Characterization of a novel androgen membrane receptor in lampreys that may regulate sexual development: Androgen binding in Lampreys may have implications for steroid ligand and receptor evolution in vertebrates

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

Characterization of a novel androgen membrane receptor in lampreys that may regulate sexual development: Androgen binding in Lampreys may have implications for steroid ligand and receptor evolution in vertebrates.

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Abstract

Lampreys are basal vertebrates, and their physiology may provide insight into the evolution of physiological systems in vertebrates. To date only progestin, corticoid and estrogen receptors, have been identified in the sea lamprey, *Petromyzonmarinus*. This is remarkable because 1) more derived vertebrates have evolved six nuclear receptors; 2) if androgen nuclear receptors are absent how might male sexual development be regulated in this ancient group of fishes; 3) androgens have been identified in lampreys so the lack of a nuclear androgen receptor may suggest alternative signaling pathways. I test the hypothesis that lampreys have an active androgen steroid receptor.

This hypothesis led to three predictions: first that lamprey synthesize the androgens dehydroepiandrosterone (DHEA) and androstenedione (Ad), second that the master sex hormone gonadotropin releasing hormone regulates androgen synthesis in the testes of male lamprey, and third that androgen signaling in the testes occurs via a novel receptor. Using high performance liquid chromatography, thin layer chromatography and radio-immunoassay I demonstrated the presence of the androgenic steroids DHEA and Ad in the circulation and tissues of sea lamprey and Pacific lamprey. Further, incubation of lamprey testes with lamprey specific GnRH I and III resulted in promoting the conversion of DHEA to Ad. Finally I have demonstrated that androstenedione binds to a membrane fraction isolated from the testes of Pacific lamprey, *Entosphenus tridentatus,* testes suggesting the presence of a putative androgen membrane receptor (mAR). The binding characteristics indicate a high-affinity (K_d = 7.548 +/- 1.455 nM, R^2 = 0.9804,) low capacity (B_{max} = 0.0.2366 +/- 0.01345 nM/mg of protein), single binding site androgen receptor. The association rate was determined by non-linear analysis to be 10.2 +/- 3.2 min, with maximum binding achieved at approximately 30 min. The dissociation rate was similar: 9.5 +/- 5.0 min, with maximum binding achieved at approximately 30 min. A partial identification of the receptor was achieved through the use of an affinity column and liquid chromatography. Identification of a novel androgen receptor may point to a novel

evolutionary pathway for androgen signaling in vertebrates. It's significant as it may indicate that this pathway is an ancestral state.

Lay Summary

Sexual development in vertebrates is regulated by hormones. The classic assumption is estrogens regulate female development and androgens regulate male development but exceptions exist. What may be of more significance is if there is a dimorphic expression of sex receptors. Lampreys are interesting for two reasons: first, because they appear to lack androgen receptors, so “how do lampreys regulate male sexual development?” and second, because lampreys are basal vertebrates they may shed insight into the physiology of an ancestral vertebrate. I hypothesized that lampreys have an unreported androgen receptor.

I measured the presence of two androgen steroids, dehydroepiandrosterone and androstenedione, in lampreys, and characterized a previously unreported androgen receptor (mAR) for androstenedione. These findings may indicate that mARs evolved before nuclear steroid hormone receptors, this has implications for the evolution of sexual development in vertebrates. A partial identification of the receptor is reported.

Preface

This dissertation is original, unpublished, independent work by the author, Wesley Didier

Identification and design of the research program: The initial idea that lampreys might have a functional unreported androgen receptor was suggested to me by Dr. David Close. This was based on his doctoral research into lamprey stress steroids and stress response. He proposed that, based on his finding that lampreys used a nontraditional stress hormone to regulate stress, I thought they might also use a nontraditional sex steroid to regulate sexual development.

Dr. Close taught me the techniques he used to characterize and describe the lamprey stress axis. Based on these techniques, we co-designed a research project predicting that dehydroepiandrosterone would be the active androgen in lampreys. Dr. Close believed we would find a cytosolic or nuclear receptor; he considered the cell membrane fraction unimportant to the project. This component of the cell fraction survey and the addition of androstenedione (Ad) as an alternative steroid of interest was my own independent theory. It was my decision to ultimately focus the investigation of my thesis on these components. The use of affinity chromatography as a purification technique was a direct result of my research on protein purification.

Performance of various parts of the research: I performed almost all of the research in this dissertation myself. The following exceptions are noted and acknowledged: my lab mate Satbir Rea and I occasionally assisted each other with radioimmunoassays; Dr. Sang Seon Yun assisted me with the mass spectrometry in Chapter 2; Dr. Geoffrey Hammond and Dr. Caroline Underhill assisted with the protein identification work. I prepared the samples used in this step, purified the sample using the affinity column I made and then Dr. Underhill loaded and ran the sample through ion exchange and sizing columns in Dr. Hammond's lab.

Analysis of the research data: I received advice on analysis of data from, Dr. Yun, Dr. Hammond and Dr. Colin Brauner, but all analyses were carried out by myself.

Previous publication: None of this work has been published at the time of submission, although manuscripts have been prepared and will be submitted in the near future.

Animal ethics: Animals used in this research were handed with respect and care in accordance with UBC animal care committee's guidelines. (UBC animal care # A11-0055 & A11-0245)

Reproductions of figures: Figures 1.1-3 are based on other published works but are original renderings made by myself to accurately convey the background information in this dissertation. Figure1.1 was based on a figure from Dorsam and Gutkind (2007). Figure 1.2 was based on a figure from Häggström and Mikael (2014). Figure 1.3 was reproduced based on a figure from Sower et al. (2009). Figures 2.1 and 2.2 contain data reproduced from Messa et al. (2009).

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List of Abbreviations

*	P>0.05
**	P>0.01
***	P>0.001
[3H]	tritiated
Ad	androstenedione
mAR	androgen membrane receptor
AR	androgen receptor
B_{max}	maximum binding
CR	corticoid receptor
CMO	-O-carboxymethyloxime
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone-sulphate
DI	deionized water
DPM	disintegrations per minute
E	estrogen
E2	estradiol
ER	estrogen receptor
ERα and ERß	alpha and beta estrogen receptors
GC	glucocorticoid
GnRH I& III	sea lamprey gonadotropin-releasing hormones I and III
GR	glucocorticoid receptor

HPLC	high performance liquid chromatography
HPG	hypothalamic–pituitary–gonadal axis
HPT	hypothalamic–pituitary–thyroid axis
Kd	dissociation constant
MC	mineralocorticoid
MR	mineralocorticoid receptor
M	mole
mwt	molecular weight
ns	no significance
Oxytet	oxytetracycline
P	percent threshold used to define statistical significance
PAGE	Polyacrylamide gel electrophoresis
PL	Pacific lamprey
PR	progestin receptor
S	11-Deoxycortisol
SEM	standard error of the mean
SD	standard deviation
SL	sea lamprey
T	testosterone
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane

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I would like to acknowledge:

Dr David Close as my initial research supervisor, who taught me the basics of endocrinology.

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My Committee members, Dr Geoffery Hammond, Dr Robert Shadwick, and Dr Kiran Soma for their support.

My lab mate, Satbir Rea, who assisted with radioimmunoassays.

Dr. Caroline, who assisted me with protein isolation work.

Dedication

This dissertation is dedicated to my wife, Antonieta Didier, and my sons Victor Didier and Erick Didier.

Chapter 1: General Introduction

The simple idea of classifying and linking organisms has been a driving force in biology for centuries. Linneaus developed the basis of our modern classification system in the eighteenth century, and Darwin further stimulated our modern exploration of evolution with his book, *On the Origin of Species* (1859), the following century. Common physical morphological characteristics drove early evolutionary theory. Mendel's work provided the basis for inheritance, demonstrating the simple inheritance of visible traits in peas and introducing the concept of genes. The latter has become the key tool of evolutionary work following the discovery of DNA, now culminating in the development of gene libraries that document the DNA sequences of entire species.

A central idea in this dissertation is that lampreys are extant basal vertebrates and thus might be representative of an ancestral vertebrate, a "living fossil." The actual origins of lamprey are unclear, but Shimeld and Donoghue (2012) indicate an origin of 450 million years ago. In their cladogram, this is indicated as the divergence of hagfish and lampreys; the divergence of gnathstomes and agnathans is demarked by a theoretical shared ancestor some 550 million years ago. Fossil evidence indicates that their morphology has changed little over 300 million years (Chang et al., 2006), and it is assumed by some that their physiology has been similarly well preserved (Janvier, 2011). Janvier discusses how morphology has historically been used to infer evolution and notes that that "physiological characters are no worse, no better than any other legacy of evolution" (p3.). This assertion is supported by our current understanding of DNA, because every morphological and physiological trait of an organism is the direct result of the expression of their genome (this includes phenotypic plasticity and epigenetic regulation of gene expression). These genes are passed on with great fidelity, evolution occurring only through low rates of mutation that tend towards stability as the trait reaches an optimized expression via natural selection. This supposition is supported by 1) the general laws of gene inheritance (Mendelian genetics); 2) natural selection against mutation ("drift barrier" in Lynch's review on evolution and mutation rates)(Lynch et al., 2016); and 3)

the fact that lampreys morphology still strongly resembles their fossilized ancestors. The fossil record shows that lampreys represent a relatively stable lineage and are therefore representative of an ancestral state, while evolutionary theory asserts that some advantageous mutations have led to the development of new inheritable traits (Lynch et al. 2016).

Based on these evolutionary principles, at some point lampreys and all other vertebrates evolved from a common ancestor. This ancestor would have passed on its DNA and genes with great fidelity, intermittently evolving new genes and traits via low rates of mutation and natural selection. Some lineages such as lampreys changed little over the millennia (hence the moniker "living fossils"), while other lineages evolved into new, stable lineages ("derived species"). Each of these lineages received ancestral DNA and therefore ancestral genes and traits. These ancestral traits are the morphologies and physiologies that some evolutionary biologists use to construct cladograms; they then infer common ancestors from which these lineages evolved (Janvier, 2011). Even if the label of "living fossil" is not entirely appropriate, lampreys still offer us a unique opportunity to learn specifically about their physiology, and thus perhaps about some inherited physiological traits of ancestral vertebrates.

Building on these ideas, the recent sequencing of the sea lampreys genome has provided evidence that 1) lampreys have a relatively small genome compared to other vertebrates; 2) lampreys have at least 224 gene families that can be traced back to the theoretical ancestor of all vertebrates (Smith et al., 2013); and 3) lampreys evolved before one of two genome duplication events in modern teleosts. The latter is significant, because gene duplication events are thought to be a driving force in evolution in that they create redundant genes that are then able to diverge (Holland et al., 1994; Kasahara, 2007; Panopoulou and Poustka, 2005). We are thus left with two conclusions: first, that lampreys have fewer genes and may therefore represent a less-complex biological system than other vertebrates; and second, that there is evidence linking this less-complex genome to the "ancestral vertebrate" from which all vertebrates originated. The lamprey's physiological systems should therefore be regarded as an opportunity to study a less-complex vertebrate physiology, with the potential to shed insight on the evolution of vertebrates by linking the lineages via shared traits. This reasoning is born out by Shimeld and Donoghue (2012) who point out in their review of the

evolutionary relationship between lamprey and hagfish, that it is the physiological comparisons (e.g. protein coding and mico-RNA) that have become the driving force that is reordering the phylogeny of these basal species.

Simplicity in a system under study is desirable for several reasons. The first is that simple systems are easier to study because there are fewer parts to observe. The second is the reduced likelihood that one process will be obscured by another process. The third is the implication that the hypothetical ancestral vertebrate was less complex than modern derived vertebrates by virtue of the fact that it must predate the DNA and gene duplication event. The relevance to this research is that biologists now have a view of how vertebrates function as well as the roles of organs, metabolic and signaling pathways, and other systems in derived vertebrates. Given the evidence that lampreys and the common ancestral vertebrate are less complex than derived vertebrates, the logical question to ask is how these vertebrates differ from each other, and what are the evolutionary implications of these differences.

The focus of this dissertation is the regulation of sexual maturation in vertebrates. As I explain below, lampreys are interesting because the full complement of 6 steroid nuclear receptors in gnathostomes has not been identified in lampreys. Thus, they are in a sense simpler (lacking some parts), as described above, yet they regulate their sexual development. In the following paragraphs, I develop and expand on these ideas, explaining generalized models of hormonal regulation of sexual maturation in derived vertebrates and hormonal control of sexual maturation in lampreys. This includes a closer look at estrogen and androgens signaling which appear to have much more complex functional variations than the simple classical view that estrogens are female hormones and androgens are male hormones.

1.1 Steroid Signaling

Steroid hormones regulate cell function through two mechanisms (Braun and Thomas, 2004).The first is the classical genomic pathway, initiated after the steroid diffuses through the membrane and binds to an intercellular transcription factor in the nuclear steroid super-family. The steroid–receptor complex then translocates to the nucleus, where it up or down regulates expression of the target gene(s) (Beato, 1993; Mangelsdorf et al., 1995). This pathway usually

requires time scales in the order of hours to be detected due to its reliance on gene transcription. The second type of signaling is more rapid (within seconds). In this signaling, steroid responses are mediated by secondary messenger systems such as G-coupled proteins (Filardo and Thomas, 2012). The implications of membrane receptors on cellular regulation have been of interest for over a century (Hollenberg and Cuatrecasas, 1978). These types of systems can be linked to gene transcription, as indicated in Figure 1.1, but they can also stimulate membrane and cytoplasmic targets. The α subunits of G-proteins are made up of four subfamilies—Gαs, Gαi, Gαq and Gα12—and can couple with other families of Gα proteins as indicated in Figure 1.1. The Gβ subunits and Gγ subunits function to activate many signaling molecules. Gα12 and Gαq can also regulate the activity of key intracellular signal-transducing molecules. Ultimately, G-protein-regulated signaling networks are known to control many cellular functions.

What is important to this investigation is not how G-coupled receptors work, but rather the increasing number of membrane androgen and estrogen receptors that are being reported, and speculation that they may be G-coupled receptor systems. What is of particular interest, in terms of this research, is to better understand how androgens regulate sexual maturation, cell development, and mating behaviors. Research will be needed to determine the specific cell signaling pathways associated with androgen binding to membrane androgen receptors. Two obvious reasons for determining the signaling pathways are to classify the newly emerging receptor types into families and to infer the evolution of these receptors. This dissertation lays out the foundation for membrane androgen receptor research in lampreys.

1.2 Lamprey Steroid Receptors

Lamprey steroid receptors are of great interest, because lampreys lack the full complement of nuclear receptors found in other vertebrates. Derived vertebrates have six related nuclear steroid receptors—the alpha and beta estrogen receptors (ERα and ERß), a progesterone receptor (PR), an androgen receptor (AR), a glucocorticoid receptor (GR), and a mineral corticoid receptor (MR)—all of which are speculated to have arisen through a series of duplications from a common ancestral receptor gene (Thornton, 2001). Lampreys have a

smaller sub-set, consisting of only three receptors: the first receptor was named lamprey PR; the second was named lamprey ER; the third was similar to both of the vertebrate corticoid receptors MR and GR and was named lamprey corticoid receptor (CR). This sub-set of receptors was named for their similarity to those of derived vertebrates (Thornton, 2001) and an assumed evolutionary link between the species. What Thornton does not point out is that these ancestral receptors may have been very promiscuous, binding multiple steroids, and that more specific binding likely evolved later as a refinement of the signaling system (Baker, 2003; Baker et al., 2009; Köhler et al., 2007). Thornton (2001) proposed that PR may regulate ovulation in hagfish and lamprey based on the presence of corpora atretica and lutea in hagfish ovaries. Ho et al. (1987) asserted that lamprey testes do not bind androgens with high affinity and that, in the absence of AR, the ER likely regulates male development. Thornton's (2001) paper built on this evidence, however, as I will discuss below, there is some evidence that androgens are active in the testes of lampreys. This brings us back to my speculation that a mAR evolved before the AR receptor. Evidence of a mAR could indicate that membrane receptors are a more ancient form of steroid controlled sexual regulation. This means that lampreys may share some components of the sex regulation pathway with other vertebrates but may also have components that have been replaced, lost, or repurposed (neofunctionalisation). Specifically, I speculate that the ancestral steroid sex regulation pathway may have consisted of a steroid synthesis pathway (Figure 1.2) that generated ligands targeting membrane bound receptors. This would mean that the sex steroid synthesis pathway coevolved with membrane receptors and was later co-opted to produce ligands for nuclear receptors. While the investigation of mAR is a relatively new area of investigation and there are not many papers on this topic, I did find a paper on steroid evolution in aquatic invertebrates. In the paper it notes that invertebrates of the deuterostome clade, such as Acraniaand Echinodermata, respond to estrogens and androgens and, at least in Branchiostoma, and an estrogen receptor has been cloned (Köhler et al., 2007). This supports my supposition of a more ancient origin for steroid signaling that predates the vertebrate system.

Lampreys may therefore represent a different evolutionary stage and/or evolutionary path in steroid receptors. If lampreys represent any alternative evolutionary path, then similarities in steroid synthesis and receptor structure or genes would have to be explained as convergent evolutionary events. The most parsimonious view would be that lampreys represent an earlier, less-complex steroid receptor system: a system inherited from a common ancestor of all vertebrates. This hypothetical ancestral steroid system would then represent the basal system from which lampreys and other extant vertebrates evolved their current steroid system. Taking this view of lamprey physiology offers us an opportunity to investigate the evolution of a physiological system such as nuclear steroid receptors (Baker, 2003; Baker et al., 2009; Filardo and Thomas, 2012; Lynch et al., 2016; Thornton, 2001).

Thornton (2001) investigated the six related nuclear steroid receptors found in more derived vertebrates—Eα and Eβ, PR, AR, GR and MR—to determine the order in which these receptors may have evolved in vertebrates. These nuclear receptors are theorized to have evolved through a series of duplications from a common ancestral receptor (Bridgham et al., 2006; Holland et al., 1994; Zhang, 2003). As previously stated, lampreys possess only three of these nuclear receptors: PR, ER and CR (Thornton, 2001). Thornton (2001) asserts that the ER was the first nuclear steroid receptor and it evolved to use an existing ligand which implies that the biosynthetic pathway evolved first. Furthermore, he states that his research supports the theory that the ER evolved to use an existing terminal ligand (in this case the sex steroid, estradiol) in this complex synthesis pathway (Figure 1.2) and that the other steroids later associated with nuclear receptors were orphan ligands (no known receptor). Bridgham et al. (2006) suggest that the ancestral steroid receptors were promiscuous, binding multiple ligands. Bridham's suggestion implies that the other steroids are not orphan ligands because they all bind to the ancestral ER. This explanation provides an evolutionary mechanism for developing the synthesis pathway, because multiple steroids could be active ligands for the ancestral ER. Thornton (2001) also points out that nuclear steroid receptors have only been reported in vertebrates, implying that this is a significant event in vertebrate evolution and in the evolution of sexual dimorphism. However, it appears that some aquatic invertebrates respond to estrogen and androgen despite the absence of nuclear receptors. The review by Köhler et al.,

(2007) acknowledges that this is an area of debate and more research is needed, but it supports the speculation that steroids had a functional role that predate nuclear receptors.

To understand the importance of sex steroid receptors and the relevance of their evolution, it is important to understand the role they play in vertebrate development. The classical view was that the default sex in vertebrate development is female and male development is regulated by the production of androgens (Norman and Litwack, 1997; Norris, 2007). Studies on fish sexual development show that classical thinking only represents one possible model of sexual development (Heule et al., 2014). Heule et al. (2014) identify eight variations on sexual development in fish, unisexuality, hermaphroditism, serial hemaphrodism, sequential hermaphrodism, simultaneous selfing, simultaneous outcrossing and gonochorism. The more salient question may therefore be, is there dimorphic expression of receptors and ligands associatedwith sexual development and maturation?

Standard models of sexual maturation of males in gnathstomes indicate that maturation is regulated by a luteinizing hormone, a follicle-stimulating hormone, and the androgenic steroid hormones testosterone (T), androstenedione (Ad), dehydroepiandrosterone (DHEA) and 5α-dihydrotestosterone (Norman & Litwack, 1997; Norris, 2007) (Figure 1.2). Sower et al. (2009) compared known components of the gnathstomes hypothalamic–pituitary–gonadal (HPG) axis with that of the hypothetical lamprey hypothalamic–pituitary–thyroid (HPT) axis. In the gnathostome model, direct cell-to-cell signaling and circulating blood hormones regulate sexual development. This means that there is a complex system of communication within gnathostomes that coordinates maturation of the organism into a sexually functional adult.

The target tissue for the HPG axis is the gonads. In testis, the generation of sperm involves three main cell types, steroidogenic Leydig cells, somatic Sertoli cells and the germ cells (Schulz et al., 2010). Schulz et al.'s review on spermatogenesis in fish provides us with the following information. The Leydig cells produce sex steroids and the Sertoli cells support the germ cells that generate the sperm. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) interact with their receptors in a highly specific manner in mammals with little overlap in biological activities at physiological hormone concentrations. LH regulates Leydig cell sex steroid production; FSH regulates Sertoli cell activities. The role of FSH in fish is less well

understood but it has effects on fish spermatogenesis and may regulate the proliferation of Sertoli cells. The Leydig cell produces progestogens, androgens and estrogens. Progestogens are present throughout the entire spermiation process and are thought to be involved in the regulation of several testicular functions. Androgens strongly influence testicular gene expression and are effective in supporting either the whole process of spermatogenesis, or at least some steps such as spermatogonial multiplication and spermatocyte formation. Estrogens, contrary to the classic "female" label appear to regulate aspects of spermatogenesis. What becomes apparent from Schulz et al.'s (2010) review is that there are a lot of variations and knowledge gaps in fish sexual development and spermatogenesis. Thus, the study of lamprey physiology is likely to yield interesting novel insight into the evolution of sexual development.

Evolutionarily, more derived vertebrate lineages are often defined by an increase in complexity as they gain more defining traits: traits that are used to define evolutionary relationships in cladograms. For example, gnathstomesare considered more derived than agnathans, and are thus placed on an evolutionary branch defined by the additional trait of having jaws. This does not mean that evolution is only defined by the acquisition of novel traits; loss or repurposing of morphological traits may also define lineages (e.g. the reduction of bones in fish skulls, and the repurposing of hyoid arches and muscles in vertebrates to serve as jaws and parts of the ear). The point is that there is a general pattern in cladograms for branching lineages to be defined by the acquisition or loss of novel traits. The development of internal traits such as the HPG axis that controls sexual maturation should be viewed in a similar manner. A change in the complexity of the HPG axis and/or changes in the messenger chemicals and receptors that participate in the control axis may, therefore, be viewed as evolutionary changes that define and separate branches of the phylogenetic trees (Bridgham et al., 2006). Recall that Janvier (2011) asserted that morphological traits are not better or worse than physiological characteristics for classifying organisms. Thornton's (2001) assertions about the evolution of nuclear receptors follows this line of reasoning, equating the presence of six nuclear receptors in jawed vertebrates as being more derived than the condition of three nuclear receptors found in lampreys. The same approach could be used when comparing the hypothetical HPG axis of lamprey and the HPG axis of other vertebrates. The lamprey HPG

axis—and by implication the ancestral state—is less complex than the gnathstomes HPG axis. Both of these examples imply that the evolutionary trend in vertebrates is that these steroid systems have become increasingly complex with time from a theoretical ancestor to modern extant vertebrates.

The model in Figure 1.3 shows these anticipated changes. The most important thing to note when comparing these models (Figure 1.3) is that the androgens and estrogens in the gnathostome model consist of a suite of blood-born hormones (*androgens:* T, Ad, DHEA and 5α-dihydrotestosterone; *estrogens:* estradiol (E2), estrone and estriol) that act through nuclear receptors. This is relevant, because there is no evidence of any seasonal increase in plasma androgen levels in Pacific lampreys (L*ampetra tridentate*) or sea lampreys (*Petromyzon marinus)* (Adams et al., 1987; Mesa et al., 2010; Sower et al., 1985). Furthermore, there is no evidence lampreys have a nuclear androgen receptor, indicating that the pathway must be very different. Both male and female Pacific and sea lampreys, however, exhibit a seasonal rise in estradiol just prior to sexual maturation (Messa et al, 2010),which raises the question of whether sexual development in both sexes is controlled by the increase in estrogen levels in the plasma or if there is an unknown androgen control axis for male development.

Bryan et al. (2007) suggested that there might be a cytoplasmic receptor in sea lamprey testes tissue. They reported that plasma levels of Ad were very low (1.55 +/–0.36 ng/ml) following stimulation by gonadotropin-releasing hormones (GnRH), and they concluded that these levels were too low to stimulate male development. They speculated that, instead, the Ad might be sequestered in the testes, and they subsequently reported that not only did the testes contain 2-3.5 times more Ad than the rest of the body, but also that the level rose in response to *in vivo* injections of GnRH. Bryan et al. (2007) also reported binding of Ad in the testes cytosol that they concluded was due to a possible high-affinity androgen receptor with multiple binding sites. The size of the receptor Bryan et al. (2007) reported in lampreys was large (440 kDa) relative to salmon (200kDa), both of which are larger than human androgen receptors (AR-A; 87 kDa, AR-B; 110 kDa). This led them to propose a previously unreported Ad cytosolic receptor in lampreys that differs markedly from other vertebrates, clearly an intriguing find and worthy of further investigation.

I therefore decided to investigate in greater detail lamprey androgens and potential androgen receptors. The first and most obvious hypothesis is that lampreys and derived vertebrates inherited their steroid biosynthesis pathway from a common ancestor. Obviously, testing this hypothesis is impossible, because the actual ancestor is not available. Nevertheless, it is important to state these broad linking hypotheses that shape evolutionary theories and consider them in the context of hypotheses that are testable. I will discuss this larger, overarching concept later.

This dissertation is written with three sequential data chapters. The central assumptions of this dissertation are that lampreys and derived vertebrates share a common ancestor from which they inherited steroids and receptors that regulate sexual maturation, and that lampreys represent an ancestral state. While it is impossible to directly test these assumptions, it is possible to test specific supporting hypotheses. With this limitation in mind, I designed experiments that would support or refute my hypotheses.

Hypotheses I-IV (outlined below) are based on accepted steroid biosynthesis pathways in jawed vertebrates and are addressed in Chapter 2. The focus in this chapter is on androgen steroid synthesis. The goal was to establish parallels between sex steroid synthesis and sexual maturation in lampreys and other vertebrates. The experiments were designed to 1) identify steroids produced by lampreys; and 2) determine if lamprey GnRHs I & III affect the production of androgens in lampreys (a prediction represented in Figure 1.3). Once these parallels are established, the dissertation moves forward into Chapter 3, in which Hypothesis V—that lampreys have an androgen receptor—is addressed and supporting evidence is reviewed. A search was conducted for androgen binding in lampreys using the precursor androgens DHEA and Ad to detect previously unreported androgen receptors. A strong membrane-binding moiety was detected in the testes tissue and became the focus of this work. Chapter 4 is based on isolation and identification of this binding moiety. The fifth and final chapter is a general discussion about the implications of this work, which includes general scientific knowledge about lampreys as well as medical and evolutionary implications.

The specific objectives of this dissertation are summarized on the following page for clarity. The justification and an explanation of the approach used for each objective follows.

Note that some hypotheses are listed as being tested in more than one chapter, through multiple techniques.

Objectives

1) to establish firmly the identity of androgenic steroids in lampreys (Chapter2)

   - Hypothesis I: Lampreys synthesize DHEA and Ad.

2) to document elements of sex steroid synthesis and the regulation of sex steroid axis by GnRH in lampreys (Chapter 2)

   - Hypothesis I: Lampreys synthesize DHEA and Ad.
   - Hypothesis II: Lamprey can convert dehydroepiandrosterone-sulphate (DHEA-S, the inactive form) to DHEA (active form) in the liver.
   - Hypothesis III: Lampreys convert DHEA to Ad in the testes.
   - Hypothesis IV: Lamprey GnRH I & III mediate production of androgens in lamprey.

3) to survey lamprey tissues for androgen binding and characterize the membrane-binding moiety (Chapter 3)

   - Hypothesis V: Lampreys have an active unreported androgen receptor.

4) to identify binding moiety detected in testis membrane fraction (Chapter 4)

   - Hypothesis V: Lampreys have an active unreported androgen receptor.
   - Hypothesis VI: The binding moiety found in lamprey testes is a putative receptor with properties similar to the androgen binding receptor reported by Braun and Thomas (2004) in the ovaries of the Atlantic croaker.

Objectives 1 and 2 (addressed in Chapter 2) are needed to definitively establish the identity and presence of DHEA and Ad in lampreys. While previous studies have reported them, they have generally been quantified by radioimmunoassay (RIA), which can be non-specific and result in false positives. My approach was to use multiple means of identification in every study to counter false-positive RIA identification results by using high performance liquid chromatography (HPLC) in conjunction with radioimmunoassay and thin layer chromatography (TLC).The integrity of this identification is superior to RIA alone, because the samples are

separated by HPLC before being subjected to RIA and TLC identification. This decreases the possibility of misidentifying a similar steroid as DHEA or Ad, because only samples that pass the HPLC identification are accepted for RIA and TLC tests. The rigor of the identification also relies on the fact that each method uses different chemical properties of the sample for identification. This identification was further strengthened because the identity of DHEA (the precursor of Ad) was also confirmed by mass spectrometry. (This data, however, was lost, and I was not able to reproduce it for this publication. My copy was lost due to computer failure, recovery from the UBC mass spectrometry computer system was not possible as the user account had expired and been purged)Mass spectrometry adds a fourth level of identification to the process; Bryan et al. (2007) reported they confirmed the identity of Ad with mass spectrometry, but did not publish the data. I submit, however, that the triple identification used at every stage in my thesis is sufficient to establish the identity of the steroids. Establishing elements of lampreys' biosynthesis pathway (Objective 2) permits comparisons of the lamprey steroid synthesis pathway with that of other vertebrates. This approach allows the identification of similarities and differences that may exist in this basal vertebrate group from other more derived vertebrates.

Objective 3 was undertaken to look for evidence of a previously unreported androgen steroid receptor and is presented in Chapter 3. Documenting evidence of a receptor provides evidence of a biological need for the synthesis of androgens. This is important because Thornton's (2001) assertion that the estrogen nuclear receptor evolved to utilize an existing ligand (estradiol) before the nuclear androgen receptor evolved implies that androgens are orphan ligands. Ligands with no purpose should be selected against due to their metabolic cost of production, rather than acting as the basis for further evolution of a more complex sex steroid synthesis pathway to produce estrogens (sex steroid synthesis proceeds as: DHEA →Ad→T→E) (Bryan et al., 2008; Liley and Stacy, 1983). Thus I arrive back at my original assertion that if lampreys produce androgens and estrogens there should be evidence that both are important in their biology.

Objective 4 was undertaken to identify the binding receptor characterized in Chapter 3. This is an important step in tracing the evolutionary history of this previously unreported

receptor. A full identification would have revealed the gene associated with the receptor. This would have allowed the search of published genomic databases for similar genes and allowed the construction of a hypothetical evolutionary history of the receptor. The partial identification achieved is less informative but does narrow the field of possible receptor–DNA matches.

1.2 Figures

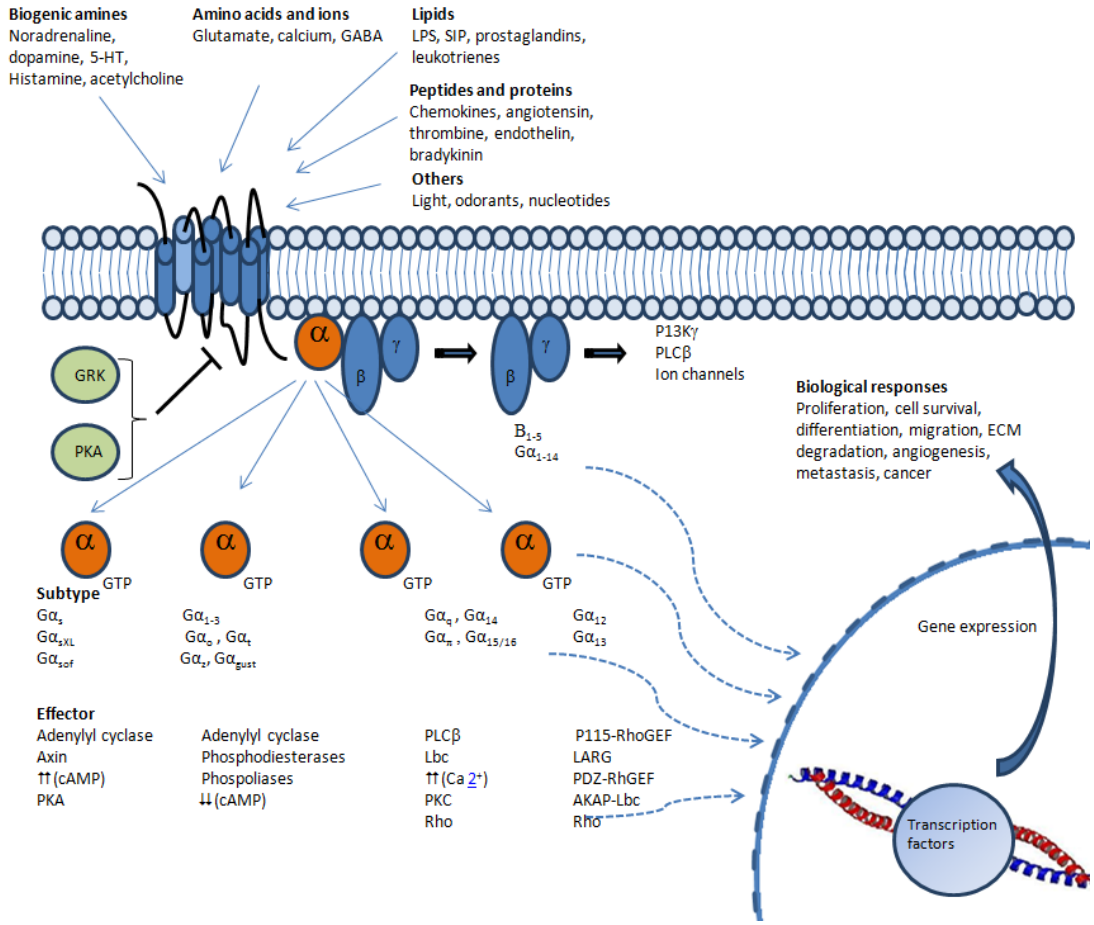

Figure 1.1 A G-protein coupled signaling cascade

This schematic indicates the many possible pathways of action. It is provided because membrane androgen receptors have been speculated by some to be G-protein receptors. The α subunits form four subfamilies—Gαs, Gαi, Gαq and Gα12—and can couple with other Gα proteins as indicated. The Gβ subunits and Gγ subunits function to activate many signaling molecules. Gα12 and Gαq can also regulate the activity of key intracellular signal-transducing molecules. The key point of this figure is not to explain how G-protein receptors function, but rather to illustrate that membrane receptors can regulate both gene expression and intercellular signaling. This means that in theory, membrane receptors, such as G-proteins, would be capable of initiating and regulating actions required to control sexual maturation. (Figure reproduced from Dorsam and Gutkind [2007]).

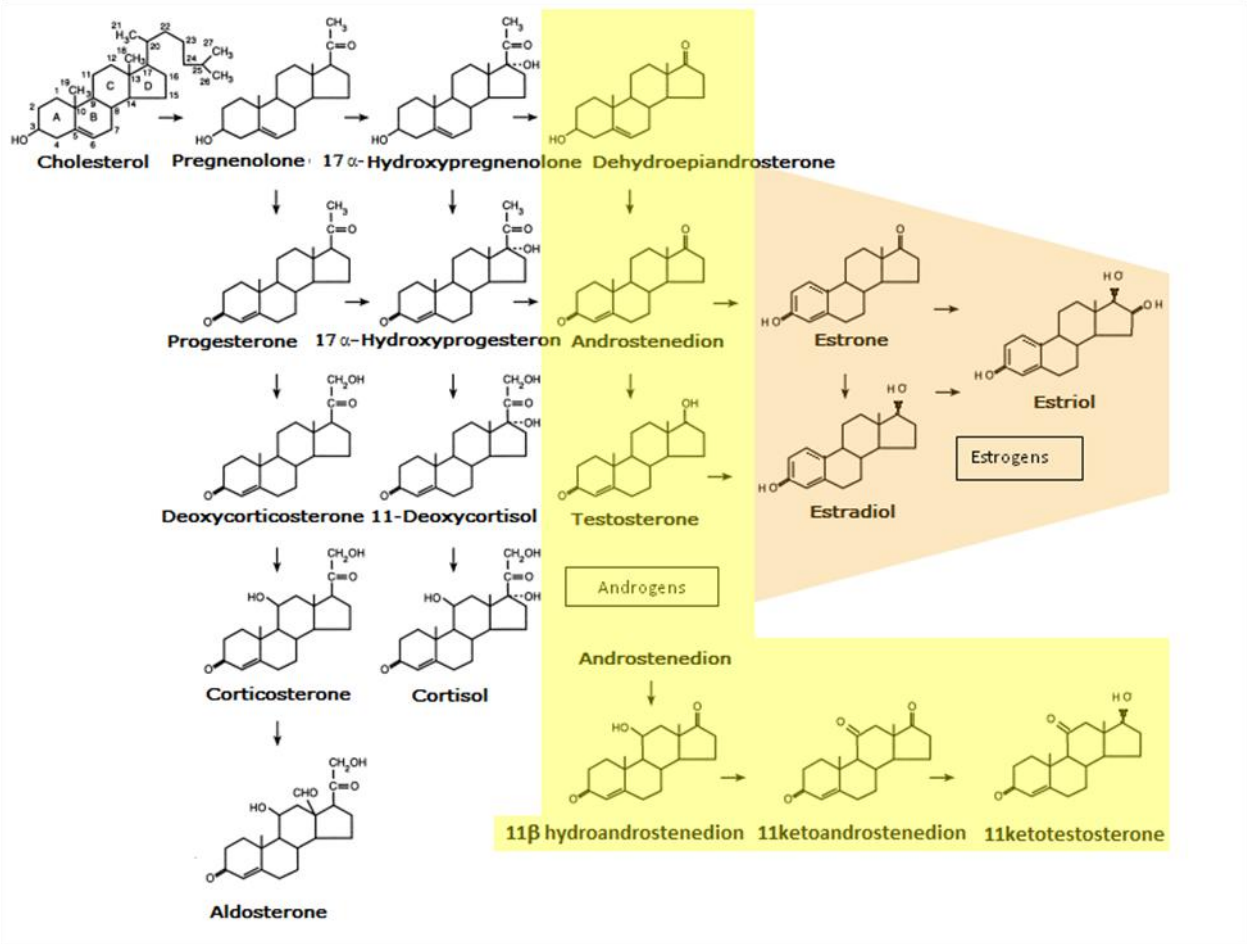

Figure 1.2 The steroid synthesis pathway in mammals

This graphical representation of the steroid synthesis pathway in mammals illustrates that androgens (yellow) are precursors of estrogens (orange). Each arrow represents an enzyme catalyzed reaction. As seen in the diagram, the production of estrogens requires the production of DHEA and Ad; thus, the evolution of androgen synthesis must predate estrogens.11ketotestosterone synthesis pathway is shown as a separate path in the bottom right corner progressing from androstenedione. (Reproduced based on figure from Häggström [2014]).

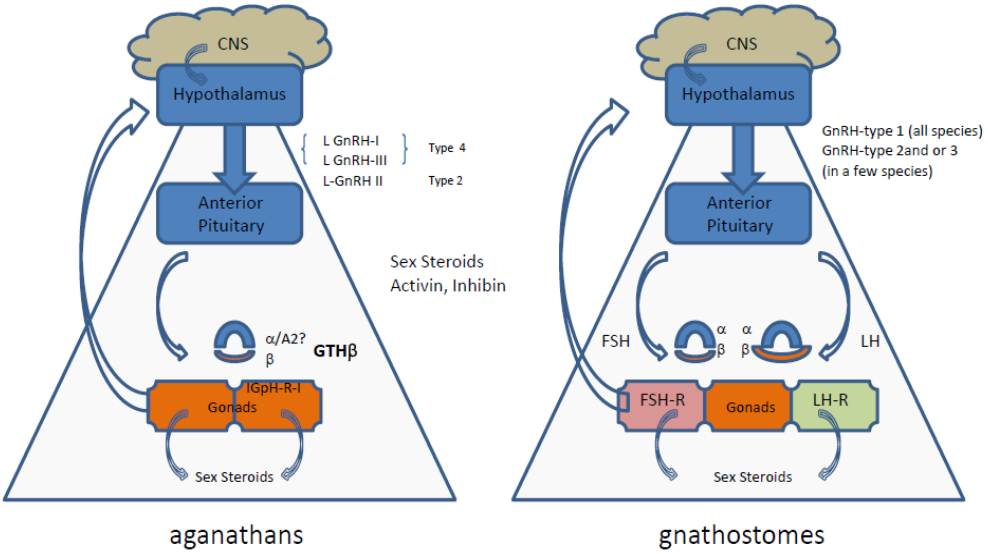

Figure 1.3 A hypothetical schematic of the hypothalamus–pituitary–gonadal axis

This diagram emphasizes that agnathans (lampreys) have two, possibly three, hypothalamic GnRHs (lamprey GnRH-II (Type 2) and lamprey GnRH-I, GnRH-III (Type 4)); one pituitary glycoprotein, gonadotropin (GTH-like) hormone; and one gonadal glycoprotein receptor; luteinizing hormone (LH) and follicle-stimulating hormone (FSH-like receptor). In comparison, gnathostomes generally have one GnRH (Type I) and/or two GnRHs (Type II and/or III); two pituitary gonadotropins (LH, FSH); and two glycoprotein receptors (Figure based on Sower et al. [2009]).

Chapter 2: Hormone Synthesis and Components of the Hypothalamus–Pituitary–Gonadal Axis in Lampreys

2.1 Introduction

This chapter focuses on steroid hormones that regulate cell function. As outlined in the introduction, establishing the identity of the steroids and elements of their biosynthesis (Objectives 1 and 2) permits the comparison of the lamprey steroid synthesis pathway with the steroid synthesis pathways of other vertebrates. This focus is paramount, because it establishes the similarities and differences that may exist in this basal vertebrate group. The lamprey lineage diverged from other vertebrate lines 450 million years ago and therefore could have developed unique steroids (Bryan et al, 2008). Establishing the presence of the sex steroid precursor dehydroepiandrosterone (DHEA) and other components of the accepted vertebrate steroid synthesis pathway would provide strong parsimonious evidence that lampreys and more derived vertebrates inherited this biosynthetic pathway. The alternative would require multiple parallel evolutionary events needed to develop these complex biosynthetic pathways.

Vertebrate sexual differentiation and maturation has already been discussed in the general introduction. The key points here are that sexual development in derived vertebrates is regulated by hormones and a complex biochemical signaling pathway. Components of this control pathway were demonstrated by Sower et al. (2009) and lead to them to hypothesize a hypothetical lamprey axis (Figure 1.2). It is also assumed that the biosynthesis of sex steroids (Figure 1.3) will be similar for lampreys and other vertebrates, because they share a common ancestor from which they inherited these synthesis pathways. One characteristic that differs between lampreys and other vertebrates is the control of male development because 1) lampreys lack a nuclear androgen receptor; and 2) lampreys appear to utilize some different hormones in the HPG axis, as indicated in Figure 1.3. The question, therefore, is how lampreys regulate masculine development.

Thornton (2001) compared the nuclear receptors in lamprey to other vertebrates and concluded that the lamprey estrogen receptor (ER) was the first nuclear receptor to evolve. He also concluded that this receptor evolved to use an existing ligand, estradiol (E2). Thornton's work raises many questions, because in many ways it is counter-intuitive. Specifically, Thornton asserts that the sex steroid synthesis pathway developed first, and that nuclear receptors developed secondarily. The evolution of a ligand prior to the evolution of a receptor is on its own a reasonable assertion, as it is a chicken and egg argument and there is no reason why a ligand couldn't precede the receptor; however, what is more difficult to understand is that a complex multi-step steroid synthesis pathway could evolve without a biological impetus. Recall from the general introduction, Figure 1.2, that steroid synthesis in gnathstomes proceeds as follows: DHEA→androstenedione (Ad)→(testosterone [T] can be bypassed at this step)→estrogens (E) (Bryan et al., 2008; Liley and Stacy, 1983). This implies that lamprey make DHEA and Ad, a supposition supported by Messa et al.'s (2010) reported estrogen steroid levels in lampreys and Bryan et al.'s (2007) reported Ad levels. If these steroids only function in conjunction with nuclear steroids, then why would they have evolved? It is reasonable to suggest that DHEA evolved as a deactivated form of the steroid 17α–hydroxypregnenolone, but what would be the driving force for the rest of the synthesis pathway to evolve?

As outlined in the general introduction, it is unlikely that lampreys would have a complex biosynthetic pathway that produces ligands with no function. This leads us back to the question: do lampreys have a functional androgen receptor? Baker (2009) provided one possible answer by suggesting that the ancestral receptors were promiscuous, binding and responding to multiple ligands. Bryan et al. (2007) suggest that there might be a functional androgen receptor in the cytoplasm of sea lamprey testes, which is discussed in greater detail in Chapter 3. Both hypothesis have merit and are not mutually exclusive. For now, it is sufficient to note that the research by Bryan et al. (2007) indicated that Ad may be a functional androgen in lampreys, a supposition that also inspired my research.

Two primary assumptions of this chapter are that lampreys and derived vertebrates share a common ancestor, from which they inherited steroids and receptors that regulate

sexual maturation, and that lampreys represent an ancestral state. This implies that lampreys and derived vertebrates have inherited a similar steroid biosynthesis pathway, and that lampreys have a functional androgen and receptor associated with male development.

Specifically this chapter will test the hypothesis that the primary androgen in lampreys is androstenedione. I aim to determine the biosynthesis of DHEA and Ad in male lampreys and examine conversion of DHEA-S to DHEA and DHEA to Ad in liver and gonads respectively. Further I will examine the regulation of testicular Ad production by lamprey GnRH I and III.

These experiments are needed to establish definitively the identity of androgen steroids in lampreys, as previous studies (Bryan et al., 2007; Mesa et al., 2010; Sower et al., 1985; Sower and Larsen, 1991) have either omitted this step or left room for a skeptical reader to question the veracity of the identification. This results from most researchers moving directly to radioimmunoassay (RIA) and reporting steroid levels in lampreys, assuming that the identity of the assayed steroid is the expected steroid. This is a significant oversight, because antibodies can cross-react with other ligands producing false positives. Antibody manufactures rate antibody cross reactivity against known steroids. For example, one DHEA-S antibody available online indicated it had 50% cross reactivity with DHEA, making it an unreliable identification tool for DHEA-S. Because it is possible that lampreys could have unknown or uncommon steroids (Bryan et al, 2008), additional verification beyond RIA is required. Separation of the biological sample by HPLC prior to determination of identity by RIA and TLC provided superior identification. Additional measurements were also conducted in my research to compare the lamprey steroid synthesis pathway with that of other vertebrates. This consisted of two pathways: 1) DHEA-S conversion to DHEA; and 2) DHEA conversion into Ad. Steroid hormones have low water solubility; plasma transport of steroids is therefore assisted by the binding of transport proteins or by the addition of sulfur (Norman and Litwack, 1997). I found no evidence of sex hormone binding globulin in lamprey literature, so I focused on DHEA-S as a possible water-soluble precursor, which is known to be an inactive form of DHEA reported in more derived vertebrates.

A key component of demonstrating hormone synthesis is identification of the tissues that produce the hormone and stimulating them to produce those hormones. In mammals

DHEA and DHEA-S are typically produced in the adrenal cortex (Norman and Litwack, 1997) and DHEA is converted into Ad in the adrenal glands (Cohan, 2011). Fish lack adrenal glands; however, the head tissues of the kidneys in teleosts is associated with steroidogenesis (Hontela et al., 2008). Unfortunately, the presence of steroidogenic tissue is not well documented in lamprey kidney. There are two 1970s papers about presumptive interrenal cells (Weisbart and Youson, 1975; Youson, 1973) and multiple references to chromaffin tissue in the heart and intestines of lampreys, but there is no published evidence of adrenal-like activity in lamprey kidneys. Furthermore, my supervisor, Dr Close, and his colleagues spent considerable effort trying to locate and dissect adrenal tissue from sea lamprey kidneys, without success, which left me with the possibility that lamprey lack steroidogenic tissue in the kidney. Lacking clear evidence that the kidney was a useful tissue to dissect for the study of endogenous DHEA-S and DHEA production, I considered the liver as a possible tissue of interest. Knobil and Neill (year) Physiology of Reproduction indicated that several steroids are modified in human livers; most relevant to my work was the conversion of DHEA to DHEA-S. Norris (2007) described human fetal livers producing DHEA-S. Knowing that these are reversible reactions; I focused on the liver as a tissue of interest. I performed in vitro incubations of DHEA-S in lamprey liver tissue to determine if DHEA-S would be converted to DHEA. This was considered an important step, because DHEA-S can act as in inactive reservoir of DHEA and the start of sex hormone synthesis.

Bryan (2007; 2008) speculated that Ad may be produced in the testes of lampreys. Incubations were therefore carried out to test if DHEA could be converted to Ad in the testes. The regulation of androgen production is also an important issue when considering Ad as a functional steroid controlling masculine development in lampreys. Typically, androgen production would be controlled by the HPG axis (Ibáñez et al., 1999; Smith et al., 1998) and should respond to GnRH. This hypothesis was tested by repeating Bryan et al.'s (2007) GnRH dose response experiments to determine if Ad is regulated in response to stimulation of the HPG axis using GnRH I & III.

For organizational purposes, this work is presented as a series of investigations: Series I: the measurement of hormones (DHEA-S, DHEA and Ad) in the plasma *in vivo* throughout sexual development; and Series II: *In vitro* incubations. Series II is divided into three parts: Part A –

DHEA-S liver incubations; Part B – Preliminary incubations to test for conversion of DHEA to Ad (where tissue and supernatant were measured as a single sample); and Part C – Refined testes incubations, in which tissue and supernatant were measured separately. The latter allows me to test Bryan's (2007) assertion that Ad is sequestered in the testes, and investigate how GnRH stimulation affects the production and distribution of Ad.

Temperature was also included as a variable because it can induce maturation in some species and moreover, thermal units can affect maturation in fish. While I do not believe lamprey maturation is queued by temperature (large North-South range and variation in glacial feed into spawning stream would not make this practical), we were able to vary maturation time by chilling and heating holding tanks. This affect is well known in sturgeon and salmon raised in captivity (Boucher et al., 2014; Jonsson et al., 2013). Our lab standard holding temperature was 7°C based on work by Dr Messa, but I measured a max temp of 19°C in Stamp falls where our Pacific lampreys were captured. Furthermore, based on concerns of the affects global warming on wildlife, temperature was considered to be a variable parameter of interest. I selected 17°C as the high experimental temperature because it was the highest temperature I could maintain in the holding tanks and it was close to the upper thermal limit I measured at stamp falls.

2.2 Methods

2.2.1 Animals

Animal collection and care protocols were conducted in accordance with University of British Columbia (UBC) policies (UBC animal care # A11-0055&A11-0245). Immature adult Pacific lampreys, *Lampetra tridentate*, were captured in June on their returning migration to their natal streams (to mature next spring) from the fish ladder at Stamp Falls, British Columbia. Each year, 100-150 fish were caught for 3 consecutive years (2009-11). Fish were collected using dip nets and transported in an 800L insulated/aerated tank (7-9°C, 90-100% air saturation) to the Department of Zoology at UBC. Lampreys were 36.6 +/- 0.73 cm in length and 85.6+/- 4.4 g. A prophylactic treatment of oxytetracycline (6.7 mg Oxytet/kg) was administered intraperitoneally within 24h of capture as a precaution against infection. The lampreys were

held in outdoor tanks at UBC until they reached sexual maturation the following June. The lampreys were in a non-feeding life stage when collected, and thus were not fed, but maintained in well-aerated flow-through fresh water (6-13°C) and kept on a natural photoperiod.

Sea lamprey, *Petromyzon marinus,* used in this work were collected from the fish ladder at Hammond Bay Biological Station, Millersburg, MI, on their returning migration to their natal streams. Fish were collected using dip nets and transported to UBC in September 2011. We received 15 male sea lamprey shipped by overnight air mail. The lampreys were transported in plastic bags; the water was saturated with oxygen prior to transport and the bags (filled 50% water 50% O_2) were packed in a cooler with ice for transport. A prophylactic treatment of oxytetracycline (6.7 mg Oxytet/kg) was administered intraperitoneally within 24h of arrival as a precaution against infection. The lampreys were 78.1 +/- 0.9 cm in length and 250.8 +/- 6.2 g in weights. These lampreys were also in a non-feeding life stage when collected, and thus were not fed in captivity and maintained in well-aerated flow-through fresh water (6-13°C) on a natural photoperiod. After 2011, I was unable to obtain permits to ship live lampreys, so tissue collection in subsequent years (2012-14) was done on site at Hammond Bay Biological Station following my protocol (described below) by Hammond Bay staff.

2.2.2Animal sampling

The Pacific lamprey and sea lamprey were anaesthetized with MS222 (0.2 g/L) (Argent Chemical Laboratories, Inc) and blood was drawn from the caudal vein into a heparinized BD vacutainer. Blood was placed on ice and then centrifuged at 1000g for 15 min to separate the plasma. Plasma was transferred to storage tubes by pipette, snap frozen in liquid nitrogen, and stored at -80°C for later measurement of endogenous circulating steroids by high performance liquid chromatography (HPLC) and RIA as described below. Lampreys were then decapitated, and placed on ice. Internal organs (brains, liver, intestine, kidneys, gonads, gills, muscle and hearts) were dissected out, placed in storage tubes, and snap frozen for subsequent analysis either by other researchers (to maximize the use of each animal) or as described below for the binding surveys and metabolic incubations in Series II.

2.2.3 Series I: Measurement of hormones in live animals over time

Sexual maturation in teleosts is typically regulated by changes in androgens and estrogens in the plasma. The levels of steroids associated with maturation typically rise during maturation (Mesa et al., 2010; Thornton, 2001). This series of measurements was made to determine if DHEA and/or Ad steroid levels in lampreys exhibited a similar rise that could be associated with sexual maturation. Pacific lampreys were separated into three groups of 20 fish, held in 200 l tanks provided with flow-through fresh water (1 l/m). All 20 lamprey were non-lethally sampled (n=20) every 2 weeks from January (prior to any signs of maturation) through to the end of March, and then weekly until the end of June, when all lampreys had matured. Fish from each sampling time were sampled from one tank and sampling was rotated sequentially through the three tanks to reduce stress on lamprey and to ensure that the lamprey had recovered from the previous sampling. Blood was drawn from the caudal vein of each animal (see animal sampling below for details). All animals in the study were tagged for identification and sexed at the end of the study, when they had reached maturation or at the time of death if they died before the end of the study. Females were considered mature when they displayed a swollen belly and were releasing eggs; males were considered mature when their cloacae were swollen and they could express milt.

2.2.4 Series II: *In vitro* tissue incubation to investigate hormone synthesis

Tracer experiments were conducted on lamprey liver and testis tissues. Tritiated [3H] precursor molecules were incubated with lamprey tissues and the conversion of the precursors into metabolic products was measured and documented. Three sets of incubations were conducted (DHEA-S in liver and DHEA or Ad in gonads). A preliminary set of gonad incubations was conducted to determine if DHEA was converted to other steroids by the ovaries and testes. After examining the results from the preliminary DHEA incubation a second set of testes, incubations tracking the distribution of Ad was done to determine if Ad is sequestered in the testis tissue. This series was done with Pacific lamprey only, as live sea lampreys were not available for this series because Fisheries and Oceans Canada did not permit further shipments

to UBC. Methodological details are listed in specific experimental methods bellow. Methods common to multiple experiment, like tissue preparation, are delineated as separate procedures

2.2.4.1 Tissue preparation for incubations

Sexually mature Pacific and sea lampreys were sedated with MS222 (0.2 g/L), decapitated, and placed on ice. All tissues (testes, ovaries, and liver) for incubations were collected immediately after euthanization as described above. Tissues were chopped with a razor blade into 1-2 mm pieces in Petri dishes on ice and incubated in L-50 Leibovitz medium (Sigma). All experimental variables of the individual incubations are noted below, including variations to incubation temperature.

2.2.4.2 Part A –DHEA-S conversion to DHEA

DHEA is the precursor for all sex steroids in more derived vertebrates and has androgenic effects in birds and mammals (Baulieu et al., 2000; Boonstra et al., 2008; Hau et al., 2004). Large reserves of DHEA and DHEA-S, a common inactive form of DHEA, are found in the plasma of humans (Baulieu, 2000). DHEA was therefore used as a precursor in incubations conducted with sea lamprey and Pacific lamprey livers to determine if lampreys can convert DHEA-S to the active form DHEA.

The livers from three male sea lampreys (56g, 57g, 62g) and Pacific lampreys (0.65g, 0.57g, 0.75g) were prepared as describe above. The location of cells that might convert DHEA-S was not known; therefore, the whole liver of each animal was used. One µCi (2.2×106 disintegrations per minute [DPM]) of [3H] DHEA-S was added to the tissue preparation. Lamprey incubations used 15 ml Brand centrifuge tubes; tubes were filled to 13 ml with incubation media. The tissue was prepared as described above and incubated at 5°C for 1h on a shaker (Oxygen was not bubbled to the media). At the end of the incubation, DHEA-S and DHEA were measured in the supernatant using the steroid extraction protocol described below.

2.2.4.2.1 Steroid extraction protocol

All steroids were isolated from plasma by ether extraction using a protocol outlined in Brenner et al., (1973) before being subjected to radioimmunoassay (RIA). Briefly, 20 µL of

plasma was ether extracted in duplicate. Two hundred µL of diethyl ether was added to each tube and tubes were vortexed for 30 sec. The tubes were centrifuged (1,000 x g for 2 min at 4°C) to allow the phases to be separated. The bottoms of the tubes were dipped into a bucket containing dry ice and methanol to freeze the aqueous layer, and then the organic phase was transferred to a clean test tube. This process was repeated three times. The organic phase was dried under a stream of N_2 gas. The dried tubes received 20 µL of RIA buffer and then were used in RIA as described above.

This protocol was used to extract steroids from supernatant for measurement. The supernatant from the tissue incubations were filtered using a 0.8 µm syringe filter, and the steroids were captured using a C-18 sep-pak (Waters). The sep-pak was activated using 5ml of methanol, and flushed with 10 ml deionized (DI) water. The supernatant or plasma samples were then passed through the sep-pack followed by 10 ml of DI water. The hormones were extracted from the sep-pak with 5ml of methanol, then dried down under nitrogen to 100µl volume. The steroids were re-suspended for separation by HPLC (600 µl solution C, 300 µl solution D, see 2.2.5.1 for complete details).

2.2.4.2.2 Tissue extraction protocol

This protocol was used to extract steroids from tissues. Briefly, tissues were weighed and then homogenized first using a tissue homogenizer in HEAD buffer and then with a Teflon homogenizer (2 tissue/10 ml 90% MeOH).The tubes were centrifuged at 1000g for 5min to pellet the tissue remnants then the supernatant was decanted. Next the sample was dried down under high-purity nitrogen flow at 50°C in a sample concentrator (TechneDri-Block DB-3) in glass culture tubes (10 mm x 75 mm, Fisher Scientific) to 100µl volume. The steroids were re-suspended for separation, by HPLC (600 µl solution C, 300 µl solution D, see 2.2.5.1 for complete details) and filtered using a 0.8 µm syringe filter in preparation for separation by HPLC.

2.2.4.3 Part B – Preliminary incubations to measure the conversion of steroids

The testis and ovary tissues from one male and one female were prepared as described above with the following differences: the tissues were divided into four incubations (0.5 g/tube) containing 100000 DPM of [3H] DHEA and one of three treatments; the control group and stimulated groups were treated with sea lamprey GnRH I and/or GnRH III (target dose 100 μg/kg of each hormone). The tissues were incubated at 7°C for 1 h on a low-speed shaker in a refrigerator (Oxygen was not bubbled into the media). At the end of the 1h incubation, the tissues were homogenized using a tissue homogenizer and the homogenate was centrifuged for 1 minute at 1000g.The supernatant was then collected and dried down under nitrogen. Steroids were re-suspended in HPLC fluid and run on the HPLC as described below.

2.2.4.3 Part C – Refined DHEA and Ad incubations to test for sequestration of Ad in testis tissue

For this set of incubations, tissues and supernatant were analyzed separately to determine if steroids were being sequestered by the testes. Briefly, 16 lampreys were sexed and separated into 4 tanks of 4 animals each. 15 lampreys were confirmed to be males at the time of dissection. Lampreys where acclimatized to their experimental tanks over a one week period. The temperature in the "hot" tanks was raised from 7°Cto17^{o}Cover 72h and then held constant at 17°C throughout the experiment. Lampreys received two *in vivo* doses of a 50/50 mix of GnRH I/GnRH III (target dose 100 μg/kg of each hormone) injected intraperitoneally, 24 h apart, prior to the extraction of testis tissue (injection 1: 7pm Day 1; injection 2: 7pm Day 2; tissue collection: 7am Day 3). Sham control animals (6) were injected with the same volume of saline. The testes were collected as described above on Day 3. One male found to have underdeveloped testes was rejected; a total of 14 males were used for the incubation. Each incubation tube was set up with one μCi (2.2 x 10 6 DPM) of [3H] DHEA or [3H] Ad and 3gof the testis tissue (GnRH groups tissue medium included GnRH I & III (100 μg/kg) to ensure any stimulatory effect of the hormones would persist throughout the incubation). The tissues were divided into two groups and incubated for 4 h at ambient temperature (7°C) or at 17°C on a low-speed shaker (Oxygen was not bubbled into the media). At the end of the 4h incubation,

tubes were centrifuged for 1 min at 1000g, and the supernatant was poured off for steroid extraction using a sep-pack, as described above. Steroids from the remaining tissue fraction were extracted separately using the tissue extraction protocol above. The isolated steroids were identified by HPLC, thin layer chromatography (TLC) and RIA, as described below.

2.2.5 Assay methods

2.2.5.1 High performance liquid chromatography

The prepared samples of endogenous steroids and standards (up to 1ml in total volume) were injected into the HPLC (Shimadzu-LC) for separation. Extracted steroids were separated on an Altima C18 reverse-phase HPLC column fitted with an Altech guard column. The HPLC was set up with four solvents: **A**: double-distilled H_2O (DDI H_2O); **B**: 100% methanol; **C**: 0.01% formic acid/DDI H_2O; and **D**: 70% acetonitrile/0.01% formic acid/DDI H_2O

Two solvents, C and D, were used to deliver a changing gradient to the column, allowing the elution of steroids from the column by subtle charge differences over a period of 90 minutes. Solvent gradients used are summarized below in the format "**flow time** and % of each solution" and "**flow time** and change to new % solution" as indicated by the arrow. The flow rate was set at 0.5 ml/minute. Less-soluble steroids were retained longer on the solid phase column until the organic solvent concentration was sufficient to elute the steroids from the column. Fractions were collected and separated into 1.5 ml tubes at 1min increments (flow rate 0.5 ml/m). All steroids elute before the 60 min, the gradient was increased to 100% MeOH to clean out all organic residue. A blank run of the HPLC was conducted before each sample run to condition the column.

Elution gradient:

0-10 min –C 70%, D 30%

10-60 min – C70%→0%,D 30%→100%

60-70 min – C 0%, D 100%

70-75 min – C 0%→70%, D 100%→30%

75-90 min – C 70%, D 30%

Cleaning gradient:

0-5 min– B0%→100%,C70%→0%, D30%→0%

5-25 min – B100%, C0%, D0%

25-30 min – B100%→0%,C0%→70%, D 0%→30%

30-50 min – C70%, D30%

Steroid standards were run on the column each time the fluids were changed and after each sample was run on the HPLC. Because T and Ad are ultraviolet (UV) detectable, they were used as the primary standards for calibration of the column. E2, DHEA, and DHEA-S have low UV absorbance. Their time of elution was therefore determined by running tritiated standards and counting the radioactivity on a LS-6500 (Beckman Coulter) scintillation counter to determine which fraction contained the standard. The column was cleaned using methanol after standards were run.

2.2.5.2 Thin layer chromatography

TLC was used as another method of steroid separation and identification that produces completely different patterns of steroid separation than HPLC, and thus provides a secondary identification of steroids. Briefly, lamprey plasma sample fractions were dried down under high-purity nitrogen flow at 50°C in a sample concentrator (TechneDri-Block DB-3) in glass culture tubes (10 mm x 75 mm, Fisher Scientific). The samples were re-suspended in 100 µl of ethyl acetate and spotted onto lanes on Whatman silica gel 60A TLC plates. Standards DHEA-S (Sigma-Aldrich) / DHEA (Sigma)/Ad (Sigma) and T (Sigma)were run in parallel lanes. The plates were placed in a glass development chamber with solvents (25 ml chloroform, 25 ml ethyl acetate, and 100 µl acetic acid) for 30 min. Samples were visualized by heating to 130°C after misting the plates with sulfuric acid. The sample lanes were scraped with an exacto-knife blade into scintillation vials in 7mm sections (by laying the plate on 7mm graph paper). The presence of radioactivity in the standard lane was confirmed by re-suspending the sample in 1 ml of ethanol in an 8-ml scintillation vial, adding 6 ml of scintillation fluid, then counting the scintillations on a LS-6500 (Beckman Coulter) scintillation counter. The DHEA identification results provided by Dr. Close followed the same protocol.

2.2.5.3 Radioimmunoassays

Radioimmunoassays were developed in the lab to measure steroids using commercially obtained anti-bodies. Standard curves were developed using steroid standards of known concentration. Assays were constructed using tritiated DHEA-S (Perkin Elmer) / DHEA (American Radio Label)/Ad (Perkin Elmer)/ combined with anti-DHEA-S (Biospacific) / anti-DHEA (abcam) /anti-Ad (Sigma)/ and DHEA-S / DHEA steroid standards (Sigma) /Ad (Sigma) /.

RIA assays were set up using the following protocol. All RIA bench work was set up in an ice bath. Antibodies were stored at $4^{o}C$ in antibody buffer (9ml deionized H_2O, 1ml 0.5 M sodium phosphate buffer (57.5g Na_2HPO_4,14.8g $NaHPO_4$ in 1l deionized H_2O), 100 µl commercially obtained anti-body and 0.05g Na Azide). Antibody dilutions were set up in duplicate and used to determine amount of antibody needed for the assay. 100 µl RIA buffer (50 ml 0.5 sodium phosphate buffer, 450 ml deionized H_2O, 4 g NaCl, 0.15g EDTA, 1 g BSA) and 100 µl antibody was added to a glass culture tube, tube 1 (10 mm x 75 mm, Fisher Scientific). Seven dilution tubes were set up with 100 µl of RIA buffer. The solution from tube 1 was then serially diluted by drawing 100 µl of the antibody solution and adding it to the next tube in line in succession until tube 8. The final 100 µl draw from tube 8 was discarded. A blank and total tube each containing 100 µl of buffer were also set up. A 100 µl aliquot of RIA buffer (containing 5000 DPM of tritiated steroid) was added to the tubes and they were incubated over night in a $5^{o}C$. The tubes were knocked down by adding 500 µl charcoal solution to all tubes (except total which received 500 µl RIA buffer) (5 ml 0.5 sodium phosphate buffer, 45 ml deionized H_2O, 0.05 gelatin, 0.5 g activated charcoal, 0.05g dextran).Tubes were then allowed to incubate for 15 min. The tubes were centrifuged at 1000 g, decanted into scintillation vials, 5ml of safety-solve (research products international) was added and the scintillations were counted on a LS-6500 (Beckman Coulter) scintillation counter. The results were plotted and the amount of antibody needed to produce a count of 2500 DPM was extrapolated.

RIAs were set up based on the antibody dilution results standard curves were set up with 5,000 DPM total and 2500 DPM maximum binding. Briefly, 12 tubes were set up and 100 µl of RIA buffer was added to each tube. Tubes were created with known steroid dilutions ranging from 500 ng/ml to 2 ng/ml, max-binding, total and blank. A 100 µl (500 ng/100 µl)

steroid solution was added to the 500 tube. The solution from tube 500 was then serially diluted by drawing 100 µl of the antibody solution and adding it to the next tube in line in succession until tube 2. The final 100 µl drawn from tube 2 was discarded. Sample tubes received 20 µl of plasma and 80 µl assay buffer. Line standard samples (large sample of plasma with known quantity of steroids) of sea lamprey plasma were added to the front and end of sample tube rows, used to standardize RIA. Radio label-buffer solution was made such that 100 µl of this solution would contain 5000 DPM. 100 µl of this solution was added to blank tubes. Antibody was added to the label-buffer solution such that 50% of the radiolabel was bound to the antibody in the absence of any standard steroid. All tubes (except blank tubes) received 100 µl of this antibody-label-buffer solution. Tubes were incubated overnight at 4°C. The tubes were knocked down and counted as described above.

2.2.5.4 Assays for incubation steroids

RIA assays were also used to confirm the chemical identity of tritiated incubation products. Because the steroids of interest were tritiated, the assay process was modified to change the competitive steroid to a cold steroid. Two tubes with 100 µl of the HPLC fraction of interest were incubated with antibodies for DHEA, DHEA-S or Ad to achieve competitive binding. The assays were done in HEAD buffer, the buffer used for tissue homogenization. The first tube contained 500 µl of HEAD buffer (25 mMHepes, 10 mM NaCl, 1 mM dithioerythritol and 1 mM EDTA, pH 7.6, Sigma-Aldrich) saturated without cold steroid standard (maximum binding), and the other tube with 500 µl of HEAD buffer with cold steroid standard (non-specific binding). Specific binding was taken as “maximum binding minus non-specific binding” and used to confirm the presence of a target steroid.

2.2.5.5 Statistical analysis of all experiments

All statistics are reported as mean +/- standard error of the mean (SEM). All statistical analyses are completed using Graphpad Prism 5.01 for Windows or Sigma Plot 11. All data were tested for homogeneity of variance and were found to conform to a Gaussian distribution. Statistical significance was set at p=0.05, and a special note was made for cases fitting a p=0.01

threshold. Unless otherwise noted, all experiments were analyzed with one-way ANOVAs with post Dunn's and Tukey's multiple-comparison tests.

2.3 Results

2.3.1 Series I: Measurement of hormones in live animals over time

2.3.1.1 Series I: Endogenous hormones

The three-step identification of DHEA and Ad in lampreys was done on plasma samples from both sea lampreys and Pacific lampreys. The results of all analyses were positive for the presence of the steroids DHEA and Ad in lamprey plasma. These results are summarized in Table A.1 in the appendix.

2.3.1.2 Series I: Plasma steroid levels

The plasma levels of DHEA and Ad were measured by RIA during the sexual maturation phase of Pacific lampreys. The DHEA and Ad levels remained low (less than 1.0 ng/ml) throughout the maturation period (Figures 2.1 and 2.2). There was no elevation in steroid production associated with maturation.

2.3.2 Series II: Part A – *In vitro* tissue incubation DHEA-S incubations

Sea lamprey liver was incubated with [3H] DHEA-S (100,000 DPM). HPLC fraction results (Figure 2.3) showed a spike in [3H]in elution fraction 52 of HPLC as indicated by DPM count (Note: These tests were run using a different HPLC at Michigan State University, which had different run times for standards than the HPLC used for this study at UBC.) DHEA in fraction 52 was confirmed with TLC. Results for both tests indicate positive results for DHEA in the fraction associated with the DHEA standard run under same conditions. (The source of this information is unpublished data from Dr. D. Close, used with permission.)

A similar set of tests was performed using Pacific lamprey livers at UBC in series II incubations. The results showed the same positive correlation, with HPLC fractions associated with DHEA-S and DHEA standards on the HPLC (Figures 2.4) and DHEA standards on TLC plates (Figure 2.5). The results for RIA testing of the HPLC fraction 60-61 for DHEA were positive; that

fraction corresponds with the DHEA standard. The specific HPLC fractions here and above differ because they were conducted on different HPLC systems. These results clearly show a conversion of the inactive DHEA-S steroid to its active form DHEA within the lamprey liver.

2.3.3 Series II: Parts B and C – *In vitro* tissue incubation

In Series II: Part B, preliminary tests were performed by incubating Pacific lamprey testes with [3H] DHEA with or without GnRH I and GnRH III. The tissue and supernatant were treated as a single sample. Results for HPLC and TLC separation of the steroids are shown in Figures2.5 and 2.6. The results indicate the presence of DHEA and Ad in the samples. Differences between treatments were visible in the TLC. The male and female subjects showed different patterns of products; the most pronounced difference being female Ad peaks at 2000 DPM while in males the lowest values were 10,000 DPM. Both ovaries and testes results have peaks at fraction 4. This is an unknown metabolic product; the position indicates that it is less water soluble than the steroid standards used. It is possibly a hydroxylated steroid; Bryan et al. (2006) reported hydroxylated steroids in sea lampreys. The low resolution of the TLC plates limited analysis of the results.

In Series II: Part C, refined DHEA–Ad incubations were conducted the following year and analyzed with HPLC for higher resolution. No females were tested in the second year. Temperature and GnRH I & III were used as variables. The tissue and supernatant were treated as separate samples. The averaged results for each treatment are shown in Figures 2.7 and 2.8.

Labels in Figures 2.7 and 2.8 indicate the fractions associated with steroid standards; groups labeled GnRH were stimulated with GnRH I & III before and during the incubation. The role and mechanism of GnRH stimulation is not known in lamprey. Pre-stimulation allows for DNA transcription up regulation in vivo. The presence in the media ensures that the cells remain stimulated for the duration of the experiment. Ambient temperature for the lampreys was 7°C. The identity of hormones associated with HPLC fractions was confirmed using RIA and TLC. The results of these tests are summarized in Table 2.2 for easy assessment of steroids found in each treatment.

The GnRH treatment produced a large variation in Ad levels in the supernatant ($p > 0.001$, Figure 2.7 and 2.9). Temperature had no significant effect on supernatant results. The GnRH treatment produced a large variation in Ad levels in the tissue ($p > 0.001$, Figure 2.7 and 2.9) and DHEA levels tissue ($p > 0.01$, Figure 2.7and 2.9). Temperature had one significant effect on tissue results ($p > 0.001$, Figure 2.8 and 2.9). The presence of [3H] Ad in DHEA incubations was verified by RIA and TLC (Table 2). Ad incubations were negative for DHEA as indicated by RIA and TLC.

2.4 Discussion

This chapter addresses four Hypotheses. Hypothesis I: Lampreys synthesize DHEA and Ad. Hypothesis II: Lamprey can convert dehydroepiandrosterone-sulphate (DHEA-S, the inactive form) to DHEA (active form) in the liver. Hypothesis III: Lampreys convert DHEA to Ad in the testes. Hypothesis IV: Lamprey GnRH I & III mediate production of androgens in lamprey.

Hypothesis I: Confirmation of the presence of DHEA and Ad in lamprey. Identification of chemical compounds in a sample can be accomplished in many ways. Because similar compounds may produce similar test results, the method of identification must be very accurate or multiple tests must be performed. In general, mass spectrometry is accepted as an accurate identification of chemical compounds, and is the preferred method of identification. The cost and access to mass spectrometry equipment, however, limited my use of this method of identification. The alternative is to have three levels of identification to determine a chemical compound. The process of triple identification was used to confirm the identities of steroids in plasma samples and in incubation products.

A single pooled sample of Pacific lamprey plasma was analyzed to confirm the presence of DHEA; its presence in sea lamprey plasma had previously been confirmed by mass spectrometry in an earlier, unpublished study by Dr. Close, and by Bryan et al. (2007). DHEA was chosen for identification because it is the precursor of all sex steroids, and therefore represents a common point of origin in the theorized metabolic synthesis pathway of lamprey sex hormones and the accepted pathway in more derived vertebrates. A single sample run of pooled plasma from many individuals was considered sufficient proof of the presence of DHEA

in lamprey plasma, because the identification was confirmed by HPLC, RIA and TLC in multiple lamprey samples, as reported below (the purpose of pooling was to create a large sample of DHEA for mass spectrometry analysis).

The three-step identification of DHEA and Ad in lampreys was performed on plasma sample from both sea lampreys and Pacific lampreys. A 22 ml sample of Pacific lamprey plasma (pooled from 25 individuals) and 34 ml of sea lamprey plasma (pooled from 18 individuals) were positive for DHEA in both samples measured using RIA in the HPLC fraction and were consistent with the DHEA standards run on the HPLC. TLC of the HPLC fractions were also consistent with those of the steroids DHEA and Ad. The results of all analyses were positive for the presence of the steroids DHEA and Ad in lamprey plasma (Table 2.1). I also identified DHEA in these samples using mass spectrometry (data were unfortunately lost and thus, not shown). N=3 was used as minimum standard for all analyses except this identification process, which was only performed once with the following justifications: 1) the sole purpose of the test was to confirm the presence of steroids already presumed to be present via RIA as indicated in other published works; 2) I had to pool all of the plasma I could spare to create a sample that would pass through the HPLC and contain enough steroids to be detected by mass spectrometry and in the three-step analysis; 3) if the assumptions of other researchers had proven false and their measurements of DHEA via RIA had been false positives, there would not have been enough DHEA in the pooled plasma sample I used to be detected by mass spectrometry; 4) the identities of steroids produced in [3H] tracer incubation experiments were also subjected to three levels of identification; and 5) mine are pooled samples that represent 22-25 individuals, implying a pseudo n>1. While my mass spectrometry results could not be replicated in time to be included here, there are two points that should be acknowledged: 1) Bryan et al. (2007) reported that they used mass spectrometry to identify Ad in their paper but did not publish the data; and 2) the steroids were identified using multiple alterative tests.

The data indicate that lampreys possess some of the androgens found in agnathans (Table 2.1). I was able to identify DHEA and Ad through multiple tests and have therefore established that lampreys produce both of these androgens.

Hypothesis II: DHEA-S can act as a precursor for DHEA synthesis in liver of Pacific lamprey and sea lamprey. DHEA-S was shown to be converted to DHEA in the Pacific lamprey and sea lamprey liver. DHEA was detected in the plasma of both sea lampreys and Pacific lampreys. These findings indicate similar pathways between lampreys and gnathostome in their sex steroid production as others have reported (Mesa, 2010; Bryan, 2007; 2008). This is confirmation of another point of similarity in steroids synthesis capability in lamprey and other vertebrates.

Hypothesis III: The hypothesis that lamprey can convert DHEA to Ad in the testis was tested by two incubation experiments. The data indicate that the testis can convert DHEA to Ad, this supports model of Ad production in the testes. This fits the Sower model of sex steroid production in the testis (Figure 1.3).

Hypothesis IV: Ad production in the testis is regulated by GnRH as indicated by the Sower HPG model. Testes were stimulated when the testis incubation included GnRH I & III *in vivo and vitro*. The results indicate that GnRH stimulation resulted in more DHEA being converted to Ad and to increased sequestering of Ad in the tissue of the testis. This finding supports the hypothalamus–pituitary–gonadal axis (HPG axis) of Sower at el (2009) (Figure 1.3), which proposed that GnRH I &III regulate sex hormone production which is consistent with Bryan et al.'s (2007) findings (see below). It is significant because the model is a simplified version of the gnathostome axis and adds to the body of evidence that lampreys may use a similar system of control to regulate sexual maturation.

Bryan et al. (2007), reported Ad was being sequestered in the lamprey testis tissue. This is consistent with a cell-to-cell signaling model in the testes. Recall, as discussed in the introduction that Leydig cells are steroidogenic and Sertoli cells are target cells. This could imply that Leydig cells are making Ad, and it is being taken up by Sertoli cells. The drop in total [3H] present indicates that Ad is being deactivated and converted to a non-steroid metabolite at a high rate (some unknown metabolites were detected in the crude Series II preliminary incubations, fraction 4, as shown in Figures 2.5 and 2.6). Note: there is no expectation that all of the [3H] tracer label would be recovered with the products represented in the steroid synthesis graphs as HPLC was configured to capture steroids, not other products of metabolism that the

tracer [3H] may have been transferred to as part of the metabolic conversion process. GnRH caused a 40% increase in the amount of Ad in the tissue. These results support Sower's HPG model and indicate that sexual maturation in male lampreys may be regulated by GnRH.

The purpose of using Ad (the expected and product of male sex steroid production) was twofold. One reason was that the DHEA ➔Ad conversion is reversible and an oversupply of Ad could push the reaction into reverse. This is supported by the small amount of DHEA detected in the Ad incubation. The second more important reason is that it is possible that DHEA was being converted to E2 and that E2 was final steroid in control of male lamprey sexual reproduction as discussed in the introduction. Note: no studies, including my own, have shown a rise in lamprey plasma androgen levels during sexual maturation. Neither Ad nor the non-classical androgen DHEA exhibit a peak during maturation in males (as indicated by the development of swollen cloacae and the ability to express sperm), indicating that it is unlikely that male development is regulated by plasma androgen levels (Bryan et al. 2007). Mesa et al. (2010) demonstrated clear rises in plasma E2 and progesterone levels associated with sexual maturation in Pacific lampreys, where the steroid levels rose by 3-5 ng/ml over the basal levels of 1 ng/ml. This is consistent with the classic model of gnathostomes sexual development (Sower 2009). It is therefore possible that male sex development is regulated by E2 and progesterone. This idea was challenged by Bryan et al. (2007), who reported large amounts of Ad being sequestered in sea lamprey testes associated with maturation. The amount of Ad was significantly increased by GnRH I & III injection *in vivo*. Bryan et al. (2007) also reported that dosing sea lampreys with Ad pellets resulted in accelerated maturation.

If E2 is the only sex steroid regulating sexually development and spermatogenesis the expected outcome from both the DHEA and Ad incubations would have been a spike in E2. No significant amount of E2 was detected in any of the incubations. This does not support a model of E2 regulation of spermatogenesis in lamprey but does not disprove E2 having a role in male sexual development. Messa et al.'s (2009) plasma sex hormone data shows a spike in E2 production April-May just before lamprey testis mature in June-July. This could indicate that E2 starts sexual maturation of the testis and Ad functions as regulator of spermatogenesis. Both theories would require further testing. The most obvious approach would be to use steroid

antagonists or possibly gene knockouts to block E2 and Ad to observe what effects the loss these steroids would have on sexual maturation.

Temperature was considered as a possible cue or driver of maturation. The warming associated with spring and summer could have been the signal to start maturation. My personal observations from holding lampreys over 5 years in the lab showed that we could slow maturation by keeping the water at 4-5°C, but the primary cue for maturation appeared to be photo period. Lamprey held in tanks at 7°C (standard holding temperature in our lab) with windows tended to mature within a 2-week period while maturation of lampreys held in the dark were spread out over a 3-5 week period (I did not specifically study this aspect of maturation). Temperature was expected to have a larger impact because a 10°C increase in body temperature should have had a large impact on metabolism, and cold water seemed to slow maturation. It is possible that the lampreys were near their thermal limit at 17°C,but I did measure a maximum temperature of 19°C in Stamp River, where Pacific lamprey were collected, indicating that 17°C was not outside of the scope of temperatures they could encounter naturally. It does, however, make more sense that lampreys would use photo period as a cue to mature given that the waterways they live in can vary in temperature depending on variations in seasonal average temperatures, seasonal snow pack and rate of melting.

Temperature had a strong effect on the Ad levels in the Ad incubations, where the amount of Ad present was significantly reduced by an increase in temperature from 7°C to 17°C. Temperature effects were much smaller in the DHEA incubations which are more relevant to the question of biological regulation of sexual maturation, because lamprey may synthesize Ad to induce maturation of the testes, if as I hypothesized, Ad is the steroid used to regulate maturation. Temperature had almost no effect on DHEA incubation. Based on these results it is unlikely that sexual maturation is driven by temperature, but the drop in Ad found in the Ad incubations does indicate thermal units accelerated the deactivation of Ad. The effect of temperature on maturation should therefore be looked at further in light of the effects of global warming on lamprey natal streams.

While all of these results for both temperature and GnRH are interesting, they must be tempered against the fact that the precursors were loaded at mg/ml concentrations, well above

biological levels ng/ml in order to produce a strong signal. Bryan et al. (2007) reported a binding moiety in the cytosolic fraction of lamprey testes for Ad and suggested that they may have found a new class of receptor. I tested for Ad biding in the testes, as well. My results indicate binding in the cytosol as reported by Bryan et al. (2007) but I found much stronger binding in the membrane fraction. This membrane binding is characterized in the next chapter.

It is thus evident from my finding that lamprey have DHEA and Ad. This is confirmed in literature (Bryan et al., 2007; Mesa et al., 2010) which report lampreys have sex steroids DHEA, Ad and E2.These findings are consistent with the hypothesis that lampreys and gnathstomes inherited the same biosynthesis pathway from a common ancestor. They are also consistent with a hypothesis of convergent evolution of steroid synthesis. Parsimony favors a shared history explanation. The effects of GnRH I & III on Ad production supports Sower et al.'s (2009) proposed HPG axis (Figure 1.3). The ultimate mechanism through which Ad exerts its control of sexual development, however, needs further exploration.

2.5 Figures

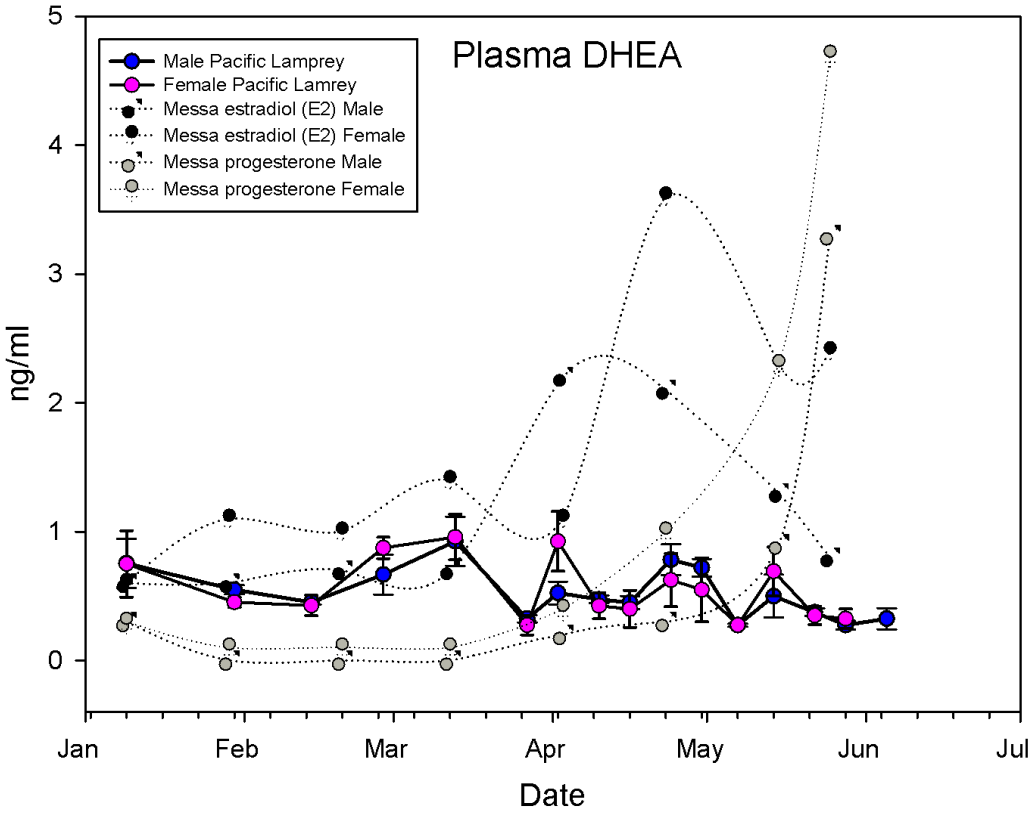

Figure 2.1 Measurement of plasma DHEA steroid levels (ng/ml) in male and female Pacific lamprey

The measurements represent time points prior to, during and following sexual maturation (Jan-June; Series I). Each time point represents a mean +/- SEM; n=10, unless otherwise indicated in brackets. A two-way ANOVA with a Tukey multiple-comparison tests indicate a significant difference between males and females. There were some significant statistical differences between spring and summer levels, but they were not considered to be biologically significant, because Messa's data exhibited similar small fluctuations in steroid level leading up to the spring peaks in steroid levels. The largest variation in DHEA was only 0.6 ng/ml, which is far less than the seasonal fluctuations of 2-5 ng/ml in progesterone and estradiol reported by Messa et al (2009), making it unlikely that sexual development is regulated by DHEA plasma steroid levels.

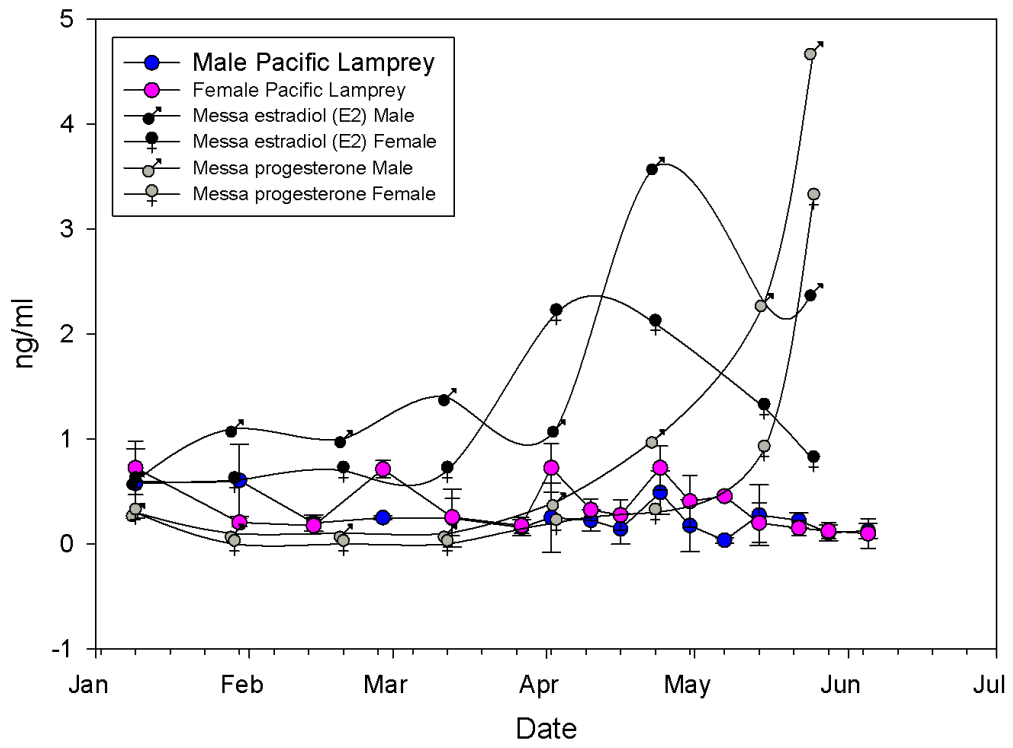

Figure 2.2 Measurement of plasma Ad steroid levels in male and female Pacific lamprey prior to, during and following sexual maturation (Series I)

The measurements represent time points prior to, during and following sexual maturation (Jan-June; Series I). This series of measurements was made to determine if Ad steroid levels in lampreys exhibited a rise that could be associated with sexual maturation. Measurements were made using the same plasma samples used for DHEA measurements in Figure 2.2 A two-way ANOVA with a Tukey multiple-comparison test indicates a significant difference between males and females at some time points. The variations were not considered to be biologically significant, because Messa's data exhibited similar small fluctuations in steroid level leading up to the spring peaks in steroid levels Bryan et al (2007&2008): the largest variation in Ad was only 0.5 ng/ml, which is far less than the seasonal fluctuations of 2-5 ng/ml in progesterone and estradiol reported by Messa et al (2009).

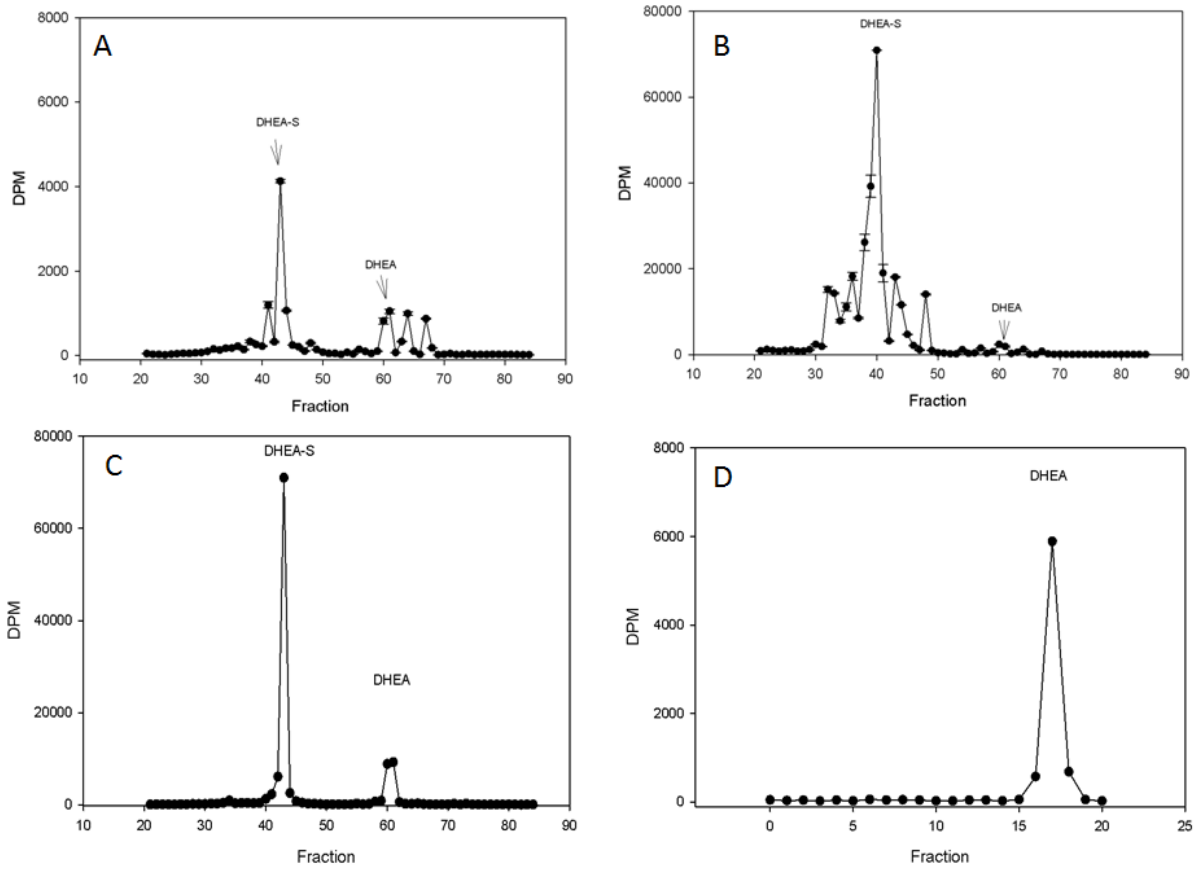

Figure 2.3 Series II: Measurement of DHEA-S conversion to DHEA in Pacific lamprey liver

A small peak is visible at the 60-61 min fraction (A and B) that corresponds to DHEA standard(C);Male(A)/female(B) Pacific lamprey DHEA-S liver incubation (n=3). Most of the DHEA-S remains sulphated. Note the scale difference between male and female: both incubations were statured with same amount of labeled DHEA-S. A large portion of the radioactivity has been lost in this incubation (2.2×10^6 to 3.1×10^5). The unlabeled peaks are unknown metabolic products. The chemical identity of the radioactive product in HPLC fraction 60-61 was confirmed as DHEA using TLC (Graph D). The HPLC graph (C) and the TLC graph (D) show typical chromatography results.

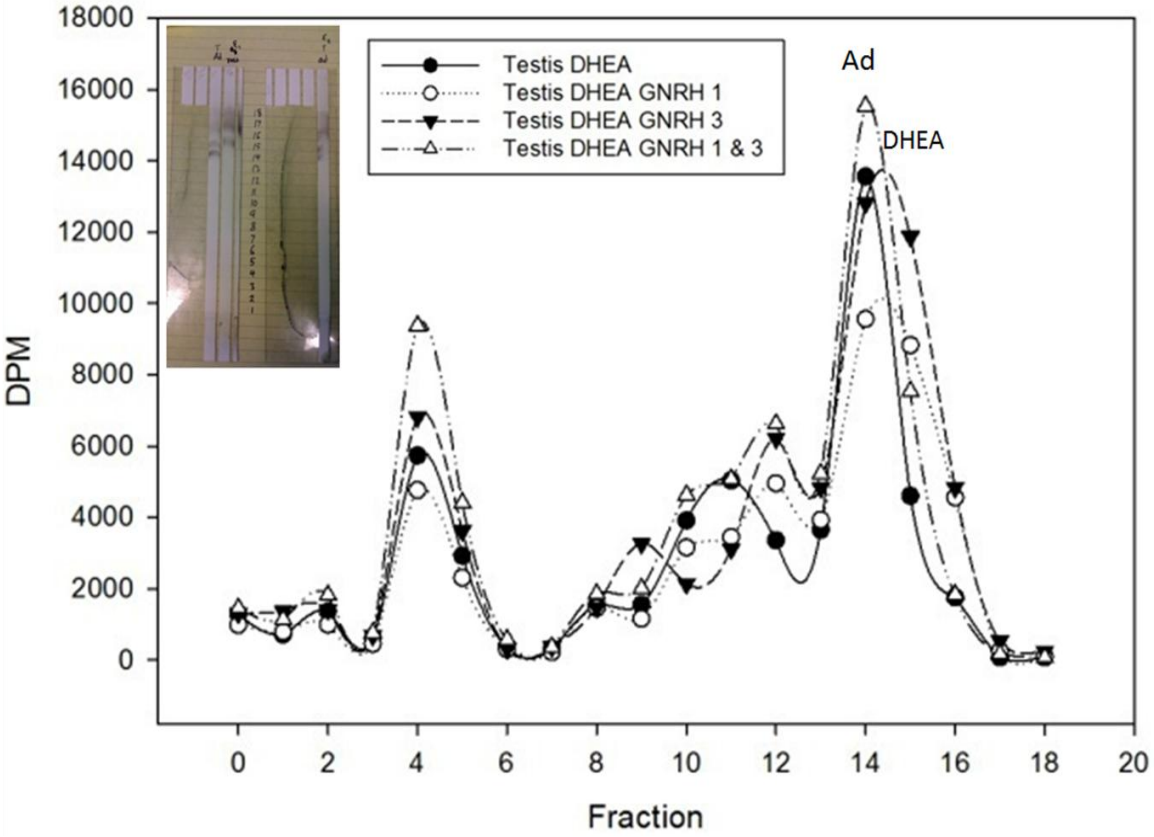

Figure 2.4 Series II: Conversion of DHEA to Ad in preliminary incubations of Pacific lamprey testis

Tissue and supernatant were combined and steroids were separated by HPLC and then TLC. Sea lamprey GnRH I & III, hypothesized to regulate sex steroids (Figure 1.3), were added to the incubation tubes. Results indicate both Ad production and that GnRH 1&3 combination stimulation increased Ad production, as indicated by this peak being 2000 DPM or higher reading for Ad than the other treatments. Ad and DHEA standards (not shown) eluted at fractions 14 and 15-16 as indicated on the graph. Fractions 4 and 8-13 are unknown metabolic products. A more refined incubation assessment was set up the following year (see Figures 2.7 and 2.8). A sample TLC is show to the left. The lane fractions have been scraped off; standards are visible in the remaining lanes.

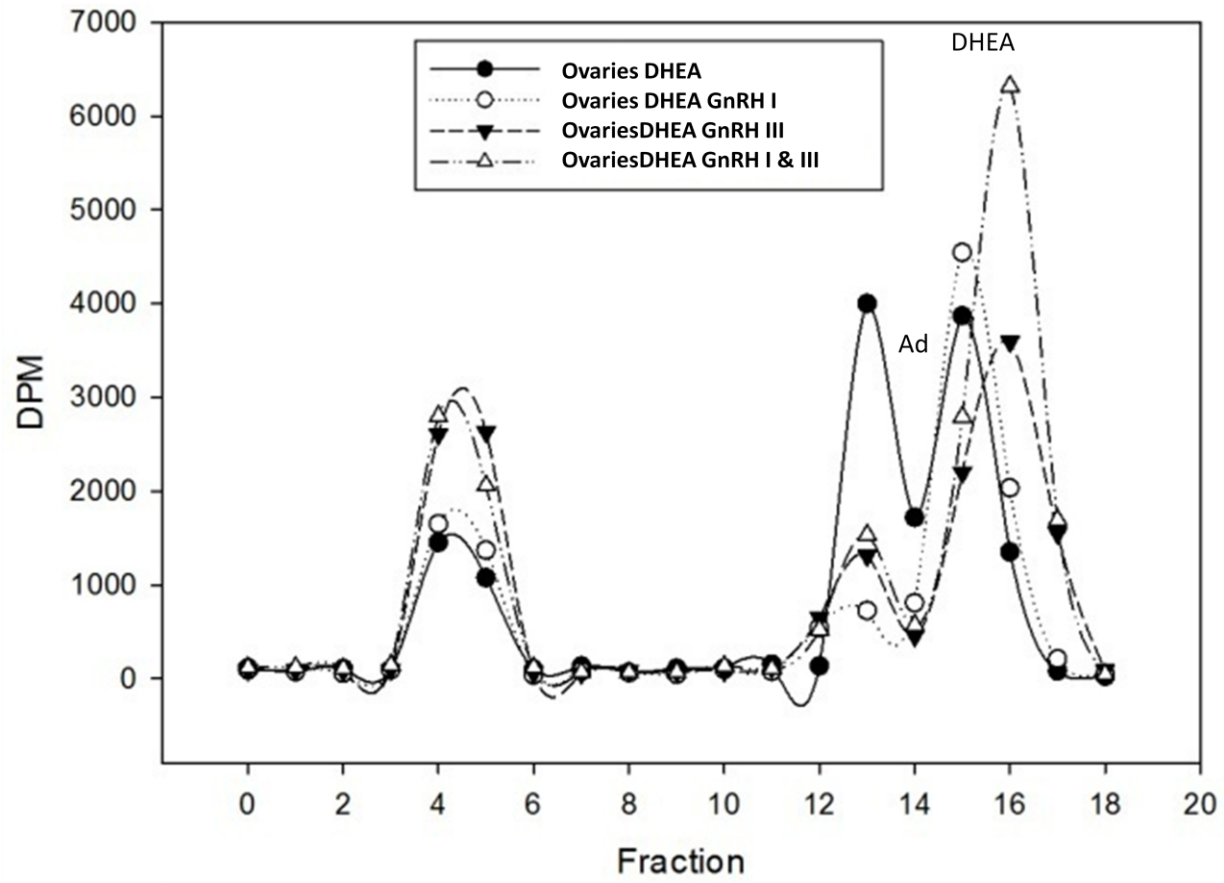

Figure 2.5 Series II: The effect of GnRH I and III on the conversion of DHEA to Ad in preliminary incubations of Pacific lamprey ovaries

Tissue and supernatant were combined and steroids were separated by HPLC and then TLC. Most of the labeled DHEA has been converted to something else that is not showing up in the TLC plate when compared to the testis (figure 2.4). A much smaller (10-20%) amount of Ad was detected compared with testis. Ad and DHEA standards (not shown) eluted in fractions 14 and 15-16, as indicated on the graph. Unlabeled peaks are unknown metabolic products.

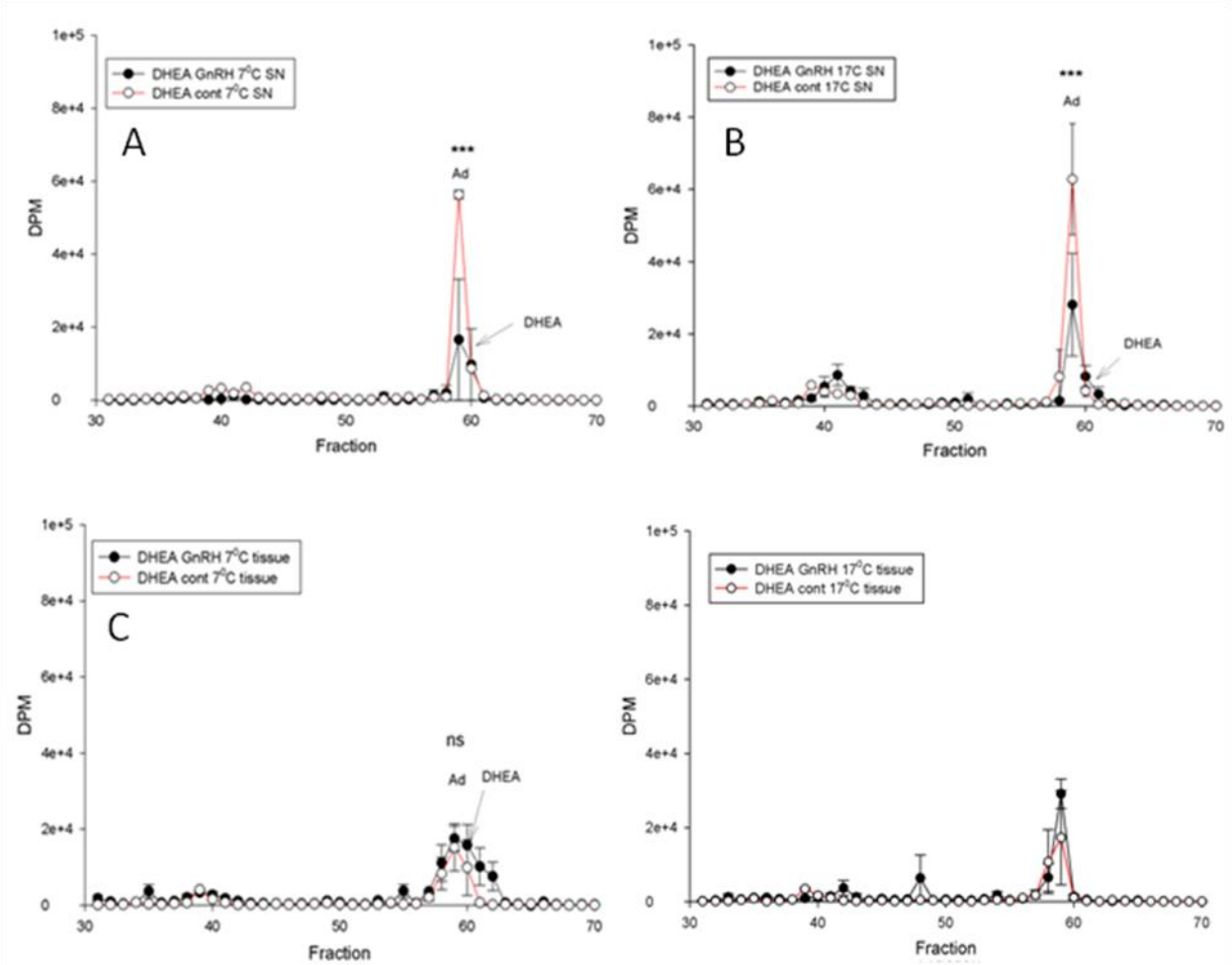

Figure 2.6 Series II: Refined incubation, the effect of GnRH and temperature on Ad production in supernatant (SN; A, B) and sequestering in tissue (C, D) of Pacific lamprey testis

Steroids were separated by HPLC and are reported as DPM counts of [3H] in each fraction. The identity of the products was confirmed by TLC and RIA. GnRH indicates that the lampreys were stimulated with GnRH I & III before (*in vivo*) and during the incubation; *cont* indicates the control group. A one-way ANOVA with and a Tukey multiple-comparison tests were performed. Statistical significance is indicated by *ns* (no significance), * (P>0.05), ** (P>0.01) and *** (P>0.001). GnRH stimulation had a strong positive effect on Ad levels in supernatant (SN) (top graphs). Temperature had no significant impact on steroid production. The DHEA precursor in the SN has been almost completely metabolized and/or sequestered in the tissue.

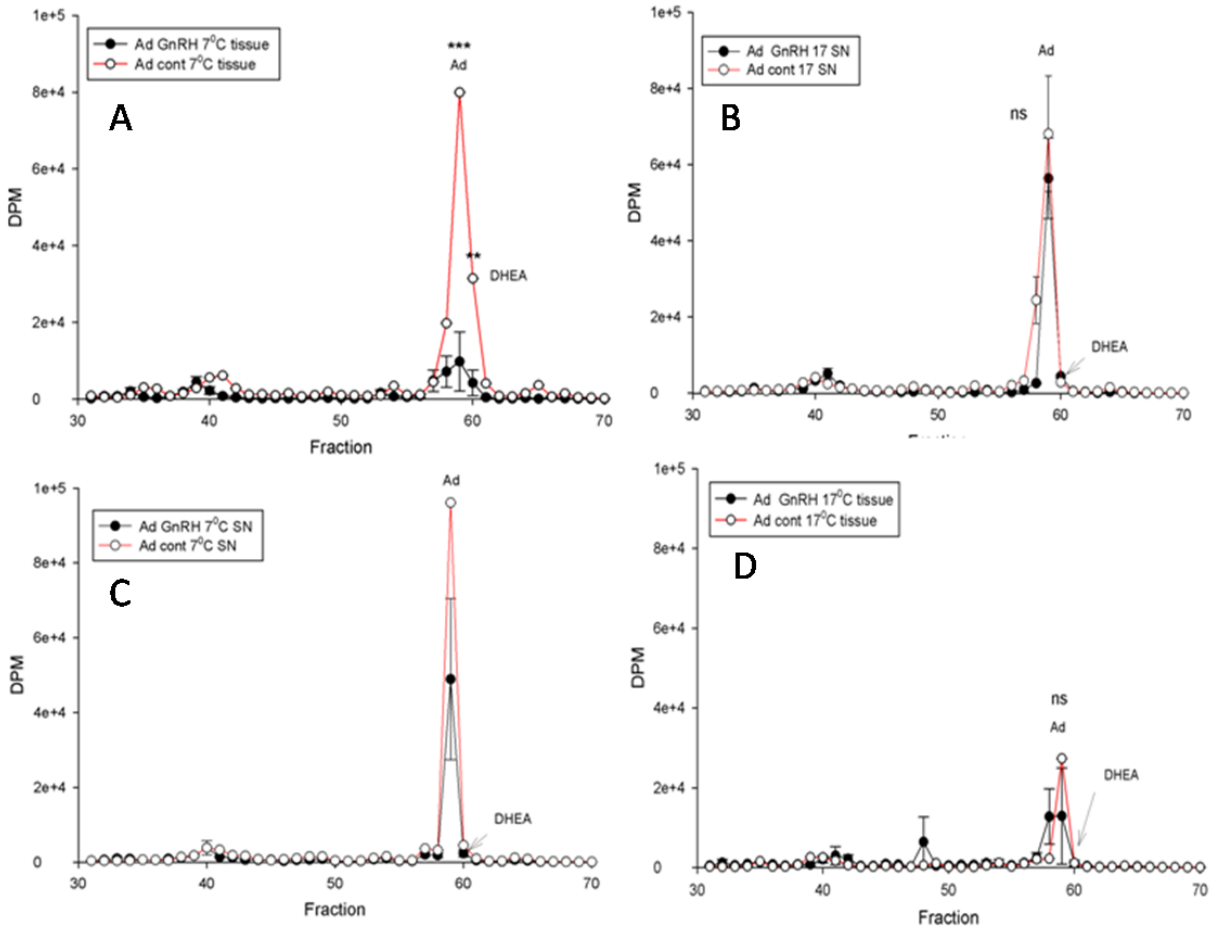

Figure 2.7 Series II:Refined incubation, the effect of GnRH and temperature on Ad conversion.

In these incubations Ad was the starting substrate. Some of the Ad was converted to DHEA, most notably in panel A. The GnRH I & III treatment resulted in satistically higher sequestering of Ad in the tissue, panel A; this is reflected in lower Ad count in the supernatant (SN) panel C. In the17^{0}C tissue (D) almost all the Ad has been converted to to a motabolite that was not captured by the HPLC fraction sample. The identity of the products was confirmed by TLC and RIA.

Chapter 3: Binding characteristics of Androstenedione to a membrane fraction isolated from Lamprey testes, implications of a possible membrane bound Androgen receptor in lamprey?

3.1 Introduction

The presence of hormones in and of itself is not indicative of hormone action. The ligand must bind to a receptor and there must be evidence of biological function. The functional component can be implied by conducting a dose-response to the ligand and interpreting relative binding that may occur under naturally occurring concentrations of the ligand, but even this is not definitive. In the case of sexual maturation in lampreys, induced maturation caused by elevations in dehydroepiandrosterone (DHEA) or androstenedione (Ad) could be the result of direct action of these steroids or the result of an indirect action: i.e., the DHEA and Ad may be converted to estradiol (E2), which could then act through the nuclear estrogen receptor described by Thornton (2001).The ligand linking to a receptor is therefore the preferred form of evidence of potential biological function. Previous studies (Bryan et al. 2007; 2008) have implicated Ad as a functional sex hormone in lampreys, where Ad implants induced sexual maturation in male lampreys in a dose-dependent manner; however, the receptor through which Ad is acting remains elusive. Bryan et al. (2007; 2008) reported cytosolic binding of Ad, but the axis or pathway of the biological response was not documented; it is thus not known whether this is actually a functional Ad receptor.

Demonstrating a functional receptor is a challenging goal. There are several components to the full identification of a receptor: 1) demonstration of binding; 2) identification of tissues where a receptor is active (target tissue); 3) characterization of the receptor binding; 4) identification of the receptor gene; 5) demonstration of up-regulation of the receptor in target tissue (e.g., during sexual maturation); and 6) identification of the axis and pathway of effect (Norris, 2007). Full characterization of a membrane receptor and its mechanism of action could

be a lifetime effort. This chapter addresses the hypothesis that there is a functional specific androgen ligand and receptor in lampreys, which would imply that lamprey development maybe induced by an androgen (Bryan et al. [2007; 2008]). Specifically, this chapter presents evidence of steroid binding to a target tissue (testis), and the characterization of this receptor binding. The importance of demonstrating a functional androgen in lampreys is discussed in the general introduction and in the following discussion sections of this dissertation.

Lampreys area basal extant vertebrate, have a small genome (Thornton 2001), and may represent an ancestral vertebrate. This supposition is based on morphological traits that have changed so little over 300 million years that their fossilized ancestors are clearly recognizable and Janvier's assertion that physiological traits are no better or worse than the morphological traits commonly used to classify organisms. We must also consider that lampreys' genome is smaller than gnathstomes' because they have not experience done of the genome duplication events that—Thornton (2001) speculated—provided the opportunity for beneficial mutations and led to the evolution of nuclear receptors. Lampreys were possibly constrained by a smaller genome that provided fewer opportunities for beneficial mutations: i.e., if having duplicate genes provides increased opportunity to evolve new traits, then the lack of duplicates must act as a constraint. This reasoning lends credence to the idea that lampreys may be physiologically similar to a theoretical ancestral vertebrate from which all vertebrate lineages arose. This further implies that the hormonal control of masculine development in lampreys may also be similar to the theoretical ancestor's hormonal control system. I therefore assume that understanding lamprey sexual development will help us understand more about the ancestral physiology and thus vertebrate evolution.

To understand the importance of sex steroid receptors and the relevance of their evolution, it is important to briefly review their role in vertebrate development.. Standard models of sexual maturation in gnathstomes indicate that maturation is regulated by luteinizing hormone, follicle-stimulating hormone, the androgenic steroid hormones testosterone (T), Ad, DHEA and 5α-dihydrotestosterone (Norman & Litwack, 1997; Norris, 2007) (Figure 1.3). Sower et al. (2009) compared known components of gnathstomes' hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) axis to a hypothetical lamprey axis. The

gnathostome model uses direct cell-to-cell signaling and circulating blood hormones to regulate sexual development. The most important point to note when comparing these models (Figure 1.3) is that the androgens and estrogens in the gnathostome model represent a suite of blood-born hormones (androgens: T, Ad, DHEA and 5α-dihydrotestosterone, estrogens (E): E2, estrone and estriol) that act through nuclear receptors. This is relevant because there is no evidence of any seasonal increase in Pacific or sea lamprey plasma androgen levels (see Chapter 2) and lamprey do not have a nuclear androgen receptor (Adams et al., 1987; Mesa et al., 2010; Sower et al., 1985; Thornton, 2001). Sea lampreys—male and female both—do however exhibit a seasonal rise in E2just prior to sexual maturation (Mesa et al., 2010). This has led to the speculation that sexual development for both species is controlled by the nuclear estrogen receptor(Thornton, 2001); however, I propose that lampreys have an active androgen receptor system.

As previously noted, Bryan et al.(2007) suggested there might be a receptor in the cytoplasm of sea lamprey testes. They specifically reported that plasma levels of Ad were very low (0.59 ng/ml) and only increased modestly (to 0.1.55 ng/ml and 1.22 ng/ml) in response to GnRH I and GnRH III respectively. They then speculated that the Ad might be sequestered in the testes, and subsequently reported that the testes contained 2-3.5 times more Ad than the rest of the body. Ad in the testes rose from 6.7 ng/g to 15.3 ng/g and 10.7 ng/g in response to *in vivo* injections of GnRH I and GnRH III respectively. They also reported a binding moiety in the testis cytosol that they thought was a possible high-affinity androgen receptor (K_d= 0.69 +/- 0.07 nM, n = 3) with a relatively high number of binding sites (Bmax=755.37 +/- 70.20 fmol/mg of protein, n=3). The size of the receptor was large: 440 kDa vs. 200 kDa in salmon (human androgen receptor AR-A 87 kDa, AR-B 110 kDa). This led them to propose a possibility of a new class of receptors (Bryan et al, 2007).

In this study, I hypothesized another possibility: my hypothesis is that lampreys have one or more unreported androgen receptors. My reasoning was that a more exhaustive search for androgen receptors was needed before postulating a completely separate evolutionary story of androgen receptors in lampreys. I focused my research on orphan steroid ligands and looked for putative ligand receptors to further investigate the interplay between the evolution

of the steroid biosynthetic pathway and their ultimate receptors. The classical pathway of androgen synthesis proceeds as follows: DHEA →Ad →T →E (Bryan et al., 2008; Liley and Stacy, 1983). In chapter 2, I have shown that lampreys produce DHEA and Ad; in this chapter, I surveyed lamprey tissues for Ad binding and characterized the membrane-binding moiety. Documenting an androgen receptor in lamprey is an important component for the evolutionary story of lamprey for two reasons: first, because it provides a biological purpose for lampreys to have a sex steroid biosynthesis pathway (recall that, as argued in the introduction, selection should favor the elimination of non-functional steroids and their biosynthesis pathway); and secondly, because it may explain how sexual maturation is regulated in basal vertebrates and thus shed insight on sexual maturation in their ancestors.

The forth objective of this dissertation was to look for evidence of unreported steroid receptors that could have driven the selection of a complex sex steroid synthesis pathway prior to the evolution of estrogen nuclear steroid receptor. The specific hypotheses tested in this chapter were Hypothesis V, that lampreys have an unreported androgen receptor, and Hypothesis VI, that the binding moiety found in lamprey testes is a putative receptor with properties similar to the androgen-binding receptor reported by Braun and Thomas (2004) in the ovaries of the Atlantic croaker (*Micropogonias undulates*).

My results indicate a putative androgen membrane receptor (mAR) in sea lamprey testis. The binding characteristics of this putative receptor are reported below. I speculate that sexual development in male lamprey may be regulated in part by this putative mAR and that the current suite of sex steroids found in modern vertebrates may have evolved in concert with membrane receptors prior to the evolution of nuclear receptors. Specifically, I propose that lamprey have membrane receptor in the testes that specifically bind Ad and that this mAR evolved before the nuclear androgen receptor found in modern vertebrates.

3.2 Methods

3.2.1 Animals

We conducted three large Pacific lamprey captures (100-150/year) from the fish ladder at Stamp Falls, British Columbia, in June (2009-2011) for use in this work and other projects. See Chapter 2 for complete details.

3.2.2 Animal Sampling

In order to maximize the utility of each lamprey and reduce the need for future animal collection, blood and organs (brains, liver, intestine, kidneys, gonads, gills and hearts) were collected from each lamprey and used in this and other studies. See Chapter 2 for complete details. Forth is chapter, all data were generated from testes collected from 24 male Pacific lampreys that were showing signs of sexual maturation—including swollen cloacae and the ability to express milt—but were not yet fully mature. Lampreys were anesthetized with MS-222 (buffered with HCO_3) and then decapitated. The body was kept on ice until the testes could be excised and prepared as described below. Any tissue that was not used immediately was snap frozen in liquid nitrogen and stored at -80°C.

3.2.3 Tissue Preparation

A key component of receptor characterization is identification of their cellular location. The tissue of interest was testis: chosen firstly because Bryan et al. (2007; 2008) found Ad binding in the testes; and secondly, because Sower's (2009) HPG axis indicates testes as a site of Ad synthesis. The testes were processed to isolate the nuclear, cytosolic and membrane cell fractions from the lamprey testis. My initial work focused on surveying steroid binding to detect putative receptors. Once the putative receptor had been detected, I focused my efforts on the characterization of the putative receptor. The methods used for each objective are outlined in sections 3.2.1 and 3.2.3 below.

3.2.3.1 Tissue fraction binding survey

The initial hypothesis that lampreys have an unreported androgen receptor was tested by conducting a steroid binding survey on lamprey testis. Radioimmunoassays (RIA) were

conducted on the purified cellular fractions to quantify the total and non-specific bindings of [3H]-labeled steroids to each fraction. Specific binding was calculated as total binding minus non-specific binding.

Tissues were prepared according to Braun and Thomas's (2004) procedures for biochemical characterization of a membrane androgen receptor. The Pacific and sea lamprey samples for the binding survey and competition binding studies required a minimum of 2 g of tissue, necessitating the pooling of brain or testis tissues from 4-5 Pacific lampreys. A total of 2g of brain or testis tissue was collected from sea lamprey; pooling was not required for muscle samples. Frozen tissue that had been stored at -80°C was thawed in HEAD buffer (25mMHepes, 10 mM NaCl, 1mMdithioerythritol and 1 mM EDTA, pH 7.6, Sigma-Aldrich) at 4°C. Briefly, testis tissue was weighed and then homogenized in HEAD buffer, first using a Polytron PT-K, (Kinematica) tissue homogenizer to separate cells and then with a Teflon homogenizer to lyse cells (2g tissue/15 ml ice-cold HEAD). The tissue was then centrifuged at 1,000g for 7 min to pellet nuclear components. The nuclear pellet was re-suspended in HEAD buffer and the process was repeated three times to wash the nuclear fraction. The final washed pellet was re-suspended in a 1:3 ratio of tissue to HEAD buffer and 10% glycerol (which stabilizes the proteins for freezing), divided into 2ml aliquots, and snap frozen in liquid nitrogen for later use. The original supernatant was removed (this is the cytosol and membrane fraction) and further centrifuged at 20,000g for 20 minat4^{o}Cto pellet out the membrane. The supernatant was pipetted off, 10% glycerol was added, and mixture was flash frozen in liquid nitrogen in 2ml aliquots for later use. The nuclear pellet was then re-suspended in 10 ml of HEAD and a sucrose pad (5 ml 1.2 M sucrose) was pipetted carefully into the bottom. The sample was then centrifuged at 6,900g for 45 min at 4°Cand the middle layer was collected, re-suspended to 15 ml with HEAD, solubilized with 287 µl Triton X-100, and shaken for 1 h. The resulting suspension was centrifuged at 100,000g using a Beckman Ultra-centrifuge for 1 h at 5°C, and the supernatant was removed. This fraction contained the solubilized membrane receptor. The sample was kept on ice or stored at 4°C for all stages. This procedure was repeated using three different tissue samples, with each replicated in triplicate (n=3 for each test) to complete the analysis. Any remaining endogenous hormone in the fraction containing the solubilized

receptor preparation was removed by charcoal treatment. A charcoal solution (100 ml HEAD, 0.125 g dextran and 1.25 g charcoal) was made, stirred for 2-4 h on ice, and then centrifuged at 1,000 g for 7 min to pellet the charcoal. This pellet was then added to the 15 ml of solubilized membrane receptor preparation, vortexed, incubated for 10 min on ice, then centrifuged 2x 1,000 g for 7 min. The supernatant was carefully pipetted out each time. The final solution was used to complete the characterization of the putative membrane receptor as described below.

Six test tubes were prepared: three to measure total binding and three to measure non-specific binding. Total binding tubes contained [3H] steroid (1.8 nM, 100,000 disintegrations per minute [DPM]); and non-specific binding tubes contained [3H] steroid (1.8 nM, 100,000 DPM) and cold steroid to displace the labeled ligand (1,000 fold higher concentration than the labeled steroid used). These tubes were dried down under nitrogen and 500 µl of the cell fraction being tested for binding was added to the tubes. Tubes were then vortexed and left to incubate for overnight at 4°C. 500 µl of dextran-coated charcoal (1.25% charcoal, 0.125% dextran in HEAD buffer, Sigma-Aldrich) was added to the tubes, and they were vortexed for 5 seconds and incubated for 5min. The tubes were then centrifuge at 1,000 g for 5 min at 4°C. The supernatant was collected in scintillation vials, and 5 ml of scintillation fluid (RPI safety-solve) was added. The samples were shaken and then counted in a Beckman Coulter LS 6500 scintillation counter. (All [3H] steroids were obtained from PerkinElmer, and cold steroids were obtained from Sigma-Aldrich.)

3.2.4 Characterization of receptor methods

The second hypothesis, that lampreys have a high-affinity membrane receptor similar to that found in Atlantic croaker, was tested by characterizing the putative membrane receptor. Cellular fractions were isolated as described. The saturation, association and dissociation tests were only conducted on sea lampreys, as Pacific lampreys were not available at that time. Three pooled samples of testis tissue (45 g each; a total of 135 sea lampreys used) were created to ensure sufficient sample volume for all tests to maintain sample consistency.

Binding assays used to characterize the receptor were conducted according to Braun and Thomas (2004) with slight modifications. Binding experiments were conducted using

Millipore FC 1.2 glass fiber Type C 96 well filter plates and a vacuum extraction manifold. Plates were treated with 100 µl of 1% polyethylinamine (PEI) overnight and then rinsed three times with deionized water, followed by one rinse of HEAD. The wells were loaded with HEAD, [3H] Ad 1.8 nM (plus 1,000foldexcess of cold Ad for non-specific binding), then 50 µl of membrane preparation was added to each of the wells at each time-point to start the binding. The final volume was 200 µl in all wells. All wells were vacuum extruded to end competitive binding. Wells were rinsed 5-7 times with 100 µl of HEAD buffer to remove unbound androstenedione. The receptor–Ad complex was retained on the cellulose filter. The filters were dried overnight, and then punched out into scintillation vials for counting. 5 ml of scintillation fluid (RPI safety-solve) was then added, the sample was shaken, stored at room temperature overnight, then shaken again and counted the next day.

All assays were run in triplicate and using three different membrane preparations. Specifically, three independent samples were prepared as describe in tissue preparation (n=3); for each sample, three total and three non-specific binding assays were run. Specific binding for each sample is reported as the average total binding minus the average non-specific binding ±the standard error of the mean (SEM). All samples were collected from sexually mature sea and Pacific lamprey. Samples were normalized using 1 mg of protein per ml of membrane prep as the benchmark before analysis. Specific variations of this procedure used to characterize the receptor are noted in sections 3.2.5.1 – 3.2.5.3 below.

3.2.4.1 Saturation curve

The radio ligand binding assays of saturation binding were run as described above, with the following variations.[3H] Ad concentration was varied to determine concentration dependent binding. Concentrations used were 1 nM, 2nM, 5 nM, 10 nM, 20 nM, 40nM and 60 nM of [3H] Ad in the test wells. Samples were incubated for 60 min on ice. These tests were carried out using sea lamprey membrane preparation as described in the tissue preparation procedure above. Sample size was n=3; each conducted in triplicate as described above. ([3H] Ad, 98.2 Ci/mM, was obtained from PerkinElmer, and cold Ad was obtained from Sigma Aldrich.) Results were used to calculate B_{max} plotting steroid concentration [Ad] vs

concentration of bound steroid [B] (SigmaPlot non-linear regression) and K_d by ploting [B] vs unbound [U] steroid (Scatchard plot intercept).

3.2.4.2 Association and dissociation

The radio ligand binding assays used the procedure described above, with the following modifications. For the association study, the time allowed for competitive binding was intentionally varied to measure the rate of association. Wells were prepared for competitive binding, and membrane preparation was added to start competitive binding in the presence of 20nM of [3H] Ad in the respective wells of a single 96-well plate. Wells were vacuum drained to end the competitive binding, resulting in the time sequence 0, 5, 10, 15, 20, 30 and60 min of competitive binding within different wells. Sample size was n=3; each time-point was conducted in triplicate.

For the dissociation study, the time allowed for cold Ad displacing [3H] Ad (20 nM) was varied. Total and non-specific binding assays were set up and allowed to come to equilibrium by incubating the plate at 4°C on a shaker for 60+min to achieve saturation binding. Disassociation of [3H]Ad was initiated by the addition of 25 μl of cold Ad (>1000 fold [3H] Ad) saturated HEAD solution to the wells at each time-point to start competitive binding. All of the assays were conducted simultaneously on a single 96-well plate by staggering the addition of the cold Ad as described above in the association study. ([3H]Ad, 98.2 Ci/mM, was obtained from PerkinElmer, and cold Ad was obtained from Sigma-Aldrich.)Sample size was n=3; each conducted in triplicate. Results were used to calculate rate constants by plotting time vs DPM bound steroid (SigmaPlot non-linear analysis).

3.2.4.3 Competition study

The ability of a receptor to bind its ligand preferentially over other endogenous steroids was determined by trying to displace the suspected ligand with alternative ligands. DHEA, 11-Deoxycortisol (S), and E2 were chosen because they are known endogenous steroids. T was tested for its value as a common androgen; sea lampreys do not produce T (Bryan et al., 2008). The specificity of the putative receptor to recognize its ligand was determined by competition

studies that used alternative cold steroids to displace[3H]Ad from the receptor ([3H] Ad, 98.2 Ci/mM, was obtained from PerkinElmer, and cold steroids were obtained from Sigma-Aldrich).

Steroids for the competition study were dissolved in ethanol and added to tubes in varying concentrations from 0.1 nM to 1 µM and dried down under nitrogen. The membrane preparation was diluted to 0.15 mg/ml protein in HEAD; 250 µl membrane prep and 250 µl of [3H] Ad label (final concentration 10nM) were added to the reaction tubes and incubated at $4^{o}C$ for 40 min. Maximum specific binding was determined by subtracting non-specific binding (10 nM [3H] Ad and 1,000foldcold Ad competitor) from total binding (10 nM [3H] Ad). Results were used to determine ligand-receptor specificity by plotting concentration of competing ligand vs [Ad] bound.

3.2.5 Statistical analysis of results

All assays were run three times, each in triplicate except where noted in the competition study (i.e., three independent samples were prepared as describe in tissue preparation (n=3); for each sample three total and three non-specific binding assays were run; specific binding for each sample is reported as the average total binding minus the average non-specific binding +/- SEM.

Maximum binding (B_{max}) and dissociation constant (K_d) were calculated by non-linear regression analysis using Graphpad Prism 5.01 for Windows. Values are reported as mean +/- SEM. All statistical analyses were completed using Graphpad Prism 5.01 for Windows (two-way ANOVA 95% confidence).

3.3 Results

3.3.1Binding survey

Brain, muscle and testes, were measured for steroid binding capacity to identify tissues that might have an unreported androgen receptor. These results are reported in Figures 3.1 and 3.2. Male and female brains were compared to highlight any dimorphism that might exist between sexes. There were statistically significant differences in steroid binding related to sex, species and steroid measured.

The nuclear fractions of lamprey brains had the highest binding. The strongest binding was E2 in the female Pacific lamprey brain; E2 binding was not statistically different for any other brain fraction. DHEA and Ad also show elevated binding in lamprey brain nuclear fractions which may warrant further investigation but the most significant binding of Ad was to the putative receptor in both Pacific and sea lamprey testis membrane fractions (*SL Ad cytosol*: 16,323+/- 2847 dpm/mg, membrane: 16,861 +/- 2,577 dpm/mg, nuclear: 3,357 +/- 1,511; *PL Ad cytosol*: 8,756 +/- 2,675dpm/mg, membrane 32,543 +/- 5,752 dpm/mg, nuclear: 2,250 +/- 375).

3.3.2 Saturation binding study

The binding study was conducted using three replicate tests on three separate samples (see Methods section 3.2.3.1). The results from each analysis were standardized to 1mg of protein/ml of membrane preparation and used to characterize the putative androgen receptor. The results for the saturation binding are shown in Figure 3.3 and the Scatchard analysis of these results is shown in Figure 3.4. B_{max} and K_d was determined using Graphpad Prism software. Non-linear regression of the specific binding of [3H] Ad indicates the presence of a high-affinity (K_d = 7.5 +/- 1.4nM, R^2 = 0.98) low-capacity (B_{max}= 0.0.23 +/- 0.013nM/mg of protein) androgen binding site. The linear Scatchard plot indicates a one-site model for androgen binding.

3.3.3 Tissue fraction binding survey

Binding surveys were conducted to probe for possible receptors in testes in nuclear, cytosolic and membrane fractions. The binding of E2, DHEA and Ad in Pacific lamprey and sea lamprey testes are compared in Figures 3.1 and 3.2. There was statistically significant binding of Ad in both Pacific and sea lamprey testes (two-way ANOVA, $p <= 0.05$). The specific binding of Pacific lamprey cell membrane fraction to Ad was significantly stronger (3-10 x stronger) than the binding of other steroids and fractions tested. Ad was also the only steroid tested on Pacific lampreys that had statistically different specific binding between nuclear, cytosol and membrane fractions. The nuclear and cytosolic specific binding of Ad did not differ statistically from any of the E2- or DHEA-specific binding results.

The sea lamprey results were very similar to Pacific lamprey, but I found high specific binding in the cytosolic fraction as well as the membrane fraction (Figure 3.2). Both the cytosol and the membrane fractions were significantly different from all other fractions for all other steroids tested, but not from each other. No other statistical differences were found between the other fractions. The plasma binding of Ad was measured to confirm that the observed binding was not binding to globulin or other plasma proteins. The assay detected no significant plasma binding (results not statistically different from 0, $p < 0.01$). This result is consistent with Bryan's (2008) results and the absence of a sex hormone binding globulin in the sea lamprey genome.

3.3.4 Competitive binding study

The endogenous steroids DHEA, S, Ad and E2 were tested for competitive binding; T was also tested. The results (Figure 3.6) show that the receptor has high selectivity for all the steroids except testosterone (which is not a competing endogenous steroid). This is clearly indicated by the fact that DHEA, S and E2 do not displace [3H] Ad from the receptor until their concentrations are 5 orders of magnitude higher than [3H] Ad, while both cold Ad and cold T displaced the [3H] Ad with only 1 order of magnitude higher concentration. Complete displacement of [3H] Ad is achieved by a 3 order of magnitude increase in cold steroid competitor.

3.3.5 Association and dissociation study

The binding kinetics for the putative receptor were determined in the presence of 20nM of androstenedione (Figure 3.5). Competitive binding between the [3H]-labeled Ad and a saturating concentration of unlabeled Ad showed a rapid change with equilibrium binding. The association half-time was determined by non-linear analysis to be 10.2 +/- 3.2 min with maximum binding being achieved after 30 min. The dissociation half-time was similarly rapid:9.5 +/- 5.0 min with maximum binding being reached after 30 min. Results were calculated using Graphpad Prism software using non-linear regression.

3.4 Discussion

My findings support my Hypotheses V, that lamprey have an unreported androgen receptor, and Hypotheses VI, that this membrane receptor has similar binding characteristics to the membrane receptor reported in Atlantic croaker ovaries. I found statistically significant binding of Ad to the putative receptor in both Pacific and sea lamprey membrane fractions. The binding was an order of magnitude larger than the E2binding lamprey testes to the nuclear fraction (*SL E2 cytosol*: 132+/- 179 dpm/mg, membrane: 263 +/- 97 dpm/mg, nuclear: 323 +/- 309; *PL E2 cytosol*: 1,012 +/- 200 dpm/mg, membrane 1175 +/- 300 dpm/mg, nuclear: 3075 +/- 210). I found this particularly interesting, given that lampreys are known to have active E2nuclear receptors (Bryan et al., 2008; Mesa et al., 2010; Thornton, 2001). This high binding clearly indicates a strong binding moiety that could be a receptor. While the results from both species were quite intriguing and worthy of further research, continuing with Pacific lampreys became impractical for two reasons. First, we were not able to obtain permits to collect sufficient Pacific lampreys for my research due to concerns about the impact on local stocks following the listing of Pacific lampreys as a species of concern in the Columbia River. Second, the genome for Pacific lampreys is not sequenced. Consequently, I switched to sea lampreys, which were readily available for tissue harvest and have a sequenced genome (https://uswest.ensembl.org/Petromyzon_marinus/Info/Index).

All of my results were consistent with the Atlantic croaker androgen membrane receptor binding characteristics, which also indicate a high-affinity single-binding site receptor. Non-linear regression of the specific [3H] Ad indicates the presence of a high-affinity (K_d = 7.5 +/- 1.5nM, R^2 = 0.9804,) low-capacity (B_{max} = 0.23 +/- 0.013nM/mg of protein) androgen binding site. The linear Scatchard plot indicates a one-site model of androgen binding. The association half-time rate was determined by non-linear analysis to be 10.22 +/- 3.239 min with maximum binding being achieved after the 30-min mark. The dissociation half-time was similarly quick: 9.5 +/- 5.0 min with maximum binding being reached after the 30-min mark. These values are more consistent with a membrane androgen receptor such as that reported by Braun and Thomas (2004) than a cytosolic receptor such as reported by Bryan et al. (2007). (Croaker

androgen receptor statistics: K_d = 15.3+/-2.7 nM; B_{max} = 2.8 +/- pM; association 3.7+/- 1.7 min; dissociation 4.7 +/- 0.2 min). These data thus indicate the presence of an Ad membrane receptor in sea lampreys.

This study is not the first to imply that sea lampreys have a receptor for Ad; as proposed a possible cytosolic receptor based on high levels of Ad in the cytosol and a binding moiety in the cytosol; however, the binding characteristics of the putative receptor that they reported differed from what I found. They reported receptor binding characteristics (K_d = 0.69 +/- 0.07 nM, n = 3) and a relatively high number of binding sites (B_{max} = 755.37 +/- 70.20 fmol/mg of protein, n=3). (My results also indicated binding in the cytosol, but I did not characterize this binding, choosing instead to focus on the membrane binding). They reported the protein to be 440 kDa, which is more than double the 200 kDa salmon receptor they compared it against. They later hypothesized that the larger protein might constitute a new class of receptor (Bryan et al., 2008). I did not investigate the cytosolic fraction, and therefore cannot comment on their receptor work, but I agree that Ad is likely a steroid of importance in lampreys. The more important result for my study was that Bryan et al. (2007) demonstrated that treatment of sea lampreys with Ad (low-dose 15 mg Ad; high-dose 150 mg Ad) induces rapid development of the testes, indicating a biological affect of Ad; this is an important component of documenting the existence of a functional receptor-ligand complex. As previously discussed, this is not definitive evidence of an Ad receptor, because the Ad could be modified to another form that is the actual active steroid. The result, however, is consistent with lampreys having an active Ad receptor mediating sexual development, which, combined with my findings, provides strong support for a putative receptor.

To summarize, my results indicate that this receptor is highly specific for Ad when compared to other endogenous steroids; the receptor was equally receptive to T and Ad. Specificity for distinguishing T from Ad in lampreys is not biologically relevant, because lampreys do not produce T. This property does, however, indicate that the binding specificity is not dependent on the functional group at carbon 17 (Ad: ketone vs. T: alcohol; Figure 1.1) and is more likely based on the carbon A ring on the steroid. I speculate that, based on low circulating levels of Ad in sea lamprey as reported by Bryan et al. (2007), combined with my

findings in Chapter 2 that the site of production and action of Ad may be limited to cell-to-cell signaling in the testes. This could imply that Leydig cells are making Ad, and it is being up taken by Sertoli cells. This hypothesis of a cell-to-cell signaling model for androgenic regulation of sexual development, however, will have to be tested in future studies. The promiscuity of the binding site also fits with Baker's hypothesis that early receptors may have accepted multiple ligands. While lampreys do not produce T, T would be active via this receptor. Thus duplication of the gene for this receptor would provide the opportunity single promiscuous receptor to evolve variations in receptor ligands, as well as receptor specificity and function. This is Baker's model of receptor evolution.

The steroid binding survey turned up a number of interesting results. Testis, brain, and muscle were measured for steroid binding capacity to identify tissues that might have an unreported androgen receptor. Testis was chosen because I was looking for an unreported androgen receptor, and Bryan et al. (2007; 2008) had identified testis as a tissue of interest. The brain seemed to be another logical tissue, because androgens have been shown to regulate mating behavior in squirrels, and song sparrows (Boonstra et al., 2008; Hau et al., 2004; Soma et al., 2002). Muscle was a less likely but possible tissue of interest, because androgens can regulate muscle development in fish (Borg, 1994; Brantley et al., 1993). Male and female brains were compared to highlight any sexual dimorphism that might exist. There appear to be differences in steroid binding relating to sex, species and steroid measured in the brain tissue (Figures 3.1 and 3.2). DHEA may have some significance in brain function based on the relatively high levels of DHEA binding relative to other steroids. Based upon this finding, I was interested in further investigating the localization and characterization of DHEA receptors in the brain. DHEA has been linked to behaviors in both birds and mammals (Boonstra et al., 2008; Hau et al., 2004; Soma et al., 2015, 2002). Soma et al. (2015) suggested that DHEA may serve as a behavior steroid for some species of birds and mammals when seasonal T levels are low. Steroid links to behavior are less well documented in fish but there is some evidence for this line of inquiry (Jalabert et al., 2015). This opens the door for speculation that DHEA could have a role in lamprey behavior. I initiated collaborations with two different laboratories but eventually abandoned this line of inquiry due to logistics and lack of resources. I ultimately

decided the [3H] Ad binding in the testes (Figure 3.2) was a more practical focus for my thesis, because it showed the highest binding capacity and the techniques needed to pursue this line of inquiry were more consistent with work in Dr. Close's lab, where I was located.

From the start of my study, I questioned the supposition that a complex biosynthesis pathway for steroids (Figure 1.1) would evolve independently from a receptor. The steroid synthesis pathway for sexual maturation (Figure 1.1) is complex, consisting of at least 17 steroids and 11enzymes. Natural selection predicts that 1) favored traits are selected for because they enhance survival or the reproductive success of the organism, and 2)traits that do not enhance survival or reproduction are not selected (but may experience neutral selection) and will be selected against if there is a cost (metabolic, reproductive, survival rate,...). The presence of an ancestral receptor would provide selective pressure to produce steroid ligands and a receptor that could be co-opted later to evolve new receptors (Mandrioli et al., 2007; Moyle et al., 1994). The independent random evolution of four specific steroid synthesis proteins and the stable inheritance of this steroid synthesis pathway (as suggested by Thornton [2001]) is possible, but less parsimonious than the sequential evolution of receptors for DHEA, Ad, and then E2. Under this hypothesis, the synthesis pathway could have evolved to produce multiple ligands for ancestral receptors. Given that receptors are well conserved across species (Mandrioli et al., 2007; Thornton, 2001), probing for a receptor for orphan ligands will likely lead to the discovery of unreported receptors and a better understanding of receptor evolution. DHEA, which was once thought of as just a precursor for other steroids for instance, has now been shown to be an active hormone in several species (Beck and Handa, 2004; Boonstra et al., 2008; Pluchino et al., 2013; Soma et al., 2002). It is quite reasonable therefore to hypothesize that androgens had ancestral receptors prior to the evolution of nuclear steroid receptors.

Based on my results, I speculate that membrane androgen receptors may have evolved prior to nuclear androgen receptors. This supposition accounts for the sex hormone synthesis pathway prior to the evolution of a nuclear androgen receptor by hypothesizing a prior biological function for orphan ligands (Baker, 2003; Mandrioli et al., 2007; Moyle et al., 1994). It opens up speculation that sexual development in vertebrates and their ancestors may be

regulated by membrane receptors. This would be a significant piece of the evolutionary puzzle when transitioning from invertebrates to vertebrates, because it provides a mechanism for control of sexual development prior to the development of nuclear receptors (Mandrioli et al., 2007; Moyle et al., 1994). It also provides an origin for steroid nuclear receptors based on the gene duplication theory and the possibility that existing steroid receptors could evolve new functions: i.e., nuclear receptors may have evolved to use membrane receptor ligands and the membrane receptors may have evolved to serve new or refined functions. This would imply that nuclear receptors represent a refinement of a pre-existing biological control system.

In conclusion, this research indicates that Ad may have an active mAR in lampreys, and this receptor may predate the development of nuclear sex steroid receptors in vertebrates. As such, the discovery of this receptor may help us develop a better understanding of receptor evolution and general endocrinology.

3.5 Figures

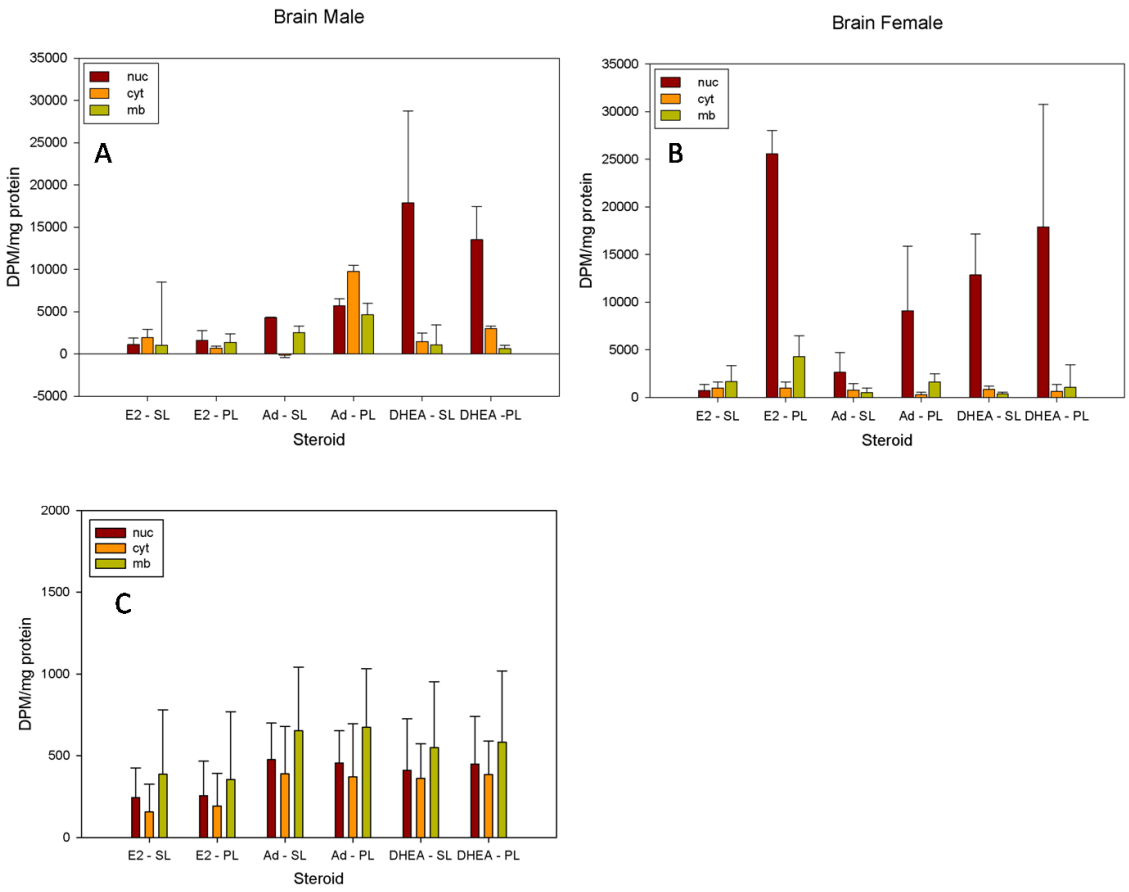

Figure 3.1 The relative specific binding of Steroids

Measurement of the binding of E2, Ad and DHEA to the isolated and purified nuclear (nuc), cytosol (cyt) and membrane (mb) fractions of sea lamprey (SL) and Pacific lamprey(PL) brain(male and female; A, and B, respectively) and male muscle tissue (C). The binding of [3H]-labeled steroids are reported as DPM/mg of protein (n=3[each conducted in triplicate], mean +/- SEM).

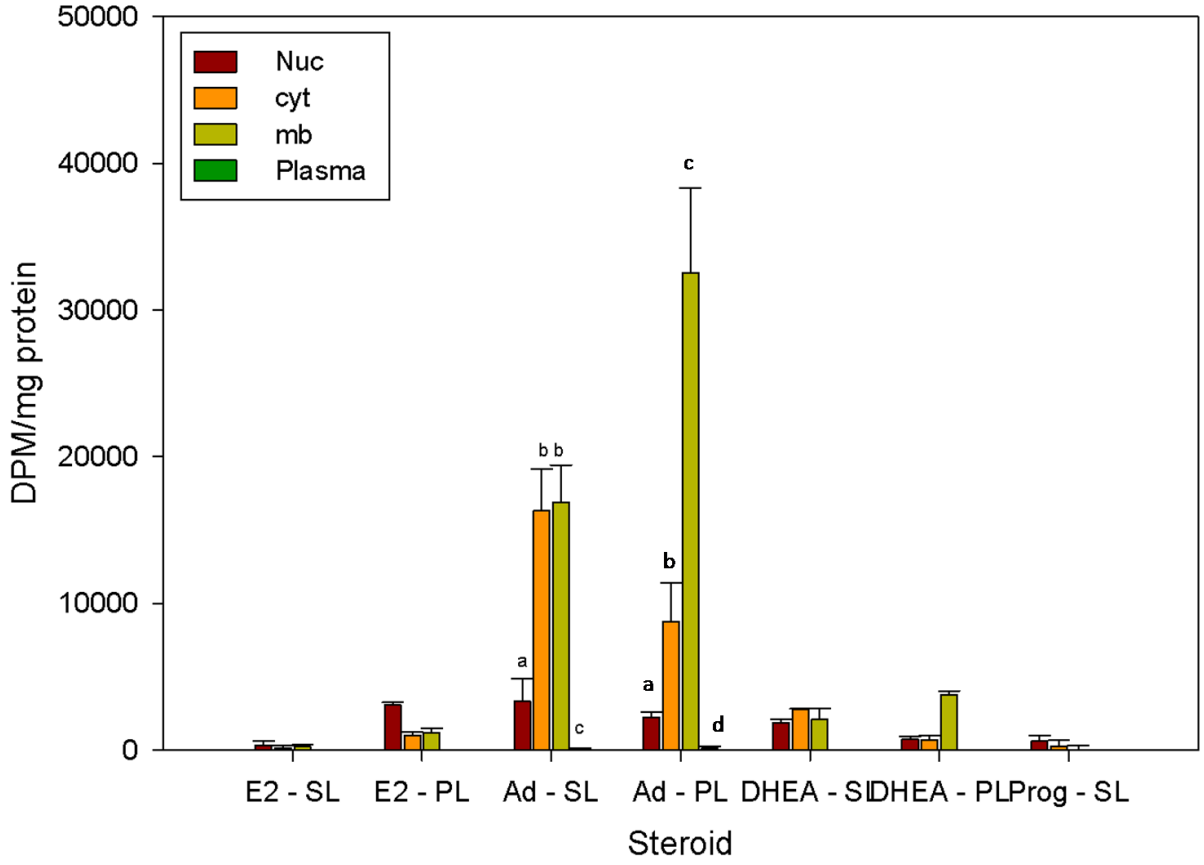

Figure 3.2 The relative specific binding of steroids to lamprey testis tissue fractions

Measurement of the binding of E2, Ad and DHEA to the plasma and purified nuclear (nuc), cytosol (cyt) and membrane (mb) fractions of sea lamprey (SL) and Pacific lamprey(PL) testis. The binding of [3H]-labeled steroids are reported as DPM/mg of protein (n=3, mean +/- SEM). Only Ad differed significantly between fractions (within species) as indicated by the letters that differ. Specific binding of Ad to nuclear and cytosol fractions were significantly higher than E2 and DHEA. Plasma binding of Ad was negligible (two-way ANOVA 95% confidence).

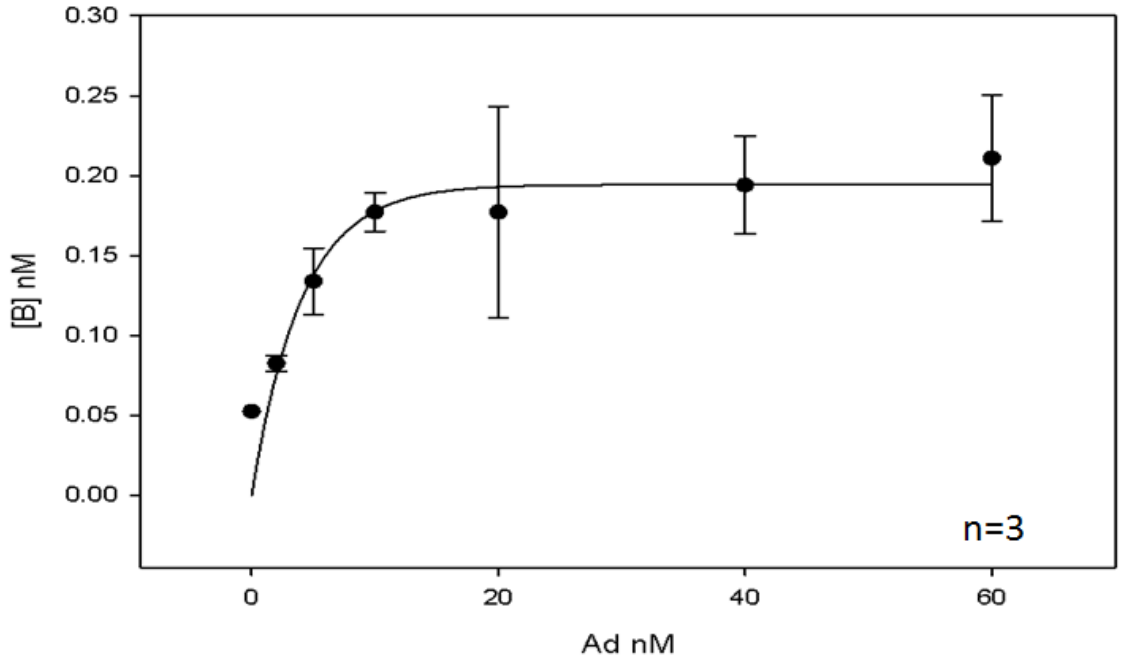

Figure 3.3 Saturation binding curve

The binding of [3H] Ad to the membrane fraction of sea lamprey testis reported as concentration of [3H] Ad in buffer vs. concentration of [3H] Ad specifically bound [B] to the putative receptor. Symbols represent mean +/- SEM. The curve of best-fit was calculated using non-linear analysis in Sigma Plot 11.0.0.77 for Windows. Maximum binding was achieved within 30 min.

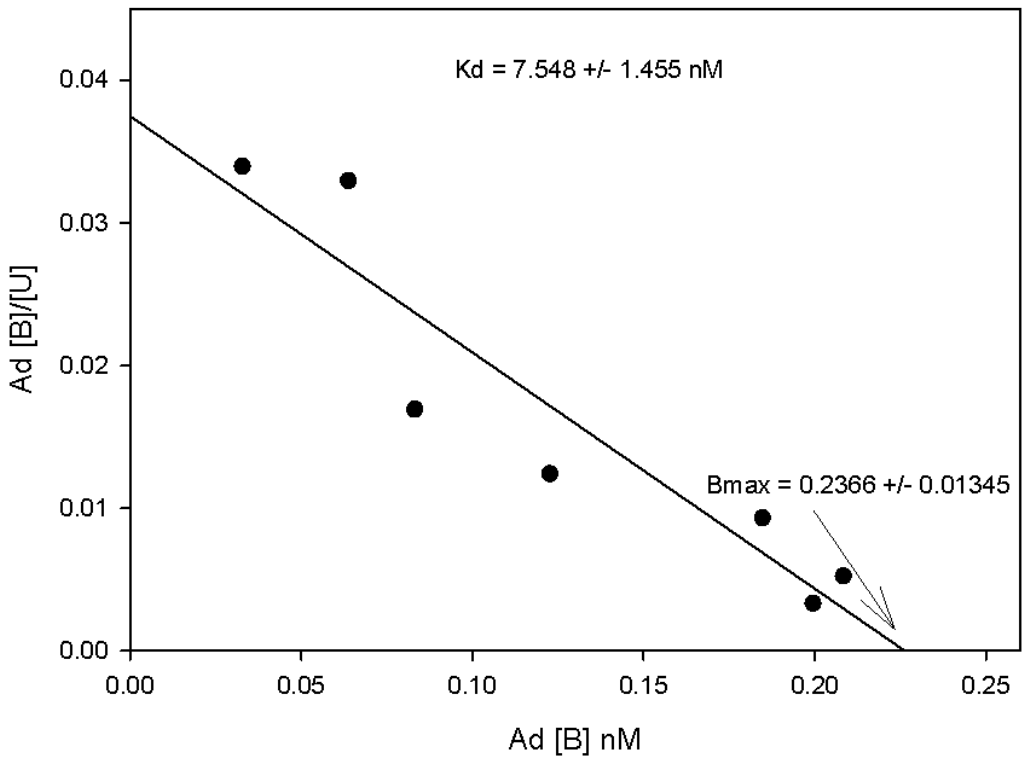

Figure 3.4 Scatchard analyses of saturation binding data

Data is reported as bound,[3H] Ad [B], vs. the ratio of bound/unbound,[3H] Ad [B]/[U](mean +/- SEM), in the membrane fraction of sea lamprey testis. Non-linear analysis of saturation data was used to calculate the dissociation constant (K_d) and maximum binding (B_{max}).

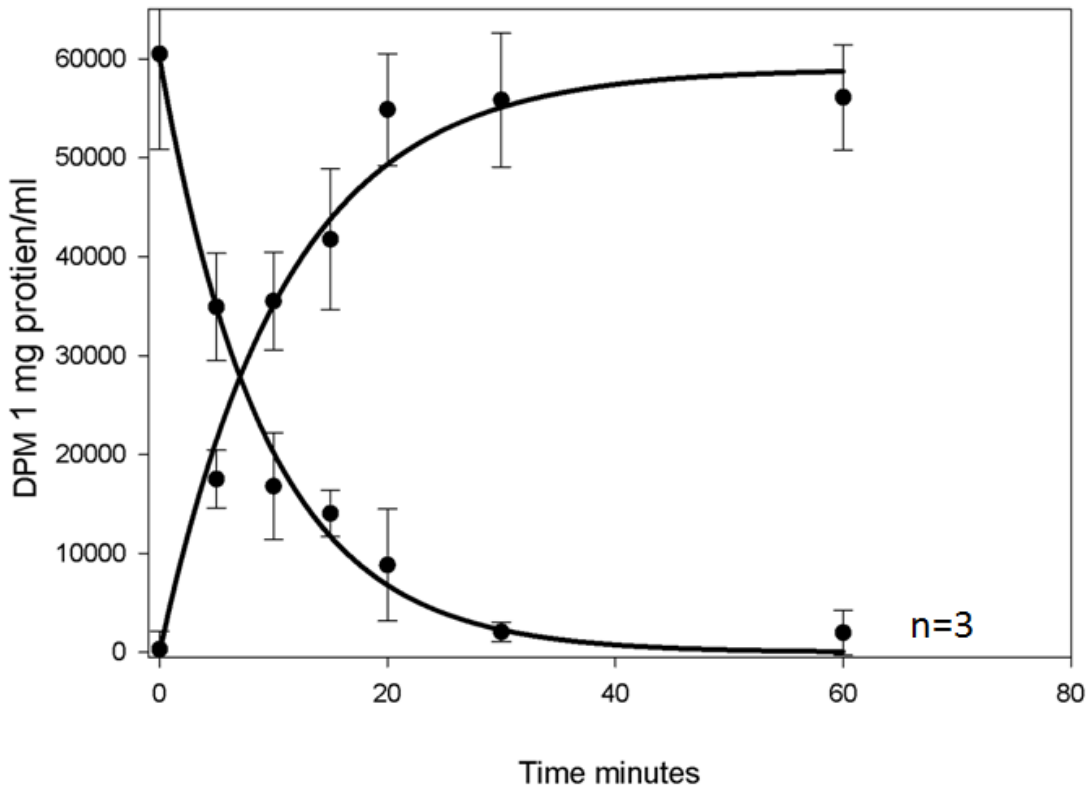

Figure 3.5 The association and dissociation rates for the [3H] Ad binding moiety

Competitive binding between the[3H]-labeled Ad and a saturating concentration of unlabeled Ad, reported as time vs. DPM in the membrane fraction of sea lamprey testis, showed a rapid change(mean +/- SEM). The association half-time was determined by non-linear analysis to be 10.22 +/- 3.23 min with maximum binding being achieved after 30 min. The dissociation half-time was also rapid: 9.49 +/- 5.01 min with maximum binding being reached at 30 min. The curves of best-fit were calculated using non-linear analysis in Sigma Plot 11.0.0.77 for Windows.

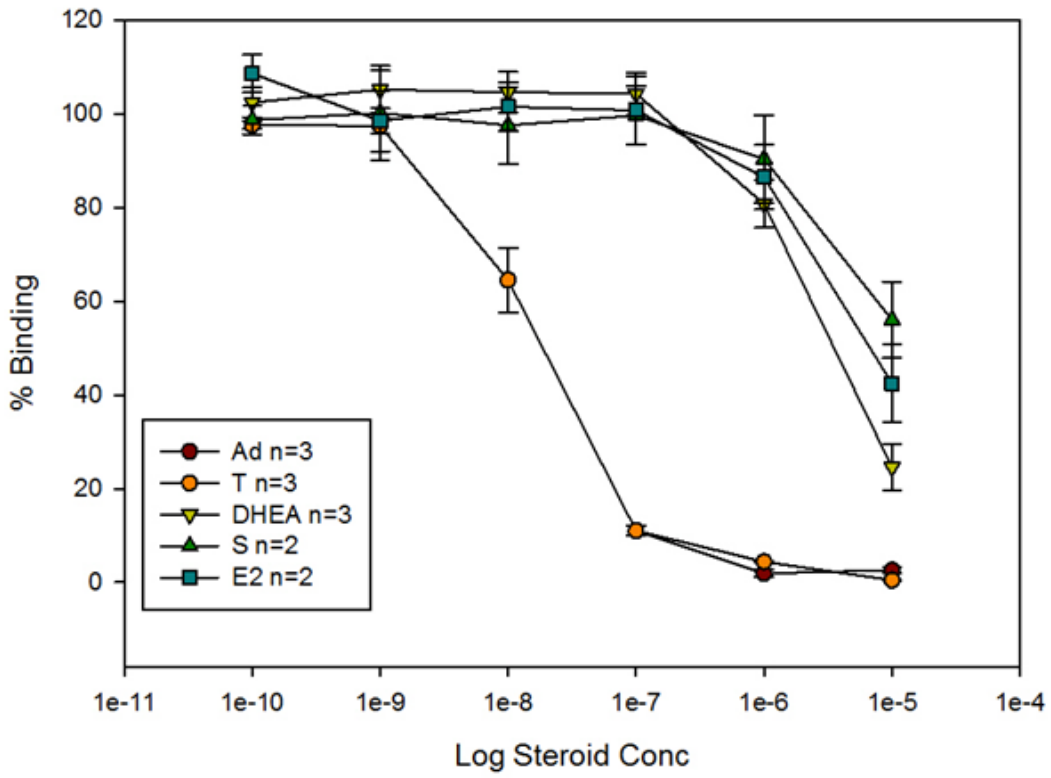

Figure 3.6 Competitive binding steroids to mAR

A graphic depicting the percent binding of DHEA, S, E2 and T to the purified membrane receptor of sea lamprey testis as a function of the respective steroid concentration. DHEA, S and E2 are known endogenous steroids in lampreys, while T is not produced in lampreys and was included as a common androgen. Symbols represent mean +/- SEM. Sample size for each steroid competition is indicated by n= in the legend.

Chapter 4: Isolation of a Putative Androgen Receptor by Affinity Chromatography

4.1 Introduction

The previous sections have already outlined the historical and biological significance of lamprey but, in brief, lampreys are the most basal extant vertebrate and may be representative of ancestral vertebrates. Neither their morphology, nor by implication their physiology, has changed much over 300 million years (Chang et al., 2006; Janvier, 2011)**.** Sea lampreys have only three known nuclear steroid receptors—progesterone receptors (PR), corticoid receptors(CR) and estrogen receptors (ER)—and apparently lack a nuclear androgen receptor (AR),which is common in most vertebrates (Thornton, 2001). The implications of Thornton's work and the androgen membrane receptor (mAR) characterized in Chapter 3 have already been discussed. This chapter details the work done to isolate and identify the mAR by purification with an affinity column and liquid chromatography.

The mAR binding kinetics were characterized in Chapter 3. In this chapter, I focus on the identification of the binding moiety. Precise identification of the protein is key information needed for testing the evolutionary hypothesis of lampreys and other vertebrate shaving a shared inheritance of androgen receptors. Ideally, the goal is to identify a protein sequence and link it to the lamprey genome. That gene sequence could then be compared to other vertebrate genomes to find similar genes and calculate the genetic drift (estimates of mutation rate and evolution of genomes).

The isolation and identification of proteins is a long established process in literature. One of the early sex steroid protein identifications was done by GL Hammond et al. (1982) using Polyacrylamide gel electrophoresis (PAGE) to isolate sex hormone binding globulin in human amniotic fluid. This simple method of separation based on gel porosity vs. molecule size and charge is still used but is now only one of a variety of purification and separation techniques used to isolate and identify proteins (Agrawal et al., 2001, Macintyre, 1988, Nunomura et al.,

1990, Pepys et al., 1977, Pepys et al., 2012, Riley and Coleman, 1970, Volanakis et al., 1978). Some of these new systems build on the principles of PAGE. MonoQ is an ion exchange column that separates molecules based on ionic charges on the molecule and a changing ionic gradient in column. Molecules are drawn off the column-matrix and eluted when there ionic charge is has higher affinity for the solution passing through the column than the matrix. Superdex 200 columns are small-scale size exclusion chromatography columns that separate molecule based on size and the ease with which the molecule move through the column. Affinity columns are a based on a completely different principle. HitrapA antibody affinity chromatography uses antibodies that are attached to the column to attract and retain proteins by increasing the concentration of imidazole in the wash buffer a decrease unspecific binding is released. This system separates known proteins from the crude protein sample using targeted antibodies. Ligand affinity binding uses the ligand associated with the protein being isolated to attract and bind the target protein. Once the target protein has been isolated onto the affinity column it may be eluted via ion exchange, with wash increasing salt concentration gradient or via ligand completion in which a ligand saturated wash is passed through the column to release the protein through competitive binding. All of these protein purification systems can be purchased from commercial producers such as GE Healthcare who produced the majority of products used in this project to purify the mAR.

Multiple attempts were made to isolate sufficient protein for sequencing. Initially ligand affinity and PAGE as purification techniques were used to isolate the protein. While results indicated a successful concentration of the protein, proteomic analysis of the isolated bands from PAGE did not identify a membrane protein. Following this initial attempt Dr GL Hammond was consulted. A multistep purification process was recommended, consisting of ligand affinity chromatography to isolate the protein, followed by increased purification via Hitrap A column, followed by protein sizing via superdex 200 column and finally proteomic analysis. The expectation was that by increasing the amount of testis membrane and using multiple isolation steps a sufficient amount of protein could be concentrated to get a protein sequence. This process was carried out in collaboration with Dr GL Hammond's lab.

My results indicate that the protein becomes very unstable when isolated from the membrane. I was not able to isolate a sufficient quantity for protein sequencing; however, I was able to get a size measurement and partially characterize the protein, which is reported in this chapter.

4.2 Methods

4.2.1 Animals

This study used testis tissue from migrating sea lampreys, collected at Hammond Bay Biological Station, Millersburg, MI (see Chapter 2 for details). The testes from 100 mature sea lampreys were collected and snap frozen at Hammond Bay. They were shipped to UBC on dry ice and held in a -80°C freezer until needed.

4.2.2 Sample preparation and purification

The putative receptor of interest was identified in the membrane fraction of the lamprey testis, as described in Chapter 3. The testis cellular membrane fraction used in the isolation procedure was prepared the same way for each isolation attempt. Different techniques of isolation were attempted in an effort to purify and isolate the binding moiety for identification. The methods used are outlined below.

4.2.2.1 Preparation testis membrane fraction

Tissues used for the isolation of the binding moiety were prepared according to Braun and Thomas's (2004) procedures (see Chapter 3 for details).

4.2.2.2 Quantification of Ad binding to purified protein in sample

Binding assays used to assess the amount of specific Ad binding in the sample after each stage in the purification and

sizing protocol were done following the methods from Braun and Thomas (2004), with slight modifications (see Chapter 3 for details).

4.2.2.2.1 Affinity column preparation

The primary isolation technique used to purify the protein of interest was an affinity column. The affinity column matrix is commercially available and is customized to bind the protein of interest by presenting the ligand (in this case Ad) at the end of a chemical "arm" attached to the Sepharose matrix (Sepharose-O-CH_2-CHOH-CH_2-NH$(CH_2)_6$-NH_2). The isolated protein binds to the ligand and becomes trapped in the column, while other components of the sample pass through the column. For this study, Ad was bound to the affinity matrix. The process required Ad to be commercially prepared (BachemInc.) with a carboxyl group attached at carbon 17 to facilitate binding to the Sepharose matrix arm.

The column was prepared following instructions provided by the manufacturer, GE Healthcare Life Sciences. The process is briefly summarized below.

EAH Sepharose 4B was supplied pre-swollen in 20% ethanol. The ethanol solution was decanted and the required amount of matrix (as determined by column size, e.g. 25ml column requires 25ml of matrix to fill it) was washed on a sintered glass filter (porosity G3) with distilled water (500ml/25mlsepharose) adjusted to pH 4.5 with HCl, followed by 0.5 M NaCl (80 ml). The androstenedione-O-carboxymethyloxime (Ad-CMO; 178 mg) ligand was dissolved into coupling solution (1 M Tris; 22.25ml; pH 4.5+50% dioxane; 13.33ml). N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydro-chloride (EDC; 290mg) was used as the coupling agent. The pH was adjusted to 4.5 during the reaction by the addition of 0.1 M sodium hydroxide or hydrochloric acid solution for the first hour. The reaction was incubated for 24 h at $4^{o}C$ on a shaker table.

The product was thoroughly washed with four cycles of solutions (100 ml) with different pH values: 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl; followed by a wash with 0.1 M Tris-HCl buffer, pH 8, containing 0.5 M NaCl; followed by a 1M Tris/ 50% dioxane wash to remove unreacted ligand; and finally by two washes of 1 M Tris-buffer. The coupled bead matrix was re-suspended in Tris-buffer and allowed to settle in a glass column and degassed.

4.2.2.2.2 Isolation of binding moiety

Two methods were used to isolate the binding moiety. The first attempt to isolate the membrane-binding moiety was performed using 6 g of sea lamprey testis on a 1ml affinity column. The membrane fraction of the sample was isolated as described above. The sample was passed through the affinity column followed by a wash with 1-2 volumes of column with Tris-buffer. GE Healthcare Life Sciences listed two ways to release the retained proteins: salt gradient and ligand competition. For this attempt, the captured protein was eluted with a 70-min run of increasing linear gradient of 0.1M KCL in 10 mM phosphate buffer. An auto-fraction collector was used to collect 1min fractions off the column. The samples were tested for binding as described in Chapter 3. There was a bimodal binding response, with binding in peaks associated with fractions 29 and49. Sample fractions 20-51 where combined and concentrated using an Amicron 10kDa centrifugal filter to a 500µl total volume. The salt was removed by adding 5ml of Tris-buffer, and the volume was again reduced to 500µl;the salt reduction step was repeated twice. The final concentrated sample was run on a mini-Protein SDS-page gel, 20µl/well.

The cytosol fraction of the testis sample was also run through the affinity column using the same procedure.

4.2.2.2.3 Proteomics

The SDS-page gel from the first isolation attempt was sent to the UBC proteomics lab for analysis. The protein bands were subjected to mass spectrometry. The results were analyzed using Mascot analytical software.

4.2.2.2.4 Isolation trials two and three

After the failure of the initial methodology to identify the receptor, an additional two attempts were made to isolate the membrane-binding moiety using a modified protocol. The methodology of the isolations was modified to protect the protein being denatured by salt during extraction from the affinity column. The salt extraction was replaced with competitive binding with free Ad through the addition of Ad saturated Tris-buffer. The Ad was removed from the extracted protein after extraction by the addition of dextran coated charcoal, using

the same procedure as was used to extract endogenous hormones for competitive binding assays in Chapter 2.

A second extraction was conducted using a new 5 ml affinity matrix column. A total of 22.0 g of sea lamprey testis were processed and the membrane extract was run out on the affinity column. A final extraction was done using 519.9g of sea lamprey testis. A new 15 ml affinity matrix gel column was used to extract the membrane-binding moiety. The protein extract was then run on an ion exchange column (HiTrapA) to concentrate and remove impurities. The fraction that had Ad-binding activity was subsequently run on a sizing column (Superdex200 column, see table 4.1).The molecular weight was calculated by plotting a standard curve of Ka against log molecular weight (mwt) of standards (indicated on figure 4.1), where Ka is defined as:

Ka= elution volume– void volume/total column volume – void volume.

(elution volume = ml elution solute passed through column; void volume = space between column matrix)

The estimated weight of the isolated receptor was calculated by extrapolating the mass associated with that fraction from a standard curve of the elution volume of the Superdex fraction with active Ad binding vs Ka.

4.2.2.3 Statistical analysis of results

All assays were run three times, each in triplicate except where noted in the competition study (i.e., three independent samples were prepared as describe in tissue preparation (n=3); for each sample three total and three non-specific binding assays were run; specific binding for each sample is reported as the average total binding minus the average non-specific binding ± SEM). All statistics are reported as mean ± SEM as calculated by Graphpad Prism 5.01 for Windows. One-way ANOVAs were completed using Graphpad Prism.

4.3 Results

4.3.1 Isolation trial one

The first attempted isolation of the binding moiety using the affinity column resulted in a successful concentration of the putative receptor. A total of 10 ml of membrane preparation was loaded onto the column (this process was repeated with cytosolic fraction). The total protein content of the membrane preparation loaded was 5.7 g, and a total of 0.78 g of protein was retained on the column and eluted in two fractions. For the cytosol preparation, a total of 6.1 g was loaded and 0.06 g of protein was eluted. The membrane-binding moiety was captured by the affinity column matrix. Figures 4.1 and 4.2 show the increase binding in the affinity extract and the corresponding drop in binding activity in the flow-through-fraction and wash-fraction from the affinity column.

4.3.2 Isolation trials two and three

The second two attempts at isolation of mAR also yield some significant results. The first attempt was made using 20.0 g of testis tissue. The protein was concentrated on the affinity column and released via competitive binding with Ad. Next, an ion exchange column was used to purify the protein recovered from the affinity column. Activity was detected in fraction A10 that was eluted from the ion exchange column and was subsequently run on a sizing column (Figure 4.3). The Superdex column run of fraction A10 (from HiTrapA) went well. There was one major peak identified by the UV absorption plot that matched the material eluting at about 20 ml. Rough calculation, based on size standards associated with this fraction, put this peak in the 7 kDa range in size (note: 7 kDa is an extrapolated out-of-range value). Almost all binding activity was lost, and the peak could not be correlated with Ad binding within the 20 ml fraction. The results were therefore considered to be inconclusive and a third isolation attempt was planned and final attempt was made the following year using a large amount of testis and completing the purification steps in rapid succession, in the hope that a detectable amount of protein would survive the process.

A final extraction was conducted, using 519.9g of sea lamprey testis and a new 15 ml affinity matrix gel column to extract the membrane-binding moiety. Because the protein rapidly

degraded in the previous isolation attempt, activity in the A10 fraction was confirmed by a single one-shot assay of 25 µl (best practice would be conduct the assay in triplicate). The remainder of the sample was immediately run on the Superdex column to maximize the amount of active binding protein run in the column. Results are tracked by elution volume for this column; a UV peak was detected in the 20 ml fraction as in the previous sample (indicating no binding activity). Given the extrapolated mwt associated with fraction 20 ml, the most likely contents of this fraction is degraded protein fragments and other cellular remnants.

Binding activity was found in 15 ml fractions (Figure 4.4).The protein content in the fractions that bound [3H] Ad was insufficient to produce a UV peak ([3H] Ad binding was quantified as described in section 3.2.4). This binding gave a size range for the molecule of 94.7-49.7 kDa. (Molecular weight calculated by standard curve; Ka against log mwt Ka = elution volume– void volume/total column volume – void volume.)

4.4 Discussion

Isolation and identification of membrane receptor proteins is a high risk project. These proteins can be extremely stable in a bilayer environment, but are often unstable and rapidly lose ligand binding activity after detergent solubilization(Bowie, 2001). This proved to be true for the Ad binding moiety: it remained stable for days at 4°C and lost less than 10% of its activity when it was snap frozen and thawed for later use. This activity was not lost when Triton-x100 detergent was used to remove the bilayer, indicating that the detergent was stabilizing the protein. The next step of concentrating the proteins by ion exchange columns, however, is affected by detergents, so Triton-x100 could not be used to stabilize the protein for this step. Without the detergent, protein stability rapidly diminished when the protein was concentrated using ion columns. Ligand binding activity in the sample was lost before the process could be completed.

The process of isolation did however reveal several properties of the Ad binding moiety. The affinity column was made using EAH Sepharose 4B beads and an Ad-CMO. The CMO was attached at the carbon 17 position on the steroid. This binding combination produces a relatively short arm (eleven atoms) that presents the ligand to the target binding protein. The

protein attached to this arm with high affinity, as indicated by the lack of binding activity in the flow-through and the junk-wash. This indicates that the binding site may be easily accessible and not deep in the protein structure, as the short arm would not allow the bound Ad to reach a deep seated binding site.

The ligand specificity of mAR is not likely based on the carbon 17 oxygen double bond of the steroid molecule (Figure 1.1) for two reasons. The first is that the binding specificity of Ad was very high in comparison with other endogenous hormones tested. Only T (not produced by lampreys) was bound with equal affinity as that of Ad (Figure 2.5). Ad differs from T at the carbon 17 position, where an oxygen double bond is converted to an OH group (>C=O vs >C-OH) changing the size, shape and charge associated with the bond. The second is that the affinity ligand was attached to the column matrix at the carbon 17 position, but did not prevent protein binding. This means that the mAR bound to the Ad ligand despite the carbon 17 position being blocked by the affinity matrix arm, again indicating that the ligand binding specificity was not affected by a change at carbon 17.

Processing a large amount of testis tissue (519.9g from100 individuals) allowed me to complete one run on the Superdex column. The results indicate that almost all of the protein had broken down into 7kDa fragments, as indicated by the UV peak at 20 ml. There is some binding in almost all fractions, which may be attributable to some of the decaying protein fragments having the binding sequence for Ad. Some protein with strong significant Ad binding activity did however survive the processing long enough to identify the fraction associated with the intact protein (Figure 4.4). This gives us a size range for the molecule of 49.7-94.7kDa. This is likely as close as we will get to a full identification using this methodology, given that the protein decays so rapidly under these conditions. Interestingly, this size range is a close match to human androgen receptors AR-A 87 kDa, and AR-B 110 kDa. (These human receptors, however, are nuclear receptors and may not be related to this mAR). Shihan et al. (2014) described G-coupled non-classical androgen receptors and demonstrated that the receptor was not blocked by nuclear receptor inhibitor small interfering Ribonucleic Acid (siRNA),but was impaired by the suppression of expression of the G-protein Gnα11using siRNA. The receptor

described was for T but seems a more likely candidate for comparison with the lamprey mAR, given that it is also a membrane androgen receptor.

Validating speculation along this line will require further testing. Briefly, this could be approached in several ways. A brute approach of simply scaling up the process to an even greater degree to that used in this dissertation could be tried. This would require up to 5 kg of testis to isolate more of the protein. Another approach would be to use SDS-page gel to separate triton X-100 stabilized protein and sequence protein bands that correspond to the 49.7-94.7 kDa size range, followed by a genomic search for matching DNA sequences. The protein was stable in the lab in the presence of triton X-100, but became de-stabilized upon removal of triton X-100 which was required before it could be passed through the sizing column in this chapter. A third approach would be to develop antibodies to the protein and use it to isolate and purify the protein. Thermo-Fisher markets commercial kits for immunoprecipitation of proteins. Once isolated the protein could be sent to proteomics for sequencing. The antibody could also be used in conjunction with commercial immunoflorescence kits (BioRad) to track down the active portions of the DNA associated with up regulation of receptor synthesis and to identify which cells express the protein. These possible future lines of inquiry are discussed in the general discussion below.

4.5 Figures

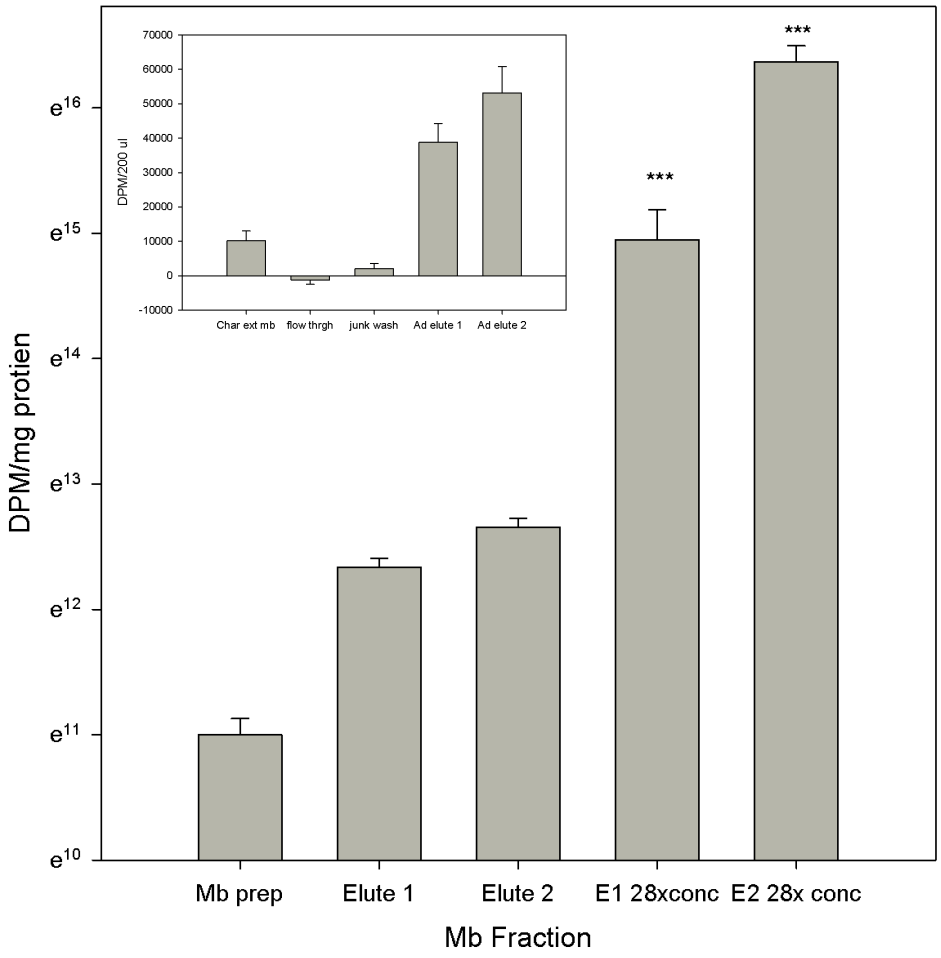

Figure 4.1 Binding assay of affinity column purified testis membrane fraction, eluted with NaCl

The concentration of Ad binding moiety released from the affinity column is expressed as DPM of [3H] Ad bound. Almost the entire binding moiety was retained on the Ad affinity column and eluted with NaCl; see the insert, which shows the raw result before concentration. Note that there is almost no activity in the flow-through and junk-wash (a flush of HEAD buffer use to remove unbound protein), indicating that the moiety is bound to the column. The elutions 1 and 2 were concentrated 28 fold (x) using a molecular sieve (E1 28xconc and E2 28xconc). This concentration process led to a significant concentration of the protein, as indicated by the *** notation for $p < 0.01$.

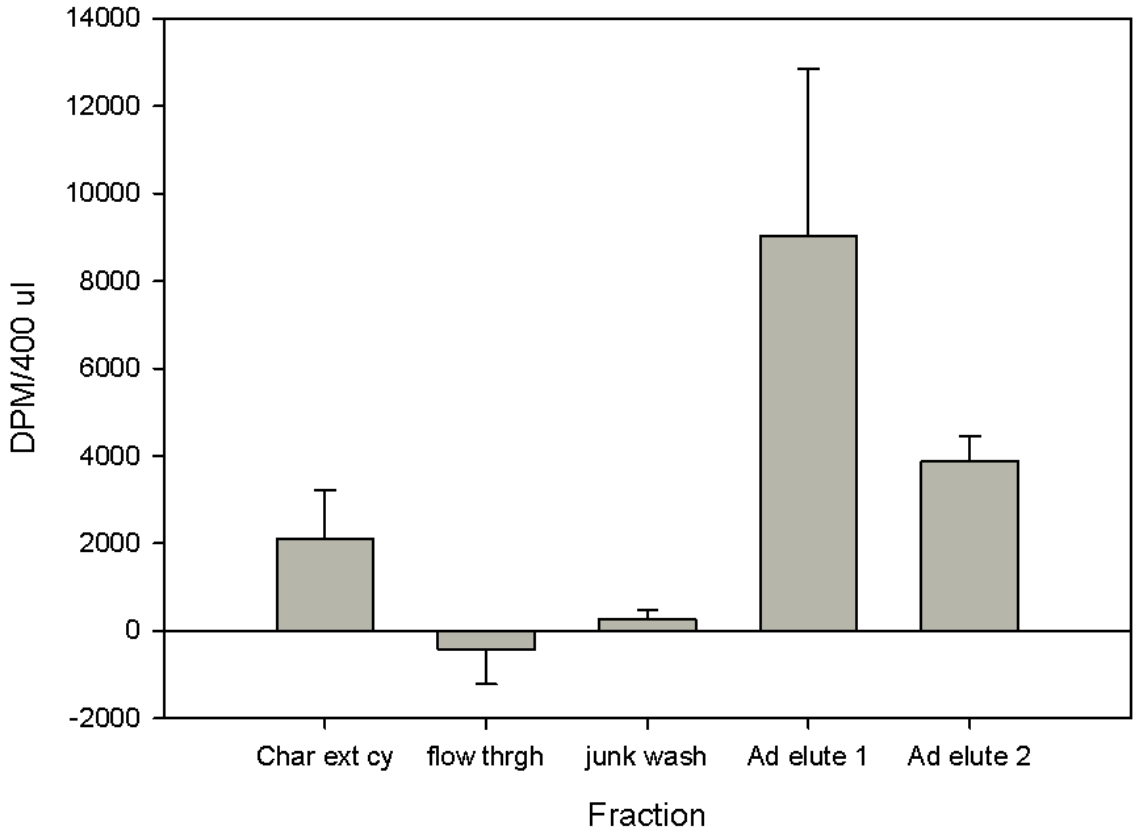

Figure 4.2 Affinity column purification results for testis cytosolic fraction

Total raw binding found in the charcoal extracted cytosol ("Char ext cy" in figure) fraction is 14 times less than the testis membrane fraction (shown in Figure 4.1). The concentration of the Ad binding moiety is reported as DPM/ 400 µl aliquot. Almost the entire binding moiety was retained on the affinity column and eluted with NaCl. Note that there is almost no activity in flow-through and junk-washing, indicating that the moiety is bound to the column. This is likely the same binding reported by Bryan et al. (2007).

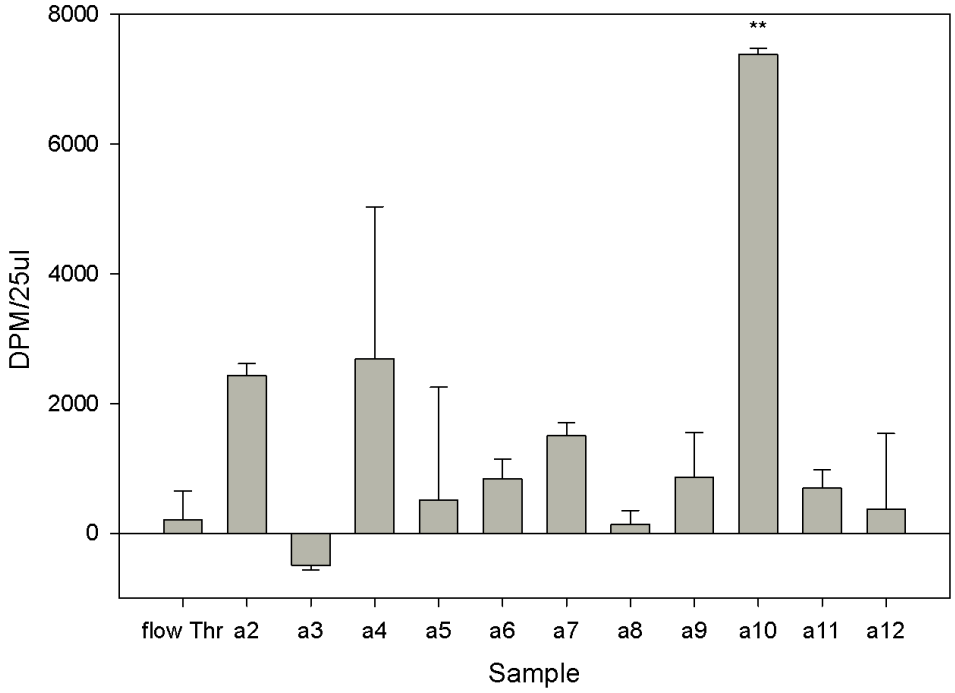

Figure 4.3 Protein isolation results reported as Ad binding activity in samples collected from HiTrap A ion column

The concentration of the Ad binding moiety is reported as DPM/ 25µl aliquot. Most fractions do not have binding that differs statistically from 0, indicating that the protein of interest is not in that fraction. Fraction a10 was the only fraction that showed statistically significant binding, indicating that the binding moiety has been concentrated into this fraction.

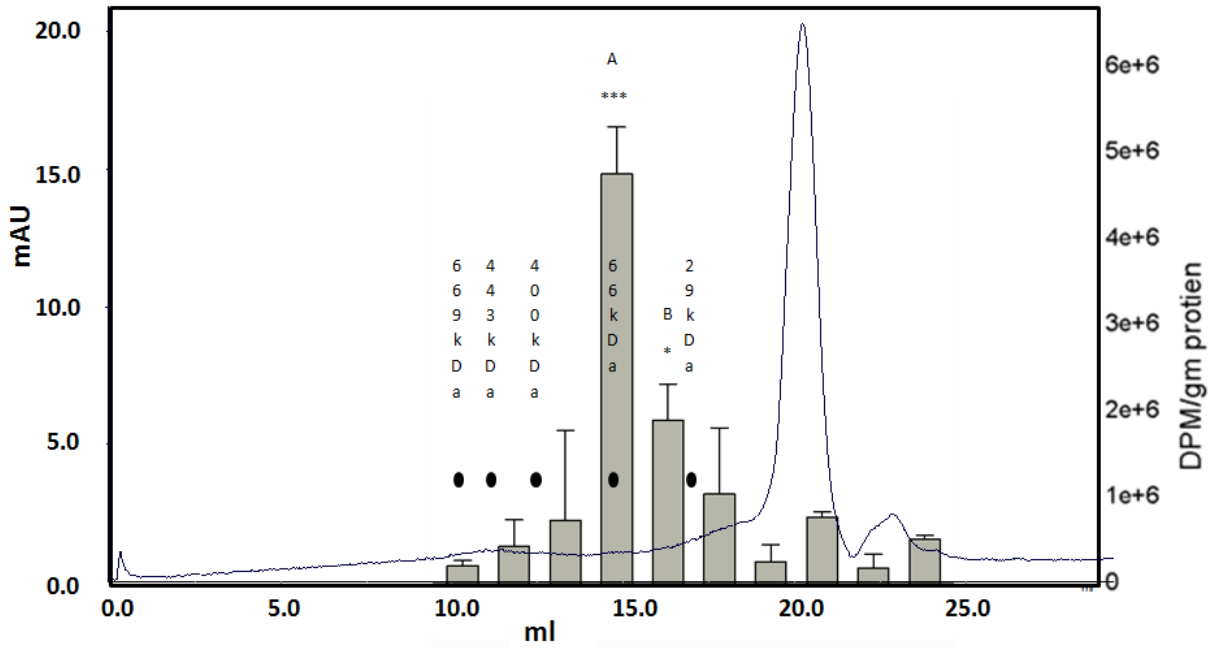

Figure 4.4 Protein sizing results from Superdex200 column

The graph shows UV absorption per ml; the strong peek at 20 ml indicates most of the protein eluted in those fractions. The fractions were tested for Ad binding (measurements were made in triplicate). Results have been displayed with a bar graph overlay (DPM/g of protein). The strongest binding activity of [3H] Ad was found in fractions labeled A and B: A was statistically different from all other fractions assayed (one-way ANOVA). Dots mark sizing standards run on the column and the corresponding masses. There is almost no binding activity in the fractions associated with the UV absorption at the 20 ml mark. Given the out-of-range extrapolated molecular weight associated with fraction 20 ml, the most likely contents of this fraction is degraded protein fragments and cellular debris; this fraction is therefore not relevant to this study.

4.6 Tables

Table 4.1

Technical specifications of Superdex200 column used.

Superdex200 column parameters	
Total column volume = 23.56 ml; Void volume = 8.54 ml, measured using Blue Dextran	
Protein standards molecular weights (mwt) and elution volumes (based on duplicate runs and averaged)	
Molecular weight calculated by standard curve; Ka against log mwt. Ka = elution volume - void volume/total column volume – void volume	
Mwt	Volume
669,000	10.25 ml
443,000	11.24 ml
200,000	12.63 ml
66,000	14.56 ml
29,000	16.94 ml

Chapter 5: General Discussion of the Findings and Implications of this Investigation

5.1 Summary of Findings

I began this dissertation by delineating a set of objectives and hypotheses. In Chapter 2, I addressed the Hypotheses I: Lampreys synthesize DHEA and Ad; Hypothesis II: Lampreys can convert DHEA-S (inactive form) to DHEA (active form) in the liver; Hypothesis III: Lampreys convert DHEA to Ad in the testes; and Hypothesis IV: Lamprey ganadotropin-releasing hormones (GnRH I & III) mediate conversion of androgens in lampreys. Chapter 2 was important, as it laid the foundation for comparing lampreys to other vertebrates. The data supported each hypothesis establishing that lamprey appear to share components of the sexual hormone synthesis pathway and GnRH hormone regulation with other vertebrates. This has implications for the much broader, hypothesis that lampreys and derived vertebrates inherited their steroid biosynthesis pathway from a common ancestor.

Chapter 3 represents the most significant findings of my thesis. Unlike Chapter 2, which confirms attributes of lampreys that have been accepted based upon speculation by many researchers; Chapter 3 opens the door for new insight into vertebrate evolution. It establishes strong evidence of an androgen membrane receptor (mAR) in lampreys, a class of receptors that has only begun to be investigated and has not yet been fully explored with regard to their evolutionary significance. Hypothesis V: Lampreys have an active unreported androgen receptor, was strongly supported by my data in Chapter 3, and should be explored further.

Chapter 4 was a high-risk endeavor to identify the putative receptor characterized in Chapter 3. Due to methodological challenges, the results were limited, ending in a size range for the mAR but not a protein sequence that could be used to search genetic databases and establish DNA links between species and evolutionary lineages. Chapter 4 was intended to provide further support for Hypothesis V and Hypothesis VI: The binding moiety found in lamprey testis is a putative receptor with properties similar to the androgen binding receptor

reported by Braun and Thomas (2004) in the ovaries of the Atlantic croaker. In the end, most of the evidence used in Chapter 4 to address Hypothesis VI was drawn from the characterization work in Chapter 3. While I did not achieve the conclusive identification of the mAR protein I was hoping for, I was able to establish that the lamprey mAR is consistent with the receptor described by Braun and Thomas (2004).

5.2 Significance of Findings

Having reviewed my finding above, I will now further address the possible impacts of my work. Recall that Chapter 2 addressed Objectives 1 and 2. Objective 1 was to firmly establish the identity of androgenic steroids in lampreys. This work established links between lamprey steroid biogenesis and the generalized biogenesis model of other vertebrates. The need for this work arose from the accepted theory that lampreys diverged from other vertebrates some 450 million years ago, since which time they have continued to evolve.

Thornton (2001; 2006) suggested that lamprey have fewer nuclear receptors than other extant vertebrates and provided evidence that the nuclear estrogen receptor evolved before the nuclear androgen receptor. He emphasized that the estrogen nuclear receptor evolved to take advantage of an already existing steroid ligand, estradiol (E2) that is at the terminal end of a complex steroid synthesis pathway. The issue of differences in lamprey steroid biochemistry and axis of action came to prominence again through the work on sea lamprey stress response by Dr Close (Close et al., 2010; Rai et al., 2015; Roberts et al., 2014). These publications demonstrated that lamprey use 11-deoxycortisol (S) as a stress hormone instead of other known corticosteroids. Close et al. (2010) noted that corticosteroids appear to be conserved, with most vertebrates sharing a stress response that includes increased glucocorticoid (GC) hormones that further regulate metabolic, endocrine, and immune functions. Hormones may be varied among species; tetrapod groups use at least two active GC hormones, either cortisol or corticosterone, and the mineralocorticoid (MC) that regulates ion balance is aldosterone. In teleosts, cortisol acts as both GC and MC(aldosterone is not present) (Liley and Stacy, 1983; Norman and Litwack, 1997).

The significance of this is that sea lamprey corticosteroid receptor (CR) does not use a known corticosteroid. Lamprey use S, the precursor of cortisol, which indicates that the lamprey stress axis is different than other vertebrates and may represent a more ancestral state (Close et al., 2010). A study of hagfish found that they have the enzymes need to produce some steroid hormones (e.g. progesterone) and response to stimuli that suggest a regulated glucocorticoid and mineralocorticoid response, but researchers were unable to identify a steroid hormone associated with the response(Clifford et al., 2017).Cortisol, deoxycortisol, 11-deoxycortisol and corticosterone were all rulled out as endogenous CR ligands. Elasmobranchs have also been identified as having a stress respone that is regulated by a non-classicle steroid. A review by Skomala and Mandelman (2012) highlighted a variety of differences between teleost and elasmobranchs stress responses including evidence that 1α-hydroxycorticosterone is the active stress hormone. All of these examples demonstrate that less derived fish have interesting variations in steroid regulation systems. These variations support Baker's (2003) theory of stepwise evolution of steroid regulation and support Janvier's assertion that physiological systems can be used to identify evolutionary events that define species.

The sexual maturation of lamprey proved equally interesting due to the apparent absence of the nuclear androgen receptor. Mesa et al. (2010) and Thornton (2001) tried to explain male sexual development by correlating it with E2 during lamprey sexual development. Bryan et al. (2007) presented an alternate theory, reporting that Ad induced male maturation and the production of hydroxylated steroids in lamprey. All of these studies raise questions surrounding the identity of active sex steroids and their associated axis of action in lampreys. This is the reason I began my dissertation by addressing these questions in Chapter 2.

The identity of endogenous steroids was accomplished through the use of high-performance liquid chromatography (HPLC), radioimmunology assay (RIA), and thin layer chromatography (TLC). These findings were further supported by the incubation of labeled steroid precursors with lamprey tissue to confirm the production of the expected steroid

products. In all cases, my results supported the hypothesis that lampreys produce DHEA and Ad.

Having established this, the investigation moved to objective 3 and established that there is evidence of a mAR in lampreys, with properties similar to the androgen binding receptor reported by Braun and Thomas (2004) in the ovaries of the Atlantic croaker. This putative receptor was characterized to test the hypothesis that it fit the characteristics of a receptor. Its selective binding of steroids, its affinity for Ad, and the characteristic of the binding were carefully measured and compared with the Braun and Thomas (2004) mAR. The results all support the hypothesis that this protein is a membrane receptor. These results are further supported by the maturation studies done in the Close lab and reported by Bryan et al. (2007); all of which confirm that male sexual development in lampreys can be induced by Ad.I have thus both the characterization of a putative receptor and evidence of binding to that receptor indicating the potential for a biological effect from its ligand, two components required to demonstrate a functional receptor(Norman and Litwack, 1997).

Based on these findings, there is evidence that supports the hypothesis that there is an active receptor for Ad in lamprey testis. I can theorize based on the lack of Ad in plasma circulation that if Ad is a functional hormone in lamprey development, it is likely functioning via cell-to-cell signaling in the testes. This is consistent with Bryan et al.'s (2007) findings of high Ad concentration in the testis. It is generally known that in bony fish and amphibians the Leydig cells are steroidogenic and regulate the development of Sertoli cells and spermatogonia. A cell-to-cell-signaling model in lamprey would likely involve Leydig cells producing Ad and Sertoli cells being the localized target tissue with the hypothesized membrane receptor.

This is as far as the current findings can take us. The next steps would be to try and confirm that the binding is localized to Sertoli cells and test the axis of action. Non-classical T-signaling by G-coupled-protein has already been proposed and investigated in the spermatogenic cell line GC-2 (Shihan et al., 2014). In fact, the number of reports of membrane androgen receptors is increasing, and it is linked to non-classical actions and cancer treatment (Hatzoglou et al., 2005; Mankoff et al., 2000; Rahman and Christian, 2007).This means that the investigations of membrane-bound androgen receptors are likely to increase in prominence and

there will be greater interest in the complete identification and characterization of these receptors. The next step in investigating mAR could be a replication of the Shihan et al. (2014) study to determine if the receptor in lamprey is homologous to the G-coupled-protein they reported.

Definitive proof of my supposition that my reported binding moiety is an androgen receptor that regulates testis maturation and its possible impact on vertebrate evolutionary theory will require further studies. There is, however, sufficient evidence to speculate about lamprey sexual development and the role of this putative receptor in the evolution of sexual maturation in vertebrates.

The receptor was partially identified in Chapter 4, but proved too unstable for complete identification. This does not diminish the results but does make it clear that a full identification by this methodology will require considerably more effort. This could be approached in several ways. The process used in Chapter 3 could be repeated using even more tissue as discussed in Chapter 3. In all practicality this work would require at least one season (collecting lamprey during spawning migration) of tissue collection at Hammond Bay Biological Station or another facility that has access to large numbers of sea lamprey. The testis of 100 lampreys were collected and processed in the final attempt reported here and in all practicality it would require 5-10x that number to boost the quantity of receptor isolated by the affinity column to justify a repeat of this method. It should be noted that given the rapid decay of the protein during my isolation attempts, there would still be a high chance of failure. An alternative could be to use affinity chromatography to purify the receptor and then separate it directly on SDS-page gel. The size data obtained in this research could then be used to identify the size band of interest for analysis by a proteomics lab. This method has the advantage of avoiding purification steps that caused the protein to decay so rapidly; the disadvantage is that there could be a large number of proteins in the band that would have to be vetted out.

A third approach could be to develop monoclonal antibodies to the protein. This would still require a large quantity of purified protein that could be used to induce antibody production. Once antibodies have been produced, they could be used to in two ways. They can be used to precipitate and concentrate the mAR from the affinity column extract. Antibodies

can also be used to make an affinity column similar to the one used in this study. This antibody column may provide a more selective binding of the mAR than the Ad ligand column used in this study (Dean and Philip, 2000 Rosenberg, 2006). The refined protein concentrate could then be submitted to a proteomics lab for sequencing. While the techniques suggested to here are well established , it would involve a considerable amount of work.

5.3 Implications

The implications of this work are diverse and at this point are primarily speculative. Some of these possible implications are identified and discussed below.

The first and most obvious implication of this work is general science knowledge. Each new discovery contributes to our overall basis of knowledge. The physiology of lamprey is still relatively unknown; thus, the information in this thesis adds to our understanding of their biology. This work verifies that lamprey produce the same steroids found in other, more-derived vertebrates.

The second implication is that these insights into lamprey sexual development might be used to control lamprey sexual development. This would be of interest for two reasons. The first is that sea lampreys are an invasive species in the great lakes, and impairing their sexual development would be one method of controlling their numbers. Unfortunately, a delivery method for wild populations would likely prove impractical because it would require dosing spawning streams with Ad or Ad blocker to alter sexual maturation. This could affect many species in the waterway in addition to the target species, lamprey. For Pacific lampreys, however, the problem is the opposite. Their numbers are in decline, and there are efforts in Canada and the United States to increase their numbers. The ability to induce sexual maturation in a hatchery setting would be of interest and useful. In both species, the knowledge about lamprey reproductive steroids will allow us to assess the impact of steroids released into the water system through human activities as more is learned about how medications and other bioactive chemicals released by human into the environment affects fish(Guillette et al., 1995; Jobling et al., 2006; Readman et al., 2005). The identification of Ad as

a bioactive regulator of lamprey sexual maturation adds Ad to a growing list of bioactive contaminants of concern for fisheries management.

This leads to the last point considered in this dissertation: the medical implications. Membrane androgen receptors have now been identified in several vertebrates. The presence of a mAR in lampreys makes it likely that these receptors exist in all more-derived vertebrate lineages, including primates. This supposition is supported by colon and prostate cancer research that have identified mAR's as paying a role in cancer (Gu et al., 2011; Papadopoulou et al., 2008).This raises the question: of what role mAR plays in human physiology, an important question because our health may be directly affected by steroids previously viewed only as precursors to testosterone and estrogen.

Receptors are important targets of medical treatments (Gustafsson, 1999; Kliewer et al., 1999). Currently, receptor-targeted treatments for breast cancer and prostate cancer are under investigation (Jordan and Brodie, 2007; Ryan and Tindall, 2011; Schiff et al., 2004). Treatments for depression can also be receptor-based therapy (Holsboer, 2001). It is also important to consider that future discoveries of unreported receptors may lead to a better understanding of the mechanism of action of different pharmaceutical chemicals. The functional pathway of most antidepressants, for instance, is currently unknown (Bhandari, 2016) and may be targeting unreported receptors. Antidepressant therapy may also be of particular relevance to future investigations of androgen receptors, because depression and mood swings have been linked to changes in hormone levels, for example in post-partum depression. There are thus a number of clinical implications associated with finding and targeting receptors.

The steroids that interact with these receptors must be considered, too. DHEA has become a supplement of interest in anti-aging, cognitive function, ovary function, muscle tone, etc., making it a popular over-the-counter supplement (Dayal et al., 2005; Gleicher and Barad, 2011). DHEA and DHEA-S have been proposed for use in treating depression, wellness and post-menopausal symptoms (Barrett-Connor et al., 1999; Baulieu et al., 2000; Bloch et al., 2003; Panjari et al., 2009; Panjari and Davis, 2007; Wolkowitz et al., 1997). Local production of DHEA in the brain has been shown to affect mating behavior in both mammals and birds (Janvier, 2011; Soma et al., 2002). Ad has been used as an anabolic steroid to supplement physical

performance (Yesalis and Bahrke, 2002). The side effects of anabolic steroids have captured public attention due to their affects on libido and aggression (Strauss et al., 1983). The documentation of receptors for DHEA and Ad may lead to theories of how these effects and side-effects are expressed. Thus, previously overlooked androgens are turning out to have important biological roles. Understanding these roles will be of significance to developmental, behavioral and clinical medicine.

In conclusion, the discovery of a functional membrane-bound Ad receptor in a basal vertebrate has the potential to alter views on evolution, environmental management and medicine. Further research is therefore both intriguing and warranted.

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Appendices

Table A.1

Series I: Summary of steroid identity presence (+) in sea lamprey and Pacific lamprey pooled plasma samples tested. Results indicate that DHEA and Ad are endogenous steroids in lampreys.

Plasma	Sea lamprey	Pacific lamprey
RIA DHEA	+	+
RIA Ad	+	+
TLC DHEA	+	+
TLC Ad	+	+

Table A.2

Series II: Summary of the effect of GnRH and temperature on DHEA and Ad production in supernatant (SN) and uptake into tissue of Pacific lamprey testes (Figures 2.7 and 2.8). The results are reported as "+" (present) or "-" (absent) for the indicated SN and tissue as identified by RIA or TLC. These results confirm the conversion of DHEA to Ad in the testis.

Fish#	1	2	3	4	5	6	7	8	9	10
Precursor	DHEA	DHEA	DHEA	DHEA	DHEA	DHEA	DHEA	DHEA	DHEA	DHEA
Treatment	GnRH	GnRH	GnRH	cont	cont	GnRH	GnRH	GnRH	cont	cont
Temp 0C	17	17	17	17	17	7	7	7	7	7
Portion	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue
RIA DHEA	+	+	+	+	+	+	+	+	+	+
RIA Ad	+	+	+	+	+	+	+	+	+	+
TLC DHEA	+	+	+	+	+	+	+	+	+	+
TLC Ad	+	+	+	+	+	+	+	+	+	+
Portion	SN	SN	SN	SN	SN	SN	SN	SN	SN	SN
RIA DHEA	+	+	+	+	+	+	+	+	+	+
RIA Ad	+	+	+	+	+	+	+	+	+	+
TLC DHEA	+	+	+	+	+	+	+	+	+	+
TLC Ad	+	+	+	+	+	+	+	+	+	+
Fish#	1	2	4	5	6	8	10	11	14	15
Precursor	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad
Treatment	GnRH	GnRH	GnRH	cont	cont	GnRH	GnRH	GnRH	cont	cont
Temp 0C	17	17	17	17	17	7	7	7	7	7
Component	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue	Tissue
RIA DHEA	-	-	-	-	-	-	-	-	-	-
RIA Ad	+	+	+	+	+	+	+	+	+	+
TLC DHEA	-	-	-	-	-	-	-	-	-	-
TLC Ad	+	+	+	+	+	+	+	+	+	+
Component	SN	SN	SN	SN	SN	SN	SN	SN	SN	SN
RIA DHEA	-	-	-	-	-	-	-	-	-	-
RIA Ad	+	+	+	+	+	+	+	+	+	+
TLC DHEA	-	-	-	-	-	-	-	-	-	-
TLC Ad	+	+	+	+	+	+	+	+	+	+