 2x SSC (Ambion).

**Prehybridization, Hybridization, and Post Hybridization**

Each section was prehybridized for 60 min at 37°C in 25 μl of hybridization buffer (Appendix III). The hybridization buffer was discarded and each section was covered with 25 μl of hybridization buffer containing 200 ng/ml of DIG-labeled antisense cRNA probe for AR and incubated for 16-24 h at 37°C. Control sections were incubated with the DIG-labeled sense cRNA probe. Unbound probe was removed by a series of washes: 5 min in 2x SSC at 37°C, 3 washes of 5 min each with 60% formamide in 0.2x SSC at 37°C, and 2 washes of 5 min each with 2x SSC at RT.

**Immunological Detection**

Immunological detection of DIG-labeled RNA probes followed the procedures outlined in the DIG Northern Starter Kit (Roche). Briefly, sections were washed for 5 min at RT in DIG washing buffer then incubated for 30 min at RT with DIG blocking buffer. The tissue sections were then incubated for 2 h at RT with alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:200 in blocking buffer. This was followed by 3 washes in washing buffer at RT for 5 min each. Each individual tissue group then received 25 μl of detection buffer for 10 min at RT. Sections were then covered with detection buffer containing BCIP and levamisole and allowed to incubate overnight at
RT. The color reaction was stopped by rinsing the sections in a washing buffer (1mM EDTA in 10mM Tris, pH 8). Sections were then washed for 5 min in reverse osmosis (RO) water and counterstained for 5 min in nuclear fast red (Vector Laboratories, Inc., Burlingame, CA). Slides were washed for 5 min in RO water, then mounted using aqueous mounting media for histological examination.

Detection of AR Protein Using Immunocytochemistry

Histology

Male *S. tiburo* reproductive organs (testes, epididymides, seminal vesicles) and embryos were fixed in 10% formalin prepared using E-PBS. Following a 48 h fixation period, the sections were transferred to 70% ethanol for long-term storage. The fixed tissue sections were trimmed then dehydrated in a graded series of alcohols (80-100%), cleared in a limonene-based xylene substitute (CitriSolv™, Fisher Scientific, Fair Lawn, NJ), and then processed for routine paraffin histology. Reproductive organs and embryos were cross-sectioned (5 μm) using a rotary microtome (‘820’ Spencer, American Optical Corporation, Buffalo, NY) and placed on microscope slides coated with 0.01% poly-L-lysine (Sigma).

Immunocytochemistry

The presence of immunoreactive AR protein in *S. tiburo* was determined according to methods outlined by Nichols *et al.* (2003). A rabbit polyclonal antibody against the first 21 amino acids of the human AR (PG-21, Upstate, Lake Placid, NY) was used as the primary antiserum. Initially, an alternative primary antibody directed against amino acids 527-541 of the rat AR was utilized (AR52, given by E. Wilson, University of North
Carolina) but was not found to be cross-reactive with *S. tiburo* AR. Tissue sections were de-paraffinized using a limonene-based solvent and re-hydrated in a descending, graded series of histology-grade alcohols (100-95%). Following a 20 min tap water rinse, the tissue sections were incubated in an antigen retrieval solution (10 mM sodium citrate, pH 6.0) for 20 min in a 95°C water bath to expose AR epitopes. The sections were cooled to RT then rinsed in RO water and incubated for 30 min at RT in a solution of 3% hydrogen peroxide and 100% methanol (1:1) to quench endogenous peroxidase activity. Afterwards, the sections were rinsed in PBS and incubated overnight at 4°C in 2% normal horse serum to block nonspecific binding. Tissue sections were brought to RT, rinsed twice with PBS, and incubated with the primary antibody (1:100) diluted in PBS containing 0.1% gelatin and 0.1% sodium azide (G-PBS) overnight at 4°C. Slides were rinsed with PBS containing 0.05% Tween-20 (PBS-T), twice rinsed with PBS, then incubated for 60 min at RT with the ImmPress™ Reagent Anti-Rabbit Ig (Vector). After 3 PBS rinses, diaminobenzidine hydrochloride (DAB) (Vector) was applied to the sections for 5-8 min to reveal reddish-brown staining as an indicator of antigen-antibody complexes. After color development, sections were rinsed in tap water and counterstained in 2% methyl green (Vector) for 45-60 min at 37°C. The fully processed sections were then rinsed in tap water for 2 min, dehydrated in an ascending, graded series of alcohols (95-100%), cleared in a limonene-based medium, and mounted using Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, MI). A series of controls were included in the immunocytochemical methods including: 1) stepwise deletion of all stages of the immunocytochemical procedure; 2) replacement of the primary antibody with nonimmune rabbit IgG or dilutant; 3) pre-absorption of the primary antibody with a 10-
fold excess of the AR21 protein (the antigen used to make the PG-21 antibody); and 4) pre-absorption of the primary antibody with a 10-fold excess of a similarly sized peptide from the androgen receptor (AR462). Once an organ was established as being AR-positive via the pre-absorbed controls, sections of this organ type were included in all subsequent ICC runs as a positive control. In addition to the antibody concentration described above, a suite of reproductive organ sections were also run with a PG-21 titer of 1:50 to assess whether increased antibody concentration would improve the sensitivity of the immunostaining.

**Immunoblotting**

Tissue sections were homogenized in 2 volumes of lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium orthovanadate, 20 mM disodium pyrophosphate, 10% glycerol and 1% Triton X-100 [pH 7.2]) (Chieffi et al., 2000) and then centrifuged at 14,000 g for 15 min at 4°C. The supernatant was mixed with 2 volumes of sample buffer and heated in a water bath for 5 min at 95°C. Proteins were separated via SDS gel electrophoresis under denaturing and reducing conditions using 10% polyacrylamide gels and the Laemmli buffer system. Gels were fixed in a standard fixation solution (40% methanol and 10% acetic acid) and stained with fixation solution with 0.25% coomassie blue. For immunoblotting, proteins were transferred from the gel to nitrocellulose membranes (Bio-Rad, Hercules, CA) which were then incubated in 10% nonfat dry milk in Tris-buffered saline (TBS) (0.138 M NaCl, 0.0027 M KCl [ph 8.0]) for 2 h to block non-specific binding. Immunoreactive AR protein was demonstrated using PG-21 as the primary antibody (diluted 1/500 in TBS containing 0.05% Tween-20 and
1% nonfat dry milk), the Mouse ExtrAvidin Alkaline Phosphatase Staining Kit (Sigma), and NBT/BCIP. Membranes were rinsed thoroughly in TBS containing 0.05% Tween-20 between each incubation. After color reaction, membranes were rinsed in RO water and air-dried.

**Northern Blotting**

The northern blotting procedure followed the methodology outlined by Bowman and Denslow (1999) with some modifications. Total RNA extracted from 5 mature male *S. tiburo* testes were pooled (20 μg) and then concentrated in a microfuge tube to near dryness in a Vacufuge™ Concentrator 5301 (Eppendorf, Westbury, NY). The RNA was then brought to a 16 μl volume with denaturing solution. An RNA marker with a range of 281 to 6,583 bases (Promega, Madison, WI) was prepared using 6 μl of marker and 10 μl denaturing solution. Both samples and markers were denatured in a 65°C water bath for 15 min. A volume of 5 μl of NorthernMax® formaldehyde loading dye (Ambion) was added to each sample for a total volume of 21 μl. The samples were then loaded onto a 1% agarose-formaldehyde gel and run at 50 V for 3 hr. A positively charged nylon membrane (BrightStar™-Plus, Ambion) was soaked in DEPC-treated water for 5 min prior to use. Following electrophoresis, the gel was soaked in DEPC-treated water for 1 min. Both gel and membrane were equilibrated separately in 20x SSC for 10 min at RT on an orbital shaker. A downward capillary transfer was performed overnight, as described by Bowman and Denslow (1999). The following day, the transfer stack was dismantled and the gel wells were marked on the nylon membrane with an ink pen. The nylon membrane was rinsed with 2x SSC and then dried in an incubator at 37°C for 10
min. The membrane was then crosslinked twice (120 mJ/cm²) using a UV Stratalinker® 1800 (Stratagene, La Jolla, CA). Methylene blue was used to stain the membrane for 45 sec and then destained for 2 min (Herrin and Schmidt, 1988). The blot was photographed and stored in a plastic bag at 4°C until used in the hybridization step. The membrane was prehybridized with prewarmed DIG Easy Hyb for 30 min at 68°C following the procedures outlined in the DIG Northern Starter Kit (Roche). The DIG-labeled RNA probe (100 ng/ml) was denatured by boiling for 5 min followed by rapid cooling in ice water. The denatured probe was then added to prewarmed DIG Easy Hyb and mixed thoroughly. The prehybridization solution was removed from the membrane and replaced with the probe/hybridization mixture and allowed to incubate overnight at 68°C with gentle agitation. The following day, a series of stringency washes were performed following the DIG Northern Starter Kit protocol (Roche). This included two 5-min washes in 2xSSC (0.1% SDS) at RT and two 15-min washes in 0.1xSSC (0.1% SDS) at 68°C. Afterwards, the membrane was rinsed again in DIG washing buffer (DIG Wash and Block Buffer Set, Roche) for 5 min followed by 30 min incubations in DIG blocking solution (Roche) and antibody solution containing an anti-DIG antibody (1:5,000) conjugated with alkaline phosphatase (Roche). The membrane was washed twice for 15 min in washing buffer and then allowed to equilibrate for 5 min in detection buffer containing NBT/BCIP (Roche). The membrane was then rinsed 3 times with DEPC-treated water to stop the color reaction and photographed.
Results

This study used molecular and immunocytochemical methods to evaluate the presence of the androgen receptor in the primary and secondary sex structures in *S. tiburo*. These results are summarized in Table 3.

Androgen Receptor Cloning and Sequencing

Four positive clones of putative AR cDNA gene fragments were obtained and sequenced. A BLASTX analysis of the sequence data (Figure 3) revealed that the cloned insert was a 324 bp fragment of *S. tiburo* AR cDNA. This portion of the gene shares a high degree of sequence homology with other species (Figure 4), but most closely resembled ARs of the spiny dogfish shark (*Squalus acanthias*) and the African clawed frog (*Xenopus laevis*). The cloned region of the *S. tiburo* AR gene corresponds to the conserved DBD, the variable hinge region, and the carboxy-terminal LBD. Additionally, 2 positive clones of putative *S. tiburo β-actin* cDNA fragments were also obtained and sequenced. BLASTX analysis of the sequence data (Figure 5) revealed that the cloned insert was a 274 bp fragment of the *S. tiburo β-actin* gene.
Table 3. Summary of organs, tissues, and cells of *Sphyrna tiburo* that were evaluated for the presence of the androgen receptor using PCR screening, immunocytochemistry (ICC), and *in situ* hybridization (ISH). Tissues/organs that were not evaluated or were evaluated but did not produce reliable results are identified as not determined (ND).

<table>
<thead>
<tr>
<th>Organ/Tissue/Cell Type</th>
<th>PCR Screening</th>
<th>mRNA (ISH)</th>
<th>Protein (ICC)</th>
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<tbody>
<tr>
<td>Testis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leydig-like cells</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sertoli cells</td>
<td></td>
<td>+</td>
<td>+</td>
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<tr>
<td>Germ cells</td>
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<tr>
<td>Spermatogonia</td>
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<td>Spermatocytes</td>
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<tr>
<td>Spermatids</td>
<td>+</td>
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<tr>
<td>Spermatozoa</td>
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<td>+</td>
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<tr>
<td>Epididymis</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Epithelial cells</td>
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<tr>
<td>Muscle cells</td>
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<tr>
<td>Connective tissue</td>
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<tr>
<td>Spermatozoa</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Leydig gland</td>
<td>ND</td>
<td>ND</td>
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<td>Seminal Vesicle</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>Epithelial cells</td>
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<tr>
<td>Smooth muscle</td>
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<td>-</td>
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<tr>
<td>Connective tissue</td>
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<td>Spermatozoa</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Clasper</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Embryos</td>
<td>N/A</td>
<td>ND</td>
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<tr>
<td>Developing gonad</td>
<td>ND</td>
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<td>Kidney</td>
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<td>Digestive tract</td>
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<td>Heart</td>
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Figure 3. Nucleotide and deduced amino acid sequence of the cloned androgen receptor gene fragment from the bonnethead shark (Sphyrna tiburo). The positions of the forward and reverse ARn degenerate primers are indicated by the solid boxes. The positions of the non-degenerate AR BH1 primers are indicated by the broken line boxes.
Figure 4. Comparison of the deduced amino acid sequences of the cloned *Sphyrna tiburo* androgen receptor along with examples from 8 other species. The sequences given were taken from the Genbank database and are referenced with accession numbers as follows: spiny dogfish *Squalus acanthias* (AY228761); African clawed frog *Xenopus laevis* (U67129); canary *Serinus canarius* (L25901), red sea bream *Chrysophrys major* (AB017158); human *Homo sapiens* (BC132975); rainbow trout (*α* *Oncorhynchus mykiss* (AB012095); Japanese eel *Anguilla japonica* (AB025361); and Burton’s mouthbrooder *Haplochromis burtoni* (AF121257).
Figure 5. Nucleotide and deduced amino acid sequences of the cloned β-actin gene fragment from *Sphyrna tiburo*. The positions of the non-degenerate BA primers are indicated by the broken line boxes.
AR Screening and Semi-Quantification by RT-PCR

Tissue screening using RT-PCR resulted in the amplification of the expected 174 bp AR cDNA product from RNA samples extracted from the testes, epididymides, seminal vesicles, claspers, and heart of *S. tiburo* (Figure 6). The clasper RNA samples produced noticeably weaker bands than those from the reproductive tract. Similarly, heart tissue samples yielded weakly positive results in the form of a faint 174 bp band.

The PCR results shown in Figure 7 demonstrate that 28 PCR cycles represented the midpoint of the log linear range of amplification. This was necessary to determine the optimal conditions for the relative (semi-quantitative) PCR procedure. Samples of cDNA from reproductive organs of mature sharks were amplified using semi-quantitative PCR with both AR and 18s ribosomal primers. The results of the multiplex PCR reactions produced the expected 174 bp AR band and a 315 bp 18s ribosomal band using cDNA from testes (n=33), epididymides (n=23), and seminal vesicles (n=24) (Figure 8). Statistically significant differences in AR mRNA expression levels were observed among the reproductive stages in all 3 of these organ types. For the testis, AR expression was higher during spermatogenesis relative to the resting stage (ANOVA and Tukey’s HSD test, p<0.01) and relative to mating (ANOVA and Tukey’s HSD test, p<0.05) (Figure 9a). In the epididymis, AR expression levels were significantly higher during mating than during the resting stage (ANOVA and Tukey’s HSD test, p<0.01) (Figure 9b). In the seminal vesicle, AR expression was higher during mating than during the resting stage (ANOVA and Tukey’s HSD test, p<0.01) and during spermatogenesis (ANOVA and Tukey’s HSD test, p<0.05) (Figure 9c).
Figure 6. Screening of androgen receptor mRNA in tissues/organs of *Sphyrna tiburo* using Titanium™ One-Step RT-PCR. Positive results are demonstrated by the amplification of a 174 bp band indicated by the arrow. The type of tissue/organ is identified below the bands. Testis (T), Epididymis (Epi), Seminal Vesicle (SV), Clasper (CL), and Heart (H).
Figure 7. Results of separate PCRs to evaluate the log-linear range of AR amplification in *Sphyra tiburo*, a necessary step to perform relative PCR. The products were separated on an agarose gel with ethidium bromide, visualized under UV light, and each band had its maximum optical density measured (see insert). The arrow indicates the mid point of the log linear range (28 cycles).
Figure 8. Examples of AR (174 bp) and 18s ribosomal (315 bp) bands amplified in a relative PCR using *Sphyrna tiburo* cDNA from testis (T), epididymis (Epi), and seminal vesicle (SV) samples. The reproductive stage of the sample is reported below the bands. Products were run on 1.5% agarose gels treated with ethidium bromide and photographed under UV light.
Figure 9. Results of relative PCR demonstrating the levels of AR mRNA expression in the reproductive tract of male *Sphyrna tiburo* during different stages of the reproductive cycle. The data are reported as mean AR/18s ratios of optical density (±SE) for the testis (A), epididymis (B), and seminal vesicle (C). Sample size per stage is indicated in the lower part of the bars. Significant difference (*).
**Northern Blotting**

Northern blot analysis of total RNA extracted from *S. tiburo* testes revealed 2 AR transcripts of about 7 and 9 kb (Figure 10). These two bands were faint which suggest a relatively low level of AR expression. Staining observed in other parts of the membrane appeared to be artifactual.

**In Situ Hybridization**

Expression of AR was observed in the Leydig-like, interstitial cells that lie between the spermatocysts of the testis of mature *S. tiburo* (Figure 11A-D). Within post-meiotic spermatocysts, AR positive staining was observed in Sertoli cells (Figure 12 A-D). AR was also expressed in the nuclei of spermatids (Figure 13A, B) and spermatozoa (Figure 13C, D) within late stage spermatocysts. There was no evidence of AR expression in the structural and secretive cells of the epididymis. However, spermatozoa within the lumen of the epididymis demonstrated a consistent positive AR reaction (Figure 14A, B). Similar to the epididymis, there was no evidence of AR expression in the epithelial cells, smooth muscle, or connective tissue that comprise the seminal vesicles. The only consistent AR staining was observed in the sperm contained within the lumen of this structure (Figure 15A-D).

**Immunoblotting**

Immunoblot analysis of protein extracts from mature male *S. tiburo* testes using the anti-AR PG-21 antibody demonstrated the presence of two weak bands corresponding to molecular weights of ~90 and ~100 kDa (Figure 16). This size is consistent with reports
Figure 10. Detection of androgen receptor (AR) transcripts by northern blotting from total RNA extracted from testis samples of *Sphyrna tiburo*. The nylon membrane has the positions and sizes of the RNA marker bands shown on the left of the membrane. The two arrows on the right of the membrane indicate the positions of positive bands detected by the AR mRNA probe.
Figure 11. Localization of AR mRNA in testis sections from a mature male *Sphyra tiburo* using *in situ* hybridization. (A) Negative control using DIG labeled sense AR probe. (B) Positive staining with DIG labeled antisense AR probe in the Leydig-like cells in between spermatocysts. (C) Negative control at higher magnification. (D) Positive staining at higher magnification demonstrating individual Leydig-like cells. SP=Spermatocyst. Arrows in B and D indicate AR positive Leydig-like cells.
Figure 12. Localization of AR mRNA in testis sections from a mature male *Sphyrna tiburo* using *in situ* hybridization. (A, C) Negative controls using DIG labeled sense AR probe. (B, D) Positive staining with DIG labeled antisense AR probe in the Sertoli cells (SC) within post-meiotic spermatocysts.
Figure 13. Localization of AR mRNA in the testis of a mature male *Sphyrna tiburo* using *in situ* hybridization. (A) Negative control using DIG labeled sense AR probe. (B) Positive staining with DIG labeled antisense AR of spermatids within stage 5 spermatocysts. (C) Negative control of a stage 7 spermatocyst. (D) AR positive staining of spermatozoa nuclei in stage 7 spermatocyst.
Figure 14. Localization of AR mRNA in the epididymis of a mature male *Sphyrna tiburo* using *in situ* hybridization. (A) Negative control using DIG labeled sense AR probe. (B) Positive staining with DIG labeled antisense AR probe in the lumen (L) containing spermatozoa.
Figure 15. Localization of AR mRNA in the seminal vesicle of a mature male *Sphyrna tiburo* using *in situ* hybridization. (A) Negative control using DIG labeled sense AR probe. (B) Positive staining in the lumen of the vesicle with DIG labeled antisense AR probe in the nuclei of spermatozoa. (C) Negative control. (D) Positive AR staining of spermatozoa nuclei.
Figure 16. Detection of androgen receptor by western blot in testis lysates from two different mature male *Sphyrrna tiburo* sampled during the spermatogenic stage. The arrows on the left side show the position of the molecular markers. Arrows on right demonstrate bands of ~90 kDa and ~100 kDa.
of AR from other vertebrate groups (Vornberger et al., 1994; Arenas et al., 2001; Zhou et al., 2002) (Figure 16). However, each of these previous studies reported only a single AR band.

**Immunocytochemistry**

*Antibody Validation*

The suitability of the PG-21 antibody to detect the AR protein in *S. tiburo* was demonstrated through a series of antibody competition controls using ICC on testis samples from both mature and immature specimens (Figures 17 and 18). Positive staining of Leydig-like cells were observed when the primary antibody (PG-21) alone was applied to testis histological sections. Similarly, AR was detected from sections of the same testis samples when the PG-21 antibody was preadsorbed with a 10-fold excess of the similarly-sized but unrelated AR462 peptide demonstrating that this protein did not bind to the antibody and hinder the positive reaction observed in the Leydig-like cells. However, AR reactivity was abolished when the PG-21 was preadsorbed with a 10-fold excess of AR21, the antigen that was injected into a rabbit to generate the PG-21 antibody. This series of controls demonstrates the specificity of the AR binding as preadsorption of the antibody with the antigen selectively abolished AR positive staining.

*AR Protein Detection*

Immunocytochemical methods demonstrated the presence of the AR protein in several different organs of *S. tiburo* (Table 3). When the antibody titer was doubled (from 1:100 to 1:50), the immunostaining intensity was not increased nor were there any additional cell types showing immunoreactivity. Positive AR staining in the interstitium of *S. tiburo*
Figure 17. Immunocytochemistry of an immature testis (A, C, E) and a mature testis (B, D, F) of *Sphyrna tiburo* demonstrating specificity of PG-21 antibody through pre-adsorbed controls. (A) Positive AR staining using PG-21 antibody alone. (C) Section from same testis with similar AR positive staining after PG-21 antibody was pre-adsorbed with AR462 peptide. (E) No positive staining when PG-21 was pre-adsorbed with AR21 peptide. (B) AR positive staining with antibody alone. (D) AR positive when antibody pre-adsorbed with AR462 peptide. (F) No AR positive staining after antibody was pre-adsorbed with AR21 peptide. Arrows indicate examples of AR-positive staining.
Figure 18. Immunostaining of AR protein in the testis of a mature *Sphyrna tiburo* demonstrating specificity of the PG-21 antibody through pre-adsorbed controls. (A) AR positive staining in the interstitium. (B) AR positive staining in the interstitium when antibody was pre-adsorbed with AR462 peptide. (C) No immunostaining when antibody was pre-adsorbed with AR21 peptide. Arrows indicate examples of AR positive staining.
testis samples was observed between spermatocysts during various stages of spermatogenesis (stages based on Parsons and Grier, 1992). Leydig-like cells demonstrated AR immunoreactivity in the vicinity of spermatocysts containing both 1º and 2º spermatocytes (Figure 19 A, B). Positive immunostaining was also observed in the interstitium of early meiotic stages while at the same time AR staining was observed in later spermatogenic stages (Figure 19 C, D). Additionally, AR staining was detected in peripheral cells of the testis near efferent ducts (Figure 19 E, F). In pre-meiotic spermatocysts, AR staining was observed in both Sertoli cells and Leydig-like cells of the interstitium (Figure 20).

There was no positive AR staining observed in any of the epithelial cells, muscle cells, or connective tissue that comprises the epididymis. Likewise, there was also no evidence of AR staining in the mature spermatozoa contained within the lumen of this structure (Figure 21 A, B). Positive AR staining was only observed in the epithelial cells of the Leydig gland which lies immediately dorsal to the anterior portion of the epididymis (Figure 21 C, D).

No evidence of AR immunoreactivity was observed in the epithelial cells, smooth muscle, or connective tissue comprising the seminal vesicle (Figure 22 A-D). During the resting stage, when the contents of this structure is minimal, there was no evidence immunostaining in the lumen (Figure 22 A, B). Spermatozoa stored within the lumen of the vesicle during the mating period also did not demonstrate any AR immunoreactivity (Figure 22 C, D).
Figure 19. AR protein detection using immunocytochemistry from testis samples from three different *Sphyrna tiburo* specimens. Control is on left in each case. (A, B) Positive-staining in the interstitium of spermatocysts containing 1º and 2º spermatocytes. (C, D) AR positive staining of Leydig-like cells around meiotic spermatocysts. (E, F) Immuno-staining in the vicinity of efferent ducts of the testis.
Figure 20. Androgen receptor protein detection using immunocytochemistry in the testis of a mature *Sphyra tiburo* during the spermatogenic stage. (A, C) Negative control showing no AR immunostaining. (B) AR positive staining in the Sertoli cells (SC) within pre-meiotic spermatocysts. (D) AR immunostaining of Sertoli cells and Leydig-like cells (LC).
Figure 21. Detection of AR protein in the *Sphyrna tiburo* epididymis using immunocytochemistry. Control sections on the left. (A,B) No positive staining in the mature epididymis. (C,D) Positive AR staining in cells of the Leydig gland that lies distally to the epididymis.
Figure 22. Immunocytochemistry to detect the AR protein in the seminal vesicle of *Sphyrna tiburo*. Control sections on the left. (A,B) No positive staining in the mature seminal vesicle from a shark captured in April (resting stage). (C,D) No evidence of the AR protein in the seminal vesicle of an October captured shark (mating stage).
Positive AR staining was observed in the developing kidney of both male (Figure 23 A, B) and female embryos (Figure 23 C, D) of *S. tiburo*. There was no evidence in either sex of the presence of the AR protein in the developing gonad or any other reproductive organs. Similarly, there was no immunostaining observed in nonreproductive structures such as the stomach, intestine, and muscle.
Figure 23. Detection of the AR protein in cross sections of 5.5 cm total length male (A,B) and 4.3 cm total length female (C,D) embryos of *Sphyrna tiburo* using immunocytochemistry. Control tissues on the left in both cases. Positive staining was observed in the developing kidney only (arrows). V, vertebrae; G, developing gonad.
Discussion

In vertebrates, androgens mediate a variety of diverse responses by binding to their cognate receptor and regulating transcription of its target genes. Androgen receptor expression, which is found in a variety of cell types, changes throughout development (Keller et al., 1996). This study provides cellular localization and a measure of relative expression levels of AR suggesting that androgens are involved in regulating some of the events associated with the seasonal reproductive cycle in male *S. tiburo*.

The immunocytochemical methods used in this study demonstrated the presence of the functional AR protein using an antibody raised against the first 21 amino acids of the human AR. The high degree of amino acid sequence conservation of this region increases the likelihood of cross-reactivity with the AR of other species. The PG-21 antibody has been previously used to identify ARs in the bullfrog *Rana catesbeiana* (Boyd et al., 1999), red-bellied newt *Cynops pyrrhogaster* (Matsumoto et al., 1996), rat *Rattus norvegicus* (Prins et al., 1991), and goat *Capra hircus* (Goyal et al., 1998). The specificity of this antibody to *S. tiburo* AR is supported by the results of the preadsorbed controls where AR staining in testis sections was abolished when the antibody was previously incubated with the peptide used to produce the antibody.
Testis

Semi-quantitative PCR of testis samples revealed that AR expression levels were lowest during the resting phase (December-April) of the reproductive cycle, highest during spermatogenesis (May-August), and intermediate during the mating period (September-November) when spermatozoa are primarily being stored and not produced (Parsons and Grier, 1992). These results suggest that the extent to which androgens regulate events in the testis of *S. tiburo* is dependent upon the seasonal stages of the reproductive cycle.

On a cellular level, evidence of AR protein in the Sertoli cells of pre-meiotic spermatocysts suggests that androgens may play a role in regulating the actions of these cells. Sertoli cell action in the shark testis, in addition to maintaining the microenvironment of the germ cells, is believed to include the production of steroid hormones (Callard *et al.*, 1978; Callard, 1991). The findings in the present study agree with reports of AR immunoreactivity in adluminal Sertoli cell nuclei of spermatogonial stage spermatocysts of the *S. acanthias* using AR52 as the primary antibody (Engel and Callard, 2005). However, the latter study presented only preliminary ICC results of AR in the testis of *S. acanthias* which precludes a detailed comparison with the present study’s findings on *S. tiburo*. This same AR52 antibody did not produce reliable immunostaining when utilized in ICC on testis sections of *S. tiburo*. The detection of AR mRNA in the Sertoli cells of *S. tiburo* is further evidence of an androgen associated role in regulating this cell type. The presence of AR mRNA in the Sertoli cells of post-meiotic spermatocysts, but not in the earlier stages, may indicate that the AR transcripts at these earlier stages were at levels below the threshold of detection of the methodology.
The lack of AR protein associated with these post-meiotic Sertoli cells may reflect a
cessation in AR mRNA translation in this cell type thus resulting in levels of transcripts
that were detectable. Sertoli cells in the mouse testis also demonstrated stage-specific
AR expression with the highest levels during stages VI-VII and the lowest levels during
stages I-III and VIII-XII of spermatogenesis. Stage-dependent expression of AR in
Sertoli cells has been similarly described in the rat (Vornberger et al., 1994) and human
(Suárez-Quian et al., 1999). By generating a knockout mouse with the AR gene deleted
only in Sertoli cells, Chang et al. (2004) demonstrated that functional AR was required in
this cell type to ensure normal spermatogenesis. Defective spermatogenesis due to the
AR-negative Sertoli cells was partly attributed to increased expression of anti-Müllerian
hormone which led to impaired steroidogenesis in Leydig cells. Also contributing to
impaired spermatogenesis and infertility in these transgenic mice was an increase in
androgen-binding protein, a decrease in cyclin A1 and sperm-1 expression, as well as an
overall reduction in serum T levels.

AR expression in the present study was also localized in the Leydig-like cells of the testis
as demonstrated by both in situ hybridization and immunocytochemistry. The zones of
spermatocysts demonstrating the greatest level of AR expression were those containing
primary and secondary spermatocytes. These observations suggest an important role for
androgens around the meiotic stage of spermatogenesis. Evidence of AR expression was
also observed in the Leydig-like cells between more advanced spermatocysts containing
early and late stage spermatids indicating an androgen-associated role later in
spermiogenesis (post-meiotic stages). These observations largely agree with the stage-
related distribution of AR-like activity found in the *S. acanthias* which demonstrated the highest levels of steroid-binding activity in the pre-meiotic stages followed by the meiotic and the post-meiotic stages (Cuevas and Callard, 1992). Stage-related AR expression of this cell type has also been observed in the marbled newt (*Triturus marmoratus Marmoratus*) where interstitial cells were shown to be AR-positive during only the periods of spermatogenesis and quiescence (Arenas *et al*., 2001). In contrast, Leydig cells in the mouse (*Mus musculus*), express AR regardless of the stage of spermatogenesis (Zhou *et al*., 2002).

A role for androgens in the post-meiotic stages of spermatogenesis in the elasmobranch testis is supported by the results of the present study as well as a study on *S. acanthias* (Cuevas and Callard, 1992). However, the observation of AR mRNA in the round and elongate spermatids coupled with the absence of the AR protein in these same cell types is unusual. Using the same PG-21 antibody, the AR protein was recently localized in the spermatocytes of *D. sabina* suggesting that androgens can act directly on germs cells to regulate spermatogenesis in an elasmobranch (J. Gelsleichter, personal communication, April 1, 2007). This type of stage-specific AR expression in developing germ cells has also been described in mammals. For example, the rat AR protein has been detected in the nuclei of elongated spermatids (Vornberger *et al*., 1994). Once chromatin condensation had occurred in these cells, the AR was no longer localized in the nuclei but was observed in the cytoplasm of the spermatids. Immunocytochemical localization of AR has also been demonstrated in the pre-meiotic and meiotic germ cells of the mouse (Zhou *et al*., 1996) and human (Kimura *et al*., 1993). It is possible that the titer of the
PG-21 antibody used in the present study may have been too low to detect the AR protein in the spermatids and spermatozoa of the testis, despite being well-suited for AR detection in other cell types. Varying the concentration of PG-21 in ICC has shown very different levels of AR immunostaining of certain cell types, such as Sertoli and Leydig cells, in the human testis (Suárez-Quian et al., 1999). This possibility was addressed in the present study by doubling the concentration of PG-21 used during ICC on sections of \textit{S. tiburo} reproductive organs. However, the results remained consistent with those from ICC using the original antibody titer. Despite the strong AR mRNA signal detected in post-meiotic germ cells, we must conclude that the AR protein in the testicular germ cells of \textit{S. tiburo} is either absent or present at levels that are below the threshold of detection of this study’s immunocytochemical methods.

**Epididymis**

On a cellular level, there was no evidence from the ICC and ISH of the presence of AR in the epithelial cells of the epididymis despite the presence of ARs. This suggests that the AR expression levels revealed through semi-quantitative RT-PCR of epididymis samples were likely from mRNA in the spermatozoa of this organ’s lumen. The lack of AR expression in \textit{S. tiburo} is in contrast to the AR localization demonstrated in the epididymis of other vertebrates. Among the AR-positive cells were epithelial and stromal cells in the rat and mouse (Yamashita, 2004) and principal and basal cells in the adult boar (\textit{Sus scrofa}) epididymis (Pearl et al., 2006). Survival of spermatozoa in this organ has also been found to be androgen dependent in the tammar wallaby (\textit{Macropus eugenii}) (Chaturapanich et al., 1992). In this same study, orchidectomy resulted in
reduced concentrations of spermatozoa, decreased luminal fluid volume, and changes in the electrophoretic pattern of proteins in the epididymis. The effects of the orchidectomy were reduced or prevented by the replacement of exogenous testosterone.

Through ultrastructural studies of the Port Jackson shark (*Heterodontus portusjacksoni*), the cells of the epididymis have been classified as ciliated cells, principal cells, and intraepithelial leucocytes and function in both protein secretion and transport of fluid and solutes (Jones and Lin, 1993). In freshwater populations of *D. sabina*, morphological changes and alterations of the histological architecture of the epididymis have been found to coincide with seasonal reproductive stages (Piercy *et al.*, 2003). However, a direct role for androgens in mediating these structural modifications is unclear as these changes do not coincide with rises in T and DHT serum concentrations (Snelson *et al.*, 1997). Failure to detect AR in the epididymal cells of *S. tiburo* suggests that this organ is not directly responsive to androgens but may be mediated by other hormonal signals.

The AR-positive tissue associated with the epididymis was identified as the Leydig gland, a specialized portion of the anterior mesonephros comprised of simple columnar epithelium with secretory and ciliated cells (Jamieson, 2005). This gland has been described in the *H. portusjacksoni* as a series of branched tubular glands that secretes eosinophilic bodies into the spermatozoa-carrying epididymis (Jones and Jones, 1982). Jones and Lin (1993) performed ultrastructural studies on this gland in *H. portusjacksoni* and determined that these secretory tubules are specialized for protein synthesis and secretion. Previous electron micrograph work indicated that some of these secreted
proteins become associated with spermatozoa and may be the basis for spermatozoa
bundle formation (Jones et al., 1984). The present study provides the first evidence that
the actions of the elasmobranch Leydig gland may be regulated by androgens. However,
the kidney of non-mammalian species (Young et al., 1995; Blasquez and Piferrer, 2005)
and the analogous mammalian prostate (Cooke et al., 1991; Prins and Birch, 1995;
Pelletier et al., 2000) have been demonstrated as targets for androgens. Since the AR
positive Leydig gland lies immediately dorsal to the epididymis, it is possible that some
of the expression observed in this study’s semi-quantitative PCR of the epididymis could
result from contamination from the Leydig gland. The possibility of Leydig gland
contamination of epididymis samples would most likely have been from samples
dissected during the resting stage when the epididymis is difficult to separate from the
adjacent Leydig gland using routine dissection procedures.

**Seminal Vesicle**

There was no definitive evidence of AR in the epithelial cells, connective tissue, and
contractile smooth muscle of the seminal vesicle in *S. tiburo*. Therefore, the AR
transcripts that were detected by semi-quantitative PCR from these tissues are most likely
from spermatozoa mRNA contained within the lumen of this organ. The responsiveness
of the seminal vesicle to androgens has not been evaluated in any other elasmobranch
species to date. However, the ultrastructure of this organ has been described in a few
chondrichthyans. For example in the ghost shark (*Callorhynchus milii*), the seminal
vesicles are characterized by spiral septa that project into the lumen (Hamlett et al., 2002;
Reardon et al., 2002). The epithelial cells in *C. milii* are simple columnar with
microvillar and ciliated cells, with no evidence of secretory vesicles (Hamlett et al., 2002; Reardon et al., 2002). This suggests the seminal vesicle does not contribute to seminal fluid in this species but rather stores and then delivers spermatozoa to the clasper (Reardon et al., 2002). However in *D. sabina*, epithelial cell proliferation in the seminal vesicle was found to be significantly elevated during both early and late periods of spermatogenesis compared to the reproductively inactive stages (Piercy et al., 2003). The latter study hypothesized that the accelerated epithelial growth observed during these spermatogenic stages is to facilitate the maintenance of spermatozoa during the protracted mating period observed in *D. sabina* and that these changes are likely to be androgen-mediated. The lack of evidence in *S. tiburo* for a direct role of androgens in the seminal vesicle contrasts a previous study of the rat where AR immunostaining was demonstrated in the epithelial and stromal cells of the seminal vesicle by ICC (Pelletier et al., 2000). Although this study did not find evidence of AR in *S. tiburo*, it cannot rule out the possibility of downstream effects of these hormones or simply that these cells are regulated differently in this species. Further, the present study focused on mature male *S. tiburo* and did not perform a detailed evaluation of ARs during embryonic and juvenile development of these structures.

**Spermatozoa**

The isolation of AR mRNA in spermatozoa of the present study does not appear to be an artifact of the ISH methodology given the consistent level of detection in the lumen of the epididymides and seminal vesicles as well as the similar detection of AR mRNA in the spermatids and spermatozoa of the testes. Spermatozoa are generally considered as being
dormant cells given the loss of their transcriptional and translational ability and specialized role in transporting the paternal genome to the oocyte (Hecht, 1998). Given this understanding, the detection of AR mRNA within the lumen of both the epididymis and seminal vesicle in this study was an unexpected but interesting finding. However, advancements in the field of molecular andrology has led to a compelling body of evidence demonstrating that human ejaculated spermatozoa retain a complex cohort of mRNAs (Dadoune et al., 2005; Zhao et al., 2006). Among the many identified transcripts in human spermatozoa are estrogen receptor α (Richer et al., 1999), estrogen receptor β (Hirata et al., 2001), and the progesterone receptor (Luconi et al., 2002). However to date, no androgen receptor transcripts have been detected in spermatozoa. In contrast to the AR mRNA localization through ISH, the ICC conducted in this study did not show evidence of the AR protein in the spermatozoa of the epididymides and seminal vesicles of S. tiburo. In an effort to address the question of antibody concentration, ICC on epididymides and seminal vesicles using twice the standard PG-21 titer resulted in similar results with no evidence of AR immunostaining in spermatozoa. These results contrast a recent finding of AR protein in the mitochondria of the mid-piece of human spermatozoa (Solakidi et al., 2005). This study utilized alternative antibodies (H280 and C19) and immunofluorescence labeling to localize the AR on a cellular level. They also detected 110- and 90-kDa protein bands from spermatozoa lysates using western blotting and an enhanced chemiluminescence system. Hence it is possible that the lack of detection of the AR protein in certain cell types of S. tiburo could reflect methodological difficulties that may be overcome with a more sensitive detection system.
Another possible explanation for the presence of AR mRNA in the sperm of *S. tiburo*, without the presence of the protein, is that these transcripts are selectively retained to play a role at a later time. Recent studies demonstrating mRNA transcripts in human ejaculated spermatozoa have created controversy over the potential role of spermatozoa RNA and led to hypotheses that these transcripts may play an important role in the establishment and maintenance of a viable paternal genome (Miller *et al.*, 2005). In a recent review, Miller and Ostermeier (2006) discussed the reports of RNA carriage in human ejaculate spermatozoa and examined various explanations for possible roles including passive retention, genomic imprinting, and a post-fertilization role for paternal RNAs. Gur and Breitbard (2006) recently demonstrated that the 55S mitochondrial ribosomes in human, mouse, rat, and bovine spermatozoa conduct nuclear encoded protein translation during capacitation. They further concluded that this translational process is essential for spermatozoa to function in fertilization. Thus the notion that AR mRNA is selectively retained in the spermatozoa of *S. tiburo* is worthy of further scrutiny given the expanding evidence that the male gamete performs a role beyond simple delivery of the paternal genome (Miller and Ostermeier, 2006).

**Claspers**

In at least 2 elasmobranch species, peak plasma T levels have been reported to coincide with increased clasper size (Garnier, 1999; Heupel *et al.*, 1999). However, there have been no studies that have demonstrated a direct androgen sensitivity of this organ. In a study measuring serum steroid hormone levels in captive pubertal *S. tiburo*, androgen concentrations were not directly correlated with the rate of clasper growth (Gelsleichter *et
Injections of T into the yolk sac of the small-spotted catshark (*Scyliorhinus canicula*) before sex differentiation demonstrated no macroscopic effect on the development of the claspers (Chieffi, 1967). The AR expression observed through RT-PCR in the present study appears to be the first direct evidence of androgen sensitivity of this secondary sex structure. In teleosts, androgen dependent development of the gonopodium, the modified anal fin, has been studied in the western mosquitofish (*Gambusia affinis*). Ogino *et al.* (2004) demonstrated ARα and ARβ expression in the distal region of the outgrowing anal fin rays. However, after observing similar AR expression in other fins, these authors further showed an androgen-dependant induction of sonic hedgehog (Shh) and Shh receptor (Ptc1) genes in the developing gonopodium. Because Shh is a signaling molecule involved in fin regeneration (Quint *et al.*, 2002), these results suggest that development of the male’s anal fin rays are mediated by downstream genes that are influenced by androgen actions.

The elasmobranch clasper is composed of cartilaginous elements that support the medial margin of the pelvic fin and extend past the posterior margin as a rod (Wourms, 1977). Upon maturity, these intromittent organs calcify and harden (Carrier *et al.*, 2004), making the histological preparation of these structures difficult. In the present study, clasper tissues processed for ICC yielded poor and inconclusive results. Furthermore, the low numbers of cells in these largely cartilaginous structures was not optimal for RNA isolation. For this reason, this study did not attempt to measure the levels of AR expression in clasper RNA. Future studies should focus on improving the methodology by decalcifying these structures prior to histological processing to overcome these
problems and gain a better understanding of the role of androgens in mediating these secondary sex characters.

**Embryos**

Though not the main focus of this study, a preliminary evaluation was conducted of AR in the embryos of *S. tiburo*. The observed AR expression in the embryonic kidney has not been described in other elasmobranchs although the adult kidney has been demonstrated to be AR-positive in a number of other non-mammalian species (Young *et al.*, 1995; Ogino *et al.*, 2004; Blasquez and Piferrer, 2005). In mammals, AR expression has been observed in the embryonic male reproductive organs including the Wolffian ducts, epididymides, ductus deferens, and seminal vesicles (Cooke *et al.*, 1991). This same study also indicated that androgen-mediated events in the embryo of the mouse have a clear temporal sequence. This is an important observation as the lack of evidence of AR expression in the embryonic reproductive structures examined in the present study may be related to the limited temporal view of these tissues that may be expressing genes in a time sensitive pattern.

**Heart**

The expression of the AR gene in heart tissue was examined by PCR with the intention of utilizing this organ as a negative control. However, the weakly positive results from the screening precluded this organ for that purpose. The finding of AR expression in the heart is not unprecedented in vertebrates. In the domestic dog (*Canis familiaris*), RT-PCR analysis demonstrated low levels of expression in this organ (Lu *et al.*, 2001).
Western blot analysis of whole tissue extracts of the heart of the bullfrog (*Rana catesbeiana*) revealed a 100 kDa band corresponding to AR (Chattopadhyay *et al*., 2003). On a cellular level, ARs have been demonstrated in the cardiac myocytes of several mammalian species including *R. norvegicus, C. familiaris*, and *Homo sapiens* (Marsh *et al*., 1998). In a study to define the biological significance of cardiac AR function, AR knock-out male mice were found to have a significant reduction in heart-to-body weight ratio compared to wild-type mice suggesting an androgen role in cardiac growth and modulation of cardiac adaptive hypertrophy (Ikeda *et al*., 2005).

**Steroid Hormones**

In mature male *S. tiburo*, circulating androgen levels follow an annual cycle that is correlated with the reproductive stage of the animal. Serum T and DHT concentrations have been shown to increase during the middle to late stages of the spermatogenic phase (late spring to summer), drop during the mating season (fall) and then remain at their lowest levels during the quiescent phase (winter to early spring) (Manire and Rasmussen, 1997). The present study has found a similar pattern with the androgen receptor which supports the notion that androgens regulate spermatogenesis but also play role in regulating other primary and secondary sex characters during other phases of the reproductive cycle. When considering the lack of evidence for a direct role of androgens in a given cell or tissue via the receptor, one cannot rule out an indirect role as downstream genes may be important in regulating certain processes. It is worth noting that estrogen (E2) receptors have also been found in male reproductive tissue in several mammalian and non-mammalian species (Goyal *et al*., 1998; Pelletier *et
al., 2000; Arenas et al., 2001) and that the existence of multiple receptors in the same cells raises questions about steroid hormone interactions in mediating the function of the male reproductive tract. In the case of S. tiburo, Manire and Rasmussen (1997) found that testicular recrudescence coincided with an elevation of serum E2 levels and postulated that this “female” hormone could function to regulate spermatogenesis in the species. This notion is supported by the localization of estrogen receptors in regions of the S. acanthias testis containing pre-meiotic spermatocysts (Callard et al., 1985; Callard, 1992).

**Future Directions**

Sertoli cells and Leydig-like cells of the testis are assumed to be the primary source of androgens in S. tiburo. Evaluating the importance of these types of cells in androgen synthesis and the possible autocrine/paracrine means of regulation would enhance the overall understanding of this steroid’s functional role in this species. One approach to address this is to use ICC with antibodies against some of the key steroidogenic enzymes, such as cytochrome P450c17 and 3β-dehydrogenase (Trant, 1996; Baker et al., 1999).

Although this study focused on identifying AR in reproductive tissues in male S. tiburo, it is likely there are other androgen-regulated tissues/organs that were not evaluated given the scope of this study. For example, serum androgen levels have been shown to increase in males during the mating season in some elasmobranch species (Snelson et al., 1997; Tricas et al., 2000) suggesting an androgenic role in mating behavior. Studies of the goldfish (Carassius auratus) have identified AR positive neurons in brain tissue (Gelinas and Callard, 1997). Hence a future study to identify ARs in the brain of S. tiburo would
contribute to our understanding of the role of androgens in mediating elasmobranch courtship and mating behavior.

This study evaluated the AR expression pattern in embryos of a size and age that corresponds to the period of sexual differentiation (J. Gelsleichter, personal communication, April 11, 2007). However, AR expression in the embryos of *S. tiburo* may be finely regulated on a temporal level as demonstrated in the mouse reproductive tract (Cooke *et al.*, 1991). To address this possibility, future studies should evaluate the expression of AR throughout all stages of embryonic development while at the same time maximizing the cross-sectional coverage of each sample in order to evaluate as many organs as possible.

To further evaluate the presence of AR mRNA in the spermatozoa of *S. tiburo*, semen samples could be collected from adult males during the mating season and purified by the swim-up technique to select for live sperm and remove somatic contaminations (Hargreaves *et al.*, 1998). Extracted RNA from the purified gametes could be evaluated for AR expression using some of the molecular tools developed in this study.

Lastly, the localization of estrogen receptors in the testis of *S. acanthias* (Callard *et al.*, 1985; Callard, 1992) raises the hypothesis that androgen and estrogen cooperate in regulating spermatogenesis in *S. tiburo*. Hence a similar study of the cellular localization of estrogen receptors in the primary and secondary sex structures would provide data toward understanding steroid-mediated control of male reproduction in this species.
Conclusions

This study demonstrates that AR is expressed in the testis of mature male *S. tiburo* during all stages of its seasonal reproductive cycle, but expression appears highest during the summer spermatogenic stage. Cell-specific localization of AR in the testis appears stage-related. The expression pattern of AR in Leydig-like cells suggests that they are primarily regulated by androgens just prior to and during the meiotic stage of spermatogenesis but there is also evidence for post-meiotic androgen regulation of this cell type. Sertoli cells appear to be androgen sensitive in the meiotic stages. There is evidence of AR mRNA in the spermatids and spermatozoa of late stage spermatozysts but the role of these transcripts is yet to be determined since the functional AR protein was not detected in these developing germ cells. RT-PCR revealed that the epididymis and seminal vesicle of mature *S. tiburo* express AR in all stages of the male’s seasonal cycle, but expression is significantly lower during the winter/spring resting stage. On the cellular level, there is no evidence of AR in either of these reproductive structures. However, AR mRNA was localized in the spermatozoa of both the epididymis and seminal vesicle. This study provides the first evidence that the action of the Leydig gland is associated with androgens. The clasper of *S. tiburo* was found to express AR but the cellular localization of the receptors in this structure was not determined. A preliminary evaluation of AR in the embryos of this species revealed that the developing kidneys may be a site of action of androgenic hormones.
Although there appears to be considerable variability in the regulatory role of androgens in the male reproductive tract, both among and within vertebrate classes, *S. tiburo* seems to share many of the same AR characteristics as more derived species. The seemingly advanced mechanism by which these ancient fishes are using steroid hormones to regulate reproductive events is an area requiring further study. Future studies should work toward gaining a deeper understanding of the effect of these hormones on their target cells. The present study has provided a basis for such research by identifying some of the cells and tissues that androgens target in *S. tiburo*. 
List of References


Appendix I: Species with published androgen receptor sequences that were aligned (Block Maker) and analyzed (CODEHOP) to develop degenerate primers for this study.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burton’s mouthbrooder</td>
<td><em>Haplochromis burtoni</em></td>
<td>AF121257</td>
</tr>
<tr>
<td>Goldfish</td>
<td><em>Carassius auratus</em></td>
<td>AY202775</td>
</tr>
<tr>
<td>Japanese eel</td>
<td><em>Anguilla japonica</em></td>
<td>AB02361</td>
</tr>
<tr>
<td>Rainbow trout (AR α)</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>AB012095</td>
</tr>
<tr>
<td>Rainbow trout (AR β)</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>AB012096</td>
</tr>
<tr>
<td>Red sea bream</td>
<td><em>Chrysophrys major</em></td>
<td>AB017158</td>
</tr>
<tr>
<td>African clawed frog</td>
<td><em>Xenopus laevis</em></td>
<td>U67129</td>
</tr>
<tr>
<td>Mouse</td>
<td><em>Mus musculus</em></td>
<td>NM013476</td>
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<tr>
<td>Boar</td>
<td><em>Sus scrofa</em></td>
<td>AF202775</td>
</tr>
<tr>
<td>Domestic dog</td>
<td><em>Canis familiaris</em></td>
<td>AF197950</td>
</tr>
<tr>
<td>Crab-eating macaque</td>
<td><em>Macaca fascicularis</em></td>
<td>MFU94179</td>
</tr>
</tbody>
</table>
Appendix II: Recipe for 100 ml elasmobranch-modified phosphate buffered saline (E-PBS) (Walsh and Luer, 2004).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.63 g</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>0.12 g</td>
</tr>
</tbody>
</table>

Adjust pH to 7.4 with 1N HCl. Filter through 0.2 μm sterile filter and store at 4°C.
Appendix III: Recipe for 1 ml of hybridization buffer.

- Formamide (50%) 500 µl
- SSPE Buffer (20x) 100 µl
- Fish Sperm DNA (10 mg/ml) 100 µl
- Yeast tRNA extract (10 mg/ml) 50 µl
- Bovine Serum Albumin (10 mg/ml) 100 µl
- DMPC-Treated Water 150 µl