1
2
3 Androgen receptors in the bonnethead, *Sphyrna tiburo*: cDNA cloning and tissue-specific
4 expression in the male reproductive tract.

5
6 John P. Tyminski¹, James J. Gelsleichter², Philip J. Motta³

7
8 ¹Center for Shark Research, Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota,
9 FL 34236, USA

10 ²Department of Biological Sciences, University of North Florida, 1 UNF Drive, Jacksonville, FL
11 32224, USA

12
13 ³Department of Integrative Biology, University of South Florida, 4202 East Fowler Avenue,
14 Tampa, FL
15 33620, USA

16
17 Keywords: sharks, androgens, steroid receptors, testis

18
19
20 Corresponding author: James J. Gelsleichter, jim.gelsleichter@unf.edu
21
22
23
24

Abstract

As demonstrated in past studies, androgens appear to play critical roles in regulating reproduction in male sharks. However, little is known about the cell-specific actions of androgens in these fishes. To address this, this study examined androgen targets in reproductive organs of a seasonally reproducing shark, the bonnethead (*Sphyrna tiburo*). A partial bonnethead AR cDNA clone was isolated and found to exhibit strong homology with known vertebrate ARs. Using RT-PCR and *in situ* hybridization, AR was found to be expressed in multiple cell types in the male bonnethead testis (premeiotic germ cells, Leydig-like interstitial cells, Sertoli cells, peritubular myoid cells, and mature spermatozoa) and gonadal ducts (stromal cells, luminal epithelial cells, mature spermatozoa). Furthermore, AR expression in these organs was found to vary temporally in relation to the seasonal reproductive cycle. Based on immunocytochemistry, the presence of AR protein in male bonnethead reproductive organs was largely consistent with patterns of AR gene expression with the single exception of mature spermatozoa, which exhibited consistently strong mRNA expression but only inconsistent and weak AR protein immunoreactivity. These results suggest important roles for androgens in regulating germ cell proliferation, hormone production, spermatid elongation, spermiation, and gonadal duct function in male bonnetheads. In addition, high abundance of AR mRNA in bonnethead spermatozoa suggest the potential for *de novo* protein synthesis following spermiation/copulation and/or a role for AR mRNA in early embryonic development, both of which have been proposed to explain the occurrence of mRNA transcripts in spermatozoa from various vertebrates.

1. Introduction

Androgens, such as testosterone (T) and dihydrotestosterone (DHT), are steroid hormones that mediate sex differentiation, sexual maturation, and post-pubertal virilization in male vertebrates (Brinkman, 2011; Murashima et al., 2014). In order for androgens to perform their physiological roles, it is essential that these hormones bind to the androgen receptor (AR) protein, which functions as a ligand-induced nuclear transcription factor (Ogino et al., 2009). Since ARs mediate the effects of androgens, the presence and distribution of ARs determines the pattern of cellular responsiveness to these hormones within an organism. In male mammals, testicular localization of ARs has been demonstrated in a number of cell types including Sertoli, Leydig, peritubular myoid, and germ cells indicating a regulatory role of androgens during spermatogenesis (Walters et al., 2010). The mammalian epididymis and seminal vesicle are also androgen target organs with ARs localized in both epithelial and stromal cells, suggesting a role for androgens in the maturation and viability of spermatozoa (Patrão et al., 2009). In comparison, less attention has been given to localizing ARs in non-mammalian vertebrates. However, recent studies have detected ARs in the male reproductive organs of a few diverse species including bony fish (e.g., zebrafish *Danio rerio*, de Waal et al., 2008), amphibians (e.g., bullfrog *Rana catesbeiana*, Chattopadhyay et al., 2003), reptiles (e.g., American alligator *Alligator mississippiensis*, Moore et al., 2010), and birds (e.g., rooster *Gallus domesticus*, Dornas et al., 2008; goose *Anser anser f. domestica*, Leska et al., 2012). Collectively, these studies suggest roles for androgens similar to those in mammals, such as in the indirect regulation of spermatogenesis through somatic cells and the maturation and maintenance of spermatozoa in the epididymis.

As in other male vertebrates, androgens appear to be associated with reproductive events in male chondrichthyans (sharks, skates, rays, and chimeras). For example, studies measuring serum T and DHT levels in male Atlantic stingrays *Dasyatis sabina* (Snelson et al., 1997; Tricas et al., 2000), epaulette sharks *Hemiscyllium ocellatum* (Heupel et al., 1999), draughtboard sharks *Cephaloscyllium laticeps* (Awruch et al., 2008), sand tiger sharks *Carcharias taurus* (Henningesen et al., 2008), and bonnetheads *Sphyrna tiburo* (Manire and Rasmussen, 1997) have demonstrated that androgen levels vary with the reproductive cycle, suggesting a possible role in regulating aspects of reproduction such as spermatogenesis and reproductive tract development. However, as with other non-mammalian species, very few studies have attempted to localize AR in elasmobranchs to identify the cell-specific roles of these hormones. In one of the only two studies to examine the distribution of AR in male elasmobranchs, Cuevas and Callard (1992) determined that ARs in the testis of the spiny dogfish (*Squalus acanthias*) were primarily localized in early stage (pre-meiotic and meiotic) spermatocysts through the use of steroid binding analyses of cytosolic and nuclear extracts. The finding that androgens may be involved in regulating early spermatogenesis was supported by a later study using semi-quantitative RT-PCR to measure AR mRNA in the same species (Betka and Callard, 1998). As *S. acanthias* is a shark species with a continuous breeding cycle, its noteworthy that there is virtually nothing known about AR distribution and androgen-regulated processes in seasonally breeding elasmobranchs, which comprise most of the species with documented androgen cycles.

The goal of this study was to investigate AR distribution in the male reproductive tract of a seasonally reproducing elasmobranch. We focused on the bonnethead (*Sphyrna*

tiburo), an abundant small hammerhead species with well-described seasonal hormone profiles (Manire et al., 1995; Manire and Rasmussen 1997; Manire et al., 1999; Gelsleichter et al. 2002). Increases in serum T and DHT concentrations occur in sexually mature male bonnetheads during late summer, a period that coincides with the peak gonad size and late spermatogenesis (Parson and Grier 1992; Manire and Rasmussen, 1997). Androgen levels decline just prior to the fall mating period and then remain low until the following spring when gonadal recrudescence begins anew (Parsons and Grier, 1992; Manire and Rasmussen, 1997). Despite the fact that patterns in plasma androgen concentrations of *S. tiburo* have been well characterized, the functional role of these hormones on a cellular and tissue-specific level is virtually unknown. To begin to address this question, we investigated AR localization in the male reproductive tract of the bonnethead through its seasonal cycle by detecting AR mRNA and AR protein at the tissue and cellular level.

2. Materials and methods

2.1. Sample Collection

Mature male bonnetheads were collected from four sites along the southwest Florida coast using set 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). The specimens were collected throughout the year during all stages of the reproductive cycle and were measured (precaudal, fork, and total length), weighed and sexed at time of capture. Reproductive stage of individual sharks was determined using several indices including

testis size, presence of semen, and histology of the reproductive organs as described in Gelsleichter et al. (2003). For mRNA detection, samples of reproductive organs (i.e., testes, epididymides, and seminal vesicles) were obtained from euthanatized or moribund sharks, flash-frozen in liquid nitrogen, and afterwards stored at -80°C until use. Additional samples, such as clasper cartilage and heart, were also collected for AR screening. To avoid RNase contamination, all instruments coming in contact with organ samples were cleaned with RNaseZap[®] decontamination solution (Ambion, Austin, TX) prior to use. For immunocytochemistry (ICC), samples of reproductive organs were fixed in 10% formalin (prepared in phosphate buffered saline modified for use with elasmobranch tissues; 10 mM NaH₂PO₄, 450 mM NaCl, pH 7.4) for 48 h, after which they were rinsed for 24 h in running tap water and then transferred to 70% ethanol for long-term storage.

2.2. PCR screening of *S. tiburo* organs for AR

Total RNA for use in cloning bonnethead AR was isolated from flash-frozen and finely ground mature male bonnethead testis using Trizol[®] reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Following isolation, the RNA pellets were briefly air-dried and re-suspended in 50 µL of water treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, MO). Total RNA concentration was quantified using spectrophotometry, and its quality evaluated based on the integrity of 28s and 18s ribosomal RNA (rRNA) following electrophoresis on 1% agarose gels.

140 Degenerate oligonucleotide primers to AR were designed based on a comparison
 141 of GenBank-published AR sequences from 10 species using Block Maker software
 142 (Henikiff et al., 1995) followed by analysis using CODEHOP software (Rose et al.,
 143 1998). Two sets of AR primers were produced with sequences as follows: AR3-Fw; 5'-
 144 CGGCTCCTGCAAGGTGTT(C/T)TT(C/T)AA(A/G)(A/C)G-3'; AR3-Rv; 5'-
 145 GAGATGATCTCGGCCATCAT(C/T)TC(deoxy-inosine) GG-3'; ARn-Fw; 5'-
 146 (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'-
 147 (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
 148 were also made to clone bonnethead *β -actin* with the intention of utilizing this
 149 constitutively expressed gene as a standard and/or a positive control during downstream
 150 applications. Non-degenerate primers for *β -actin* were constructed using sequences
 151 successfully used to amplify a portion of this gene in the houndshark (*Triakis scyllium*)
 152 (GenBank Accession No. AB084472). The actin primers used were BA-Fw; 5'-
 153 GGATGATGAAATTGCAGC-3' and BA-Rv; 5'-CGTTGTAGAAAGTGTGATGC-3')
 154 (C. Haruta, Nihon University, Japan, personal communication, July 31, 2002).

155 Extracted RNA was reverse transcribed producing complementary DNA (cDNA)
 156 using random hexamer primers and murine leukemia virus (MuLV) reverse transcriptase
 157 using the GeneAmp[®] RNA PCR Core Kit (Applied Biosystems, Foster City, CA). A
 158 nested PCR strategy was employed in which the two sets of AR primers were used in two
 159 successive reactions. The product of the first reaction (AR3 primers) was used as the
 160 cDNA template for re-amplification in a second reaction using the ARn primers, which
 161 annealed to internal sites of the initial product. All PCR reactions included 10 pmol of
 162 the respective degenerate primers in a 25- μ L reaction performed using a Techgene

compact thermocycler (Techne, Cambridge, UK). The PCR conditions for both reactions were identical: 94°C for 2 min, 20 cycles of 95°C for 30s, 60°C for 30 s with decreasing temperature proportionately each cycle to 51°C, 72°C for 1 min followed by 15 additional cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and hold at 72°C for 7 min.

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 95° for 45 s, 50° for 45 s, and 72° for 45 s then 72° for 7 min. All PCR products were analyzed on 1.2% agarose gels stained with ethidium bromide.

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.

2.3. Northern Blotting

Northern blotting was performed to confirm the appropriate size of AR mRNA. The northern blotting procedure followed the methodology outlined by Bowman and Denslow (1999). Twenty microliters of total RNA was extracted from pooled samples of mature male bonnethead testis, concentrated in microcentrifuge tubes to near dryness using a Vacufuge™ Concentrator 5301 (Eppendorf, Westbury, NY), denatured, and separated on 1% agarose-formaldehyde gels at 50 V for 3 h. RNA was transferred to positively-charged nylon membranes (BrightStar™-Plus, Ambion) via overnight downward capillary transfer. Membranes were rinsed and crosslinked twice (120 mJ/cm²) using a UV Stratalinker® 1800 (Stratagene, La Jolla, CA). The presence of AR transcripts was determined using a digoxigenin (DIG)-labeled AR antisense cRNA probe constructed by *in vitro* transcription of linearized plasmid containing the AR fragment as DNA template, DIG-labeled UTP (Roche Applied Science, Indianapolis, IN), and the MEGAscript® High Yield Transcription Kit (Ambion). The antisense probe and a similarly prepared sense probe were evaluated for their labeling efficiency using the direct detection method described in the DIG Northern Starter Kit (Roche). Northern blot was performed following the procedure described in the DIG Northern Starter Kit (Roche) using the antisense probe at a concentration of 100 ng/mL, the DIG Wash and Block Buffer Set (Roche), anti-DIG-alkaline phosphatase conjugate (Roche), and the chromogen BCIP/NBT (Roche). Transcript size was determined by comparison with RNA markers ranging in size from 281 to 6,583 bases (Promega, Madison, WI).

2.4. Relative PCR

208 AR screening and reproductive stage-related changes in the expression of AR
 209 mRNA were measured in male bonnethead organs using relative PCR. Two species-
 210 specific, non-degenerate primers for bonnethead AR were designed based on the
 211 sequence data obtained via cloning using Primer3 software (Rozen and Skaletsky, 2000)
 212 (AR-BH1-Fw; 5'-ATGCCGTCTGAGAAAGTGCT-3'; AR-BH1-Rv; 5'-
 213 AAATTGCTGCATCCTCGGTA-3'). These primers were tested in PCR reactions using
 214 cDNA from bonnethead testis and resulted in the amplification of the expected 174-bp
 215 fragment.

216 Total RNA for use in relative PCR was isolated from bonnethead testes,
 217 epididymides, seminal vesicles, clasper, and heart using the RNeasy[®] Mini kit (Qiagen)
 218 following the manufacturer's protocol. The extracted RNA was quantified by
 219 spectrophotometry and examined for quality via agarose gel electrophoresis. The RNA
 220 was reverse transcribed using the Advantage[®] RT-for-PCR Kit (Clontech, Mountain
 221 View, CA) with 0.5 µg of total RNA and random hexamer primers. The cDNA samples
 222 were aliquoted and stored at -80°C. Quantification of AR expression was achieved
 223 through the use of multiplex PCR reactions using bonnethead-specific AR primers and
 224 Competimer[™]/18s rRNA oligonucleotides (Ambion) as described by Lattier et al.
 225 (2001). These universal oligonucleotide primers are a more effective standard than the
 226 more commonly used *β-actin* because rRNA levels do not vary significantly from tissue
 227 to tissue. However, since rRNA is such a major component of total RNA, the addition of
 228 the Competimers was necessary to modulate the efficiency of the PCR reaction while not
 229 affecting the amplification of the AR target within the multiplex PCR.

230 As described by Lattier (2001), accurate measurement of the amount of a target
231 mRNA expressed in a given tissue can only be accomplished using this approach if the
232 amplification cycle number is within the log-linear range of formation of the gene-
233 specific product. To evaluate the log-linear range of AR product formation, mature male
234 testis cDNA was used in a series of identical PCR reactions using the AR primers.
235 Individual reaction tubes were sequentially removed from the thermocycler at two cycle
236 intervals starting with the 21st and ending with the 39th cycle. Subsequent gel
237 electrophoresis and image analysis of these PCR products indicated that the mid-point of
238 the detectable log-linear range occurred after 28 cycles of amplification.

239 An annealing temperature of 68°C was found to be optimal for the bonnethead-
240 specific AR primers. Since the 18s primers function efficiently with annealing
241 temperatures from 55-68 C°, it was determined that a 68°C annealing temperature could
242 be used for all multiplex relative PCR reactions. Hence, the relative PCR conditions
243 were: 94°C for 3 min followed by 28 cycles of 94° for 45 s, 68° for 1 min, and 72° for 1
244 min with a final extension of 72° for 5 min.

245 Once the appropriate annealing temperature and ideal number of cycles were
246 determined, another series of identical multiplex reactions were conducted to assess the
247 appropriate Competimer to 18s primer ratio. Increasing concentrations of 18s
248 Competimers were added to a series of reactions containing both the 18s and AR primers
249 and run using the aforementioned PCR conditions. A 9:1 ratio of Competimer to 18s
250 primer was found to be optimal for producing an 18s rRNA band of the same relative
251 intensity as that of the amplified AR product.

cDNA samples from bonnethead organs were processed using the optimized relative PCR procedure and PCR products were 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 rRNA ratios by stage were analyzed for significance using a one-way analysis of variance (ANOVA) followed by 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 natural log-transformed prior to analysis.

2.5. RNA *In Situ* Hybridization

RNA *in situ* hybridization (RISH) was performed to localize AR mRNA in bonnethead reproductive organs. Testis, epididymis, and seminal vesicle samples for RISH were fixed for 48 h in 4% paraformaldehyde prepared using phosphate buffered saline modified for use with elasmobranch tissues (E-PBS), and then transferred to 100% ethanol for long-term storage at -20°C. Fixed samples were removed from ethanol and incubated overnight in a 30% sucrose solution made with water treated with 0.1% dimethyl pyrocarbonate (DMPC) (Sigma). After embedding in Tissue-Tek[®] O.C.T.

compound (Sakura Finetek U.S.A., Inc., Torrance, CA), 5- μ m 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 for 16-24 h at 40°C to fix the RNA in the tissue. Tissue sections were then used immediately for RISH or stored for later use at -80°C.

The RISH protocol followed the procedures described by Dijkman et al. (1995) with some modifications. Cryostat sections were heat-fixed and dried for 2 h at 40°C, after which each tissue was circled using a PAP pen (Electron Microscopy Sciences, Hatfield, PA). Tissue sections were then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 5 min at room temperature (RT). The sections were then washed once with PBS for 3 min then twice for 5 min with 2x SSC (Ambion).

Following fixation and rinsing, each section was prehybridized for 60 min at 37°C in 25 μ L of hybridization buffer (50% formamide, 2x SSPE buffer, 1 mg/mL fish sperm DNA, 0.5 mg/mL yeast tRNA, 1 mg/mL BSA in DMPC-treated water). Afterwards, the hybridization buffer was discarded and each section was incubated in 25 μ L of fresh hybridization buffer containing 200 ng/mL of the previously described DIG-labeled antisense cRNA probe for AR 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 low and high stringency 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 of DIG-labeled RNA probes was performed using solutions from the DIG Wash and Block Buffer Set (Roche), which are compatible with the Dijkman et al. (1995) protocol. Unless otherwise noted, all incubations took place at

RT. Sections were washed for 5 min in DIG washing buffer, followed by blocking for 30 min with DIG blocking buffer. Sections were then incubated for 2 h in DIG blocking buffer containing alkaline phosphatase-conjugated anti-DIG antibody (Roche) at a dilution level of 1:200. This was followed by 3 washes in DIG washing buffer for 5 min each. Each section was then incubated in 25 μ L of DIG detection buffer for 10 min. Last, sections were incubated in DIG detection buffer containing NBT/BCIP and 240 μ g/mL levamisole for 16-20 h in the dark. The color reaction was stopped by rinsing sections in washing buffer (1 mM EDTA in 10 mM Tris, pH 8). Sections were then rinsed for 5 min in reverse osmosis (RO) water and counterstained for 5 min with Nuclear Fast Red (Vector Laboratories, Inc., Burlingame, CA). Slides were washed for 5 min in RO water, then mounted using aqueous mounting media for microscopic examination.

2.6. Immunocytochemistry

ICC was performed to localize the presence of AR protein in bonnethead reproductive organs. Samples of testis, epididymis, and seminal vesicle previously fixed and stored for use in AR ICC were trimmed then dehydrated in a graded series of alcohol (80-100%), cleared in a limonene-based xylene substitute (CitriSolv™, Fisher Scientific, Fair Lawn, NJ), and processed for routine paraffin histology. Reproductive organs were cross-sectioned (5 μ m) using a rotary microtome and adhered to microscope slides coated with 0.01% poly-L-lysine (Sigma).

ICC was performed using rabbit polyclonal antibody against the first 21 amino acids of the human AR (PG-21, Upstate, Lake Placid, NY) as primary antibody. Tissue

sections were deparaffinized using CitriSolv, re-hydrated in a descending, graded series of histological grade alcohols (100-95%), and rinsed in tap water. Afterwards, sections were incubated in antigen retrieval solution (10 mM sodium citrate, pH 6.0) for 20 min at 95°C to expose AR epitopes that may have been masked by the fixation process. Following antigen retrieval, sections were cooled to RT, rinsed in PBS and blocked for nonspecific reactivity with the primary antibody via overnight incubation at 4°C in 2% normal horse serum in PBS (Vector). The following day, slides were rinsed twice in PBS and incubated overnight at 4°C in primary antibody diluted 1/100 in PBS containing 0.1% gelatin and 0.1% sodium azide. On the final day of the protocol, sections were rinsed once in PBS containing 0.05% Tween-20 (PBS-T), twice rinsed with PBS, and antibody-antigen complexes were revealed using the ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer Detection Kit (Vector) and the peroxidase substrate 3,3'-diaminobenzidine (ImmPACT DAB, Vector) or Vector ImmPACT NovaRED Peroxidase (HRP) Substrate following the manufacturer's instructions. Endogenous peroxidase activity was quenched by incubation of sections in a 1:1 mixture of 3% hydrogen peroxide and 100% methanol prior to incubation with the primary antibody. Following color development, slides were counter-stained with either 2% methyl green (Vector) for 45-60 min at 37°C or Vector Hematoxylin QS for 45 s. Afterwards, sections were 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 diluent; 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 confirmed as being AR-positive using all controls, sections of this organ type were included in all subsequent immunocytochemical runs as a positive control. Negative controls incubated with diluent only were conducted for every slide. In addition to the antibody concentration described above, a series of immunocytochemical runs were also performed using a PG-21 titer of 1:50 to assess whether increased antibody concentration would improve the sensitivity of the immunostaining.

2.7. Immunoblotting

Immunoblotting was performed to confirm the molecular weight of immunoreactive AR detected in bonnethead reproductive organs. Proteins were extracted and fractionated from bonnethead testis using the Qproteome Cell Compartment Kit (Qiagen) following the manufacturer's instructions. Acetone-precipitated proteins from 100- μ L cytosolic, membrane, and nuclear fractions were re-suspended in 25 μ L of Laemmli 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) (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 8.0) for 2 h to block non-specific binding. Immunoreactive AR protein was detected 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 antibody incubations. Following color reaction, membranes were rinsed in RO water and air-dried.

3. Results

3.1. 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 (Fig. 1) revealed that the cloned insert was a 324 bp fragment of bonnethead AR cDNA. This portion of the gene shares a high degree of sequence homology with other species (Fig. 2), but most closely resembled ARs of the spiny dogfish shark (*Squalus acanthias*) and the African clawed frog (*Xenopus laevis*). The cloned region of the bonnethead AR gene corresponds to the conserved DNA-binding domain (DBD), the variable hinge region, and the carboxy-terminal ligand-binding domain (LBD). Additionally, 2 positive clones of putative bonnethead β -actin cDNA fragments were also obtained and sequenced. BLASTX analysis of the sequence data revealed that the cloned insert was a 274 bp fragment of the bonnethead β -actin gene (data not shown).

3.2. AR Screening and Semi-Quantification by RT-PCR

Tissue screening using standard RT-PCR resulted in the amplification of the expected 174 bp AR cDNA product from RNA samples extracted from the bonnethead testes, epididymides, seminal vesicles, claspers, and heart (Fig. 3). The heart RNA samples generally produced noticeably weaker bands than those from the reproductive tract.

Multiplex PCR reactions performed using bonnethead-specific AR primers and Competimer™/18s rRNA oligonucleotides 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) (Fig. 4). Statistically significant differences in AR mRNA expression levels were observed among the reproductive stages in all 3 of these organs (Fig. 5). For the testis, AR expression was higher during spermatogenesis compared to the resting stage and mating period (ANOVA and Tukey's HSD test, $p = 0.004$). In the epididymis, AR expression levels were significantly higher during spermatogenesis and mating than during the resting stage (ANOVA and Tukey's HSD test, $p = 0.01$). Last, in the seminal vesicle, AR expression was higher during mating than during the resting stage and the period of spermatogenesis (ANOVA and Tukey's HSD test, $p = 0.004$).

3.3. Northern Blotting

Northern blot analysis of total RNA extracted from bonnethead testes revealed 2 AR transcripts of about 7 and 9 kb (Fig. 6). These two bands were faint (and difficult to scan/photograph), which suggests a low level of AR expression.

3.4. RNA *In Situ* Hybridization

The presence of AR mRNA was observed in multiple cell types in the bonnethead testis and there were stage-associated differences in AR expression patterns (Fig. 7). Testicular AR expression during the pre-meiotic and meiotic stages of spermatogenesis was most prominent in the Leydig-like, interstitial cells lying between spermatocysts although there also was some evidence of AR expression in germ cells and Sertoli cells during these stages. AR expression was noticeably greater in Sertoli cell nuclei during spermiogenesis, particularly in spermatocysts containing germ cells undergoing late stages of spermatid elongation. AR expression was prominent in mature spermatozoa. Last, AR expression was also highly visible in peritubular myoid cells surrounding spermatocysts containing mature spermatozoa.

AR expression in the bonnethead epididymis was observed in the stroma and epithelial cells of the lumen, but was greatest in spermatozoa, when present (Fig. 8). A similar pattern of expression was observed in bonnethead seminal vesicle, in which the typical aggregation of spermatozoa in this organ resulted in intense hybridization signals (Fig. 8).

3.5. Immunocytochemistry

The presence of the AR protein was observed in multiple cell types in the bonnethead testis and there were stage-associated differences in AR immunoreactivity patterns (Fig. 9). The most prominent AR immunoreactivity was observed in Leydig-like, interstitial cells surrounding spermatocysts containing pre-meiotic and meiotic stage germ cells. AR protein was also notably visible in Sertoli cells of spermatocysts containing elongating spermatids and in peritubular myoid cells of spermatocysts

438 containing mature spermatozoa. Lower amounts of AR protein were observed in both
439 germ cells and Sertoli cells of pre-meiotic spermatocysts. AR protein was detected in the
440 midpiece of mature spermatozoa in some samples, but staining was far less consistent
441 than that observed for all other cell types and not congruent with the intense staining
442 observed for mRNA detection.

443 AR immunoreactivity was observed in both the luminal epithelium and stroma of
444 the epididymis and seminal vesicle, but immunostaining was generally weaker and less
445 consistent than that observed in the testis (Fig. 10). Greater and more consistent AR
446 immunoreactivity was observed in Leydig's gland, the modified anterior portion of the
447 kidney in male elasmobranchs, which is highly associated with the epididymis and was
448 generally included in histological preparations of this organ (Fig. 10). AR protein was
449 not detected in mature spermatozoa present in gonaducts.

450 The suitability of the PG-21 antibody for detecting AR protein in the bonnethead
451 reproductive tract was validated through the use of pre-absorption controls. Positive
452 immunostaining was consistently observed when the primary antibody alone was applied
453 to histological sections or when it was pre-absorbed with a 10-fold excess of the
454 similarly-sized but unrelated AR462 peptide. However, AR reactivity was reliably
455 abolished when PG-21 was pre-absorbed with a 10-fold excess of the AR21 protein, the
456 antigen used to generate this antibody.

458 **3.6. Immunoblotting**

459 Immunoblot analysis of protein extracts from mature male *S. tiburo* testes using
460 the anti-AR PG-21 antibody demonstrated the presence of a nuclear protein

corresponding to a molecular weights of ~110 kDa (Fig. 11). This size is consistent with reports of AR from other vertebrate groups (Vornberger et al., 1994; Arenas et al., 2001; Zhou et al., 2002).

4. Discussion

This research provides evidence for AR expression and protein production in multiple cell types in the bonnethead reproductive tract, suggesting that androgens play important roles in the function of these cells and in the overall process of male reproduction. To accomplish this, a partial cDNA of bonnethead AR was cloned from testis and found to exhibit strong homology with spiny dogfish AR and other vertebrate ARs, particularly in the highly conserved DBD and LBD. In addition, isolation and partial characterization of bonnethead AR permitted the development of molecular tools for determining that the testis, epididymis, seminal vesicle, and clasper express AR, and localizing putative cell-specific androgen targets within most of these organs. Last, immunocytochemical analysis using the PG-21 antibody confirmed that all cell types found to express AR mRNA also appear to produce AR protein. Although other studies have presented data on androgen-binding activity and AR expression in the shark (i.e., *S. acanthias*) testis (Cuevas and Callard, 1992; Betka and Callard, 1998), this is the first study to broadly examine AR presence in multiple components of the male shark reproductive tract. It is also the first study to examine androgen targets in seasonally reproducing elasmobranchs, which have been well studied with regards to plasma androgen profiles, and in general, temporal patterns in AR expression and production coincide well with circulating levels of these hormones (Manire and Rasmussen, 1997; Gelsleichter et al., 2002).

The presence of both AR mRNA and protein in Leydig-like, Sertoli, and peritubular myoid cells in the bonnethead testis is similar to findings in fishes, birds, and mammals (e.g., de Waal et al., 2008; Liu et al., 2009; Patrão et al., 2009; Leska et al.,

2012). These data suggest that androgens are critical for regulating the normal functions of these cell types in male vertebrates in general, a premise that is well supported by recent studies on the phenotypic alterations in male mouse reproduction that occur in cell-selective AR knockout models. For example, loss of AR in Sertoli cells, which nourish and shape the development of germ cells, results in infertility due to spermatogenic arrest at meiosis or the round spermatid stage depending on the knockout model in question (O'Shaughnessy et al., 2009; Walters et al., 2010). Spermatogenic arrest at meiosis has also been observed following partial AR deletion in Leydig cells, which are responsible for androgen synthesis and secretion, likely due in part to reduced production of testosterone (Kerkhofs et al., 2009; O'Shaughnessy et al., 2009; Walters et al., 2010). Partial deletion of the AR gene in peritubular myoid cells has also been shown to result in infertility, perhaps due to altered function of Leydig and/or Sertoli cells, both of which appear to be influenced by peritubular myoid cells via paracrine mechanisms (Walters et al., 2010; Welsh et al., 2012). While the exact roles of these cells remain poorly understood in most sharks, it is reasonable to consider that androgens play comparable roles in the male bonnethead. This is based on the increased levels of AR immunoreactivity in Leydig-like cells near pre-meiotic/meiotic-stage spermatocysts and in Sertoli cells associated with elongating spermatids, suggesting important roles for androgens in specifically regulating meiosis and spermiogenesis. The greater AR immunoreactivity observed in peritubular myoid cells surrounding spermatocysts containing mature spermatozoa may suggest an androgen-mediated role for these cells in spermiation as they have been shown to have the ability to contract and are believed to play comparable roles in the testis of other male vertebrates (Fernandez et al., 2008).

Positive detection of both AR mRNA and protein in epithelial cells and stroma of male bonnethead gonaducts is also consistent with earlier studies on male mammals, which have reported strong AR expression and immunoreactivity in these cell types (Patrão et al., 2009). These data suggest that androgens influence the roles that the reproductive ducts play in regulating sperm function, a notion that is supported by observations on mouse fertility following post-testicular AR inactivation. Male mice with epithelial AR inactivation in the reproductive ducts exhibit reduced fertility despite normal sperm production in the testis, presumably due in part to lowered secretory activity of these cells (Walters et al., 2010). This is likely to have significant impacts on survival, motility, and other aspects of the fertilizing capability of mature spermatozoa as the mammalian epididymis has been shown to express several androgen-regulated genes encoding proteins involved in cell viability, metabolism, and sperm maturation although these have yet to be confirmed in male chondrichthyans. Androgen effects on stromal cells in the reproductive ducts are also expected to have indirect effects on sperm function as these cells have been shown to influence epithelial differentiation and function via paracrine mechanisms (Cunha et al., 2003). Last, the consistent, strong AR immunoreactivity observed in epithelial cells of Leydig's gland is noteworthy because this organ is believed to contribute to the production of seminal fluid proteins in male sharks (Jones and Hamlett, 2006).

The occurrence of AR mRNA and protein in early-stage germ cells in the bonnethead testis does not agree well with most studies on other male vertebrates, many of which have found no evidence for AR expression or immunoreactivity in these cells (Wang et al., 2009). This suggests that direct androgen effects on developing germ cells

are unlikely in most male vertebrates, a premise supported by the lack of effect of AR gene deletion in mouse testis germ cells. In fact, mice with germ cell-specific AR deletion exhibit normal testis size and circulating androgen levels, complete spermatogenesis, and unimpaired fertility (Kerkhofs et al., 2009; Walters et al., 2010). However, it is important to note that there may be species- and stage-dependent differences in AR expression in male vertebrate germ cells as studies on some mammals (i.e., human, rat, monkey, and even mouse) have observed AR presence in germ cells, particularly in elongated spermatids (Tsai et al., 2006; Wang et al., 2009). Therefore, additional work is needed to clarify the potential importance of androgens in developing shark germ cells.

The consistent and abundant presence of AR mRNA but only sporadic and limited occurrence of AR protein in mature spermatozoa in the bonnethead reproductive tract is even more surprising than their presence in testicular germ cells as mature and ejaculated spermatozoa have long been considered to be transcriptionally silent cells, incapable of protein synthesis (Grunewald et al., 2005; Miller et al., 2005). Because of this, previous observations of various mRNA transcripts in sperm have been considered by some to merely represent residual mRNA left over from protein synthesized during sperm development and maturation rather than evidence for *de novo* production (Pessot et al., 1989; Naz and Sellamethu, 2006). However, more recently, it has been shown that mature spermatozoa can in fact use mRNA transcripts to produce proteins via translation by mitochondrial-associated ribosomes (Gur and Breitbart, 2006; 2008; Miller and Ostermeier, 2005). Furthermore, this process can occur as late as the period just prior to fertilization, suggesting the potential for *de novo* protein synthesis in mature spermatozoa

in the female as well as male reproductive tract (Gur and Breitbart, 2008). This is interesting considering the reproductive biology of the bonnethead because females store male spermatozoa in a specialized compartment of the reproductive ducts (i.e., the oviducal glands) for a period of up to 6 months following the copulatory period, after which ovulation and fertilization finally occur (Manire et al., 1995). Therefore, there is reason to consider that androgen-mediated effects on bonnethead spermatozoa may not occur until after these cells have exited the male reproductive tract, and perhaps that the occurrence of AR protein may be more likely to visualize in female rather than male histological samples. The potential for AR immunoreactivity in post-ejaculated bonnethead spermatozoa is also possible because, notwithstanding the controversy regarding AR presence in developing germ cells, a growing number of studies have recently demonstrated AR presence in mature spermatozoa as well as androgen-mediated effects on sperm function (Solakidi et al., 2005; Aquila et al., 2007; Zuccarello et al., 2009; Kotula-Balak et al., 2012; Aquila and De Amicis, 2014).

In conclusion, the presence of AR mRNA and protein have been demonstrated to occur in developing germ cells, Leydig-like interstitial cells, Sertoli cells, and peritubular myoid cells in the male bonnethead testis, along with stromal and epithelial cells in components of the reproductive ducts. This suggests that androgens regulate the functions of these cells at various stages in the reproductive cycle and contribute significantly to male fertility in this species. Evidence for AR expression but not substantial protein production in mature, non-ejaculated spermatozoa may also indicate that androgen-mediated effects continue to contribute to male fertility beyond the stages

of sperm production and maturation (e.g., during sperm storage in the female oviducal gland), and require further investigation.

Acknowledgements

The authors acknowledge the staff and volunteers at Mote Marine Laboratory including S. Leggett, C. Collie, R. Hueter, C. Luer, C. Manire, and C. Walsh, as well as the numerous undergraduate student interns that participated in field sampling and animal collection procedures. We are also grateful to G. Prins and L. Birch at the University of Illinois at Chicago for their donation of the AR21 and AR462 peptides, and to Melissa Gonzalez De Acevedo for assistance in the preparation of some samples. Samples used in this study were acquired through field collections funded through grants from NOAA/NMFS to the National Shark Research Consortium, Environmental Protection Agency grant #R826128-01-0 to C. Manire, the Mote Scientific Foundation to Mote Marine Laboratory's Charlotte Harbor Initiative, and the NOAA/NMFS Cooperative Research Program award #NA12NMF4540080 to J. Gelsleichter. Additional portions of this study were funded by National Science Foundation award 0416454 to J. Gelsleichter. We also thank J. Lindzey, J. Moore, and J. Yoder for their support and useful assistance during the early stages of this project. Although the research described in this article has been funded in part by the United States Environmental Protection Agency, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389-3402.
- Aquila, S., De Amicis, F., 2014. Steroid receptors and their ligands: effect on male gamete functions. *Exp. Cell Res.* 328, 303-313.
- Aquila, S., Middea, E., Catalano, S., Marsico, S., Lanzino, M., Casaburi, I., Barone, I., Bruno, R., Zupo, S., Andò, S., 2007. Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Hum. Reprod.* 22, 2594-2605.
- Arenas, M.I., Royuela, M., Lobo, M.V.T., Alfaro, J.M., Fraile, B., Paniagua, R., 2001. Androgen receptor (AR), estrogen receptor-alpha (ER- α) and estrogen receptor-beta (ER- β) expression in the testis of the newt, *Triturus marmoratus marmoratus* during the annual cycle. *J. Anat.* 199, 465-472.
- Awruch, C.A., Pankhurst, N.W., Frushe, S.D., Stevens, J.D. 2008. Endocrine and morphological correlates of reproduction in the draughtboard sharks *Cephaloscyllium laticeps* (Elasmobranchii: Scyliorhinidae). *J. Exp. Zool. A* 309, 184-197.
- Betka, M., Callard, G.V., 1998. Cloning of an androgen receptor cDNA from *Squalus acanthias* testis. *Bull. Mt. Desert Isl. Biol. Lab.* 37, 111.
- Bowman, C.J., Denslow, N.D., 1999. Development and validation of a species- and gene specific molecular biomarker: vitellogenin mRNA in largemouth bass (*Micropterus salmoides*). *Ecotoxicology* 8, 399-416.
- Brinkmann, A.O., 2011. Molecular mechanisms of androgen action – a historical perspective. *Methods Mol. Biol.* 776, 3-24.

628 Chattopadhyay, S., Park, J.H., Seong, J.Y., Kwon, H.B., Lee, K. 2003. Cloning and
629 characterization of androgen receptor from bullfrog, *Rana catesbeiana*. Gen. Comp.
630 Endocrinol. 134, 10-17.

631 Cuevas, M.E., Callard, G.V., 1992. Androgen and progesterone receptors in shark
632 (*Squalus*) testis: characteristics and stage-related distribution. Endocrinology 130,
633 2173-2182.

634 Cunha, G.R., Hayward, S.W., Wang, Y.Z., Ricke, W.A., 2003. Role of the stromal
635 microenvironment in carcinogenesis of the prostate. Int. J. Cancer 107, 1-10.

636 de Waal, P.P., Wang, D.S., Nijenhuis, W.A., Schulz, R.W., Bogerd, J., 2008. Functional
637 characterization and expression analysis of the androgen receptor in zebrafish (*Danio*
638 *rerio*) testis. Reproduction 136, 225-234.

639 Dijkman, H.B.P.M., Mentzel, S., de Jong, A.S., Assmann, K.J.M., 1995. RNA *in situ*
640 hybridization using digoxigenin-labeled cRNA probes. Biochemica 2, 23-27.

641 Dornas, R.A.P., Oliveira, A.G., Dias, M.O., Mahecha, G.A.B., Oliveira, C.A., 2008.
642 Comparative expression of androgen receptor in the testis and epididymal region of
643 roosters (*Gallus domesticus*) and drakes (*Anas platyrhynchos*). Gen. Comp.
644 Endocrinol. 155, 773-779.

645 Fernández, D., Bertoldi, M.V., Gómez, L., Morales, A., Callegari, E., Lopez, L.A., 2008.
646 Identification and characterization of myosin from rat testicular peritubular myoid cells.
647 Biol. Reprod. 79, 1210-1218.

648 Gelsleichter, J., Rasmussen, L.E.L., Manire, C.A., Tyminski, J., Chang, B., Lombardi-

649 Carlson, L., 2002. Serum steroid concentrations and development of the reproductive
 650 system during puberty in male bonnethead sharks, *Sphyrna tiburo*. Fish Physiol.
 651 Biochem. 26, 389-401.

652 Gelsleichter, J., Steinetz, B.G., Manire, C.A., Ange, C., 2003. Serum relaxin
 653 concentrations and reproduction in male bonnethead sharks, *Sphyrna tiburo*. Gen.
 654 Comp. Endocrinol. 132, 27-34.

655 Grunewald, S., Paasch, U., Glander, H.J., Anderegg, U., 2005. Mature human
 656 spermatozoa do not transcribe novel RNA. Andrologia 37, 69-71.

657 Gur, Y., Breitbart, H., 2006. Mammalian sperm translate nuclear-encoded proteins by
 658 mitochondrial-type ribosomes. Genes Dev. 20, 411-416.

659 Gur, Y., Breitbart, H., 2008. Protein synthesis in sperm: dialog between mitochondria
 660 and cytoplasm. Mol. Cell. Endocrinol. 282, 45-55.

661 Henikoff, S., Henikoff, J.G., Alford, W.J., Pietrokovski, S., 1995. Automated
 662 construction and graphical presentation of protein blocks from unaligned sequences.
 663 Gene 163, GC17-26.

664 Henningsen, A.D., Murru, F.L., Rasmussen, L.E., Whitaker, B.R., Violetta, G.C., 2008.
 665 Serum levels of reproductive steroid hormones in captive sand tiger sharks, *Carcharias*
 666 *taurus* (Rafinesque), and comments on their relation to sexual conflicts. Fish Physiol.
 667 Biochem. 34, 437-446.

668 Heupel, M.R., Whittier, J.M., Bennett, M.B., 1999. Plasma steroid hormone profiles and
 669 reproductive biology of the epaulette shark, *Hemiscylium ocellatum*. J. Exp. Zool. 284,
 670 586-594.

671 Jones, C.J., Hamlett, W.C., 2006. Ultrastructure of the male genital ducts of the

672 clearnose skate *Raja eglanteria*. J. Exp. Zool. 30, 1018-1029.
 673 Kerhofs S., Denayer, S., Haelens, A., Claessens, F., 2009. Androgen receptor knockout
 674 and knock-in mouse models. J. Mol. Endocrinol. 42, 11-17.
 675 Kotula-Balak, M., Hejmej, A., Lydka, M., Cierpich, A., Bilinska, B., 2012. Detection of
 676 aromatase, androgen, and estrogen receptors in bank vole spermatozoa.
 677 Theriogenology, 78, 385-392.
 678 Lattier, D.L., Gordon, D.A., Burks, D.J., Toth, G.P., 2001. Vitellogenin gene
 679 transcription: a relative quantitative exposure indicator of environmental estrogens.
 680 Environ. Toxicol. Chem. 20, 1979-1985.
 681 Leska A., Kiezun, J., Kaminska, B., Dusza, L., 2012. Seasonal changes in the expression
 682 of the androgen receptor in the testes of the domestic goose (*Anser anser f.*
 683 *domestica*). Gen. Comp. Endocrinol. 179, 63-70.
 684 Liu X., Su, H., Zhu, P., Zhang, Y., Huang, J., Lin, H., 2009. Molecular cloning,
 685 characterization and expression pattern of androgen receptor in *Spinibarbus*
 686 *denticulatus*. Gen. Comp. Endocrinol. 160, 93-101.
 687 Manire, C.A., Rasmussen, L.E.L., Hess, D.L., Hueter, R.E., 1995. Serum steroid
 688 hormones and the reproductive cycle of the female bonnethead shark, *Sphyrna tiburo*.
 689 Gen. Comp. Endocrinol. 97, 366-376.
 690 Manire, C.A., Rasmussen, L.E.L., 1997. Serum concentrations of steroid hormones in
 691 the mature male bonnethead shark, *Sphyrna tiburo*. Gen. Comp. Endocrinol. 107, 414-
 692 420.
 693 Manire, C.A., Rasmussen, L.E.L., Gross, T.S., 1999. Serum steroid hormones including

694 11-ketotestosterone, 11-ketoandrostenedione and dihydroprogesterone in juvenile and
695 adult bonnethead sharks, *Sphyrna tiburo*. J. Exp. Zool. 284, 595-603.

696 Miller, D., Ostermeier, G.C., Krawetz, S.A., 2005. The controversy, potential and roles of
697 spermatozoal RNA. Trends Mol. Med. 11, 156-163.

698 Moore, B.C., Milnes, M.R., Kohno, S., Katsu, Y., Iguchi, T., Guillette L.J., 2010.
699 Influences of sex, incubation temperature, and environmental quality on gonadal
700 estrogen and androgen receptor messenger RNA expression in juvenile American
701 alligators (*Alligator mississippiensis*). Biol Reprod. 82, 194-201.

702 Murashima, A., Kishigami, S., Thomson, A., Yamada, G., 2014. Androgens and
703 mammalian male reproductive tract development. Biochim. Biophys. Acta. May 27. pii:
704 S1874-9399(14)00126-6. doi: 10.1016/j.bbagr.2014.05.020.

705 Naz, R.K., Sellamuthu, R., 2006. Receptors in spermatozoa: are they real? J. Androl. 27,
706 627-636.

707 Ogino, Y., Katoh, H., Kuraku, S., Yamada, G., 2009. Evolutionary history and functional
708 characterization of androgen receptor genes in jawed vertebrates. Endocrinology 150,
709 5415-5427.

710 O'Shaughnessy, P.J., Morris, I.D., Huhtaniemi, I., Baker, P.J., Abel, M.H., 2009. Role of
711 androgen and gonadotrophins in the development and function of the Sertoli cells and
712 Leydig cells: data from mutant and genetically modified mice. Mol. Cell. Endocrinol.
713 306, 2-8.

714 Parsons, G.R., Grier, H., 1992. Seasonal changes in shark testicular structure and
715 spermatogenesis. J. Exp. Zool. 261, 173-184.

716 Patrão, M.T.C.C., Silva, E.J.R., Avellar, M.C.W., 2009. Androgens and the male

717 reproductive tract: an overview of classical roles and current perspectives. *Arq. Bras.*
 718 *Endocrinol. Metab.* 53, 934-945.
 719 Pessot, C.A., Brito, M., Figueroa, J., Concha, I.I., Yanez, A., Burzio, L.O., 1989.
 720 Presence of RNA in the sperm nucleus. *Biochem. Biophys. Res. Commun.* 158, 272-
 721 278.
 722 Rose TM, Schultz ER, Henikoff JG, Pietrokovski S, McCallum CM, Henikoff S., 1998.
 723 Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly
 724 related sequences. *Nucleic Acids Res.* 26, 1628-1635.
 725 Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist
 726 programmers. *Methods Mol. Biol.* 132, 365-386.
 727 Snelson, F.F., Jr., Rasmussen, L.E., Johnson, M.R., Hess, D.L., 1997. Serum
 728 concentrations of steroid hormones during reproduction in the Atlantic stingray,
 729 *Dasyatis sabina*. *Gen. Comp. Endocrinol.* 108, 67-79.
 730 Solakidi, S., Psarra, A-M.G., Nikolaropoulos, S., Sekeris, C.E., 2005. Estrogen receptors
 731 α and β (ER α and ER β) and androgen receptor (AR) in human sperm: localization of
 732 ER β and AR in mitochondria of the midpiece. *Hum. Reprod.* 20, 3481-3487.
 733 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the
 734 sensitivity of progressive multiple sequence alignment through sequence weighting,
 735 position-specific gap penalties and weight matrix choice. *Nucleic Acid. Res.* 22, 4673-
 736 4680.
 737 Tricas, T.C., Maruska, K.P., Rasmussen, L.E., 2000. Annual cycles of steroid hormone
 738 production, gonad development, and reproductive behavior in the Atlantic stingray.
 739 *Gen. Comp. Endocrinol.* 118, 209-225.

740 Tsai, M.Y., Yeh, S.D., Wang, R.S., Yeh, S., Zhang, C., Lin, H.Y., Tzeng, C.R., Chang,
741 C., 2006. Differential effects of spermatogenesis and fertility in mice lacking androgen
742 receptor in individual testis cells. *Proc. Natl. Acad. Sci.*, 103, 18975-18980.

743 Vornberger, W., Prins, G., Musto, N.A., Suarez-Quian, C.A., 1994. Androgen receptor
744 distribution in rat testis: new implications for androgen regulation of spermatogenesis.
745 *Endocrinology* 134, 2307-2316.

746 Walters, K.A., Simanainen, U., Handelsman, D.J., 2010. Molecular insights into
747 androgen actions in male and female reproductive function from androgen receptor
748 knockout models. *Hum. Reprod. Update* 16, 543-558.

749 Wang R.-S., Yeh, S., Tzeng, C.-R., Chang, C., 2009. Androgen receptor roles in
750 spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor
751 knockout mice. *Endocr. Rev.* 30, 119-132.

752 Welsh M., Moffat, L., Belling, K., de Franca, L.R., Segatelli, T.M., Saunders, P.T.K.,
753 Sharpe, R.M., Smith, L.B., 2012. Androgen receptor signaling in peritubular myoid
754 cells is essential for normal differentiation and function of adult Leydig cells. *Int. J.*
755 *Androl.* 35, 25-40.

756 Zhou, Q., Nie, R., Prins, G.S., Saunders, P.T.K., Katzenellenbogen, S., Hess, R.A., 2002.
757 Localization of androgen and estrogen receptors in adult male mouse reproductive tract.
758 *J. Androl.* 23, 870-881.

759 Zuccarello, D., Garolla, A., Ferlin, A., Menegazzo, M., De Toni, L., Carraro, M.,
760 Veronese, C., Foresta, C., 2009. Androgen receptor is expressed in both X- and Y-
761 carrier human spermatozoa. *Fertil. Steril.* 91, 193-200.

762

LIST OF FIGURES

Figure 1. Nucleotide and deduced amino acid sequence of the cloned androgen receptor gene fragment from the bonnethead (*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 2. Comparison of the deduced amino acid sequence of the cloned bonnethead (*Sphyrna tiburo*) androgen receptor along with examples from eight other vertebrate 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 3. Screening of androgen receptor mRNA in tissues/organs of bonnethead (*Sphyrna tiburo*) using RT-PCR. Positive results are demonstrated by the amplification of a 174-bp band, which is indicated by the arrow. The type of organ is identified below the bands. Testis (T), Epididymis (Epi), Seminal Vesicle (SV), Clasper (CL), and Heart (H).

Figure 4. Examples of AR (174 bp) and 18s ribosomal (315 bp) bands amplified in a relative PCR using bonnethead (*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 5. Results of relative PCR demonstrating the levels of AR mRNA expression in the reproductive tract of male bonnethead (*Sphyrna tiburo*) during different stages of the reproductive cycle. The data are reported as mean AR/18s ratios of optical density ($\pm$ SE) for the a) testis, b) epididymis, and c) seminal vesicle. Sample size per stage is indicated in the lower part of the bars. Homogenous subset are identified by letters above the bars (ANOVA and Tukey's HSD test, $P < 0.05$).

Figure 6. Detection of androgen receptor (AR) transcripts via Northern blotting using total RNA extracted from testis samples of bonnethead (*Sphyrna tiburo*). The positions and sizes of RNA molecular weight marker bands are shown on the left of the nylon membrane. The two arrows on the right of the membrane indicate the relative positions of faintly positive bands detected using a digoxigenin-labeled AR antisense cRNA probe.

Figure 7. Localization of androgen receptor (AR) mRNA (arrows) in testis of mature male bonnethead (*Sphyrna tiburo*) using RNA *in situ* hybridization. AR was detected in nuclei of a) Leydig-like interstitial cells (ILC), b) Sertoli cells (SerC), c) mature spermatozoa (SZ), and myoid cells (MC).

Figure 8. Localization of androgen receptor (AR) mRNA (arrows) in genital ducts of mature male bonnethead (*Sphyrna tiburo*) using RNA *in situ* hybridization. AR was detected in a) spermatozoa (SZ) and c) luminal epithelial cells (E) of the epididymis, and b) spermatozoa (SZ) and d) luminal epithelial cells (E) of the seminal vesicle.

Figure 9. Localization of androgen receptor protein in testis of mature male bonnethead (*Sphyrna tiburo*) via immunocytochemistry using the AR-21 antibody. Positive immunostaining is indicated by arrows. Images include: a) low-magnification (100x) view of a testis section from an individual undergoing late spermatogenesis, demonstrating the presence of primary and secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa along with AR immunoreactivity in interstitial Leydig-like cells and myoid cells; b) high magnification (1000x) view of spermatocysts containing secondary spermatogonia from an individual undergoing early spermatogenesis, demonstrating AR immunoreactivity in secondary spermatogonia and Sertoli cells; c) high magnification (1000x) view of spermatocysts containing primary spermatocytes from an individual undergoing early spermatogenesis, demonstrating strong AR immunoreactivity in interstitial Leydig-like cells; d) 400x magnification view of spermatocysts containing elongating spermatids, demonstrating strong AR immunoreactivity in Sertoli cells; e) 400x magnification view of spermatocysts containing mature spermatozoa, demonstrating strong AR immunoreactivity in sperm midpiece; and f) 400x magnification view of spermatocysts containing mature spermatozoa, demonstrating AR immunoreactivity in myoid cells.

Chromogen/counterstain are DAB/Methyl Green in a, d, e, f and Vector
NOVARed/hemotoxylin in b and c. SG₁: primary spermatogonia, SG₂: secondary
spermatogonia, SC₁: primary spermatocytes, SC₂: secondary spermatocytes, St:
spermatids, SZ: spermatozoa. SerC: Sertoli cell, ILC: interstitial Leydig-like cell, MC:
myoid cell.

Figure 10. Localization of androgen receptor protein in genital ducts of mature male
bonnethead (*Sphyrna tiburo*) via immunocytochemistry using the AR-21 antibody.
Positive immunostaining is indicated by arrows. Images include: a) 400x magnification
view of transverse section of the epididymis from an individual undergoing late
spermatogenesis, demonstrating moderate to strong AR immunoreactivity in epithelial
cells of the epididymal lumen (E), strong immunostaining in the stroma, and a lack of AR
immunoreactivity in luminal spermatozoa (SZ); b) 100x magnification view of transverse
section of the epididymis from an individual undergoing mid-spermatogenesis,
demonstrating moderate AR immunoreactivity in the epithelial cells of Leydig's gland
(LG); and c) 400x magnification view of transverse section of the seminal vesicle from
an individual undergoing late spermatogenesis, demonstrating AR immunoreactivity in
cells of the luminal epithelium (E) and lack of AR immunoreactivity in luminal
spermatozoa (SZ). Chromogen/counterstain are DAB/Methyl Green.

Figure 11. Detection of androgen receptor protein in testis homogenates of mature male
bonnethead (*Sphyrna tiburo*) via Western Blotting using the AR-21 antibody. A protein
band approximately 110-kDa in molecular weight exhibited AR immunoreactivity in
nuclear (N) fractions of testis homogenates (right arrow). AR-like immunoreactivity was

856 absent in cytosolic (C) and membrane (M) fractions. St: Bio-Rad Precision Plus Protein

857 Standards.

858

859

860

861

862

863

864

865

Figure 1

```
aggcgcaaaaactgcccatcatgccgtctgagaaagtgcctttgcagctggaatgacactt 60
R R K N C P S C R L R K C F A A G M T L 20

ggaggtcgaaaattaaagaatacacgaccattccaaaccacagatgaaaccgactctcca 120
G G R K L K N T R P F Q T T D E T D S P 40

gttgtaaaaagcaacaggacagcacacactctgttgtagcaagaattgggtgtaccgagg 180
V V Q K Q Q D S T H S V V P R I G V P R 60

atgcagcaatttcagtatcagccactgtttctcactgtgctacaatctattgagcctgat 240
M Q Q F Q Y Q P L F L T V L Q S I E P D 80

atggatatattcaggctatgacaacacacagcctgatacatctgccagtttggttaacaagc 300
M V Y S G Y D N T Q P D T S A S L L T S 100

ctcaatgagcttggcgaacgtcaa 324
L N E L G E R Q 108
```


Figure 2

Bonnethead shark	RRKNCPSCLRLKCF AAGMTLGGRKLKNTRPFQTTEETLSPVVQKQODSTHSVVPRIGVPRMQCFQ
Spiny dogfish shark	RRKNCPSCLRLKCF AAGMTLGGRKLKNTRPFQTAEETDSSVIQKQODTTLSEVPRIGVPRMQKFQ
African clawed frog	RRKNCPSCLRLKCF YEAGMTLGARKLKKLGNLKAQEELDGSSVQGE--SKELSPGMGIPQLEGYS
Canary	RRKNCPSCLRLKCF YEAGMTLGARKLKKLGNLKAQDDIEGASSSSP---TEEQAPKLVNTRIDGYE
Red sea bream	RRKNCPSCLRLKCFEAGMTLGARKLKKIGQHKNSDED--HPLQEPAEVMPNISPKSGLS----FN
Human	RRKNCPSCLRLKCF YEAGMTLGARKLKKLGNLKLQEEGEASSTTSP---TEETTCKLTVSHIEGYE
Rainbow trout	RRKNCPSCLRLRCFEAGMTLGRKLKKIVQLKSPEED--LPTQGPDTTIQGVSPQSGLS----FH
Japanese eel	RRKNCPSCLRLKCFEAGMTLGARKLKKIGQLKPPDE---LPTQAPTDAIQCISPKPGLS----FP
Burton's mouthbrooder	RRKNCPSCLRLKCFEAGMTLGARKLKKIGQKSPEEE--HGAQDP--VDPHVSPKSGVQ----LN

Bonnethead shark	YQPLFLT VLOSIEPDH VYSGYDNTQP DTSASLLTSLNELGERQ-----
Spiny dogfish shark	CQPLFLAVLOSIEPDH VYAGYDNTXP DTSASLLTSLNELGERQLVHVVKWAKVLP GFRNLHVDDQ
African clawed frog	CQPIFLNVLEAIEPVVVCAGHDNNQP DTSFALLLSLNELGERQLVHVVKWAKALP GFRNLHVSDQ
Canary	CQPIFLNVLEAIEPGVVCAGHDNSQP DTSFNNLLTSLNELGERQLVHVVKWAKALP GFRNLHVDDQ
Red sea bream	SCVVFLNVLESIEPEVVNAGHDYGP DSAATLLTSLNELGERQLVKVVKWAKGLP GFRNLHVDDQ
Human	CQPIFLNVLEAIEPGVVCAGHDNNQP DTSFAALLLSLNELGERQLVHVVKWAKALPGLRNLHVDDQ
Rainbow trout	SQLVFLNILESIEPEVVHAGHDCCQP DSAATLLTSLNELGERQLVKVVKWAKGLP GFRNLHVDDQ
Japanese eel	TQSVFLNILESIEPEVVNAGHDYGC TDSAAATLLTSLNELGERQLFKVVKWAKGLP GFRNLHVDDQ
Burton's mouthbrooder	SHLVFLNILESIEPEVVNAGHDYGP DSAATLLTSLNELGERQLVKVVKWAKGLP GFRNLHVDDQ

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11