January 2012

Sex Determination And Differentiation In The Common Snapping Turtle - A Reptile With Temperature-Dependent Sex Determination

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SEX DETERMINATION AND DIFFERENTIATION IN THE COMMON SNAPPING
TURTLE – A REPTILE WITH TEMPERATURE-DEPENDENT SEX
DETERMINATION

by

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Bachelor of Science, University of North Dakota, 2006

A Dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota
December
2012

This dissertation, submitted by Anthony Loren Schroeder in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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ABSTRACT

Temperature-dependent sex determination (TSD) was first discovered in an African lizard over 40 years ago. TSD has since been shown to be exhibited by many vertebrates including some fish and amphibians, numerous lizards, turtles, and all crocodilians studied to date. Although numerous questions surround TSD, a major question focuses on understanding the genetic, physiological and molecular mechanisms underlying this process. However, the molecular mechanisms underlying TSD are not well known and the gene(s) that transduces a signal for ovary or testis development is not known in any species. Furthermore, it is well established that sex steroid hormones, androgens and estrogens, are important for sex determination and differentiation in TSD species. Yet, the role of androgens in these processes is not well understood. This dissertation addresses these questions in two parts. First, to identify unique, thermo-sensitive, genes involved in TSD and secondly, determine the role of androgens in sex determination and differentiation in the common snapping turtle, a reptile with TSD.

I used differential-display PCR to clone a candidate gene involved in TSD, the cold-inducible RNA binding protein (Cirbp). The temporal and spatial patterns of Cirbp mRNA and protein expression during and after sex determination were determined using quantitative real-time PCR, *in situ* hybridization, and immunohistochemistry. I used next-generation Illumina sequencing to identify small nucleotide polymorphisms (SNPs) to test for associations between Cirbp genotype, mRNA expression, and sex ratios.

To determine the role of androgens in sex determination and differentiation, snapping turtle embryos were treated with the androgen, dihydrotestosterone (DHT), the anti-androgen, flutamide, or a vehicle (ethanol) control. Whole mount *in situ* hybridization and immunohistochemistry were used to determine the effect of the treatments on sex differentiation of the male reproductive tracts.   Quantitative real-time PCR was used to measure expression patterns of ovary-specific genes, testis-specific genes and steroidogenic genes in gonads from embryos treated with vehicle, DHT, or flutamide.

I found Cirbp was induced at a high female-producing temperature, but not at a low female-producing temperature. Cirbp is associated with TSD and expression of alternative Cirbp alleles is capable of transducing temperature differently for establishing a signal that directs ovaries versus testes development. I observed allelic specific expression and differences in allele frequencies between turtle embryos from northern and southern Minnesota, suggesting genetic adaptation to local thermal regime. I also found significant genetic associations between Cirbp genotype, Cirbp expression and sexual phenotype in a study that produced mixed sex ratios.

Androgens were capable of inducing ovarian development even at an all-male producing temperature, presumably by inducing aromatase expression and increasing endogenous estrogen production. I also observed among clutch variation in androgen responsiveness to produce females, suggesting there is a genetic basis for the response.

My findings provide the strongest evidence to date for a unique gene involved in TSD. Additional studies are required to define the functional role of Cirbp in sex determination. Androgens appear critical for ovarian development, but ongoing research is needed to understand androgen signaling and the genetic variation underlying this process.

CHAPTER I

INTRODUCTION TO SEX DETERMINATION AND DIFFERENTIATION IN
AMNIOTIC VERTEBRATES

Sex determination and differentiation are important developmental processes because they shape every aspect of the organism's biology, from the ecological to the molecular levels (Charnov, 1982; Shine, 1989; Ranz et al., 2003; Ellegren and Parsch, 2007). Sex determination is the developmental decision to form testes or ovaries. Sex differentiation is the development of sex differences in non-gonadal tissues due to sex hormone signaling.

Although sex determination and differentiation are fundamental biological processes, the modes of sex determination exhibited by amniotic vertebrates vary dramatically. The variation in sex determination between mammals, birds, and reptiles makes these organisms useful for studying the conserved or unique mechanisms of sex determination and differentiation.

Here I provide a brief synopsis of sex determination and differentiation in amniotic vertebrates. First, I describe the varying sex determining mechanisms exhibited by amniotes, followed by the cellular, morphological, and molecular development of the gonads. Next, I discuss sex differentiation, using the development of the male and female reproductive tracts as an example. Finally, I establish research objectives to be addressed in proceeding chapters of this dissertation.

Sex Determining Mechanisms of Amniotic Vertebrates

The modes of sex determination are generally classified into two broad

categories: genotypic or environmental. The mechanisms of sex determination exhibited

by amniotic vertebrates are summarized in Figure 1.1 and described in more detail below.

Genotypic Sex Determination

An individual's genetic composition, or genotype, can influence the organism to

develop as male or female. Species that display this mode of sex determination are said

to have genotypic sex determination (GSD) (Bull 1983; Janzen and Paukstis, 1991;

Valenzuela 2004). In GSD species, sex is permanently fixed at conception by inherited

genetic factors.   The most well known forms of GSD depend on the organization of

inherited sex chromosomes. Mammals, some lizards, and some turtles exhibit GSD with

heterogametic males (XY) and homogametic females (XX). Birds, snakes, some lizards,

and some turtles exhibit GSD with homogametic females (ZZ) and heterogametic males

(ZW).

Environmental Sex Determination

Environmental sex determination (ESD) is an alternative to GSD. Species with

ESD determine their sex after fertilization depending on various environmental factors,

such as pH, photoperiod, social environment, nutritional status, and temperature (Bull,

1983; Janzen and Paukstis, 1991). Temperature is the only known environmental factor

to determine sex in amniotic vertebrates, known as temperature-dependent sex

determination (TSD). TSD was originally discovered in the African lizard, *Agama*

agama (Charnier, 1966). It has since been shown that temperature determines sex in

some lizards, numerous turtles, and all crocodilians studied to date (Viets et al., 1993; Lang and Andrews, 1994; Deeming 2004; Ewert et al., 2004; Harlow, 2004).

Species with TSD determine sex during a critical period of development when the embryo is sensitive to the incubation temperature, known as the temperature sensitive period (TSP). The TSP varies with species, but generally occurs around the middle third of embryonic development (Janzen and Paukstis, 1991; Wibbels et al., 1991a; Lang and Andrews, 1994; Ewert et al., 2004). Three thermal patterns of TSD are commonly observed (Bull, 1983; Ewert et al., 2004, Valenzuela, 2004). Type Ia produces males at low temperatures and females at warm temperatures. This pattern of TSD is primarily observed in turtles, including snapping, green, leatherback, and painted (Standora and Spotila, 1985). Type Ib produces males at high temperatures and females at low temperatures. This pattern of TSD occurs in some lizards, some turtles, and crocodilians (Valenzuela, 2004). Type II produces males at intermediate temperatures and females at both cool and warm extremes around the intermediate temperature. Some turtles and lizards, such as the leopard gecko (*Eublepharis macularius*) exhibit this latter pattern of TSD (Janes and Wayne, 2006; Rhen et al., 2011).

<div align="center">Differentiation of the Gonads</div>

Development of the gonads is unique because they are derived from a bipotential tissue, meaning the tissue can differentiate into either testes or ovaries. The bipotential tissue, known as the genital ridge or bipotential gonad, initially develops from the intermediate mesoderm and then as an outgrowth of cells from the mesonephric kidney (Wilhelm et al., 2007). The bipotential gonad is histologically indistinguishable between the sexes, consisting of an outer cortex and inner medulla composed of undifferentiated

<div align="center">3</div>

mesenchymal cells and an overlying coelomic epithelium. Primordial germ cells separate

into two populations and migrate from the yolk sac to the right or left genital ridge.

Germ cells in mammals migrate through the hindgut and up the dorsal mesentery to the

coelomic epithelium, but the germ cells in reptiles and birds migrate through the blood

stream to the coelomic epithelium (Kuwana, 1993; Starz-Gaiano and Lehmann, 2001).

The genital ridge grows as a bipotential tissue until the appropriate signals for

differentiation of testes or ovaries occurs. Regardless of the mode of sex determination,

the general morphological and cellular development of the gonads appears similar across

vertebrate groups, but the timing of these events during development can vary between

species (Morrish and Sinclair, 2002; Carmona et al., 2009). A summary of the

histological development of the testes and ovaries is provided in figure 1.2.

Morphological and Cellular Development of the Testes

Testis organogenesis has been reviewed repeatedly in the last decade (Smith and

Sinclair, 2001; Morrish and Sinclair, 2002; Tilmann and Capel, 2002; Barsoum and Yao,

2006; Wilhelm et al., 2007; DeFalco and Capel, 2009), but a brief overview is provided.

The testes develop from the inner medulla of the genital ridge, while the outer cortex

regresses (Raynaud and Pieau, 1985; Wibbels et al., 1991). The inner medulla grows due

to differentiation of somatic cells into Sertoli, peritubular myoid, or Leydig cells. Germ

cells eventually differentiate to form mature sperm. Each of these cell types plays an

important role in testes development.

Pre-Sertoli cells are derived from cells that originate in the coelomic epithelium

and migrate to the medulla after commitment to testes development (Karl and Capel,

1998). The pre-Sertoli cells will differentiate further to form mature Sertoli cells. The

presence of Sertoli cells is the first indicator of testes development. Sertoli cells enclose the germ cells and assist with the formation of testes cords. Sertoli cells function to support germ cells, regulate spermatogenesis, and secrete certain hormones.

Other somatic cells will migrate from the mesonephros to the gonad and differentiate to form modified myofibroblastic cells called peritubular myoid cells (Capel et al., 1999). These cells surround Sertoli and germ cells to finalize the formation of the testes cords, or seminiferous tubules. These cells have contractile properties needed for moving sperm and other fluids into the lumen of the seminiferous tubules in the adult testes.

Leydig cells are the final somatic cell type that differentiates in the testes. These cells develop in the interstitium between the seminiferous tubules (Doddamani, 2006). Leydig cells are steroidogenic cells that secrete male hormones, or androgens, necessary for developing male secondary sex characteristics.

Germ cells proliferate early in testis development, then leave the cell cycle, and arrest as spermatogonia. Spermatogonia remain arrested until sexual maturity when signals from the brain and testes allow for spermatogenesis to occur (Pudney, 1995).

After these cell types have been established, endocrine signals will influence sex differentiation of other tissues including the brain and reproductive tracts. Sertoli and Leydig cells secrete hormones necessary for the development of the male reproductive tract and will be discussed later. The final developmental feature of testes development is the formation of testis-specific vasculature (Morrish and Sinclair, 2002).

Morphological and Cellular Development of the Ovaries

Numerous reviews have been published describing ovary organogenesis (Ditewig and Yao, 2005; Yao, 2005; DeFalco and Capel, 2009; Edson et al., 2009; Jiménez, 2009), and a brief overview will be provided. Ovaries develop from the outer cortex of the genital ridge and the inner medulla regresses (Morrish and Sinclair, 2002). The somatic and germ cells proliferate which leads to thickening of the cortex. The development of germ cells influences the differentiation of two somatic cells: granulosa and theca cells.

Germ cells continue to proliferate in the cortex and form a nest of germ cells called oogonia (Raynaud and Pieau, 1985). Formation of oogonia usually occurs before birth/hatching. Oogonia proliferate by meiosis and form primary oocytes. Stromal cells will differentiate and form a single layer of cells around the oocytes to form primordial follicles. These cells are called pre-granulosa or follicular cells. Primordial follicles arrest at the diplotene stage of meiotic prophase I and remain arrested until sexual maturity (McLaren and Southee, 1997). At sexual maturity, a subset of follicles is recruited to continue developing. The pre-granulosa cells undergo mitosis and form larger cells, now called granulosa cells (Ditewig and Yao, 2005). Granulosa cells are important for normal development of the ovum and converting androgens into estradiol. Stromal cells differentiate to form cells that surround the granulosa cells and follicles called theca cells. Two layers of thecal cells will develop, an internal and an external layer. These layers have distinct functions involved in hormone production and support of the follicle. Follicles continue to develop until they mature and a single ovum is expelled from the ovary at ovulation. Follicles that do not mature or expel an ovum will degenerate and be re-absorbed in a process called atresia (Wilhelm et al., 2007).

Molecular Mechanisms of Sex Determination

What allows for commitment to testes or ovaries? It is the molecular signaling that occurs in the genital ridge to both turn on testes-specific genes and suppress ovarian genes or vice versa. Given that the modes of sex determination exhibited by amniotic vertebrates vary, it seems likely the molecular mechanisms underlying sex determination also vary. For example, a single gene called the <u>s</u>ex-determining <u>r</u>egion on the <u>Y</u> chromosome (*Sry*) determines the sex of the embryo in eutherian mammals with GSD (Sinclair et al., 1990; Koopman et al., 1990; Goodfellow and Lovell-Badge, 1993). However, Sry or an equivalent gene has not been identified in any other GSD species, nor has the gene(s) that transduces temperature into a signal for testis or ovary development been identified in any TSD species.

Conservation in morphogenesis of the gonads would also suggest the presence of evolutionarily conserved genes in sex determination. Studies examining the molecular mechanisms of sex determination in non-mammalian species have focused on cloning orthologs of sex determining genes first identified in mammals and examining their expression in developing gonads. To date, approximately 18 genes are known to be conserved in either incipient testes or ovaries within amniotic vertebrates, but the levels and timing of expression of these genes varies between species (Pieau et al., 2001; Rhen and Schroeder 2010). A summary of the molecular mechanisms underlying sex determination is provided in figure 1.2.

Conserved Testis-specific Genes

Of the approximately 18 conserved sex determining genes, *Sox9*, anti-müllerian hormone (*Amh*), and doublesex and mab-related transcription factor-1 (*Dmrt1*) have been

studied extensively and involved in testes development in almost all amniotic vertebrates. Members of the platelet-derived growth factor (*Pdgf*) signaling pathway also appear to be conserved in testes development, but these have only been examined in a few mammals, chicken, and one species of turtle (Basciani et al., 2010). Other testis-specific genes in mammals include: *Fgf9, Sox8, SF-1*, and *WT1*, but these genes have not been well studied within amniotic vertebrates and many of these genes have not shown higher expression in incipient testes relative to ovaries (Rhen and Schroeder, 2010).

Sox9 is a transcription factor and the first marker for Sertoli cell differentiation in the testes (Wilhelm et al., 2007). *Sox9* is a direct target of *Sry* in mammals and is expressed early in the testis-specific gene cascade (Morais da Silva et al., 1996; De Santa Barbara et al., 2000; Sekido and Lovell-Badge, 2008). *Sox9* functions in the developing testes to regulate expression of other testis-specific genes, including *Amh*, and may also repress ovary-specific genes (de Santa Barbara et al., 1998). Expression of *Sox9* is greater in incipient testes and gonads at a male-producing temperature in chicken and most reptiles with GSD or TSD (Oreal, 1998; Smith et al., 1999; Western et al., 1999; Torres Maldonado et al., 2003; Shoemaker et al., 2007a; Rhen et al., 2007; Valenzuela, 2010). However, unlike mammals, *Sox9* expression occurs after induction of *Amh* in chickens and many reptiles, suggesting that *Amh* is not regulated by *Sox9* or an equivalent *Sox* gene in non-mammalian species (Oreal et al., 1998; Western et al., 1999; Takada et al., 2005; Rhen, unpublished data).

Amh is a protein hormone that is secreted by Sertoli cells and important for regression of the female reproductive tracts in males (Vigier et al., 1987; Behringer et al., 1990). *Amh* is expressed in the developing Sertoli and granulosa cells in embryonic and

adult mammalian testes and ovaries, respectively. Expression of *Amh* is higher in the

developing testes than ovaries in chickens and reptiles (Oreal et al., 1998; Smith et al.,

1999; Western et al., 1999; Takada et al., 2004; Shoemaker et al., 2007a; Rhen,

unpublished results).

Dmrt1 is a transcription factor and the only gene that plays a conserved role in sex

determination from invertebrates to vertebrates (Raymond et al., 1999; Zarkower, 2001;

Volff et al., 2003). *Dmrt1* is expressed in germ cells and differentiating Sertoli cells

throughout testicular development. *Dmrt1* is up-regulated in the developing mammalian

testes after the appearance of sexual dimorphism between the gonads (De Grandi et al.,

2000; Raymond et al., 1999; 2000). Expression of *Dmrt1* is higher in bipotential gonads

incubated at male-producing temperatures than female-producing temperatures and in

incipient testes in reptiles with TSD and GSD, respectively (Smith et al., 1999; Kettlewell

et al., 2000; Torres Malonado et al., 2003; Murdock and Wibbels, 2003a; Shoemaker et

al., 2007a; Rhen et al., 2007 Valenzuela, 2010). *Dmrt1* is considered the candidate sex

determining gene in chickens because it is required for testis development and linked to

the Z chromosome (Smith et al., 2009).

Conserved Ovary-specific Genes

Fewer genes have been identified to be involved in early ovary development

compared to early testis development in mammals. Furthermore, there are limited studies

in non-mammalian species cloning mammalian orthologs of ovary-specific genes. For

example, *Wnt4, R-spondin1* (*Rspo1*), and *FoxL2* are involved in ovarian development in

mammals, however, these genes have only been studied in chicken and a subset of reptile

species (Rhen and Schroeder, 2010).

FoxL2 has been investigated the most of these three candidate genes. *FoxL2* is a transcription factor necessary for granulosa cell differentiation in the ovaries (Ottolenghi et al., 2005; Duffin, 2009; Uhlenhaut et al., 2009). *FoxL2* is expressed in a female-specific pattern in the gonads of mammals and chickens (Cocquet; 2002; Govoroun et al., 2004; Hudson et al., 2005; Uhlenhaut and Treier , 2006). Expression of *FoxL2* is higher in bipotential gonads incubated at female-producing temperatures than male-producing temperature and incipient ovaries in reptiles with TSD.

There is a likely explanation for the lack of studies attempting to clone ovary-specific genes in other species. Sex steroid hormones, particularly estrogens, play a conserved role in sex determination in non-placental mammals and other non-mammalian species (Crews et al., 1991; Crews, 1996; Pieau and Dorizza, 2004; Lance 2009; Ramsey and Crews, 2009; Pask, 2012). Sex steroid hormones are not critical for sex determination in mammals, but are important for maintenance of the ovary after sex determination.

Exogenous estrogen treatments induce ovary development in genetically male chickens and reptilian embryos incubated at male-producing temperatures (Gutzke and Bull, 1986; Bull et al., 1988; Dorizzi et al., 1991; Crews et al., 1991; Wibbels et al., 1991; Wibbels et al., 1993; Rhen and Lang, 1994; Nakabayashi et al., 1998; Akazome and Mori, 1999). Furthermore, expression of aromatase, the gene that converts testosterone into estrogen, is higher in the developing chicken ovary and incipient ovaries in most reptiles with TSD and GSD (Desvages et al., 1993; Jeyasuriya et al., 1994; Smith et al., 1997; Nakabayashi et al., 1998; Gabriel et al., 2001; Place et al., 2001; Murdock and

Wibbels, 2003b; Endo and Park, 2005; Rhen et al., 2007; Ramsey et al., 2007; Valenzuela and Shikano, 2007).

Examination of the two estrogen receptor mRNAs showed the receptors are expressed in both incipient testes and ovaries and at both male- and female-producing temperatures in chickens and reptiles with TSD, respectively (Smith et al., 1997; Bergeron et al., 1998; Nakabayashi et al., 1998; Ramsey and Crews, 2007; Rhen et al., 2007). These observations support the hypothesis that estrogens are important for sex determination and males are responsive to estrogens early in sex determination.

There is also a growing hypothesis that androgens are important for sex determination in non-mammalian species and maintenance of the ovary in mammals (Ramsey and Crews; 2009). Mutations in the androgen receptor (*Ar*) and low levels of circulating androgens lead to the disease phenotype of premature ovarian syndrome (Kimura et al., 2007). Furthermore, exposure to high levels of androgens leads to polycystic ovarian syndrome (Walters et al., 2008). Expression of *Ar* is higher in the incipient ovary and in gonads incubated at female-producing temperatures in chickens and reptiles with TSD, respectively (Katoh et al., 2006; Ramsey and Crews, 2007; Rhen et al., 2007). Indeed, treatments with dihydrotestosterone, a nonaromatizable androgen can feminize snapping turtle embryos incubated at or near a temperature that produces a 1:1 sex ratio (Rhen and Lang, 1994; Rhen and Schroeder; 2010). These data also support the role of androgens in ovarian development.

Sex Steroid Hormones in the Gonads:

Synthesis, Regulation, and Mechanism of Action

Sex steroid hormones are critical for sex determination or maintenance of the gonads in vertebrates. The synthesis of sex steroid hormones, estradiol or testosterone, by the gonads is also critical for growth and differentiation of other tissues, including the brain and reproductive tracts (Norris, 2007). Given that hormones are essential for development, it is important to understand the biosynthesis, regulation, and mechanism of action of sex steroid hormones.

The biosynthesis of active steroid hormones is under tight regulation by follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) from the pituitary gland and gonadotropin-releasing hormone (GnRH) from the hypothalamus (Stocco, 2001). Through interactions with membrane-bound receptors on the surface of steroidogenic cells for LH and FSH, these hormones play a key role in regulating steroidogenic gene expression and steroid-dependent feedback loops. The detailed description of regulation of steroidogenesis by hormones from the brain will not be discussed here, but a brief overview of the genes necessary for the conversion of cholesterol to steroids and mechanism of action of steroids will be provided.

They synthesis of steroids is facilitated by steroidogenic acute regulatory protein (StAR). StAR controls the rate-limiting step of steroidogenesis by supplying the substrate cholesterol to the inner mitochondrial membrane (Stocco, 2001). After the delivery of cholesterol to the mitochondria, steroids are synthesized through a series of reactions that are catalyzed by cytochrome P450s including CYP11A (P450scc; cholesterol side-chain cleavage), CYP17 (cytochrome P450 c17α-hydroxylase, 17, 20-

lyase), and CYP19A (cytochrome P450 aromatase); and hydroxysteroid dehydrogenases (HSDs), including 3β and 17β-HSD (Miller, 1988; Figure 1.3). 5α-reductase, although not critical for formation of testosterone or estradiol, is an enzyme that converts testosterone into dihydrotesterone (DHT) (Andersson et al. 1989). DHT is an important sex steroid hormone because it is a more potent androgen than testosterone and cannot be aromatized to estrogens.

Estrogens and androgens elicit their responses by binding to their specific receptors: estrogen receptor (ER) or androgen receptor (AR), respectively. Both receptors are members of the steroid hormone superfamily of nuclear receptors and function as transcription factors to regulate target genes (Ribeiro et al., 1995). Structurally, members of this family of nuclear receptors are very similar. They are composed of three interacting functional domains: an amino-terminal domain, DNA-binding domain, and the ligand-binding domain. The amino-terminal domain encodes a ligand-independent activation function necessary for protein-protein interactions and transcriptional activation. The DNA-binding domain contains a zinc finger structure that is necessary for the binding of the receptors to specific DNA sequences. Finally, the ligand-binding domain is necessary for the binding of the specific hormone and mediates nuclear translocation and transactivation of target gene expression.

ER has two subtypes, ERα and ERβ, and both bind estrogens with high specificity. AR is capable of binding testosterone or DHT, but binds DHT more readily. Both AR and ER function in a similar manner. Binding of estrogens to ER or androgens to AR triggers a conformational change that activates the receptor. The receptor will form a dimer, translocate to the nucleus, bind to estrogen or androgen response elements

on target genes, and influence gene transcription (Jänne et al., 1993; Hiipakka and Liao, 1998; Nilsson et al., 2001). It is noteworthy that ER and AR can influence transcription of genes without binding to DNA, so called non-genomic actions, but these will not be discussed here (Revelli et al., 1998).

Morphological and Cellular Development of the Reproductive Tracts

The original formation of the male and female reproductive tracts occurs during embryonic development and is independent of sex. After sex determination, either the male or female reproductive tracts will continue to develop and differentiate, while the other reproductive tracts regress. An overview of the morphological and cellular development of the reproductive tracts is described below.   The histological development of the male and female reproductive tracts is presented in Figure 1.4.

Male Reproductive Tract Development and Differentiation

There have been many reviews describing the development of the male reproductive tract (Nef and Parada, 2000; Barsoum and Yao, 2005; Hannema and Hughes; 2007; Wilhelm et al., 2007; Joseph et al., 2009). The anlagen of the male reproductive tracts are derived from the intermediate mesoderm of the urogenital ridge. These structures are called the Wolffian or mesonepric ducts. The Wolffian ducts develop as short segments within the rudimentary embryonic kidney, the pronephros (Staack et al., 2003). The pronephros will regress during development, but the ducts remain. The second embryonic kidney, the mesonephros, will develop, and the ducts develop from the mesonephric tubules within the mesonephros. Here the ducts will grow caudally until they reach the cloaca (Barosum and Yao, 2005). Wolffian ducts will not grow further unless differentiation of the testes has occurred.

Wolffian ducts are stabilized by hormones secreted by cells in the testes. Leydig cells produce androgens or testosterone, which prevents the Wolffian ducts from regressing and causes cells of the ducts to proliferate and the ducts to begin coiling (Dyche, 1979). Sertoli cells secrete AMH, which binds the AMH receptor on the female reproductive tracts and causes the ducts to regress (Kuroda et al., 1990; Munsterberg and Lovell-Badge, 1991). Androgens are necessary for stabilization of the Wolffian ducts, however, the molecular mechanisms underlying androgen signaling in the Wolffian ducts are not well known (Hannema and Hughes, 2007).

After stabilization, Wolffian ducts differentiate into the morphologically and functionally distinct structures of the male reproductive tract: the efferent ductules, epididymis, vas deferens, and the seminal vesicles (mammals only) (Dyche, 1979). The efferent ductules connect the testes to the epididymis so that sperm can be moved from the gonad to the reproductive tract. The epididymis is a highly coiled structure that allows for the maturation and storage of sperm. The vas deferens is a tube that moves sperm from the epididymis to the ejaculatory duct, where sperm can be released from the body.

Female Reproductive Tract Development and Differentiation

A number of reviews have been published about Müllerian duct development (Nef, 2000; Kobayashi and Behringer, 2003; Klattig and Englert, 200**7**; Wilhelm et al, 2007). The anlagen of the female reproductive tracts are called the Müllerian or paramesonephric ducts. Müllerian ducts develop parallel to the Wolffian ducts in the embryonic kidney and develop in an anterior to posterior and craniocaudal manner.

The Müllerian ducts only persist if the gonads differentiate into ovaries. Ovaries do not produce sufficient anti-müllerian hormone to signal for regression of the Müllerian ducts. Wolffian ducts regress in the females due to insufficient testosterone production from the ovaries to stabilize the Wolffian ducts. The Müllerian ducts will differentiate to form the fallopian tubes, uterus and upper vagina (Dyche, 1979).

Study Objectives

Scientific investigation has focused on understanding many aspects of TSD including the evolution, adaptive significance, physiology, genetic, and molecular mechanisms underlying TSD. Although all of these aspects are important, the overall goal of this dissertation is to understand the genetic, molecular, and physiological mechanisms underlying TSD. To achieve this goal, this study has two main objectives: 1) Identify the unique, thermo-sensitive genes involved in TSD and 2) Determine the role of androgens in sex determination and differentiation in a TSD species. The first objective will provide information about what gene(s) are involved in TSD and how temperature transduces a signal for ovary or testis development. The second objective will provide information about how androgens signal to influence development of the reproductive tracts and ovary.

The snapping turtle (*Chelydra serpentina*) will be used as the model organism to achieve the study objectives. The snapping turtle exhibits TSD and has several characteristics making it a useful model for studying TSD and sex differentiation. Snapping turtles have relatively large clutch sizes (averaging ~40 eggs/clutch) and a relatively short temperature-sensitive period, lasting only 3-5 days of embryonic development (Rhen et al., 2007; 2009). Furthermore, studies have shown there is a

latitudinal cline in TSD pattern for snapping turtle populations, indicating snapping

turtles have adapted to their local thermal regimes (Ewert et al., 2005). This suggests

there is an underlying genetic basis for TSD. Lastly, snapping turtles can be easily

treated with exogenous hormones and hormone antagonists by spotting the chemical on

the eggshell, allowing the chemical to diffuse through the egg and the circulation to carry

the chemical to the embryo.

Overall, the findings from this dissertation will provide a greater understanding of

the molecular mechanisms that are critical for sexual development and fertility in

amniotic vertebrates.

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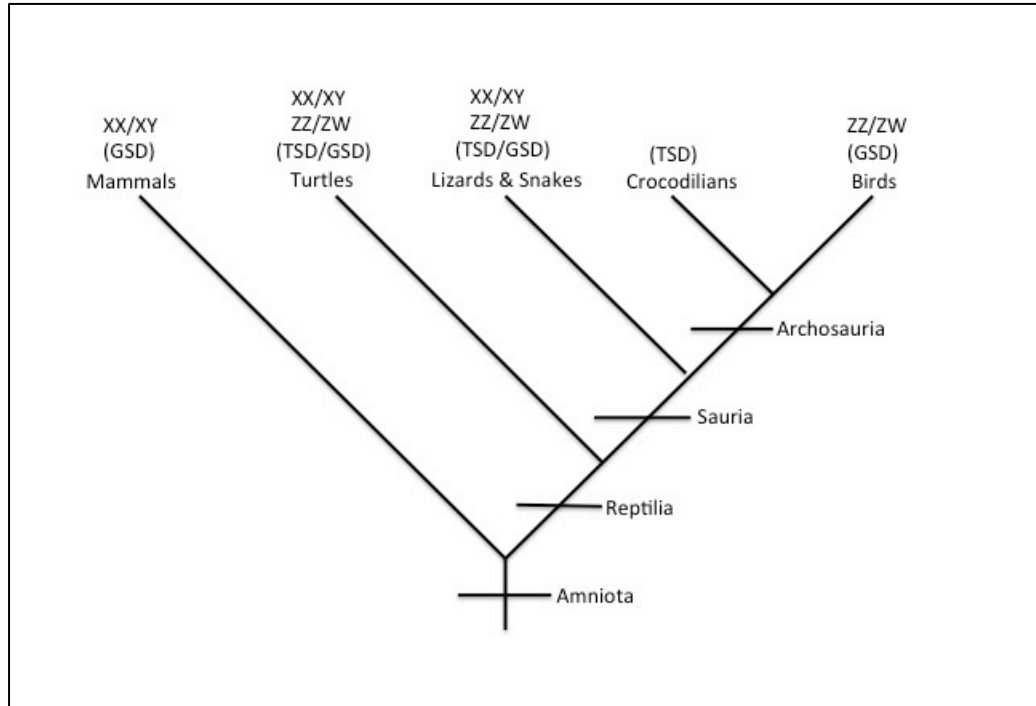
Figure 1.1. Cladogram representing the evolution of mechanisms of sex determination within amniotic vertebrates (adapted from Eernisse and Kluge, 1993). TSD = Temperature-dependent sex determination. GSD = Genotypic sex determination.
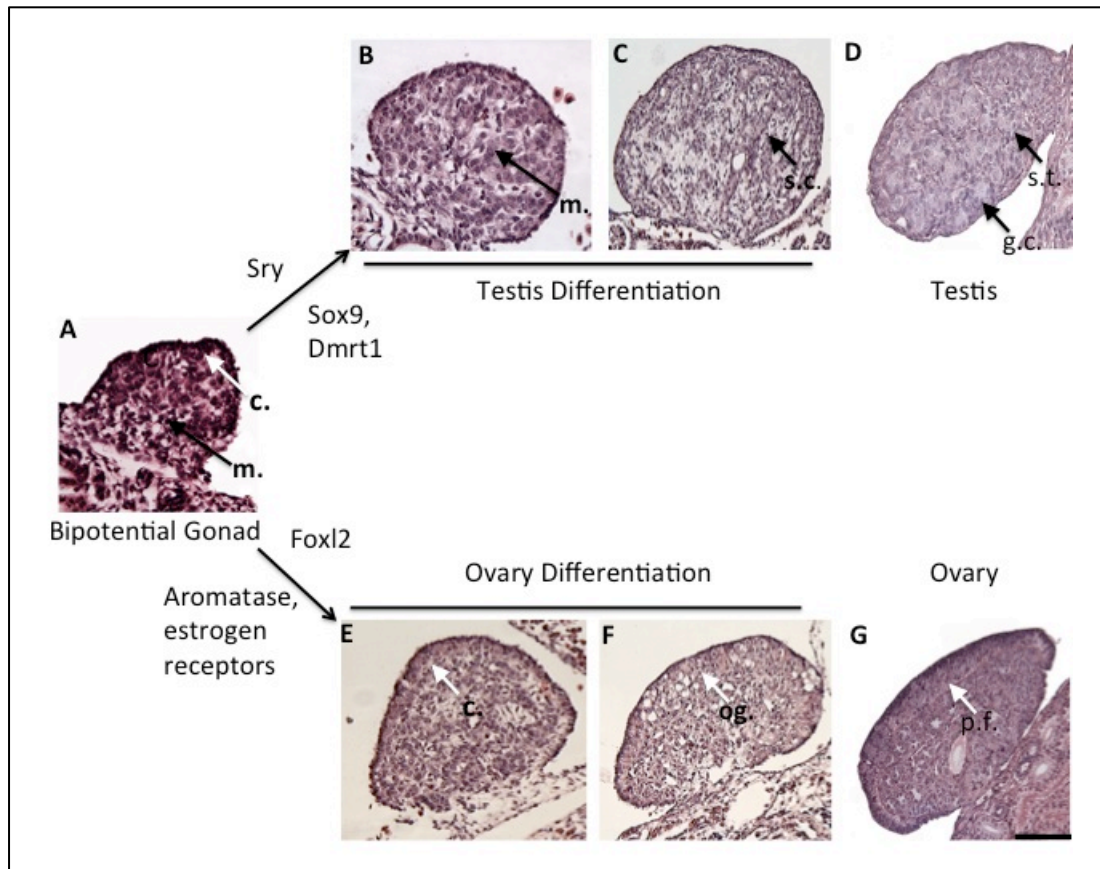
Figure 1.2. Overview of the morphological and molecular mechanisms of testis (B-D) and ovary differentiation (E-G) from a bipotential primordia (A). The black arrows in each panel indicate the medulla of the gonad and the white arrows indicate the cortex in the gonad. c. = cortex. m. = medulla. s.c. = sex cords. s.t. = seminiferous tubulues. g.c. = germ cells. og. = oogonia. p.f. = primordial follicles. Scale bar = 50 μm.
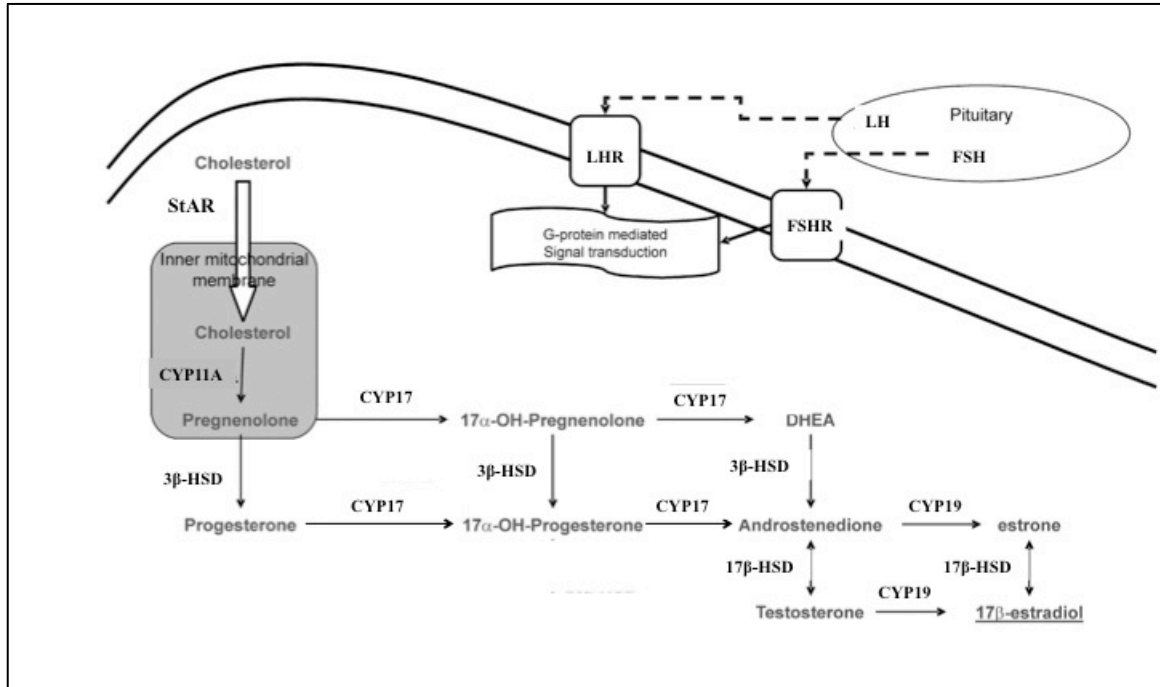
Figure 1.3. Generalized overview of gonadal steroidogenesis and the roles of various genes involved in regulating steroid biosynthesis (adapted from Villeneuve et al., 2007). StAR = steroidogenic acute regulatory protein; CYP11A = cytochrome P450 cholesterol side-chain cleavage; CYP17 = cytochrome P450 17α-hydroxylase, 17,20-lyase; CYP19 = aromatase; 3β-HSD = 3β-hydroxysteroid dehydrogenase; 17β-Hsd = 17β-hydroxysteroid dehydrogenase; LH = luteinizing hormone; FSH = follicle-stimulating hormone; LHR = luteinizing hormone receptor; FSHR = follicle-stimulating hormone receptor.
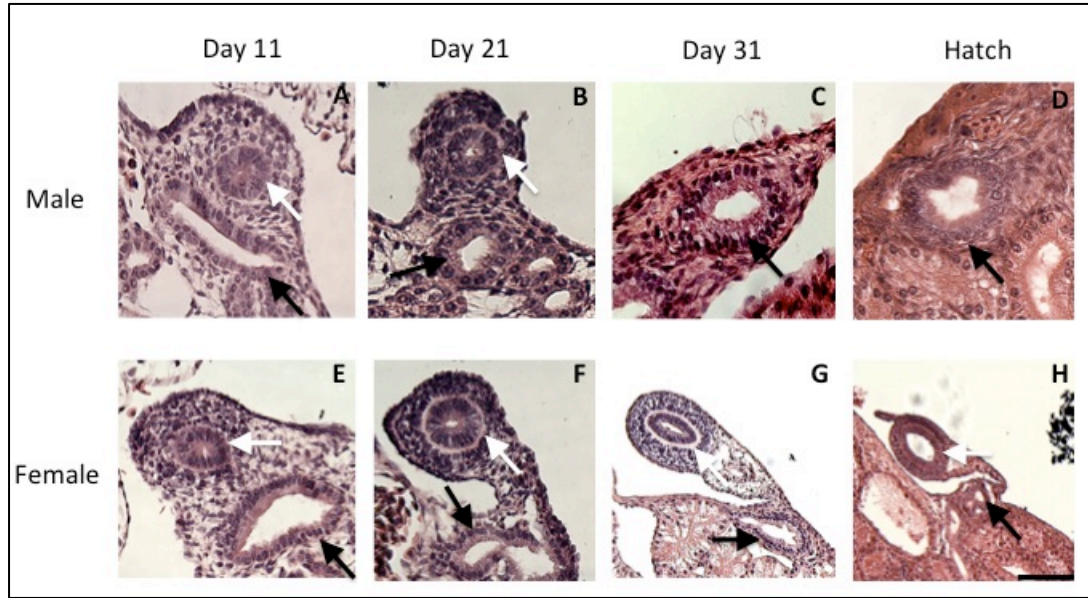
Figure 1.4. Histological development of the Wolffian ducts (A-D) in males and Müllerian ducts (E-H) in females days after sex determination in the common snapping turtle. White arrows indicate the Müllerian ducts in each panel. Black arrows indicated the Wolffian ducts in each panel. Notice the regression of the Müllerian ducts in the males and growth of the Müllerian ducts in the females. Scale bar = 50 μm.

CHAPTER II

IDENTIFICATION OF A CANDIDATE GENE INVOLVED IN TEMPERATURE-
DEPENDENT SEX DETERMINATION IN THE COMMON SNAPPING TURTLE

Abstract

Temperature-dependent sex determination (TSD) was first discovered in a lizard
species more than 40 years ago. Since its discovery, the molecular mechanisms
underlying this process have been studied extensively. Even so, the initial gene(s) that
transduces temperature into a signal for testes versus ovarian development is not known
in any species with TSD. To identify genes involved in TSD, I used differential display
PCR to clone and identify differentially expressed genes between bipotential gonads from
embryos incubated at a male- or female-producing temperature. I identified the cold-
inducible RNA binding protein (Cirbp) as a strong candidate gene in the common
snapping turtle, *Cheldyra serpentina*, a species with TSD. *Cirbp* mRNA expression was
induced at a high female-producing temperature early in the TSP, but not at a low female-
producing temperature. I examined the spatial distribution of *Cirbp* mRNA in hatchling
gonads as well as the spatial distribution of Cirbp protein in bipotential and hatchling
gonads. Cirbp protein expression localized to the cytoplasm during the thermo-sensitive
period, but translocated to the nucleus after the thermo-sensitive period. I also
demonstrate a significant genetic correlation between *Cirbp* mRNA expression in the
bipotential gonads and sex ratios produced within clutches. These findings suggest

Cirbp is involved in commitment of the bipotential gonads to an ovarian fate and appears to be playing different roles in gonad development during and after the thermo-sensitive period in the common snapping turtle.

Introduction

Sex determination is one of the most important developmental processes because the decision to develop as male or female impacts every aspect of an organism's biology. This decision influences the genetics, physiology, morphology, and social interactions of the organism, which translates into the sex differences that we observe in nature. Natural and sexual selection favors the evolution of sex differences and has led to the evolution of a variety of modes of sex determination in vertebrates. In amniotic vertebrates, sex is determined by an individual's genotype, referred to as genotypic sex determination (GSD), or by various environmental variables, referred to as environmental sex determination (ESD). A variety of environmental variables have been found to influence sex determination in animals, but temperature is the only known environmental variable to influence sex determination in amniotic vertebrates. Temperature-dependent sex determination (TSD) occurs when incubation temperature during early development determines the sex of the embryo (Janzen and Paukstis, 1991). Among amniotic vertebrates, TSD is only observed in reptiles, being exhibited by numerous lizards, turtles, and all crocodilians studied to date (Viets et al., 1993; Lang and Andrews, 1994; Deeming 2004; Ewert et al., 2004; Harlow, 2004).

Although sex-determining mechanisms are not conserved among amniotic vertebrates, morphological differentiation of the gonads does appear to be conserved (Wibbels et al., 1991; Yao and Capel, 2005). The gonads are unique in that they develop

from a bipotential tissue that can differentiate into either testes or ovaries. The bipotential tissue, known as the genital ridge, originally develops from the intermediate mesoderm and then as a thickening of the coelomic epithelium that overlies the mesonephric kidney. The genital ridge will continue to grow to form the bipotential gonad that consists of an outer cortex and inner medulla. Testes develop when the inner medulla grows, seminiferous tubules differentiate and the outer cortex regresses. Somatic cells of the bipotential gonad will differentiate into Sertoli, peritubular myoid, or Leydig cells in the testes and germ cells will eventually differentiate into sperm. In contrast, ovaries develop when the outer cortex grows and the inner medulla regresses. In ovaries a reorganization of germ cells and somatic cells occurs to form primordial follicles. The somatic cells in the bipotential gonad will differentiate into granulosa and thecal cells and the germ cells will differentiate into oocytes.

The key event during sex determination is irreversible commitment of the bipotential gonads to testicular or ovarian development. In species with TSD, sex is determined during the middle of embryogenesis when the gonads are sensitive to temperature, which is referred to as the temperature sensitive period (TSP; Crews, 1996; Pieu and Dorizzi, 2004; Place and Lance, 2004). During the TSP, temperature induces expression of genes involved in testis or ovary commitment. To date, most studies in TSD species have focused on cloning orthologous genes known to be important in sex determination in mammals. Many of these orthologs are temperature sensitive and appear to play a conserved role in gonad development. For example, expression of *Sox9*, anti-müllerian hormone (*Amh*), *Pdgf-ß* and *Dmrt1* is temperature sensitive with higher levels in incipient testes in all TSD species studied (reviewed in Shoemaker and Crews,

2009; Rhen and Schroeder, 2010). In contrast, aromatase, *Foxl2* and estrogen receptors have been identified as temperature sensitive genes that are expressed at higher levels in incipient ovaries (reviewed in Shoemaker and Crews, 2009; Rhen and Schroeder 2010). While the identification and characterization of orthologous genes is important for defining a conserved sex-determining pathway among vertebrates, it will not lead to the identification of novel genes involved in TSD. To date, the initial thermo-sensitive gene(s) that transduces temperature into a biological signal has not been identified in any TSD species.

One reptile that exhibits TSD is the common snapping turtle, *Cheldrya serpentina*. This species produces females at low and high temperatures, while males are produced at intermediate temperatures (Yntema, 1976; Rhen and Lang, 1994; Ewert et al., 2005). Eggs incubated between 23°C and 26.5^{o}C throughout development produce all males in the population we study. In contrast, eggs incubated at 31°C produce exclusively females. Mixed sex ratios are produced at temperatures below 23°C and between 27.0^{o}C and 29.5^{o}C, with 28.2^{o}C producing a 50:50 sex ratio. The snapping turtle is a unique TSD species, because its TSP comprises a narrow window of embryogenesis (~7%; Yntema, 1979; Rhen et al., 2012), while in other TSD species the TSP comprises a significantly longer period of development (Mrosovsky and Pieu, 1991; Lang and Andrews, 1994; Crews, 1996). We have previously reported that shifting eggs from a male- to a female-producing temperature for six days is sufficient to induce commitment of the bipotential gonad to ovarian fate (Rhen et al., 2007; 2009). This short TSP makes the snapping turtle a unique model to identify the thermo-sensitive genes during the sex-determining period.

The goal of this study was to identify unique thermo-sensitive genes involved in TSD in the snapping turtle. I used differential display PCR (DD-PCR) to clone a novel gene that plays a role in TSD. DD-PCR has been used to discover novel genes in a variety of organisms and to study numerous developmental processes, including sex determination (Nordqvist and Töhönen, 1997; Dcotta et al., 2001). Here, I identify the cold-inducible RNA binding protein (*Cirbp*) as a strong TSD candidate in the snapping turtle. *Cirbp* was induced at a high female-producing temperature early in the TSP, but not at a low female-producing temperature. I examined the spatial distribution of *Cirbp* mRNA in hatchling gonads as well as the spatial distribution of *Cirbp* protein in bipotential and hatchling gonads. I also demonstrated a significant genetic correlation between *Cirbp* mRNA expression in the bipotential gonads and sex ratios produced within clutches. I hypothesized that *Cirbp* plays a role in TSD in the snapping turtle and more specifically that *Cirbp* is involved in commitment of the bipotential gonads to an ovarian fate.

Material and Methods

Embryos and Incubation for Differential Display PCR

Animal experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol #0905-1). I collected snapping turtle eggs within 24 hours of laying from snapping turtle nests along the Clearwater and Mississippi Rivers in north-central Minnesota. Eggs were covered with soil and transported to the animal quarters in the Biology Department at the University of North Dakota. Eggs were washed in tepid water, candled for embryo viability and infertile eggs were removed. Equal or

approximately equal numbers of viable eggs from each clutch were assigned to one of two thermal treatments. To produce males, one group of eggs was incubated at 26.5°C. To produce females, the other group of eggs was incubated at 26.5°C until embryos reached stage 16, at which point eggs were shifted to 31°C for 6 days and then returned to 26.5°C. Eggs were placed in containers filled with moist vermiculite and then randomly positioned within foam box incubators as described by Rhen and Lang (1994).

I harvested gonads from embryos at 26.5°C and from clutch mates that had been shifted to 31°C. Embryos were sampled on days 2, 3, 4, and 5 after the start of the temperature shift. Eggs were opened and embryos euthanized as described in Rhen et al. (2007). The adrenal-kidney-gonad (AKG) complex was removed, placed in RNA*later* solution (Ambion, Austin, TX), and stored at -20 $^{\circ}$C. Gonads were dissected from the AKG complex. Total RNA was isolated from gonads and used for cDNA synthesis. This cDNA was used as template for differential display (DD)-PCR. A subset of eggs from each thermal treatment was allowed to hatch to confirm that the 26.5 $^{\circ}$C treatment produced only males and the 31°C treatment produced exclusively females, as well as to compare gene expression between embryos and hatchlings.

mRNA Differential Display

I extracted total RNA from the pair of gonads isolated from individual embryos or hatchlings from each treatment group using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA). I adapted the manufacturer's protocol for use with tissue as described in Rhen et al. (2007). Total RNA was eluted in 11 µl of elution buffer and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Total RNA (100 ng) from each pair of gonads was reverse transcribed using the 3'

anchoring primer, H-T$_{11}$A, per the manufacturer's protocol (GenHunter, Nashville, TN).

The thermal profile was 65°C for 5 min, 37°C for 60 min, 75°C for 5 min, and

indefinitely at 4 ^{o}C. This cDNA was used as template for PCR to identify differentially

expressed genes. I followed the manufacturer's protocol using H-T$_{11}$A as the reverse

primer and the arbitrary 13-mer primer, H-AP 03, as the forward primer (Table 2.1). I

used a 3-step thermal profile with 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec. This

thermal profile was reapeated for 40 cycles. The final extension was at 72°C for 5 min.

and then at 4°C.

I ran PCR products on a 6% polyacrylamide gel for approximately 2 hours at 60

volts in TBE buffer (Invitrogen, Carlsbad, CA). After electrophoresis, Sybr Gold was

used to stain the gel and resolve the PCR bands, instead of using radiolabeling as

described by the manufacturer. I identified differentially expressed bands by measuring

the optical density of the PCR products using LabWorks software on the AutoChemi gel

visualization system (UVP, LCC, Upland, CA). Differentially expressed PCR bands

were cut out of polyacrylamide gels using a sterile scalpel, and placed in 1.5 ml

microfuge tubes. I extracted DNA from gels and used PCR to reamplify the cDNA

fragment as described by the manufacturer (GenHunter). I analyzed reamplified PCR

products via agarose gel electrophoresis. Single PCR products were excised from the gel

and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Cloning and Sequencing Analysis

The TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) was used to clone the DD-

PCR product into the pCR4-TOPO plasmid according to the manufacturer's protocol.

Plasmids containing an insert were used as template in sequencing reactions with the

BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Sequences from multiple clones were aligned and a consensus sequence was generated

using Sequencher software (Gene Codes Corp, Ann Arbor, MI). The novel snapping

turtle sequence was then used in BLAST search of the non-redundant nucleotide database

to identify orthologous genes in other species.

5' Rapid Amplification of cDNA Ends (RACE)

The full-length sequence for *Cirbp* cDNA was determined by 5' RACE according

to the manufacturer's protocol (5' RACE System for Rapid Amplication of cDNA Ends;

Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 1 μg of total RNA

from gonads. The primary 5'RACE PCR used the abridged anchor primer (AAP) and a

reverse primer for the DD-PCR product described above. The secondary PCR used the

abridged universal anchored primer (AUAP) and a semi-nested reverse primer (Table

2.1). The 5'RACE PCR products were cloned and sequenced as described. If the full-

length sequence was not obtained, new primers were designed and used in another 5'

RACE until the full-length cDNA was obtained. The full-length cDNA sequence was

used in a BLAST search to identify orthologous genes.

Once full-length cDNA was obtained, we used the online ExPASy tool

(http://ca.expasy.org/tools/dna.html) for *in silico* translation of the nucleotide sequence. I

used the predicted snapping turtle *Cirbp* amino acid sequence in a protein BLAST to

retrieve orthologous amino acid sequences. I used BioEdit software to align the snapping

turtle *Cirbp* amino acid sequence with the *Cirbp* sequences from other vertebrates.

Egg Incubation and Collection for Determining mRNA Expression of Cirbp during TSP

A second experiment was set up to confirm that *Cirbp* mRNA was differentially expressed between the 26.5^{o}C and the 26.5^{o}C-31°C-26.5^{o}C treatment groups by using quantitative real-time PCR. Bipotential gonads were isolated from each treatment group (26.5^{o}C or 31°C) on days 2, 3, 4, and 5 after the start of the temperature shift. RNA was extracted as described previously (Rhen et al., 2007). Total RNA was reverse transcribed using the iScript cDNA Synthesis Kit following the manufacturer's recommendations (BiodRad, Hercules, CA). The cDNA was used as template in real-time PCR reactions.

Short-Term Shift Experiment

Previous experiments examined *Cirbp* mRNA expression two or more days after the start of the temperature shift, so I conducted an experiment to determine the time course for *Cirbp* mRNA induction. We collected gonads from embryos incubated at 26.5^{o}C and from clutch mates shifted from 26.5^{o}C to 31°C. Approximately equal numbers of embryos from each temperature treatment were sampled 6, 12, 24, and 48 hours after the start of the temperature shift. Gonads were dissected, RNA was isolated and reverse transcribed, and quantitative PCR performed as described above.

Cold-Shift Experiment

Because females can also be produced at cooler temperatures, I conducted an experiment to determine if *Cirbp* was induced at cool temperatures. Approximately equal numbers of eggs from three different clutches were separated into two temperature treatment groups. Eggs from both treatments were incubated at 26.5^{o}C until stage 14, when half the eggs were shifted to 20°C. After stage 19, a subset of embryos from the 20°C treatment were shifted back to 26.5^{o}C and embryos from both temperatures were allowed to hatch to determine sex ratios from each temperature treatment.

I collected gonads from embryos incubated at 20°C (also referred to as 26-20-26C) and from clutch mates at 26.5°C (also referred to as 26C). Approximately twelve embryos (4 embryos x 3 clutches) were sampled from each temperature at stages 15 through 19 after the temperature shift. Gonads were dissected, RNA was isolated and reverse transcribed, and quantitative real-time PCR performed as described previously.

Quantitative Real-Time PCR

Real-time PCR was performed to measure *Cirbp* mRNA and 18S rRNA expression in gonads from the experiments described above. SYBR Green solution was used for real time reactions with the primers in Table 2.1. Reactions were run on a 7300 Real-Time PCR System (Applied Biosystems). The thermal profile was 95°C for 10 min to activate the DNA polymerase followed by 40 cycles of two-step PCR (94°C for 15 sec and 60°C for 1 min). Rigorous standard curves across eight orders of magnitude were used to quantify gene expression in absolute terms as described in Rhen et al. (2007). Controls lacking reverse transcriptase or RNA template were also prepared to demonstrate there was no contaminating DNA, RNA, or PCR products. A melting temperature analysis was added at the end of the real time PCR to verify that a single product was amplified for each gene analyzed.

In situ hybridization

A 354 base pair fragment of snapping turtle *Cirbp* was amplified using cDNA from embryonic gonads as template. PCR primers for probe generation are shown in Table 2.1. The PCR product was cloned using the TOPO TA Cloning Kit. The orientation of the insert was determined by sequencing. The plasmid containing the *Cirbp* fragment was used as a template to synthesize antisense or sense RNA probes with

digoxigenin-UTP and T3 or T7 RNA polymerase according to the manufacturer's protocol (Dig RNA Labeling Kit; Roche Applied Sciences, Indianapolis, IN).

AKGs isolated from male and female hatchling turtles were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Tissues were washed in PBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. AKGs were sectioned at 6-μm and mounted on Histobond slides (VWR, Radnor, PA). Slides were deparaffinized in xylene, rehydrated in graded ethanol, and washed in PBS.

Sections were washed and prehybridized for 2 h at 55°C in hybridization solution (50% formamide, 5x SSC, 1x Denhardts (2% Polyvinylpyrrolidone, 2% Ficoll 400, 2% BSA), 250 μg/ml yeast tRNA, 500 μg/ml salmon sperm DNA, 50 μg/ml heparin, 2.5 mM EDTA, 0.1% tween-20, 0.25% CHAPS). *Cirbp* antisense or sense riboprobes were dissolved in hybridization solution at a concentration of 100 ng/ml and hybridized to slides overnight.

After hybridization, slides were moved through a series of post-hybridization washes to remove unbound riboprobe. Slides were washed once in 50% formamide with 5x SSC and 0.1% tween-20 (SSCT), twice in 50% formamide with 2x SSCT, and twice in 50% formamide with 0.2x SSCT. Slides were incubated in each wash solution for 30 minutes at 65°C. Slides were washed twice in 1x maleic acid buffer, 0.1% Tween (MABT) for 15 minutes at room temperature. Sections were blocked in 2% blocking solution (Roche Applied Sciences) with 10% sheep serum, incubated with pre-absorbed anti-dioxigenin-AP Fab fragment (diluted 1:2000, Roche Applied Sciences), and stained with BM Purple AP substrate (Roche Applied Sciences). Images were taken using an

Olympus BX-51 microscope equipped with an Infinity 2 digital camera (Lumenera Corp., Ottawa, ON, CA) using Rincon HD software (Imaging Planet, Goleta, CA).

Immunohistochemistry

AKGs were isolated from embryos incubated at male- and female-producing temperatures on days 1 through 6, 11, 21, and 31 after the temperature shift to examine *Cirbp* protein expression during the TSP and after sex determination. Hatchling gonads were also isolated. Tissues were fixed overnight at 4°C in 10% buffered formalin. Tissues were processed as described above for *in situ* hybridization.

Endogenous peroxidase was quenched by placing deparaffinized, re-hydrated slides in 3% hydrogen peroxide in methanol for 15 min at room temperature (RT). Slides were then washed 3 times in PBS for 5 min each. Antigens were unmasked by incubating slides in 10 mM sodium citrate buffer (pH 6.0) and heating the buffer in a microwave for 5 minutes. The buffer was allowed to cool for 2 minutes and then heated again for 5 minutes. I heated and cooled slides once more before slides were washed twice with distilled water for 5 min and then once in PBS for 5 min.
Sections were permeabilized and blocked in 3% goat serum, 0.1% Triton X-100, 1% bovine serum albumin (BSA) in PBS for 2 hours at RT or overnight at 4°C. The primary Cirbp antibody was diluted 1:500 (Aviva Systems Biology, San Diego, CA) in 1x PBS, added to tissue sections, and incubated overnight at 4°C. Negative controls were incubated in blocking solution without the primary antibody. Sections were washed three times in 1x PBS and then incubated in a biotinylated goat-anti rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA) for 2 h at RT. The VectaStain ABC Elite

kit (Vector Laboratories) was used to detect the primary/secondary antibody complex. Staining was performed using freshly prepared 3, 3'-diaminobenzidine (DAB; Vector Laboratories) for 5 minutes and counterstained with hematoxylin. The sections were dehydrated, cleared in xylene, and permanently mounted with Vectamount. Images were captured as described above.

<div align="center">

Relationship between Cirbp mRNA expression and sex ratios

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I conducted an experiment to test whether there is a genetic correlation between *Cirbp* mRNA expression in gonads during the TSP and sex ratio at hatching. Snapping turtle eggs from nine clutches were incubated at 26.5^{o}C until stage 17.5 of development. At stage 17.5, eggs from each clutch were shifted to 31°C for 2.5 days and then shifted back to $26.5\,^{o}$C. Approximately equal numbers of embryos from each of the 9 clutches were sampled at 24 and 48 hours of the 31°C exposure. Gonads were dissected and total RNA extracted as previously described. Total RNA was reverse transcribed to measure *Cirbp* mRNA expression using quantitative PCR. A subset of eggs from each clutch was allowed to hatch to determine sex ratios for each clutch.

<div align="center">

Statistical Analysis

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I used two-way analysis of variance (ANOVA) to compare optical density of the PCR products on the polyacrylamide gel from the DD-PCR. Independent variables were temperature, day of the temperature shift, and the interaction between these variables.

For the quantitative real-time PCR, I analyzed patterns of gene expression using clutch, temperature treatment, and sampling day as main effects in a three-way ANOVA using type III sums of squares. All cycle threshold (Ct) values were log10 transformed to meet the assumptions of the ANOVA. I used Ct values for 18S rRNA as a covariate to

control for potential variation in the quality of input RNA as well as variation in efficiency of the reverse transcription reaction. Given significant main effects or interactions between independent variables ($\alpha < 0.05$), I used the Dunn-Sidak method (SAS Institute, Cary, NC) to correct for multiple comparisons among treatment groups.

Chi-squared tests were used to determine if there was significant variation in sex ratios amongst clutches. To determine if there was a relationship between *Cirbp* mRNA levels in embryonic gonads and the sex ratios produced within each clutch, I performed the nonparametric Spearman's rank correlation, because our data didn't meet the assumptions of the parametric test. All statistics were performed using JMP 5.0.1.2 software (SAS Institute, Cary, NC).

Results

Hatchling Sex Ratios

I examined the gross morphology and the histological structure of AKGs from hatchling turtles that had been exposed to different thermal regimes. Hatchling snapping turtles that had been incubated at $26.5^\circ C$ throughout embryonic development had testes and no visible signs of oviducts. In contrast, all hatchlings that had been exposed to $31^\circ C$ had ovaries and oviducts, indicating that the six-day temperature shift was sufficient to produce exclusively females.

I also examined hatchling gonads and reproductive tracts from the cold shift experiment. The sex ratio for snapping turtles exposed to $20^\circ C$ from stage 14 to stage 19 was 25% male (n = 12). I was unable to diagnose the sex of eight embryos from the $20^\circ C$ group that didn't hatch. In contrast, all of the hatchlings from $26.5^\circ C$ developed testes (n = 18).

Differential Display PCR

I identified a PCR product of 150 base pairs (bp) that was differentially expressed between the 26.5°C and 31°C treatment groups (Figure 2.1). This PCR product had higher optical density values at 31°C compared to 26.5°C ($F_{(1, 16)}$ = 11.02, p = 0.0043). The optical density of this PCR product did not change over time ($F_{(3,16)}$ = 0.9712, p = 0.4306) and was not affected by the temperature x day interaction ($F_{(3,16)}$ = 2.69, p = 0.0810).

Identification of the Full-Length cDNA and Amino Acid Sequence

I used 5' RACE to obtain the full-length sequence for this DD-PCR product. The full-length cDNA was 851 bp long, and displayed 86 % nucleotide identity with the chicken cold-inducible RNA binding protein (*Cirbp*) cDNA (GenBank Accession No. NM_001031347). The full-length cDNA sequence contained a 501 bp open reading frame that encodes a 169 amino acid sequence (Figure 2.2). The predicted amino acid sequence for snapping turtle Cirbp displayed the two conserved domains; a single recognition motif (RRM) at the amino-terminal and an arginine and glycine-rich carboxyl-terminal. Within the RRM, two highly conserved sequences, a hexamer called RNP2 and an octamer called RNP1, were present as well as many aromatic residues dispersed throughout the motif.

The predicted amino acid sequence for snapping turtle Cirbp displayed high homology to the amino acid sequences of other vertebrates (Figure 2.3). For example, snapping turtle Cirbp displayed 93% amino acid identity with the chicken Cirbp. Even greater homology was observed in putative functional domains. The RNA recognition motif (RRM) displayed 99% amino acid identity among amniotes, with mouse and rat

Cirbp containing alanine in place of serine at amino acid 21. The RRM was not as highly conserved among non-amniotes: homology was less than 90% in this region.

<p align="center">*Cirbp mRNA Expression During the TSP*</p>

After obtaining the full-length cDNA sequence for snapping turtle *Cirbp*, I conducted an independent experiment to confirm that *Cirbp* was differentially expressed between male- and female-producing temperatures. Expression of *Cirbp* was influenced by clutch ($F_{(2,65)}$ = 22.16, p < 0.0001) and temperature treatment ($F_{(1,65)}$ = 124.48, p < 0.0001). Expression of 18S rRNA was a significant covariate ($F_{(1,65)}$ = 37.25, p < 0.0001). *Cirbp* expression did not vary with sampling day and was not influenced by interactions among independent variables. *Cirbp* mRNA levels were twice as high in gonads from embryos at 31°C than in gonads from embryos at 26.5°C (Figure 2.4). However, there was no difference in *Cirbp* mRNA expression between hatchling testes and ovaries.

Given that the temperature effect was already significant on day 2, I conducted another experiment and sampled embryos from both temperatures at 6, 12, 24, and 48 hours of the temperature shift. Expression of *Cirbp* mRNA was influenced by clutch ($F_{(2,130)}$ = 12.7564, p < 0.0001), incubation temperature ($F_{(1,130)}$ = 10.1723, p = 0.0019), and sampling time ($F_{(3,130)}$ = 49.6173, p < 0.0001). There were no significant interactions between any of the independent variables. *Cirbp* was induced at both 26C and 26-31-26C, but did not increase significantly until 48 hours (Figure 2.5). At 48 hours, *Cirbp* mRNA levels were dimorphic with expression levels twice as high at 26-31-26C than at 26C.

I conducted another study to determine if *Cirbp* mRNA expression was induced at a cooler temperature that produces females. Eggs were incubated at 26.5°C until stage 14, half the eggs were then shifted to 20°C. I sampled embryos from both temperatures at stages 15-19. Expression of *Cirbp* mRNA was influenced by clutch ($F_{(2,87)} = 15.03$, $p < 0.0001$), incubation temperature ($F_{(1,87)} = 54.18$, $p < 0.0001$), and developmental stage ($F_{(4,87)} = 7.71$, $p < 0.0001$). There were no interactions between any of the independent variables. *Cirbp* mRNA expression was significantly higher in the embryonic gonads incubated at 26.5°C than it was in embryos at 20.0°C from stage 17 through the end of the TSP (Figure 2.6).

Spatial distribution of Cirbp *mRNA and protein in the snapping turtle gonads*

The spatial distribution of *Cirbp* mRNA was observed in snapping turtle hatchling testes and ovaries using *in situ* hybridization. Localization of *Cirbp* mRNA was sexually dimorphic in the snapping turtle. *Cirbp* mRNA was localized to the cortex of the ovary and appears to be expressed in somatic cells surrounding developing germ cells (Figure 2.7B). Strong *Cirbp* mRNA staining was localized to seminiferous tubules in the testes. Light *Cirbp* mRNA staining was also seen within the seminiferous tubules, likely in the developing germ cells (Figure 2.7A). In the testis sample there is an ovarian-like cortex at the surface of the male-gonad and staining of *Cirbp* mRNA appears similar to that of the ovary. Other species of turtles have also reported the presence of a cortex on the male gonad, but commonly this will degenerate over time to form normal testes (Pieau et al., 1998).

I used IHC to examine the spatial distribution of Cirbp protein during sex determination (Days 1-6 of the temperature shift) and gonad differentiation (Days 11, 21,

31, and hatch). Cirbp protein expression was sexually dimorphic as early as day 1 after the temperature shift. Staining in cortical epithelial cells was very strong at the female-producing temperature (31°C) but was not detectable in epithelium at the male-producing temperature (26.5°C) (Figure 2.8D and C, respectively). Cirbp protein expression became more localized to the cortex of gonads at 31°C, while Cirbp protein expression remained localized in the medulla in gonads at 26.5°C (Figure 2.9G and H, respectively) The spatial pattern of Cirbp protein expression continued to diverge during gonad differentiation. Expression was localized to the cortex and germ cells of the developing ovaries (Figure 2.7D and Figure 2.9B, D, F, H). Moreover, staining appeared cytoplasmic during the TSP, but nuclear during gonad differentiation.

The developing testes showed Cirbp protein expression within the medulla and in a circular pattern indicating the protein is localized to the developing germs cells in the seminiferous tubules (Figure 2.7C and Figure 2.9A, C, E, G).

Correlation between Cirbp Expression and Sex Ratio

I tested whether there was a genetic correlation between *Cirbp* expression and sex determination in an experiment that produced mixed sex ratios. Eggs from 9 clutches were incubated at 26.5°C until embryos reached stage 17.5. Eggs were then shifted to 31°C for 2.5 days and shifted back to 26.5°C until eggs hatched. This brief exposure to a female-producing temperature produced an overall sex ratio of 28% males. There was significant variation in sex ratio among clutches, ranging from exclusively females in clutch 28 to mostly males in clutch 15 (66.7%) ($\chi^2 = 25.9$, p = 0.0011; Figure 2.10).

Expression of *Cirbp* mRNA did not differ between day 1 and day 2 (One-way ANOVA; $F_{(1,86)} = 0.59$, p = 0.4441). Average *Cirbp* mRNA levels (mean $\pm$ SEM) in this

experiment were very similar to those described above for embryos shifted to 31°C (day 1 = 534.62 ± 76.30 attograms (ag)/2.5ng RNA; day 2 = 580.92 ± 62.57 ag/2.5ng RNA, respectively).

There was a significant negative correlation between *Cirbp* expression and hatchling sex ratios among clutches (Spearman's Rho = -0.70, r = -0.63, p = 0.0358; Figure 2.11). Clutches with lower *Cirbp* expression produced more males, while clutches with higher *Cirbp* expression produced more females. For example, clutch 20 had the lowest *Cirbp* expression and produced 58.3% males. In contrast, clutch 26 had the highest *Cirbp* expression and produced only female hatchlings.

Discussion

Many researchers are interested in understanding the molecular mechanisms underlying TSD. Two main questions have been posed: 1) How conserved are the sex-determining genes between TSD species and other non-TSD species? 2) What are the thermo-sensitive genes unique to TSD? To date, numerous studies in TSD species have defined the spatial and/or temporal pattern of expression of conserved sex-determining genes. However, studies designed to identify novel thermo-sensitive gene(s) are lacking, and in fact, the thermo-sensitive switch for testes versus ovary development has not been discovered in any TSD species. The purpose of this study was to identify novel genes involved in TSD in the snapping turtle. I isolated, cloned, and sequenced a snapping turtle gene that is induced by exposure to female-producing temperatures. This gene is an ortholog of the cold-inducible RNA binding protein (*Cirbp*) found in other organisms.

*Cirbp (*also known as A18 hnRNP) is a member of a large RNA binding protein family that contains a highly conserved RNA recognition motif (RRM, also known as

RNA binding domain (RBD) and glycine-rich domain known as the glycine-rich RNA binding protein family (GRP). Members of this family are evolutionarily conserved from plants to humans and are involved in almost every aspect of RNA biology, including RNA processing, export, translation and stability (Dreyfuss et al., 2002; Maris et al., 2005). Snapping turtle Cirbp contains a single RRM at the amino-terminus and glycine-rich carboxy-terminus that is highly conserved among vertebrates. Snapping turtle Cirbp RRM also contains two highly conserved sequences, RNP1 and RNP2, which allow for interactions between Cirbp and pre-mRNAs and mRNAs to regulate translation or mRNA stability (Yang et al., 2006; De Leeuw et al., 2007; Xia et al., 2012). Many members of this family are responsive to environmental stressors and can influence a variety of cellular and developmental processes, including sex determination in invertebrates (Amrein et al. 1988; Bell et al. 1988, Nicoll et al., 1997; Skipper et al., 1999).

I used several methods to characterize the temporal and spatial pattern of *Cirbp* expression in bipotential gonads during the TSP, in differentiating gonads after the TSP, and in hatchling testes and ovaries. I observed dimorphic *Cirbp* expression during the TSP, when mRNA levels were roughly two-fold higher at the female-producing temperature than the male-producing temperature. I also observed clear differences in protein expression in gonadal epithelium during the TSP. Exposure to the female-producing temperature dramatically increased Cirbp expression in epithelial cells, suggesting that Cirbp may be involved in commitment of the bipotential gonads to an ovarian fate. Indeed, recent work has shown that a sub-population of granulosa cells develops from epithelial cells in fetal mouse ovaries (Mork et al., 2012). Previous studies

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in endotherms have shown that *Cirbp* is constitutively expressed in almost every tissue, but is induced in response to various environmental stressors, including mild hypothermia (Nishiyama et al., 1997a; Sheikh et al, 1998; Nishiyama et al., 1998; Pan et al., 2004; Wellman et al., 2004). Studies in mammalian somatic cells indicate that *Cirbp* is induced at temperatures between 25°C and 31°C and as early as 12 hours after a shift from 37°C to 32°C (Nishiyama et al., 1997).

Cirbp has recently been identified as a temperature sensitive gene in the red-eared slider, another TSD turtle (Chojnowski & Braun, 2012). Suppression-subtractive hybridization was used to make cDNA libraries from whole embryos incubated at male- or female-producing temperatures. *Cirbp* was one of the cDNAs in the library enriched in transcripts from a female-producing temperature. Unfortunately, *Cirbp* expression was not measured in gonads or localized by *in situ* hybridization or immunohistochemistry. Given that *Cirbp* is expressed in most tissues, it not clear whether this gene is differentially expressed in embryonic gonads of the red-eared slider.

I measured *Cirbp* expression in hatching testes and ovaries, but did not observe any difference in transcript levels. This result is similar to what has been reported in gonads from neonatal and one-month old alligators (Kohno et al., 2010). Although transcript levels are comparable in RNA extracted from whole gonads, there is a clear difference in protein expression and localization between hatchling ovaries and testes.

Because *Cirbp* appears to be involved in ovarian development, I also examined *Cirbp* expression at a cooler temperature that produces females in our population. *Cirbp* mRNA was expressed at a higher level in gonads from embryos at 26.5°C versus 20°C, yet 20°C still produced females. This finding suggests that *Cirbp* may only play a role in

ovary formation at warm temperatures, while other gene(s) could be involved in ovary specification at cool temperatures. In fact, previous studies have shown clutch by temperature interactions for TSD in the snapping turtle, indicating that different mechanisms are involved in sex determination at high versus low temperatures (Rhen et al., 1994, but see Janzen 1992). Moreover, studies in mice have shown that *Cirbp* is inducible between 25°C and 32°C, but not at cooler temperatures (i.e. 15°C), although studies in *Xenopus* have shown that *Cirbp* is inducible at temperatures as low as 4°C (Nishiyama et al. 1997; Saito et al., 2000). A caveat of these results is the three clutches for the cold shift experiment were previously assigned to a separate experiment that exposed the eggs to cycling temperatures. Clutches were removed from the cycling temperatures at the beginning of the TSP; yet this temperature exposure may have induced *Cirbp* expression. This may also explain why *Cirbp* mRNA levels at 26.5^{o}C were higher than we had previously observed when *Cirbp* was induced at 31°C.

The temporal expression pattern for conserved sex-determining genes in gonads from snapping turtle embryos has been described (Rhen et al., 2007). I found that *Cirbp* was induced before any of the ovarian genes measured in that study. Aromatase and *Foxl2* were not induced until the third day of a shift from 26.5^{o}C to 31°C, which is after most gonads have committed to an ovarian fate (~70% of embryos in the current study developed ovaries after just 2.5 days at 31°C). I detected a significant correlation between *Cirbp* expression and sex ratio in siblings exposed to the same temperature regime (i.e., embryos at 26.5^{o}C were exposed to 31°C for 2.5 days). Families with lower *Cirbp* expression during the temperature shift produced more males, while families with

higher *Cirbp* expression produced more females. In contrast, aromatase and Foxl2

expression were not correlated with sex ratio in the same study (data not shown).

Taken together, these results suggest that *Cirbp* is upstream, while *Foxl2* and aromatase

are downstream in the gene network for ovary determination.

Expression of Cirbp protein was dimorphic in bipotential gonads on the first day

of the temperature shift: staining in gonad epithelial cells was stronger at the female-

producing temperature than it was at the male-producing temperature. This difference

became more pronounced by the end of the TSP. In addition, Cirbp staining appeared to

be cytoplasmic during the TSP, but nuclear after the TSP. This suggests Cirbp could be

playing distinct roles during sex determination and gonad differentiation. In *Xenopus*,

Cirbp displays nucleocytoplasmic shuttling and Cirbp has distinct roles in the nucleus and

cytoplasm (Aoki et al., 2002). In response to environmental stressors, Cirbp moves from

the nucleus to the cytoplasm where it associates with ribosomes to regulate expression of

specific mRNAs (Yang et al., 2001; De Leeuw et al., 2007). Because Cirbp is primarily

found in the cytoplasm during the TSP, it may be regulating mRNAs involved in testis or

ovary determination. Nuclear Cirbp seems to be associated with cell growth and

differentiation in other systems and may play a similar role in the gonad after sex

determination.

I also used *in situ* hybridization and immunohistochemistry to examine *Cirbp*

mRNA and protein expression in hatchling testes and ovaries. *Cirbp* mRNA and protein

were concentrated in the seminiferous tubules of hatchling testes indicating that *Cirbp* is

expressed in germ cells. *Cirbp* is also expressed in human, mouse and American alligator

testes. Studies in mice have shown that *Cirbp* is constitutively expressed in the testes.

Cirbp expression is up-regulated at cooler temperatures and down-regulated at elevated temperatures, testicular varicocele, and experimental cryptorchidism (Nishiyama et al., 1998; Zhou et al., 2009). More specifically, *Cirbp* is expressed in germ cells and appears to be involved in spermatogenesis, which is a temperature sensitive process in mammals. Xia et al. (2012) recently reported that *Cirbp* has many mRNA targets in the testes, including azoospermia factor mRNAs, which are essential for spermatogenesis. In mice, primary spermatocytes, secondary spermatocytes, and round spermatids express *Cirbp*. In contrast, *Cirbp* is not expressed in Sertoli cells or Leydig cells of mice. Although the exact role *Cirbp* plays in spermatogenesis is not known, *Cirbp* has been reported to regulate the cell cycle in spermatogonia (Masuda et al., 2012). It is important to note there were no major gross abnormalities or fertility problems in *Cirbp* knockout mice despite having fewer spermatogonia. However, the down-regulation of *Cirbp* in the testes by heat stress may contribute to male infertility (Xia et al., 2012).

  Cirbp mRNA and protein were concentrated in the ovarian cortex of hatchling turtles. Cirbp protein appeared to be mainly detected in the nucleus of the oocytes in the snapping turtle, supporting the idea that Cirbp is important for cell growth, differentiation, and maintenance of the gonad after sex determination. *Cirbp* is also expressed in the ovaries of numerous mammals, fish, *Xenopus*, and American alligator. *Cirbp* is expressed in granulosa cells and oocytes and appears to be involved in folliculogenesis (Knoll-Gellida et al., 2006). Three isoforms of the cold-inducible RNA binding protein (referred to as CIRP) have been identified in *Xenopus*, XCIRP, XCIRP-1 and XCIRP-2, and all three are expressed in oocytes. XCIRP2 is the most abundant isoform and it expression is cytoplasmic, suggesting that XCIRP2 may be involved in

regulating translation (Matsumoto et al., 2000). It has been suggested that *Cirbp* is a

proto-oncogene involved in ovarian cancer (Lleonart, 2010; Emmanuel et al., 2011).

Thus, *Cirbp* may play a role in regulating the cell cycle in ovaries and testes.

We hypothesize that *Cirbp* interacts with other sex-determining genes in the

bipotential gonads of snapping turtle embryos. In *Xenopus*, *Cirbp* is a target of TCF

transcription factors, which are important Wnt/ß-catenin signaling and have been

implicated in mammalian sex determination. TCF transcription factors are known to

interact with stabilized ß-catenin to activate target genes necessary for ovarian

development in many species (Van Venrooy et al., 2008; Bernard et al., 2008). Cirbp

also interacts with and stabilizes ß-catenin (Peng et al., 2006). It would therefore be

interesting to directly test the hypothesis that Cirbp interacts with the Wnt/ß-catenin

pathway in snapping turtle embryos.

Cirbp has also been shown to target Wilm's Tumor 1 (WT1) in *Xenopus* (Peng et

al., 2000). WT1 is a zinc finger transcription factor essential for gonadogenesis and sex

determination in mammals (reviewed in Swain and Lovell-Badge, 1999; Capel 2000).

Several WT1 isoforms are produced by alternative splicing of mRNA and alternative

translation initiation sites. For instance, the WT1 +KTS and −KTS isoforms play

different roles in gonad development. The -KTS isoform is essential for initial

development of the bipotential gonads, while the +KTS isoform is required for testis

determination in mice (reviewed in Bowles and Koopman, 2001; Wagner et al., 2003).

Studies in the American alligator and red-eared slider turtle demonstrate that WT1

mRNA and protein are also expressed in embryonic AKGs during the TSP (Spotila et al.,

1998; Western et al., 2000; Schmahl et al., 2002). To our knowledge, no one has examined potential interactions between *Cirbp* and WT1 in TSD species.

Another cold inducible RNA binding protein, RNA-binding motif protein 3 (RBM3), is involved in microRNA (miRNA) biogenesis by regulating Dicer (Pilotte et al., 2011). RBM3 is a member of the RRM protein family and is structurally similar to Cirbp, suggesting that RBM3 and Cirbp may have similar molecular functions. miRNAs are short, noncoding RNAs that regulate gene expression by binding to complementary mRNA sequences and causing their degradation. miRNAs play key roles in development, differentiation and plasticity (Pauli et al., 2011). Recently, miRNAs have been reported to be important players in sex determination and differentiation in mammals and chickens (Bannister et al., 2009; Torley et al., 2011; Cutting et al., 2012; Li et al., 2012). Thus, Cirbp could regulate expression of sex-determining genes by influencing the formation of miRNAs.

In summary, I found that a high, female-producing temperature increases *Cirbp* expression in the bipotential gonads of snapping turtle embryos. I also detected a significant familial correlation between the *Cirbp* induction during the TSP and sex ratios at hatching. Taken together, these results strongly suggest that *Cirbp* plays a role in mediating temperature effects on sex determination in the snapping turtle. Yet, *Cirbp* is probably not the only TSD gene because we would expect elevated *Cirbp* expression at both cool and warm female-producing temperatures. Indeed, prior studies in the snapping turtle (and other TSD reptiles) suggest polygenic inheritance of thermal sensitivity and genotype by temperature interactions (Rhen and Lang, 1998; Rhen et al., 2011). Although I provide hypothetical mechanisms by which Cirbp might participate in

TSD, additional experiments are required. Given that the main functional domain in Cirbp is the RRM, it would be informative to identify RNAs that physically interact with Cirbp in bipotential gonads at male versus female temperatures. However, the ultimate test will involve manipulation of *Cirbp* expression (i.e., overexpression and knockdown experiments) and phenotypic analysis of gonad differentiation at male and female temperatures.

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Table 2.1. List of primers used for isolating, measuring mRNA expression levels, and obtaining the full-length cDNA of Cirbp. H-T$_{11}$A and H-AP 03 primers were used for mRNA Differential Display PCR to clone Cirbp. The Real Time Forward and Reverse primers were used for quantitative PCR to measure the mRNA expression of Cirbp. The 5' RACE Semi-nested primers were used for the 5' RACE to obtain the full-length cDNA of Cirbp.   The riboprobe primers were used for synthesizing antisense and sense probes for *in situ* hybridization.

Primer	Primer Sequence
H-T$_{11}$A	5' - AAGCTTTTTTTTTTTA - 3'
H-AP 03	5' - AAGCTTTGGTCAG – 3'
Real-Time Forward	5' – CAAGTGAACAATCTGACTTGTAACAG – 3'
Real-Time Reverse	5' – TTTTTACATCGATCTTTCTTGCAT – 3'
5' RACE Semi-nested 1	5' – TTTCCCGTCCATTTATTCAACGGT – 3'
5' RACE Semi-nested 2	5' – CTCAGGAATGCTGTTTCAGGCTCA – 3'
5' RACE Semi-nested 3	5' – CCTGCTGCTGCTATAGTAGTCTCT – 3'
Riboprobe Forward	5' – TGAAAGACAGAGAGACCCAGAGGTCCA-3'
Riboprobe Reverse	5' – ACCTGTCACCATAGCCACCTTGATTCCT-3'
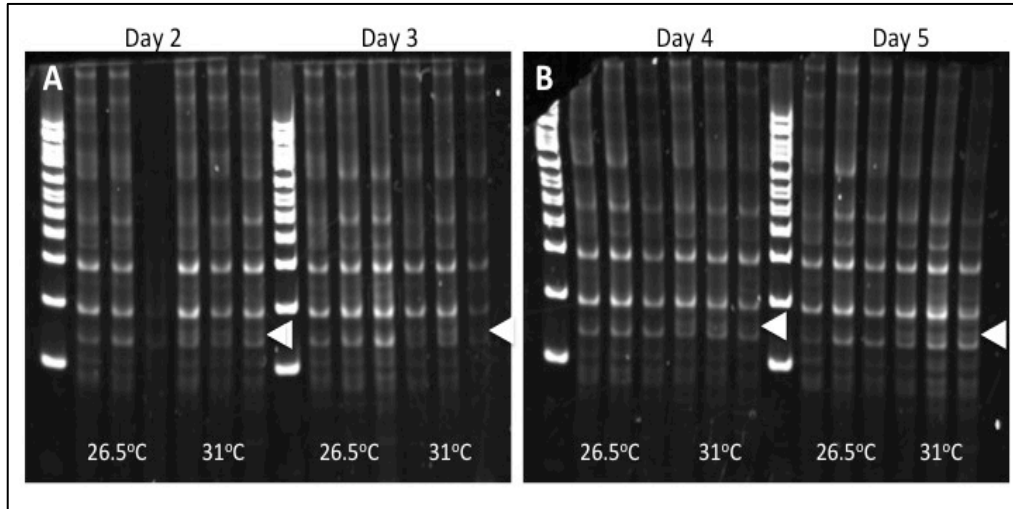
Figure 2.1. 6% polyacrylamide gel using H-T$_{11}$A and H-AP 03 for Differential
Display PCR. A) Bands present from the polyacrylamide gel on days 2 and 3 after
the temperature shift. B) Bands present from the polyacrylamide gel on days 4 and
5 after the temperature shift. The male-producing (26.5°C) and female-producing
(31°C) thermal groups are indicated in the middle of the three replicates for each
treatment. The day after the temperature shift is listed above the lanes. The white
arrowheads indicate the differentially expressed band at approximately 150 base
pairs in the female-producing (31°C) treatment group. The ladders shown on the
ends and middle of the gels are 100 base pair ladders.

```
tacatcgtgctgggactttgaagcattgtgctgcccttgcactcaccgaggaccgaacaaa        61
atggcatcagatgagggcaagctctttgttggtggactgagttttgataccaatgaacag       121
 M  A  S  D  E  G  K  L  F  V  G  G  L  S  F  D  T  N  E  Q

tcactggagcaagtcttttctaaatacggacagatctctgaagttgtcgtggtgaaagac       181
 S  L  E  Q  V  F  S  K  Y  G  Q  I  S  E  V  V  V  V  K  D

agagagacccagaggtccagaggctttggatttgttacttttgagaacatagatgatgct       241
 R  E  T  Q  R  S  R  G  F  G  F  V  T  F  E  N  I  D  D  A

aaagatgcaatgatggctatgaatggaaagtctgttgatgggcgtcagattagagttgac       301
 K  D  A  M  M  A  M  N  G  K  S  V  D  G  R  Q  I  R  V  D

caggcaggtaaatcatccgaaaatagatcccgtggatacagaggggggttcatcaggtggc       361
 Q  A  G  K  S  S  E  N  R  S  R  G  Y  R  G  G  S  S  G  G

agaggctttttccgtggaggcagaggtcgggggggccgtggcttctctagaggaggtgga       421
 R  G  F  F  R  G  G  R  G  R  G  G  R  G  F  S  R  G  G  G

gacagaggctatggaggaagcagattcgattccagaagtgggggatataatgggtctaga       481
 D  R  G  Y  G  G  S  R  F  D  S  R  S  G  G  Y  N  G  S  R

gactactatagcagcagcaggaatcaaggtggctatggtgacaggtcttcaggagggtcc       541
 D  Y  Y  S  S  S  R  N  Q  G  G  Y  G  D  R  S  S  G  G  S

tacagagacaactatgacagttacggttgaagagaagatggacatttgagcctgaaacagc       601
 Y  R  D  N  Y  D  S  Y  G  *

attcctgagaatttcgtgaaagcagtacctacaatcaactttctaagcagcagtgagctgca       663
tcgaagaggattgatacagctgttgactgagcagtggagttcaagagctttgagcagtgtat      725
aatatccgatatagatccaagtgaacaatcggacttgtaaccgttgaataaatggacgggaa      787
aaagcaatgctttttaaaataattctataaataaatgcaagaaagatcgatgtaaaaaaaaaa     850
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Figure 2.2. Full-length cDNA nucleotide and deduced amino acid sequences for snapping turtle *Cirbp*. Amino acid sequence is shown in single-letter code below the nucleotide sequence. The RNA recognition motif sequence is in bold and the putative RNP sequences are underlined. The arginine and glycine rich regions are doubly underlined. The stop codon is indicated by an asterisk.

```
                            RNP2                                                              RNP1
Snapping Turtle  MASDEGKLFVGGLSFDTNEQSLEQVFSKYGQISEVVVKDRETQRSRGFGFVTFENIDDAKDAMMAMNGKSVDGRQIRVDQAGKSSENRSR  91
Human            ..........................................................................D........  91
Mouse            ....................A.....................................................D........  91
Rat              ....................A.....................................................D........  91
Alligator        ...................................................................................  91
Chicken          ...................................................................................  91
AC Frog xCIRP    .-......I..N.E..DC...A.T..R..............K...............V...............GE.RG  90
AC Frog xCIRP-1  .-......I..N.E..DC...A.T..R..............K...............V...............GE.RG  90
AC Frog xCIRP-2  .-......I..N..E.........K...............P............A............GD.RG  90
Zebrafish        .-.......T....DA......V.TN.H.ARN..N........LEG.......T...E..GGGGGGG  90
Atlantic Salmon  .-.......T...AEA.....N.AKCD.IM...G.P......KYD.AE.....EG...Q.L..T..NE.QGGGRGGG  90

Snapping Turtle  ------GYRGGSSG---GRGFFRGGRGRGRGFSRGGGDRGYGGSRFDSRSGGYNGS---RDYYSSRNQGG-YGDRSSGGSYRDNYDSYG----  169
Human            ------...A---.............N.E........G.....N.E....G......-S.S.G.S...........S....ATHNE  172
Mouse            ------...A---....S..-.............G.E........G......A.-S...S..Y...........S...ATHNE  172
Rat              ------...A---....S..-.......W...G.E........G......A.-S...S..Y...........S...ATHNE  172
Alligator        ------...A---...............S...............S.........S..G..............S...ATHNE  165
Chicken          ------...T---...............-.............N-.S..-............S...KSWF  171
AC Frog xCIRP    ------...---.............S....N....G.SGS....G.G.S..S-....-...........S....----  163
AC Frog xCIRP-1  ------...---.............S....N....G.SGS....G.G.S..S-.....-...........S...ATHE-  166
AC Frog xCIRP-2  ------...---.............S....N....G.SGS.....G.S..S-....A-...........S...ATHE-  166
Zebrafish        RSGGGGS....GG.RGG.G...G.GGY....S.DRSYGGGG...KSG-----GGGY.SGG..G.NRDR-.SG.G.RSS..KDGYD  179
Atlantic Salmon  ---GGG.F..SRG.GGY.G----.Y--.G.R.Y..E....---GG..RSG-----GGG--GGR..G.SS---..G...N------  154
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Figure 2.3. Amino acid sequence alignment for snapping turtle, human (NP_001271), mouse (NP_031731), Rat (NP_112409), alligator (BAF94149), chicken (NP_001026518), three splice variants of African clawed frog (BAA31861, AF278702_1, NP_001079794, respectively), zebrafish (NP_001017797), and Atlantic salmon (NP_001133148) Cirbp. Whereas differences from the snapping turtle sequences are shown, identities are indicated with dots. The bolded sequence for the snapping sequence is the RNA recognition motif. The single underlined nucleotides are the consensus RNA binding motifs   RNP1 and RNP2. The specific RNPs are labeled above their nucleotide sequence.
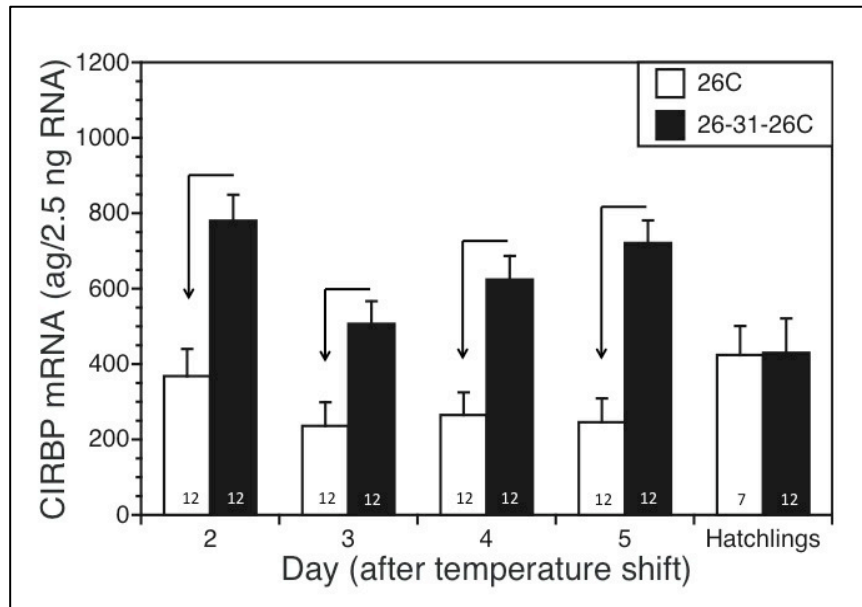
Figure 2.4. Cold inducible RNA-binding protein (*Cirbp*) expression in gonads from snapping turtle embryos and hatchlings. One group of eggs was incubated at 26.5°C throughout development, which produces only males (26C, open bars). Another group was incubated at 26.5°C until stage16, shifted to a female producing temperature of 31°C for 6 days and returned to 26.5°C for the remainder of development (26-31-26C, solid bars). This brief exposure to 31°C is enough to produce exclusively females. Levels of *Cirbp* mRNA are adjusted least square means ($\pm$ 1 SE) for each temperature regime and time point. Arrows indicate a significant ($p \leq 0.05$) difference in *Cirbp* expression between incubation temperatures based on the Dunn-Sidak post hoc test.
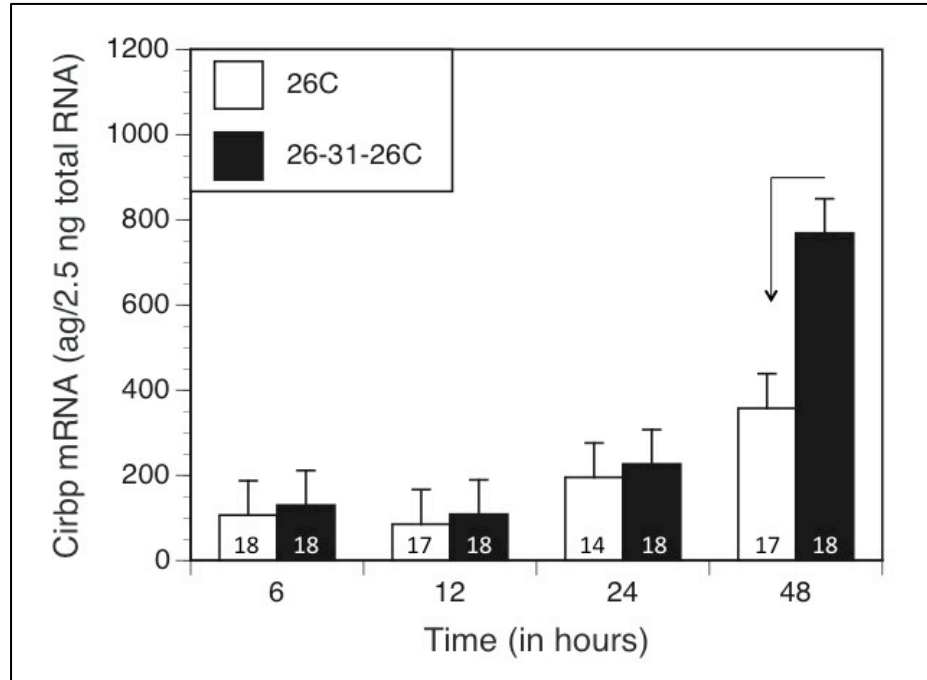
Figure 2.5. Cold Inducible RNA binding protein (*Cirbp*) expression in gonads from snapping turtle embryos. One group of eggs was incubated at 26.5°C throughout development, which produces only males (26C, open bars). Another group was incubated at 26.5°C until stage 16, shifted to a female producing temperature of 31°C (26-31-26C, solid). Gonads from each temperature regime were sampled at 6, 12, 24, and 48 hours after the temperature shift. Levels of *Cirbp* mRNA are adjusted least square means ($\pm$ 1 SE) for each temperature regime and time point. Arrows indicate a significant ($p \leq 0.05$) difference in *Cirbp* expression between incubation temperatures based on the Dunn-Sidak post hoc test.
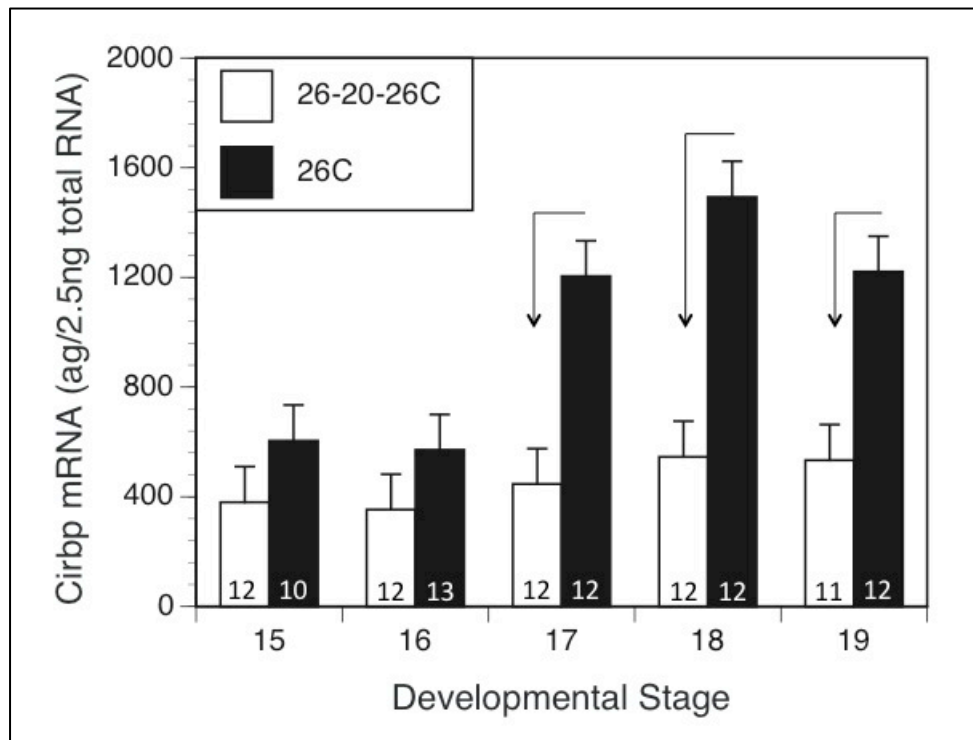
Figure 2.6. Cold Inducible RNA binding protein (*Cirbp*) expression in gonads from snapping turtle embryos. One group of eggs was incubated at 26.5°C throughout development, which produces only males (26C, open bars). Another group was incubated at 26.5°C until stage 14, shifted to a female-biased temperature of 20°C until stage 20 and returned to 26.5°C for the remainder of development (26-20-26C, solid bars). Gonads from each temperature regime were sampled at stages 15 through 19 after the temperature shift. Levels of *Cirbp* mRNA are adjusted least square means ($\pm$ 1 SE) for each stage and temperature regime. Arrows indicate a significant ($p \leq 0.05$) difference in *Cirbp* expression between incubation temperatures based on the Dunn-Sidak post hoc test.
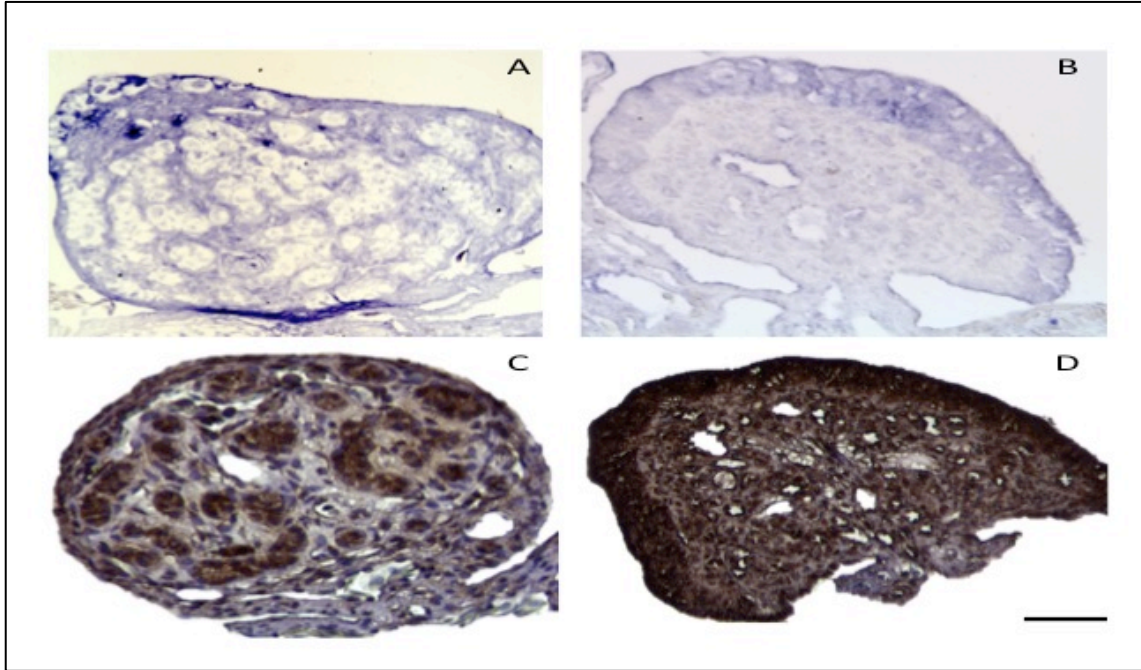
Figure 2.7. The distribution of cold inducible RNA binding protein (Cirbp*)* mRNA and protein in hatchling testes and ovaries using *in situ hybridization* and immunohistochemistry, respectively. A) *Cirbp* mRNA expression in hatchling testes incubated at 26.5^{o}C throughout development. B) *Cirbp* mRNA expression in hatchling ovaries incubated at 31°C for 6 days during the TSP. C) Cirbp protein expression in hatchling testes incubated at 26.5^{o}C throughout development. D) Cirbp protein expression in hatchling ovaries incubated at 31°C for 6 days during the TSP. Scale bar = 100 μm.
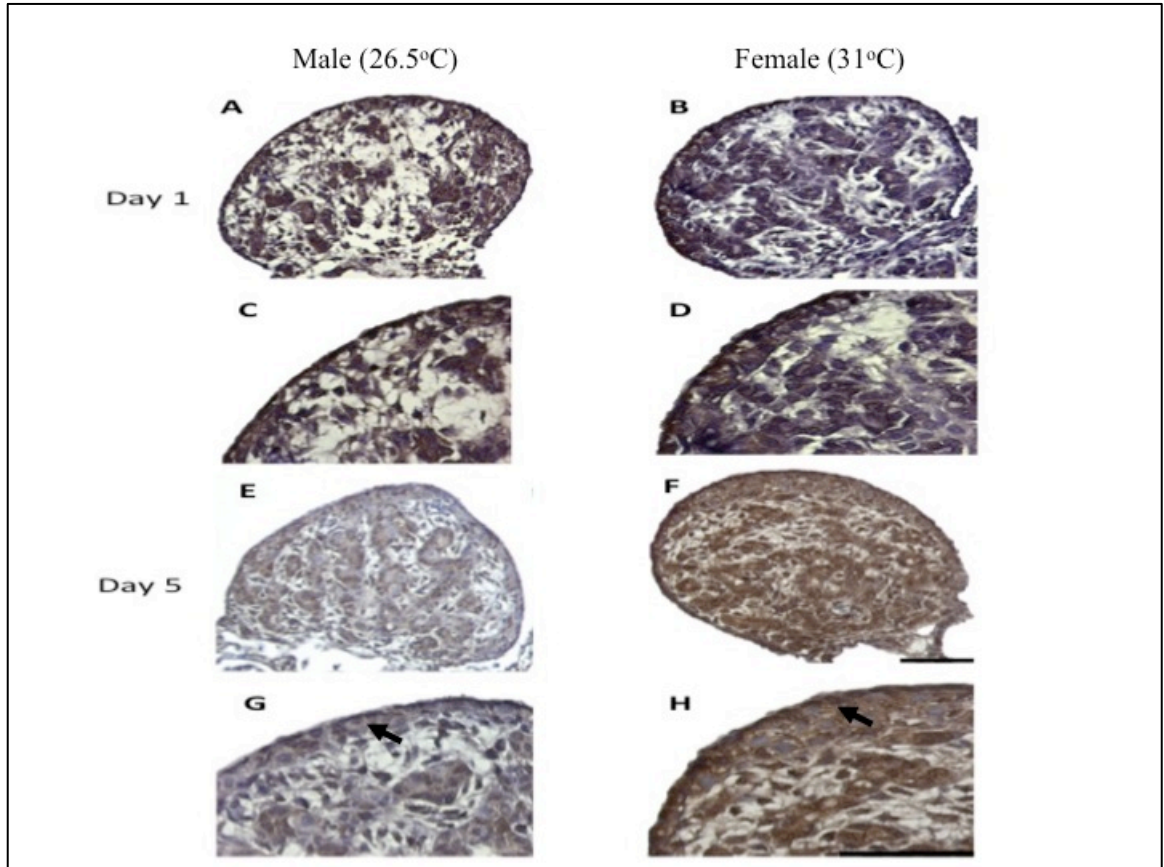
Figure 2.8. The distribution of Cirbp protein in bipotential gonads during the TSP incubated at male-producing (26.5°C; A, C, E, G) or female-producing (31°C; B, D, F, H) temperatures on days 1 (A-D) and 5 (E-H) after the temperature shift. Images were taken at either 20x magnification (A, B, E, F) or 40x magnification (C, D, G, H). The arrows in (G) and (H) indicate the cytoplasmic staining of Cirbp in the cortex of the bipotential gonad. Scale bars = 100 μm.
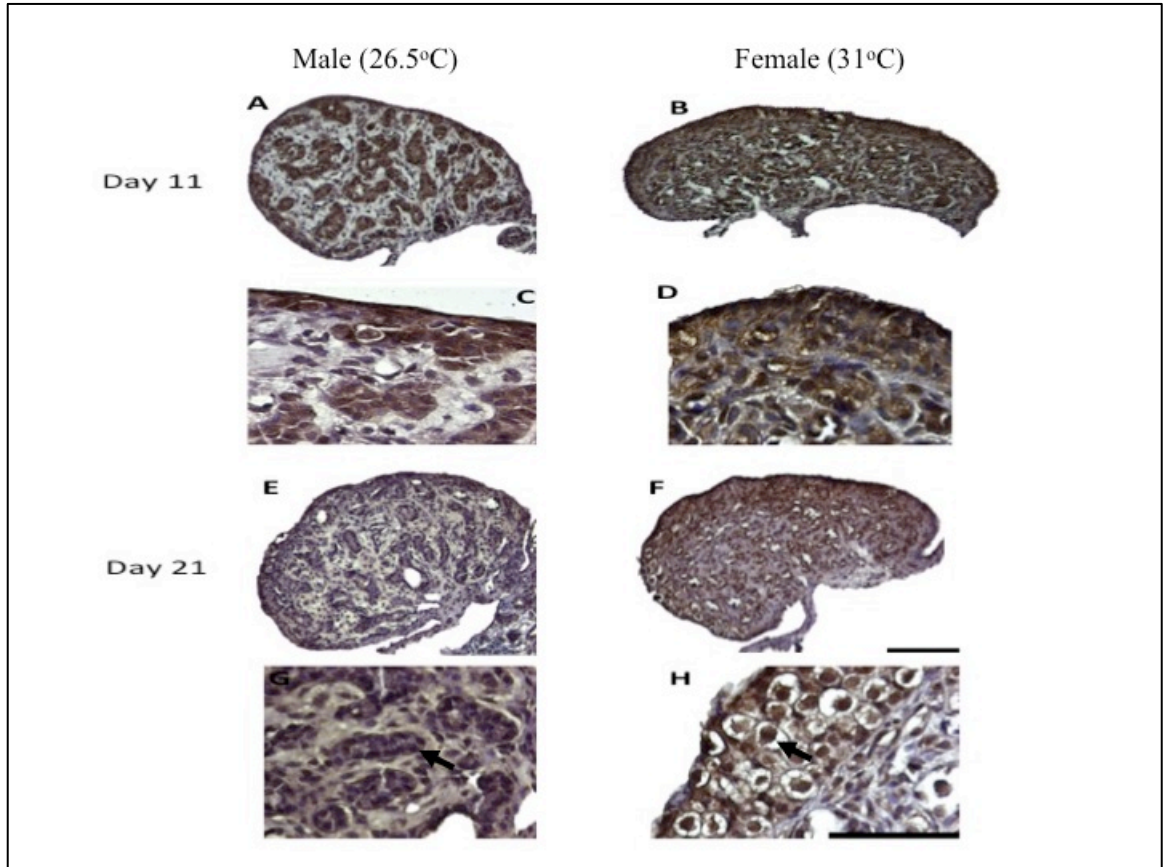
Figure 2.9. The distribution of Cirbp protein in differentiating gonads incubated at male-producing (26.5°C; A, C, E, G) or female-producing (31°C; B, D, F, H) temperatures on days 11 (A-D) and 21 (E-H) after the temperature shift. Images were taken at either 20x magnification (A, B, E, F) or 40x magnification (C, D, G, H). The arrows in (G) and (H) indicate the nuclear staining of Cirbp in the medulla of the developing testes (G) and the cortex of the developing ovaries (H). Scale bars = 100 μm.
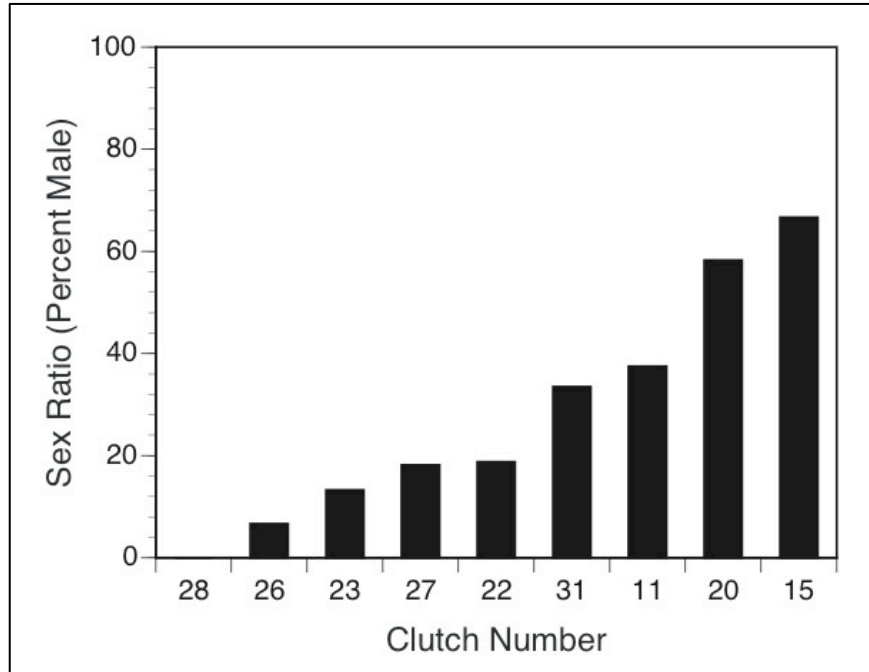
Figure 2.10. The sex ratios produced (in percent males) for 9 clutches that were shifted from a male-producing (26.5°C) to a female-producing (31°C) temperature for 2.5 days at stage 17.5.
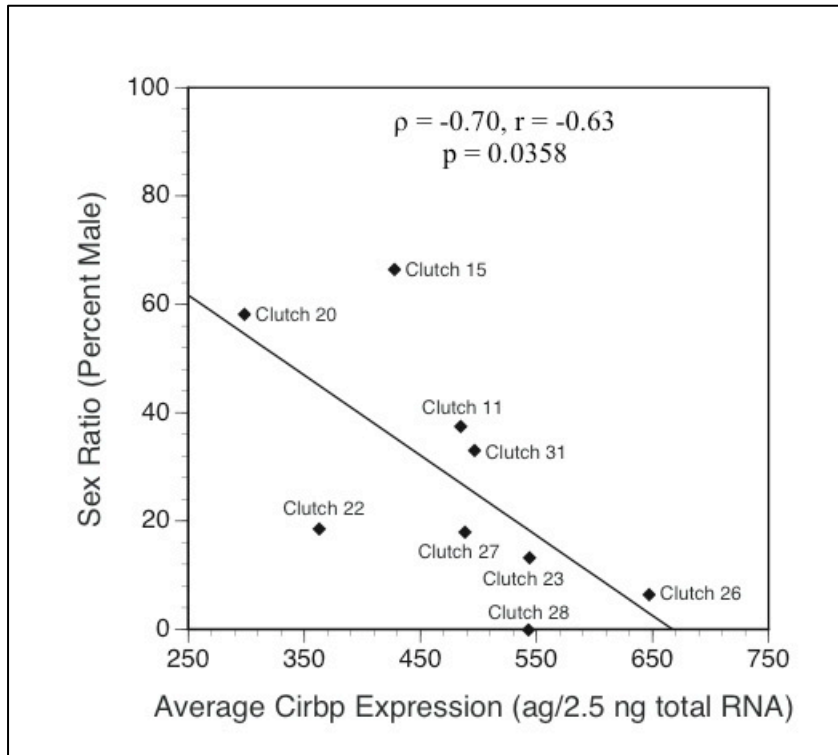
Figure 2.11. The relationship between the average *Cirbp mRNA* expression on days 1 and 2 and the sex ratios produced for 9 different clutches following a shift from a male-producing (26.5°C) to a female-producing (31°C) temperature for 2.5 days at embryonic stage 17.5. The statistics in the figure are based on the Spearman's rank correlation test.

CHAPTER III

NOVEL GENETIC ASSOCIATION BETWEEN COLD INDUCIBLE RNA-BINDING
PROTEIN AND TEMPERATURE-DEPENDENT SEX DETERMINATION IN THE
COMMON SNAPPING TURTLE

Abstract

The gene(s) that transduces temperature in a signal for ovary versus testis development is not known in any species that displays temperature-dependent sex determination (TSD). We recently identified cold-inducible RNA-binding protein (Cirbp) as a TSD candidate gene in the snapping turtle based on dimorphic expression in gonads during the sex-determining period. In this study, we test for associations between Cirbp genotype, mRNA expression, and sex ratios. We used next generation sequencing to identify single nucleotide polymorphisms (SNPs) within the coding sequence of Cirbp in snapping turtles from northern and southern Minnesota. We observed allelic specific expression and differences in allele frequencies between turtle embryos from northern and southern Minnesota, suggesting genetic adaptation to local thermal regime. We synthesized TaqMan probes to detect alternative alleles for one SNP and used quantitative PCR to verify allelic specific Cirbp expression. We also found significant genetic associations between Cirbp genotype, Cirbp expression and sexual phenotype in a study that produced mixed sex ratios. These results provide the strongest evidence to date for a TSD gene. Additional studies are required to define the functional role of Cirbp in sex determination.

Introduction

Sex determination in amniotic vertebrates can be divided into two broad categories, either genotypic or environmental. Genotypic sex determination (GSD) occurs at fertilization and is determined by the individual's genotype. GSD species often, but not always, display morphologically distinct sex chromosomes, as observed in mammals, birds, snakes, some lizards, and some turtles. Environmental sex determination occurs when certain environmental factors influence gonad development and the sex of an embryo. Various environment factors are known to determine sex in animals, but temperature is the only one to influence sex determination in amniotic vertebrates. This phenomenon is referred to as temperature-dependent sex determination (TSD). Among amniotes, TSD is only observed in reptiles, including many lizards, numerous turtles and all crocodilians studied to date (Viets et al., 1993; Lang and Andrews, 1994; Deeming 2004; Ewert et al., 2004; Harlow, 2004).

The critical period when temperature induces commitment of the bipotential gonads to testicular or ovarian fate is referred to as the temperature-sensitive period (TSP) (Crews, 1996; Pieu and Dorizzi, 2004; Place and Lance, 2004). The specific temperatures that produce males and females and length of the TSP vary among TSD species (Yntema, 1979; Wibbels et al., 1991; Lang and Andrews, 1994; Rhen and Lang, 1998; Pieau et al., 2004). Incubation temperature influences the expression of genes involved in testis and ovary development. Although the initial trigger for sex determination is different between GSD and TSD species, it appears that many orthologous genes are involved in vertebrate sex determination. For example, *Dmrt1*,

Sox9, *Pdgf-B*, and anti-Müllerian hormone (AMH) display temperature sensitive expression with higher levels in incipient testes in all species studied (reviewed in Shoemaker and Crews, 2009; Rhen and Schroeder, 2010). In contrast, aromatase and *Foxl2* have been identified as temperature sensitive genes that are expressed at higher levels in incipient ovaries (reviewed in Shoemaker and Crews, 2009; Rhen and Schroeder 2010). While these downstream genes are conserved, the initial switch for testis versus ovary development is not conserved between GSD and TSD species. For example, a single gene on the Y chromosome, *Sry*, is the trigger for testis development in mammals. However, an exact ortholog of SRY has not been identified in non-mammalian vertebrates. This suggests that the gene(s) transducing temperature into a signal for testes versus ovary development may not be orthologs of mammalian sex-determining genes. Thus, a candidate gene approach dependent on orthologs of mammalian genes may not identify the initial trigger for TSD (Lance, 2009).

One reptile that exhibits TSD is the common snapping turtle, *Cheldrya serpentina*. I have been using this species as a model for studying mechanisms underlying TSD. The snapping turtle is a useful model because it has a very short TSP: exposure to a female-producing temperature for six days is sufficient to induce ovarian development (Rhen et al., 2007; 2009). This allows identification of thermo-sensitive gene(s) that may trigger commitment to ovarian development. This species produces females at low and high temperatures, while males are produced at intermediate temperatures (Yntema, 1976; Rhen and Lang, 1994; Ewert et al., 2005). Eggs incubated between 23°C and 27°C produce all males in the population we study. In contrast, eggs incubated at 31°C produce exclusively females. Mixed sex ratios are produced at

temperatures below 23°C and between 27.0°C and 29.5°C, with 28.2°C producing a 50:50 sex ratio.

While this pattern of thermal sensitivity is observed in the population we study, Ewert et al. (2005) demonstrated a latitudinal cline for TSD in the snapping turtle. This suggests populations of snapping turtles are adapted to their local thermal regimes. Studies within snapping turtle populations have found among-family variation at temperatures that produce mixed sex ratios, indicating broad-sense heritability for TSD (Jansen, 1992; Rhen and Lang; 1998). More recent studies in other TSD species have demonstrated a heritable genetic basis for TSD (Rhen et al., 2011; McGaugh et al., 2011; McGaugh and Janzen, 2011). The presence of genetic variation for TSD makes genetic association studies a feasible approach to identify TSD candidate genes.

Understanding the genetic basis of phenotypic plasticity is essential for understanding variation in sex ratios observed among families and populations of TSD species.   Single nucleotide polymorphisms (SNPs) represent the most common DNA sequence alteration in humans (Cargill et al., 1999; Nadeau, 2002). SNPs are classified into two broad categories, either synonymous or non-synonymous. Non-synonymous SNPs lead to changes in the amino acid sequence of a protein, which may alter protein function. Non-synonymous mutations are less common than synonymous mutations, which do not change the amino acid sequence of a protein and do not directly alter protein structure or function. However, synonymous SNPs can influence gene transcription, mRNA processing, mRNA stability, or translation, which in turn have phenotypic consequences (Goymer, 2007). Synonymous mutations that influence gene expression account for much of the phenotypic variation within individuals, among

individuals, and among species (Whitehead and Crawford, 2006; Stranger et al., 2007). Therefore, the determination of variation in gene expression due to synonymous SNPs in biologically relevant loci is essential for understanding phenotypic variation.

Allelic specific expression (ASE) assays have become a common method for associating gene expression with variation in phenotype. ASE assays require a heterozygous marker, usually SNPs, within a gene to discriminate between two alleles (Germer et al., 2000; Wittkopp et al., 2004; Ronald et al., 2005). Differences in expression of alternative alleles may be responsible for the phenotype of interest. This approach has been used in genome-wide association studies of complex phenotypes in humans (Dixon et al., 2007). This approach has also been used to study phenotypic variation in natural populations (Tung et al., 2011; Storey et al., 2007). ASE assays may provide a powerful way to associate candidate genes with variation in TSD in natural snapping turtle populations.

Recently, I identified cold-inducible RNA-binding protein (Cirbp) as a candidate gene for TSD in the common snapping turtle (Rhen and Schroeder, 2010). Cirbp contains a single RNA recognition motif and glycine-rich motif that is plays a crucial role in mRNA processing, RNA export, translation, and stability (Dreyfuss et al., 2002; Maris et al., 2005). Cirbp is known to regulate many temperature-dependent cellular processes by regulating mRNAs through translational repression and stabilization (Yang et al., 2006; De Leeuw et al., 2007; Xia et al., 2012). Cirbp was more strongly expressed at a female-producing temperature (31^oC) than a male-producing temperature (26.5^oC). I also found a significant correlation between Cirbp expression and sex ratios within families. Clutches with higher Cirbp expression produced more females, while clutches with

blunted Cirbp expression produced more males, suggesting Cirbp is involved in the commitment to the ovarian fate.

It is challenging to test the function of differentially expressed genes in TSD species because, unlike mice and other model organisms, it is not possible to produce gene knockouts. Yet, with the development of next generation sequencing, researchers can begin to identify variants in candidate genes that may explain variation in TSD. In this study, I tested for genetic associations between Cirbp expression and gonad phenotype. I identified SNPs in snapping turtle Cirbp using next-generation Illumina sequencing.   I determined allele frequencies and allele-specific expression from RNA-Seq data. Probes were synthesized to detect alternative alleles for one of the SNPs and measure allelic specific expression of Cirbp in a natural snapping turtle population. I used this data to test the hypothesis that variation in Cirbp expression is genetically associated with commitment to ovarian fate.

Materials and Methods

Animal experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol #0905-1). In June 2008, 2009, and 2010, I collected eggs from snapping turtles nests throughout the state of Minnesota, USA. extending from the Iowa border to the Canadian border. We transported eggs to the animal quarters in the Biology Department at the University of North Dakota. I held eggs were held at ~20°C for less than one week before clutches were assigned to experimental treatments. Eggs were washed in tepid water, candled for viability and infertile eggs removed. Egg collection and incubation

protocols have been provided in extensive detail previously (Rhen et al., 2007, 2009), but a summary of our procedures for each experiment is provided below.

SNP Identification and Allelic-Specific Expression Assay Using Illumina Sequencing

Approximately equal numbers of eggs from 25 clutches were separated into two experimental groups. Eggs were placed in containers filled with moist vermiculite and containers were randomly positioned in foam incubators (Rhen and Lang, 1994, Rhen et al., 2007, 2009). One group of eggs was incubated at 26.5°C throughout development to produce males. The other group was incubated at 26.5°C until stage 17.5, at which point eggs were then shifted to 31°C for 6 days to produce females, and returned to 26.5°C for the rest of development. This brief exposure to 31°C is sufficient to induce ovary development in all embryos (Rhen et al., 2007). I dissected adrenal-kidney-gonad complexes (AKGs) from embryos incubated at 26.5°C and from clutch mates that had been shifted to 31°C. Tissues were collected on days 1, 2, 3, 4, and 5 of the temperature shift, placed in RNAlater (Ambion, Austin, TX), and stored at -20°C until RNA was extracted.

Genetic Association Experiment

Snapping turtle eggs from 15 clutches were incubated at 26.5°C until stage 17.5 of development. Eggs were shifted to 31°C for 2.5 days and then shifted back to 26.5 $^{\circ}$C. Approximately equal numbers of embryos were sampled from each clutch at 24 and 48 hours of the 31°C exposure. Gonads were dissected and total RNA extracted as previously described. A subset of eggs was allowed to hatch to determine sex ratios for each clutch. Hatchlings were sexed, AKGs dissected, snap-frozen, and stored at -80°C until genomic DNA isolation.

RNA Extraction, DNA Extraction, cDNA Synthesis

Embryonic gonads were carefully micro-dissected from AKGs. I extracted total RNA from gonad pairs isolated from individual embryos or hatchlings using the PicoPure RNA Isolation Kit (Life Technologies, Carlsbad, CA) as described in Rhen et al. (2007). I combined total RNA from individual embryos to make 20 pools for RNA-Seq: 2 temperatures x 5 days x 2 biological replicates. While ten of these pools (2 temperatures x 5 days) were from eggs collected in southern Minnesota, the other ten pools (2 temperatures x 5 days) were from eggs collected in northern Minnesota. The northern and southern populations were separated by the latitude of 46° 55' N. The concentration and quantity of each RNA pool was determined using the BioRad Experion System (Bio-Rad, Hercules, CA). Total RNA was sent to the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign for sequencing. Twenty individual cDNA libraries were prepared at the Keck Center and each library was sequenced using the Illumina HiSeq 2000 platform (Illumina Inc, San Diego, CA).

Embryonic gonads for the genetic association study were carefully micro-dissected from AKGs. Total RNA was extracted using RNAzol RT (Molecular Research Center, Cincinnati, OH). The RNAzol RT protocol was modified for the small amount of tissue from a pair of gonads. We used one-quarter of the liquid recommended by the manufacturer for tissue homogenization, RNA isolation, and recovery. For example, I homogenized tissue in 250 µl of RNAzol RT instead of 1 ml. I added 1 µl of precipitation carrier (Molecular Research Center, Cincinnati, OH) to the homogenate to assist with RNA isolation because the expected yield was less than 10 µg. Total RNA was dissolved in 15 µl of RNase-free water and quantified with a NanoDrop ND-1000

spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genomic DNA was

removed by DNase treatment following the RNA extraction to ensure RNA purity.

Total RNA (150 ng) from each pair of gonads was reverse transcribed in a 20 µl

reaction using the iScript cDNA Synthesis Kit, which contains a blend of oligo dT and

random hexamers (BioRad, Hercules, CA). I diluted cDNA to a concentration of 1 ng

input RNA/µl for use in real-time PCR reactions.

One half of the frozen hatchling AKGs was used to isolate genomic DNA for SNP

genotyping. I isolated genomic DNA using TRI Reagent according to the manufacturer's

recommendations (Molecular Research Center, Cincinnati, OH). I resuspended DNA in

100 µl of water. The DNA was quantified with a NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmington, DE). The integrity of DNA was determined by

gel electrophoresis. Purified genomic DNA was stored at -20°C until it was used for

genotyping.

Bioinformatics, SNP Base Calling, and mRNA-Seq

I used CLC Genomics Workbench 4 (CLC bio, Cambridge, MA) for read

mapping, SNP identification, and RNA-Seq analyses.   I mapped reads to full-length

Cirbp cDNA and determined the number of reads that were mapped and not mapped from

each of the 20 cDNA libraries. Reads uniquely mapped to Cirbp were used to identify

SNPs and to determine allele frequencies in populations of snapping turtles from northern

and southern MN. I used the SNP detection tool to identify SNPs in Cirbp cDNA.

Parameters for SNP detection required a minimum coverage of 4 reads for each SNP and

a minimum variant frequency of 35 percent.

I used RNA-seq to measure ASE: read counts for each allele were determined for each of the twenty cDNA libraries. Allelic-specific expression was standardized as reads per kilobase of exon per million mapped reads (RPKM) (Mortazavi et al., 2008). The calculation for the RPKM is provided below.

$$= \frac{\text{Number of Reads for Cirbp Allele}}{\text{Length of Cirbp x Total number of reads (in millions)}}$$

Quantitative Real-time PCR for Alleles and Genotyping

I designed PCR primers and highly specific TaqMan (major groove binder) probes for real-time PCR. I used these primers and probes to measure ASE of Cirbp mRNA and to genotype hatchling turtles (Applied Biosystems, Carlsbad, CA). The sequence of primers and probes for the 124A>C SNP are shown below:

> Forward Primer 5'- GGTGGACTGAGTTTTGATACCAATG – 3'
> Reverse Primer 5'-TTCACCACGACAACTTCAGAGATC- 3'
> Reporter 1 (Fam) 5'- AACAGTC**A**CTGGAGCAA-3'
> Reporter 2 (Vic) 5'- AACAGTC**C**CTGGAGCA-3'

I used cDNA from 15 clutches as template in our ASE assay. In brief, each reaction tube contained 5 µl SsoFast probes supermix (Bio-Rad, Hercules, CA), 300 nM of forward and reverse primers, 200 nM of each probe, 2 µl of cDNA from one individual (equivalent to 1 ng of total RNA), and water to bring the total reaction volume to 10 µl. PCR reactions were run on the Bio-Rad CFX 384 Real-Time System (Bio-Rad). The thermal profile was 95°C for 30 sec to activate the DNA polymerase followed by 40 cycles of two-step PCR (95°C for 5 sec and 64.3^{o}C for 10 sec). I used 64.3^{o}C as the annealing and extension temperature because it was the optimal temperature for distinguishing between the alleles. I made standard curves for each allele as described previously (Rhen et al., 2007, 2009).

Genotyping was performed using genomic DNA isolated from hatchlings for all 15 clutches. Primers and probes were the same as for the ASE assay. Reactions were set up as described above. Instead of using cDNA as template, I used 1 μg of genomic DNA as template for each individual. The thermal profile was $95^{o}C$ for 3 min to activate the DNA polymerase enzyme followed by 50 cycles of two-step PCR ($95^{o}C$ for 5 sec and $64.3^{o}C$ for 10 sec). Genotyping reactions were run on the Bio-Rad CFX 384 Real-Time PCR System (Bio-Rad).

Statistical Analysis

I used logistic regression to compare allele frequencies for each SNP in the 20 cDNA libraries described above. I used temperature, population, and the interaction between temperature and population as independent variables in one model. In a second model, I analyzed ASE for each SNP using temperature, allele and the interaction between temperature and allele as independent variables. Given significant effects ($\alpha <$ 0.05), I used the Dunn-Sidak method to correct for multiple comparisons among groups. Chi-square tests were used to test for an association between the hatchling genotype and gonadal sex. We also performed a stratified categorical analysis using the Cochran-Mantel-Haenszel (CMH) test to control for clutch identity, which could be confounded with hatchling genotype. I performed the nonparametric Spearman's rank correlation test to examine relationships among latitude, sex ratio, and allele frequency. All statistics were performed using JMP 5.0.1.2 software (SAS Institute, Cary, NC).

Results

Illumina Sequencing and SNP Identification

I obtained 153,568,937 reads (100bp/read) from the 20 cDNA libraries. I mapped 15,337 sequence reads to the full-length Cirbp cDNA sequence, with 5,379 reads mapping from the $26.5^{o}C$ libraries and 9,958 reads mapping from the $31^{o}C$ libraries. A summary of the number of Illumina sequencing reads that were mapped or were not mapped back to the snapping turtle Cirbp for each population and thermal regime is provided in Table 3.1.

From the 15,337 reads mapped, I identified four SNPs within the snapping turtle Cirbp sequence. SNP at nucleotide (nt) 124 (A->C) occurred within the open reading frame of Cirbp and was a synonymous mutation at the codon for amino acid 21, which coded for serine. Three other SNPs were identified: nt 618 (G->A), 644 (T->C), 802 (T ->A), which were located within the 3'UTR of the snapping turtle Cirbp. I determined the allele frequencies for each SNP from the two populations and thermal regimes and a summary of the allele frequencies is provided in Table 3.2.

Allele Frequencies and ASE for each SNP using mRNA-Seq

Logistic regression showed the allele frequency of SNP 124A>C did not vary significantly between the incubation temperatures, but did vary significantly by population, with the C allele being more common in families of turtles collected from northern Minnesota and the A allele occurring more frequently in families from southern Minnesota (Table 3.3). There was also a significant temperature x population interaction, with the A allele occurring more frequently in the $31^{o}C$ libraries than $26.5^{o}C$ libraries in both the northern and southern populations.

Cirbp expression was significantly influenced by temperature, allelic state (SNP 124A>C), and the temperature x allele interaction (Table 3.4). The A allele was induced by the shift to 31°C, while expression of the C allele did not differ between 31°C and 26.5°C (Figure 3.1).

The allele frequency of SNP 618G>A varied significantly between the two populations and the temperature x population interaction, with the G allele occurring more frequently in families from southern Minnesota and in the 31°C libraries in both populations (Table 3.3). Allele frequencies did not differ between temperatures.

Cirbp expression was significantly influenced by temperature and allelic state (SNP 618G>A) (Table 3.4). Allelic expression was not significantly influenced by the temperature x allele interaction. The shift to 31°C induced expression of both alleles with the G allele being more frequent at both temperatures (Figure 3.2).

The allele frequency of SNP 644T>C varied significantly with temperature and differed between populations, with the T allele being more common in families collected from southern Minnesota than from northern Minnesota (Table 3.3). The allele frequency did not vary significantly by the temperature x allele interaction.

Cirbp expression was significantly influenced by temperature, allelic state (SNP 644T>C), and the temperature x allele interaction (Table 3.4). The T allele was induced by the shift to 31°C, while expression of the C allele did not differ between 31°C and 26.5°C (Figure 3.3).

Allele frequency of SNP 802T>A varied significantly by temperature, population, and the temperature x population interaction (Table 3.3). The T allele was more common

in families collected from northern Minnesota than families collected in southern Minnesota and appeared more strongly induced at 31°C (Table 3.2).

Cirbp expression was significantly influenced by temperature and allelic state (SNP 802T>A), but not by the temperature x allele interaction (Table 3.4). The T allele was induced at 31°C, while expression of the A allele was not affected by the temperature shift (Figure 3.4).

Genetic Association with Sexual Phenotype

I tested whether there was a genetic association between expression of alternative Cirbp alleles and sex determination in an experiment that produced mixed sex ratios. Eggs from 15 clutches were incubated at 26.5°C until embryos reached stage 17.5°C. Eggs were then shifted to 31°C for 2.5 days and shifted back to $26.5\,^{\circ}$C until eggs hatched. This brief exposure to a female-producing temperature produced an overall sex ratio of 75% males. There was significant variation in sex ratios among the 15 clutches (LR $\chi^2 = 85.64$, DF = 1, p < 0.0001), ranging from exclusively females in clutch 36 to exclusively males in clutch 15 (Figure 3.5).

I used TaqMan MGB probes for SNP 124A>C in a quantitative PCR to measure allelic specific expression of Cirbp in embryonic gonads. There was a significant difference in Cirbp expression between different genotypes, with A/A homozygotes having the higher expression and C/C homozygotes having significantly lower expression. There was not a difference in expression of the A and C alleles in heterozygotes (Figure 3.6). This pattern suggests a trans-acting factor may be driving allele specific expression.

I also determined the genotype and sex of each hatchling. I found a significant association between Cirbp genotype and the sex of hatchlings (LR χ^2 = 10.42, DF = 2, p = 0.0055). I found that 69.7% of A/A homozygotes were males, 73.6% of A/C heterozygotes were males, while 100% of C/C homozygotes were males (Figure 3.7). However, when I controlled for clutch identity, I found there was not a significant association between Cirbp genotype and sex (Cochran-Mantel-Haenszel χ^2 = 4.65, DF = 2, p = 0.0976). This again suggests that a trans-acting factor may be driving allele specific expression and the genetic association between Cirbp and sexual phenotype.

Because there was a significant association between hatchling genotype and hatchling sex, but not when controlling for variation between clutches, I determined if there was a correlation between SNP frequency and sex ratio among families. There was a significant positive correlation between the number of Cirbp C alleles in a family and the family sex ratio (Spearman's rho = 0.501, r = 0.49, p = 0.0044), but only 24 percent of the variance was explained by this relationship. In general, families with a higher frequency of Cirbp C alleles had more male-biased sex ratios (Figure 3.8).

Although clutches collected for this study spanned a relatively small portion of the latitudinal distribution of this species, we tested whether there was any relationship between latitude and sex ratios. There was a significant correlation between the latitude where individual clutches were collected and sex ratios (Spearman's rho = 0.575, r = 0.40, p = 0.0014), but only 16 percent of the variance was explained by this relationship (Figure 3.9). The clutches collected from slightly more southern latitudes produced mixed sex ratios, with a tendency to be more female-biased. Clutches that were collected from more northern latitudes had more male-biased sex ratios. Despite the correlation

between allele frequency and sex ratio among clutches and latitude and sex ratio, there was not a significant relationship between allele frequency and latitude (Spearman's Rho = 0.147, r = 0.041, p = 0.4548).

<div align="center">Discussion</div>

Genetic association studies have become widely used to provide links between candidate genes and complex disease phenotypes (Hirshhorn and Daly, 2005), but association studies can also be useful for linking genetic variants to any common phenotype, including sex. I previously identified Cirbp as a strong TSD candidate gene. To the best of our knowledge, this is the first study to 1) identify variation in the coding sequence of a thermo-sensitive gene, 2) report temperature-dependent expression of alternative alleles, and 3) detect a significant genetic association between a TSD candidate gene and gonadal sex. Taken together, these results strongly suggest that Cirbp plays a functional role in TSD. In other words, Cirbp may be involved in transducing temperature into a molecular signal for testis versus ovary development. The Cirbp A allele at nucleotide 124 is associated with ovarian development, while the Cirbp C allele is associated with testis development. Furthermore, differences in allele frequencies between snapping turtle populations may provide a mechanistic basis for adaptation of each population to their local thermal regime.

I used next generation Illumina sequencing to identify single nucleotide polymorphisms in the snapping turtle Cirbp sequence. For this study, I mapped reads to full-length cDNA sequence that we previously reported (Chapter I). I have yet to determine if alternative splice forms of Cirbp exist in the common snapping turtle. Studies in mammals have demonstrated Cirbp expression is influenced by alternative

splicing (Al-Fageeh and Smales, 2009). Furthermore, studies in *Xenopus* have identified three Cirbp isoforms that are differentially expressed over time and in a tissue-specific pattern (Uochi and Asashima, 1998; Matsumoto et al., 2000; Peng et al., 2000). This opens the possibility that alternative splicing of Cirbp may be implicated in TSD in the snapping turtle, but this was outside of the scope of our study.

I identified a total of four SNPs in the snapping turtle Cirbp transcript. One SNP (124 A>C) is at codon 21 in the open reading frame of Cirbp, while the others were located in the 3' UTR. The 124A>C transversion is a synonymous mutation for serine within the highly conserved RRM. A serine is found at codon 21 in human, alligator and chicken, but not in mice, rat, or Xenopus (Chapter I). In mice and rats, alanine occurs at codon 21 and in Xenopus the amino acid cysteine occurs. The serine at codon 21 is found in a motif that makes it likely to be phosphorylated by kinase ATM (Wong et al., 2007). It is not known whether post-translational modifications play a role in activation of Cirbp under various environmental conditions. Prior studies of Cirbp have reported putative phosphorylation sites outside of the RRM, but the authors suggest that more research on kinases and Cirbp phosphorylation is needed (Yang et al., 2006; Lleonart, 2010).

A latitudinal cline in TSD pattern among snapping turtle populations strongly suggests adaptation to their local thermal regime (Ewert et al., 2005). Populations in northern and southern Minnesota differ in their pivotal temperatures. Incubation of eggs at 28.2^{o}C produces a 50:50 sex ratio in the northern population while 27.8^{o}C produces a 50:50 sex ratio in the southern population (T.Rhen, unpublished results). In this study we also found a significant relationship between latitude and sex ratios for families that were

collected less than 1° latitude away from each other. These medium and small-scale clines are perfectly consistent with the broader, continental scale cline reported by Ewert et al. (2005). If Cirbp is involved in sex determination, I would expect variation in allele frequencies among populations due to local adaptation. I found significant differences in allele frequency between northern versus southern populations in the Illumina study and a significant correlation with latitude in the genetic association study, suggesting that Cirbp may play a role in adaptation to thermal regimes in different locations. Furthermore, allelic specific expression for several SNPs showed differences in temperature sensitivity.

I used one SNP as a marker for testing genetic association with gonad phenotype in a natural population of snapping turtles in Minnesota. The Illumina data allowed identification of an *a priori* candidate, SNP 124A>C, that showed significant differences in expression between male-producing and female-producing temperatures. Furthermore, SNP 124A>C was located in the open reading frame and therefore a likely candidate to be functional in the context of sex determination. Examination of allelic expression for each genotype revealed that homozygotes for the A allele had higher Cirbp expression when shifted to 31°C than homozygotes for the C allele. There was also a significant difference in sex ratio between genotypes, with A homozygotes being more likely to develop ovaries and C homozygotes being more likely to develop testes. Cirbp may therefore play a role in transducing high temperatures into a signal for ovarian development.

Although I found allele specific expression that was associated with sex determination, I do not know whether *cis* regulatory elements or *trans*-acting factors are causing this variation. Analysis of ASE in inbred parental lines and hybrids provides a

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powerful method for dissecting these mechanisms. Although I did not have such information, I was able to compare Cirbp expression in heterozygotes to Cirbp expression in homozygotes.

Cis-regulatory polymorphisms are often in or near the gene being regulated. These elements can affect transcription rate, transcript stability, or other aspects of gene expression. *Trans*-regulatory polymorphisms occur at another locus, which may encode a transcription factor, co-activator, co-repressor, or some other factor that influences expression of the gene being regulated. When variation in expression of a gene is strongly influenced by *cis*-regulatory polymorphisms, alternative alleles are expressed at different levels in heterozygotes, referred to as allelic imbalance. In contrast, when variation in expression is due to *trans*-regulatory polymorphisms, both alleles are expressed at the same level in heterozygotes. There was no difference in expression of different alleles in heterozygotes, suggesting that expression of Cirbp is regulated *in trans*. Although I did not observe allelic imbalance in heterozygotes, this does not prove that a *cis*-regulatory polymorphism does not exist. Rhen et al. are currently breeding adult snapping turtles from various latitudes in Minnesota. This will allow for a more rigorous test for allelic imbalance. We will also be able to compare allele specific expression in heterozygotes versus homozygotes. Such comparisons can reveal additive versus dominance effects in a *trans*-acting factor.

If variation in Cirbp expression is in fact regulated by a *trans*-acting factor, it would indicate polymorphism in an upstream gene in the TSD pathway. One could hypothesize that such a factor only activates Cirbp expression under the appropriate environmental conditions (i.e., at specific temperatures). Studies in other species reveal

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that Tcf transcription factors regulate Cirbp expression (van Venrooy et al., 2008). We have evidence that the shift from 26.5°C to 31°C activates the canonical Wnt signaling pathway, which involves β-catenin and Tcf transcription factors (Rhen, unpublished results).

Other trans-acting factors, like microRNAs (miRNA), could be regulating Cirbp expression. This is important to note because the A to C transversion at nucleotide 124 creates a putative miRNA binding site (data not shown). Although most functional target sites for miRNAs occur in the 3' UTR, there is evidence that some target sites occur in the 5' UTR and open reading frame of mRNAs (Farh et al., 2005; Lewis et al., 2005; Lytle et al., 2007). Recent studies have identified several miRNAs with sexually dimorphic expression patterns in the bipotential gonads of chickens and mice (Bannister et al., 2009; Huang et al., 2010; Cutting et al., 2012). These findings suggest that miRNAs may be a conserved regulatory mechanism for sex determination in amniotic vertebrates.

The genetic association between Cirbp and sexual phenotype could also be explained by codon usage bias and differences in protein expression. Codon usage bias occurs when there is an unequal use of synonymous codons within a species (Grantham et al., 1980; Moriyama, 2003). Variation in codon usage is associated with gene length, GC-content, GC-content at the third nucleotide (referred to as GC3), recombination rate, and density of genes (Duret and Mouchiroud, 1999; Kreitman and Comeron, 1999; Duret, 2000; Marais et al., 2001; Versteeg et al., 2003). Bias has been observed most often in highly expressed proteins involved in cell growth and cell division. Particular codons may be selected for fast responses to environmental changes (Bangoli and Lio, 1995).

Cirbp is temperature-responsive and influences cell growth and division in many species, making it a candidate for codon usage bias. The SNP 124A>C at position 3 of the codon increases the GC content of the gene and the GC3 content. One could speculate that the increase in GC3 makes this codon suboptimal, thereby decreasing the efficiency of Cirbp translation. It is noteworthy that patterns and degrees of codon usage bias can vary by species and among genes within species. For example, the gene copy number for serine tRNAs varies significantly in humans: there are 5 tRNA genes that recognize codon TCA, but no tRNA genes that recognize the codon TCC (Lavner and Kotlar, 2005). In contrast, TCC was the optimal codon and TCA was less optimal in nematodes (Cutter et al., 2006). The possibility of differences in Cirbp translation due to codon usage bias in the snapping turtle warrants further investigation.

This study provides the strongest evidence to date for a TSD candidate gene. However, genetic association alone does not prove causation. Additional studies are required to define the functional role of Cirbp in TSD. For instance, Cirbp target mRNAs could be identified by RNA-binding immunoprecipitation combined with next generation sequencing (RIP-Seq). This data would help us understand how Cirbp may be regulating expression of other genes involved in sex determination. RNA-binding proteins are capable of regulating numerous mRNAs in a coordinated fashion (Keene, 2007). These mRNAs often encode proteins with similar function. This suggests that Cirbp may have multiple targets that regulate ovarian commitment and a post-transcriptional RNA regulon for TSD. However, the ultimate test will involve manipulation of Cirbp expression and phenotypic analysis of gonad development at male and female temperatures. siRNA could be used to knockdown Cirbp expression in bipotential

gonads. Based on results of the current study, I hypothesize siRNA mediated knockdown

of Cirbp at female-producing temperatures would cause sex reversal. I could test whether

a decrease in Cirbp expression leads to an increase in expression of testis-specific genes

like *Dmrt1* and *Sox9* and commitment to testis development. Conversely, over

expression of Cirbp at a male-producing temperature should result in activation of ovary-

specific genes.

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Table 3.1. Summary of the number of Illumina sequencing reads from each population and temperature for the 15,337 Illumina sequencing reads that were mapped to Cirbp or the 153,553,961 reads that were not mapped to Cirbp.

	Temperature(^{o}C)	Population	# of Reads
Mapped to Cirbp	26.5	Southern	2,425
	26.5	Northern	2,953
	31	Southern	5,109
	31	Northern	4,489
Not mapped to Cirbp	26.5	Southern	36,177,000
	26.5	Northern	39,968,446
	31	Southern	40,791,720
	31	Northern	36,616,795

Table 3.2. Summary of the allele frequencies (in number of reads) for the four Cirbp SNPs identified from the 20 Illumina sequencing libraries from each temperature regime and population.

Position (bp)	Temperature (°C)	Population	Allele	Frequency (# of Reads)
124	26.5	South	A	835
124	26.5	South	C	110
124	26.5	North	A	826
124	26.5	North	C	253
124	31	South	A	1264
124	31	South	C	201
124	31	North	A	1123
124	31	North	C	222
618	26.5	South	G	487
618	26.5	South	A	194
618	26.5	North	G	474
618	26.5	North	A	379
618	31	South	G	1103
618	31	South	A	521
618	31	North	G	978
618	31	North	A	653
644	26.5	South	T	510
644	26.5	South	C	77
644	26.5	North	T	629
644	26.5	North	C	135
644	31	South	T	1347
644	31	South	C	137
644	31	North	T	1135
644	31	North	C	219
802	26.5	South	T	107
802	26.5	South	A	106
802	26.5	North	T	193
802	26.5	North	A	64
802	31	South	T	340
802	31	South	A	196
802	31	North	T	391
802	31	North	A	128

Table 3.3. Logistic regression model of the influence of temperature and population on the allele frequencies for snapping turtle Cirbp single nucleotide polymorphisms (SNP). Significant terms in the model at p ≤ 0.05 are bolded.

Cirbp SNP	Source	LR χ^2	df	p
	Temperature	2.33	1	0.1272
124 A>C	**Population**	**43.52**	**1**	**<0.0001**
	Temperature x Population	**14.96**	**1**	**0.0001**
	Temperature	0.0056	1	0.9401
618 G>A	**Population**	**63.96**	**1**	**<0.0001**
	Temperature x Population	**7.14**	**1**	**0.0075**
	Temperature	**6.59**	**1**	**0.0102**
644 T>C	**Population**	**27.00**	**1**	**<0.0001**
	Temperature x Population	2.21	1	0.1370
	Temperature	**5.30**	**1**	**0.0214**
802 T>A	**Population**	**47.53**	**1**	**<0.0001**
	Temperature x Population	**4.82**	**1**	**0.0282**

Table 3.4. ANOVA model of the influence of temperature and allele on the allelic specific expression for each snapping turtle Cirbp single nucleotide polymorphisms (SNP). Significant terms in the model at p ≤ 0.05 are bolded.

Cirbp SNP	Source	*df*	*F*	*p*
	Temperature	**1**	**21.139**	**0.0100**
124 A>C	**Allele**	**1**	**400.884**	**<0.0001**
	Temperature x Allele	**1**	**14.708**	**0.0185**
	Temperature	**1**	**43.139**	**0.0028**
628 G>A	**Allele**	**1**	**24.970**	**0.0075**
	Temperature x Allele	1	3.545	0.1328
	Temperature	**1**	**94.912**	**0.0006**
644 T>C	**Allele**	**1**	**409.924**	**<0.0001**
	Temperature x Allele	**1**	**60.491**	**0.0015**
	Temperature	**1**	**18.033**	**0.0132**
802 T>A	**Allele**	**1**	**15.645**	**0.0167**
	Temperature x Allele	1	4.460	0.1023
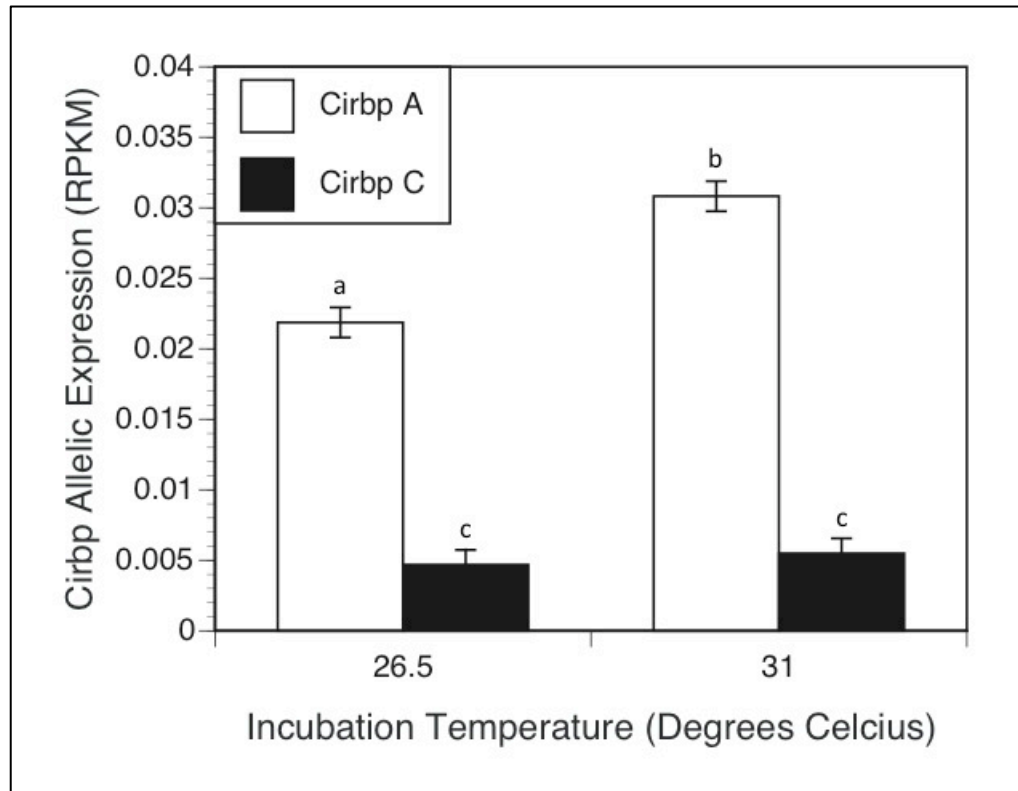
Figure 3.1. Allele specific Cirbp expression in reads per kilobase per million mapped reads (RPKM) at male-producing (26.5^{o}C) or female-producing (31°C) temperatures. Expression levels are least square means ($\pm$ SE) for each temperature regime and allele (SNP 124A>C). Different letters indicate a significant ($p \leq 0.05$) difference in Cirbp allelic expression between incubation temperatures and alleles based on the Dunn-Sidak post hoc test.
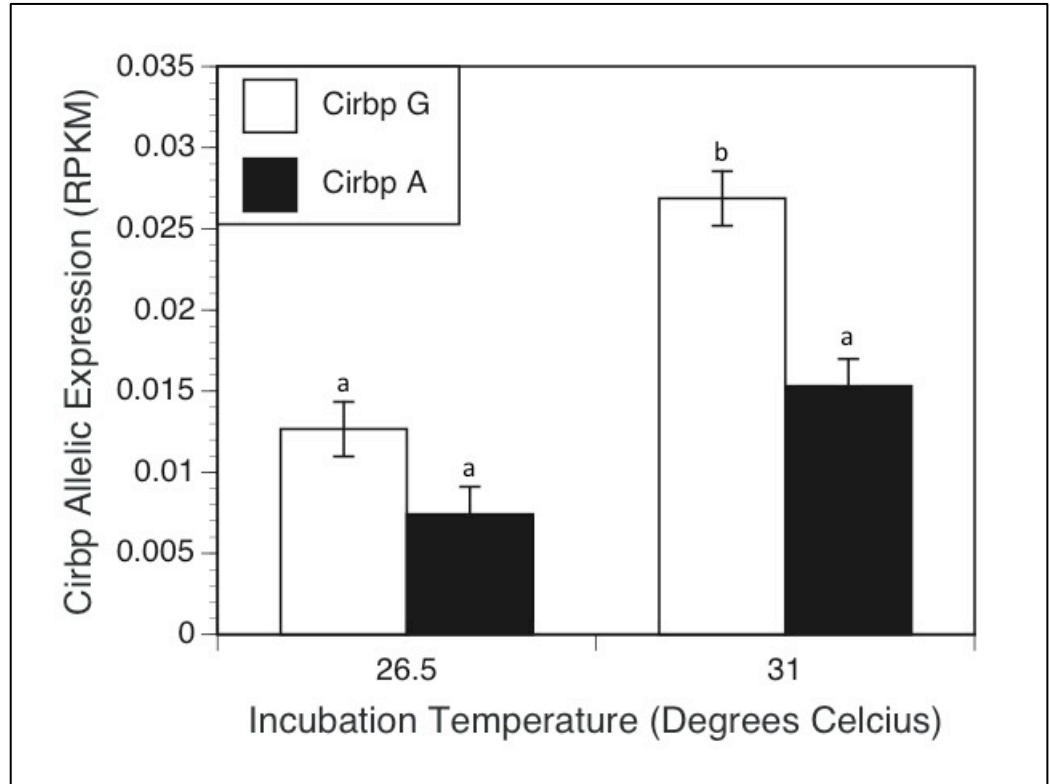
Figure 3.2. Allele specific Cirbp expression in reads per kilobase per million mapped reads (RPKM) at male-producing (26.5^{o}C) or female-producing (31°C) temperatures. Expression levels are least square means ($\pm$ SE) for each temperature regime and allele (SNP 618G>C). Different letters indicate a significant ($p \leq 0.05$) difference in Cirbp allelic expression between incubation temperatures and alleles based on the Dunn-Sidak post hoc test.
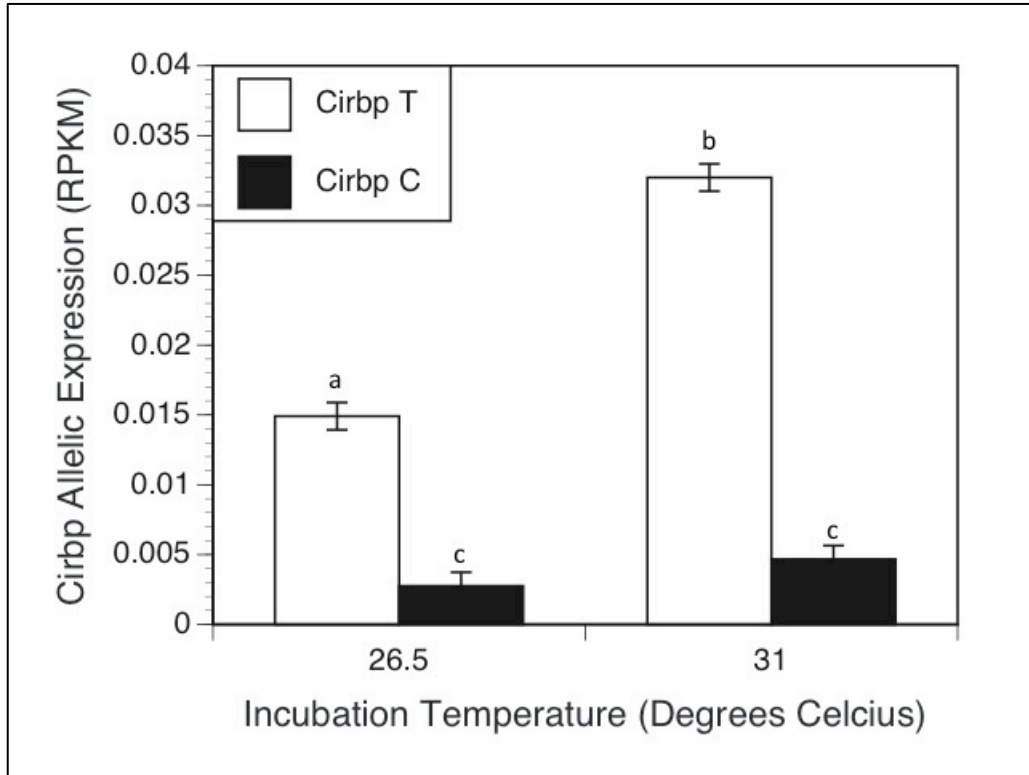
Figure 3.3. Cirbp expression in reads per kilobase per million mapped reads (RPKM) at male-producing (26.5°C) or female-producing (31°C) temperatures. Allelic expression levels are least square means ($\pm$ SE) for each temperature regime and allele (SNP 644T>C). Different letters indicate a significant ($p \leq 0.05$) difference in Cirbp allelic expression between incubation temperatures and alleles based on the Dunn-Sidak post hoc test.
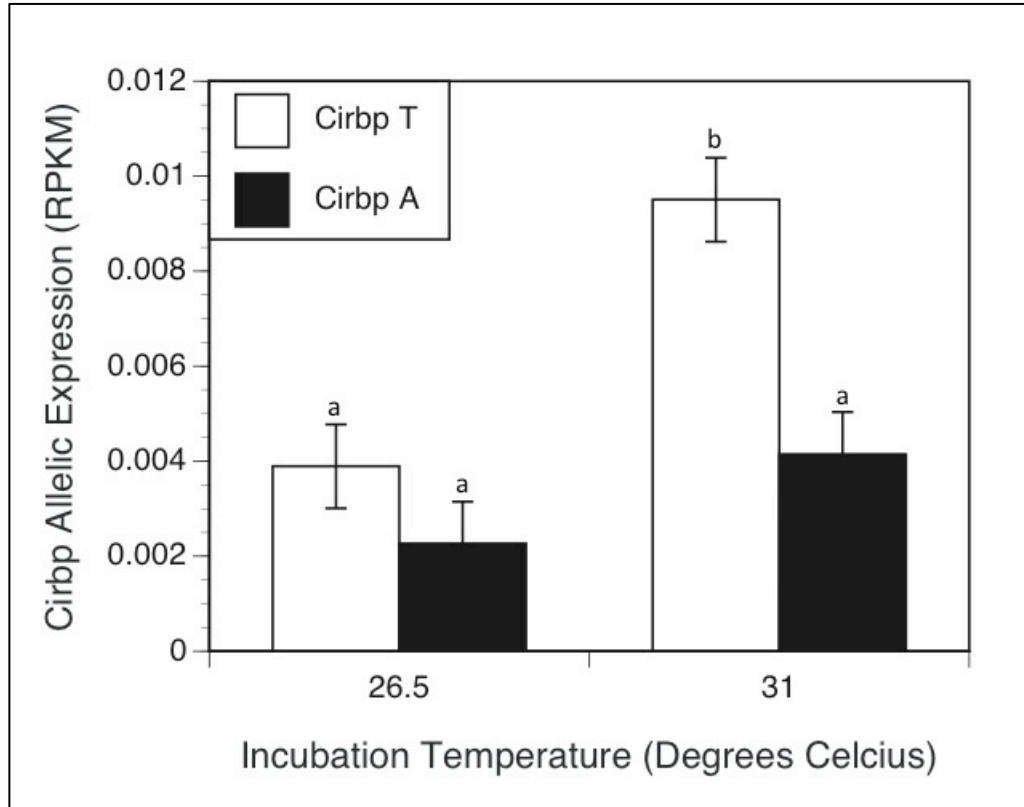
Figure 3.4. Cirbp expression in reads per kilobase per million mapped reads (RPKM) at male-producing (26.5^{o}C) or female-producing (31°C) temperatures. Allelic expression levels are least square means ($\pm$ SE) for each temperature regime and allele (SNP 802T>A). Different letters indicate a significant ($p \leq 0.05$) difference in Cirbp allelic expression between incubation temperatures and alleles based on the Dunn-Sidak post hoc test.
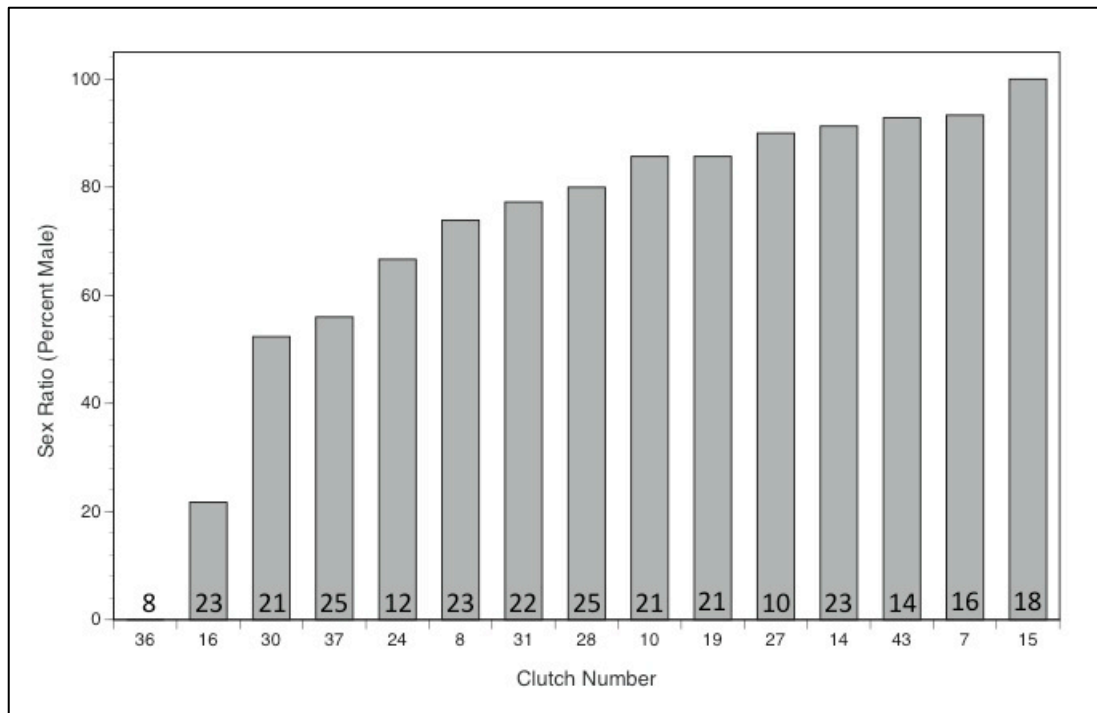
Figure 3.5. The sex ratios produced (in percent males) for 15 clutches that were shifted for 2.5 days from a male-producing (26.5°C) to a female-producing (31°C) temperature at stage 17.5.
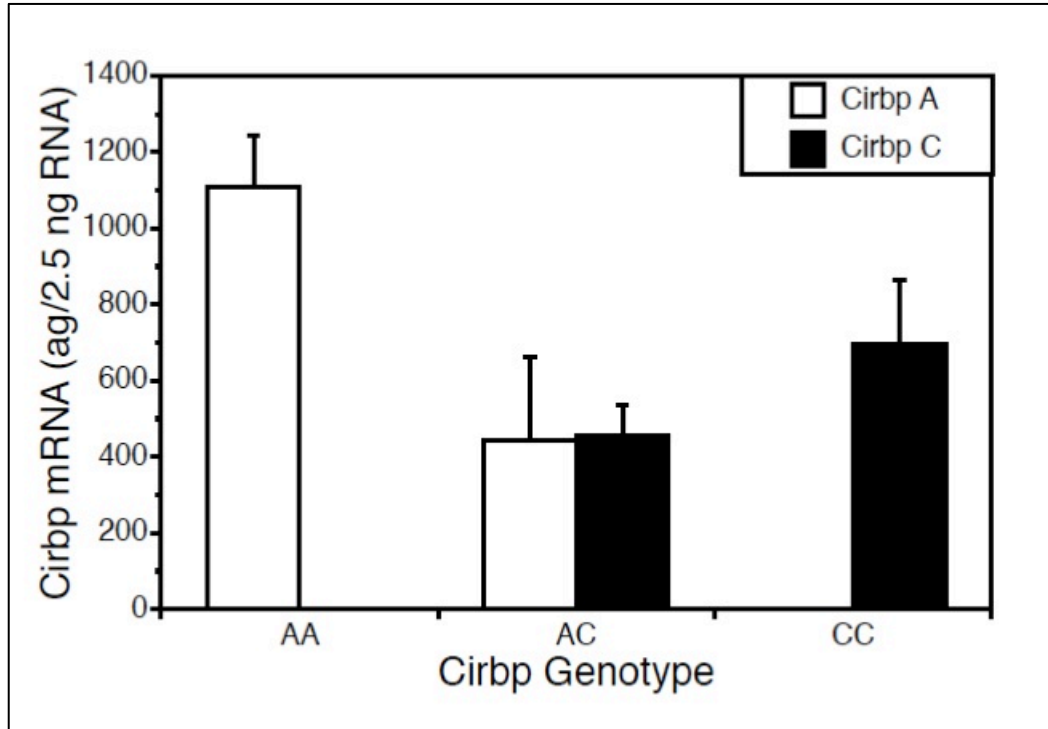
Figure 3.6. Average expression of different Cirbp genotypes AA (n = 129), AC
(n = 53), and CC (n = 15) for SNP 124A>C in gonads from snapping turtle
embryos incubated at 31°C for 24 and 48 hours. Levels of Cirbp mRNA are least
square means ($\pm$SE) for each genotype.
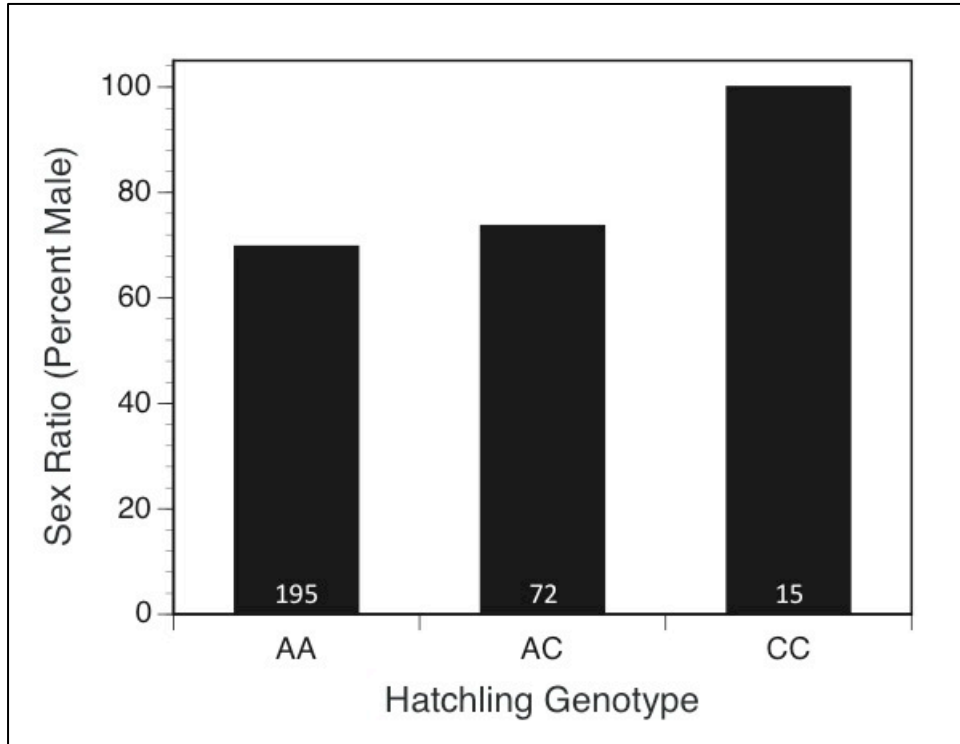
Figure 3.7. The overall sex ratio from hatchlings from each clutch for each Cirbp genotype for SNP 124A>C. The total number of hatchlings with each genotype is presented in each column.
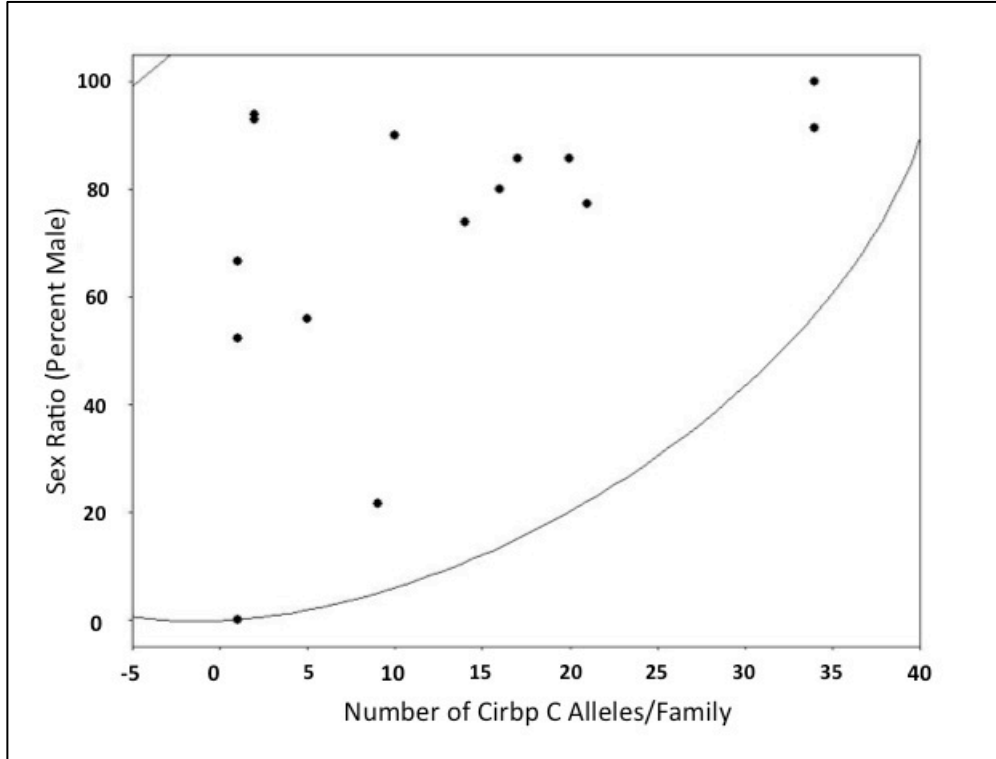
Figure 3.8. Scatterplot of the relationship between the number of Cirbp C alleles and the sex ratio produced (in percent male) in each snapping turtle family. The ellipse around the data represents the 95% confidence band.
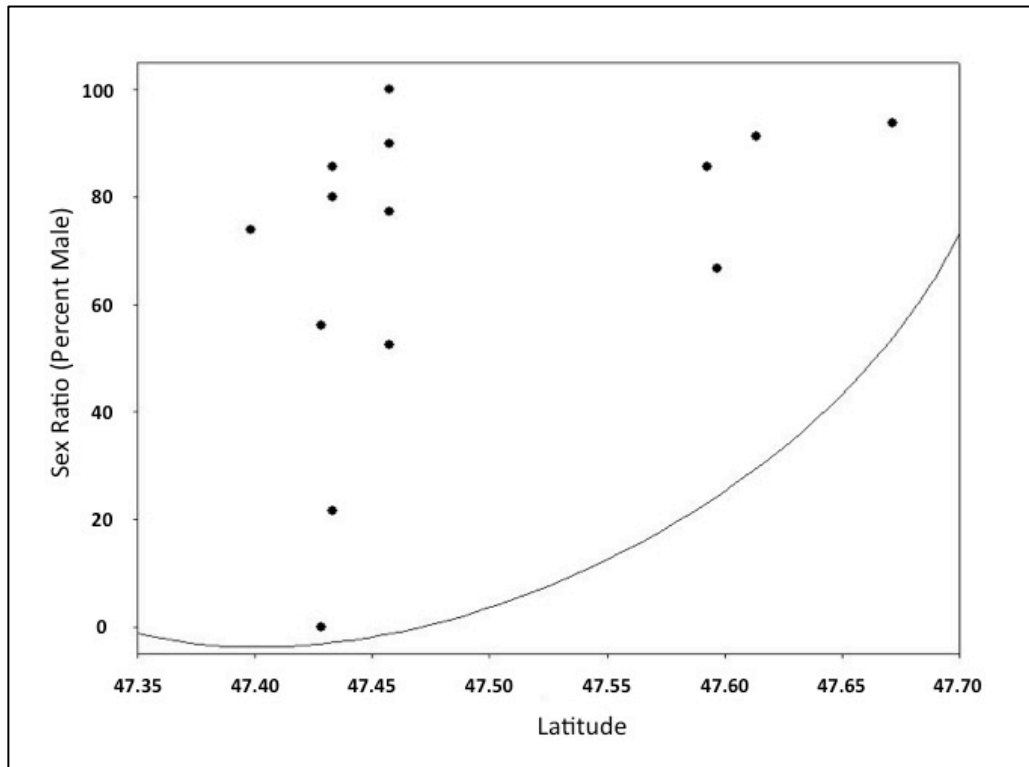
Figure 3.9. Scatterplot of the relationship between the latitude clutches were collected at and the sex ratio produced (in percent male) within each clutch. The ellipse around the data represents the 95% confidence band. Clutch 43 was not included in the figure due to the extreme southern latitude at which the clutch was collected.

CHAPTER IV

EFFECTS OF DIHYDROTESTOSTERONE AND FLUTAMIDE ON THE DEVELOPMENT OF THE MALE REPRODUCTIVE TRACTS IN THE COMMON SNAPPING TURTLE, A REPTILE WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

Abstract

The development of the male reproductive tracts is essential for reproduction and fertility in vertebrates. The Wolffian ducts are the embryonic structures that give rise to the male reproductive tracts in mammals, birds and reptiles. It is well established that androgens are critical for the stabilizing the Wolffian ducts and preventing their regression in males. Although androgens are critical, many of the morphological and molecular mechanisms of androgen signaling in the Wolffian ducts are not known. In this study, I administered a non-aromatizable androgen (DHT), flutamide (an anti-androgen) or a vehicle control to snapping turtle eggs to determine when androgens are stabilizing the Wolffian ducts. I used whole mount *in situ* hybridization to examine how these treatments affected androgen receptor mRNA expression after sex had been determined. I also used immunohistochemistry to examine cell proliferation and apopotosis in the Wolffian ducts. Design-based stereology was performed to provide unbiased measures of androgen-dependent morphological changes in the Wolffian ducts. Androgen receptor mRNA was present in the Wolffian ducts at stages 22 and stages 25 of embryonic development of the snapping turtle, indicating the ducts are stabilized before

hatching. I did not observe any differences in cell proliferation or apoptosis between the different treatments. I observed the development of ovaries in many of the embryos that were treated with DHT or flutamide, although the embryos were incubated at an all-male producing temperature. Although these results provided little information about Wolffian duct stabilization in the snapping turtle, it suggests treatments with DHT or flutamide are capable of producing females in snapping turtles incubated at an all-male producing temperature.

Introduction

Normal development and differentiation of reproductive tracts is essential for reproduction and fertility in vertebrates. In males, the reproductive tracts are derived from tissues known as Wolffian ducts (WDs), while female reproductive tracts are derived from tissues known as Müllerian ducts (MDs) (Drews, 2000; Kobayashi and Behringer, 2003; Hannema and Hughes, 2006). The reproductive tracts arise from the intermediate mesoderm early in development as a pair of straight tubules that develop in both sexes. After sex determination, the developing testes will secrete two hormones, anti-Müllerian hormone (AMH) and androgens, which are necessary for normal development of the male reproductive tracts. Sertoli cells in the testes secrete AMH, which binds to its receptor on the MDs and causes the MDs to regress (Jost 1947; Blanchard and Josso, 1974). The testes will also secrete androgens that stabilize the WDs and prevent their regression (Jost, 1970; Capel, 2000; Renfree et al., 2009). In females, developing ovaries do not produce androgens or AMH, so the MDs remain and differentiate into the mature female reproductive tract, while the WDs regress (Huhtaniemi, 1994; Kobayashi and Behringer, 2003; Yin and Ma, 2005). After

androgen-dependent stabilization, the WDs undergo significant morphological changes including tubular elongation and coiling that lead to the differentiation of various regions of the male reproductive tract, the epididymis, vas deferens, and in mammals, the seminal vesicles (Wilson et al., 1981; Hannema and Hughes, 2007; Wilhelm et al., 2007). Despite the importance of the male reproductive tracts for proper reproduction, limited research has been conducted in mammals and almost nil in other species (see Joseph et al., 2009). The cellular and molecular processes associated with androgen-dependent WD stabilization remain unclear in any species.

Secretion of testosterone and expression and activation of the androgen receptor (AR) are necessary for stabilization of the WDs. Testosterone is produced and secreted by Leydig cells within the testes. Testosterone passes into the lumen of seminiferous tubules and moves down the WD by diffusion (Tong et al., 1996). Testosterone can bind to AR directly or testosterone can be modified in some cell types to the more potent androgen, dihydrotestosterone (DHT), by the enzyme 5-alpha-reductase (Andersson et al. 1989). AR is a transcription factor and member of the nuclear hormone receptor family. The members of this family are activated by the binding of steroid hormones to their receptor and influence a variety of cellular events, including cell growth, differentiation, and tissue development (Mangelsdorf et al., 1995; Ralff et al., 1995). Once activated, AR moves to the nucleus, binds to androgen responsive elements in target genes and influences their expression (Claessens et al., 2008). AR binds both testosterone and DHT with high specificity and affinity, but DHT is more capable of stabilizing the receptor because it dissociates less readily (Grino et al., 1994). In mammals, DHT has been found to have little effect on the early stages of stabilization, but is important for the

differentiation of the Wolffian ducts (Bentvelsen et al., 1995; Dean et al., 2012). It is unknown if DHT may be involved in earlier stabilization or only in the differentiation of the WD in other species. AR is first expressed in the WD stroma and then at a low level within the epithelium of the WD (Majdic et al., 1995; Bentvelsen et al., 1995). Studies suggest that AR expressing cells in the stroma are the primary target of androgens and the proliferation and differentiation of epithelial cells occurs through stromal-epithelial interactions. The proliferation and differentiation of the epithelial cells lead to the formation of the functional regions of the male reproductive tract (Dyche, 1979; Cunha et al., 1992; reviewed in Archambeault et al., 2009).

While perturbations at any stage of sexual development can lead to infertility, the stabilization of the WDs by androgens is particularly important, because genetic mutations or exposure to endocrine-disrupting chemicals could interfere with androgen signaling. In humans, patients with loss-of-function mutations in AR have shown an incomplete development or a lack of WD-derived structures and often the female phenotype is displayed in adults (Brinkmann, 2001; McPhaul, 2002). AR knockout mice have also shown that males have incomplete or absent epididymis, vas deferens, or seminal vesicles (Yeh et al., 2002). Studies administering anti-androgens, like flutamide, in rats have shown incomplete or even a lack of WD-derived structures in adults, but only at high doses (Imperato-McGinley et al., 1992; Welsh et al., 2006). Flutamide is a synthetic endocrine-disrupting chemical that competes with testosterone and DHT for binding to AR, but prevents AR activation and stabilization of the WDs. Studies using flutamide have established the timing of WD stabilization in embryonic mammals, because exposure to anti-androgens during a specific developmental window leads to

abnormalities in the WD-derived structures (Welsh et al., 2006; Welsh et al., 2007). The WDs are stabilized between embryonic days 15.5 and 17.5 and differentiate between 19.5 and 21.5 in mice and rats (Welsh et al., 2007). However, it is unknown when the WDs are stabilized in other species or whether this process is androgen-dependent.

The goal of this study was to determine when WDs in the common snapping turtle are stabilized by androgens. The snapping turtle is a long-lived species that uses temperature-dependent sex determination and has been a model for studying the effect of endocrine-disrupting chemicals in nature (de Solla et al., 2008; Kelly et al., 2008; Eisenreich et al., 2009). Endocrine-disrupting chemicals can be passed to the offspring by maternal transfer or the egg can be exposed to exogenous factors after laying. This exposure can have adverse affects on androgen signaling that can influence the sex and development of the WDs in the snapping turtle. In this study, I exposed turtles to DHT and flutamide by topical dosing of eggs to determine their effects on WDs during embryonic development. This study provides the foundation needed to begin exploring the molecular mechanisms of androgen signaling in WD stabilization in non-mammalian vertebrates.

<div align="center">Materials and Methods</div>

<div align="center">*Egg Collection, Incubation, and Hormone Treatments*</div>

Animal experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol #0905-1). Eggs were collected within 24 hours of laying from 8 snapping turtles nests throughout Minnesota in early June of 2010. Clutch sizes ranged from 29 to 76 eggs. Eggs were transported to the animal quarters in the Biology Department at the

<div align="center">122</div>

University of North Dakota and kept at 20°C for less than one week. Eggs were washed in tepid water, candled for embryo viability and infertile eggs removed from the study. Equal or approximately equal numbers of viable eggs from each clutch were randomly assigned to one of three treatment groups to control for clutch effects. Eggs were placed in containers filled with moist vermiculite and containers were randomly positioned within foam box incubators as previous described (Rhen and Lang, 1994). Eggs were incubated at 26.5°C, which produces males in this population (Rhen and Lang, 1994; Ewert et al., 2005). Prior to hormone treatments, eggs from each clutch and treatment group were randomly sampled to determine the developmental stage of the embryos (Yntema, 1968). Eggs were candled again for viability and eggs containing dead embryos were eliminated from the study.

Stage 17 is the middle of the temperature-sensitive period and is considered the stage when embryos are most sensitive to temperature to produce a sex in this species (J. Lang, unpublished data). Hormone manipulations were performed at stage 17 of embryonic development to determine if DHT or flutamide had any influence on development of the WDs at stages after sex determination.

Treatment groups included one vehicle-treated (ethanol group) only and two hormone groups, either DHT or flutamide. Eggs in the vehicle (control) treatment received a single dose of 5 µl of 95% ethanol. Eggs from the experimental groups received a single 50 µg dose of dihyrotestosterone dissolved in 5 µl 95% ethanol or a single 100 µg dose of flutamide dissolved in 5 µl 95% ethanol. Dosages chosen for each chemical were based on previous studies with turtles (Rhen and Lang, 1994; Rhen and Schroeder, 2010). All solutions were topically applied to the vascularized upper surface

of the eggshell as previously described (Crews et al., 1991). After receiving treatments, all eggs were returned to incubators at a constant male-producing temperature of 26.5^{o}C. Only a subset of the treated eggs was sampled for this experiment.

Tissue Collection, Processing, Sectioning, and Histology

I sampled embryos from each treatment group at stages 20 through 26, which spans from the end of the temperature sensitive period to hatching in the snapping turtle (Yntema, 1968). For this study, between 6 and 8 embryos from each stage and treatment group were opened and embryos quickly euthanized via decapitation. The adrenal-kidney-gonad (AKG) complex, containing the WDs, was dissected and fixed in 4% paraformaldehyde-PBS solution. Tissue was fixed overnight at 4°C and then rinsed in PBS. Between three and five samples from each treatment and stage were saturated through an increasing sucrose gradient (10-30%), embedded in OCT, and stored at -80°C until the blocks were ready to be sectioned. The remaining samples were dehydrated through a methanol gradient and stored in 100% methanol at -20°C until they were processed for whole-mount *in situ* hybridization.

OCT blocks were serially sectioned at 20 μm and 10 sections were mounted on 20 Histobond slides (VWR, Radnor, PA). Slides were stored at -80°C until they were used for immunohistochemistry, TUNEL assays, hematoxylin and eosin staining, and stereology. I examined the morphological development of the gonads and reproductive tracts from each individual.

Whole-Mount In Situ Hybridization (WISH)

A 333 base pair fragment of the snapping turtle androgen receptor was generated by PCR using cDNA from embryonic gonads incubated at a male-producing temperature.

PCR primers for riboprobe generation were:

Forward 5'ATGACAACAACCAGCCTAACTCCT-3'

Reverse 5'- TGATTTGAAGCCACCCGAACTCCT-3'

I cloned the PCR product into the pCR4-TOPO plasmid using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). I amplified the AR insert by PCR using M13 Forward and M13 Reverse primers. The identity of the PCR fragment and the fragment orientation within the plasmid were determined by sequencing. The amplified AR product was used as template to synthesize digoxigenin-labeled antisense or sense probes with digoxigenin-UTP and T3 or T7 RNA polymerase according to the manufacturer's recommendations (Roche Applied Sciences, Indianapolis, IN). The sense probe was synthesized to use as a negative control in the whole mount *in situ* hybridization.

AKGs used for WISH were removed from -20°C and rehydrated in graded methanol diluted in PBS, 0.1% Tween (PBT).   WISH was performed using standard methods (Streit and Stern, 2001; Wilkinson and Nieto, 1993), with some modifications for our tissues.

Briefly, endogenous peroxidases in AKGs were quenched in 6% H_2O_2 in PBT for 1 hour with rocking. AKGs were washed 3 times in PBT and then incubated in 10 μg/ml proteinase K in PBT for 20 mins at room temperature (RT). Tissues were rinsed twice in PBT, post-fixed at room temperature for 30 minutes in 4% PFA with 0.1% glutaraldehyde, and washed three times for 10 min in PBT. AKGs were incubated in a 1:1 mixture of prehybridization buffer and PBT and then incubated in pre-hybridization buffer for at least 1 hour at 65°C. Prehybridization buffer was removed and hybridization buffer (50% formamide, 5x SSC (pH 4.5), 50 μg/ml yeast tRNA, 100 μg/ml heparin, 5

mM EDTA, 0.2% Tween-20, 0.5% CHAPS) containing 400 ng of AR antisense or sense riboprobes were added to the AKGs and hybridized overnight at 55°C.

After hybridization, the tissues were washed twice in hybridization buffer without the riboprobe for 20 mins each at 65°C. All post-hybridization washes were for 20 mins at 65°C, unless otherwise noted. Tissues were incubated in pre-warmed 1:1 solution of hybridization buffer and 5x saline-sodium citrate, 0.1% Tween (SSCT). Tissues were incubated in graded SSCT washes (5x and 2x), followed by incubation in RNase A (100 μg) and RNase T1 (100U) dissolved in 2x SSC for 30 mins at 37°C. Tissues were then washed twice in prewarmed 2x SSCT, followed by incubation in prewarmed 0.2x SSCT. Tissues were then washed three times for 10 mins each in 1x maleic acid buffer, 0.1% Tween (MABT) at RT.

AKGs were blocked in 2% blocking solution (Roche Applied Sciences) with 10% sheep serum diluted in MABT and incubated for at least 2 hours at RT. AKGs were then incubated overnight at 4°C in blocking solution with preabsorbed anti-dioxigenin-AP antibody (diluted 1:2000, Roche Applied Sciences). The following morning, AKGs were removed from the antibody solution and rinsed three times in MABT, followed by four, 30 min washes in MABT at RT. After MABT washes, AKGs were washed twice for 10 mins in NTMT developing solution (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl$_2$, 0.1% Tween) at RT. AKGs were incubated in NTMT containing 4.5 μl nitro blue tetrazolium (NBT), 3.5 μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), and 2 mM levamisole. AKGs were incubated in the dark and monitored for color development. Color development was stopped by washing AKGs in PBT containing 1 mM EDTA twice for 10 mins each at room temperature. AKGs were post-fixed in 4%

paraformaldehyde with 0.1% gluteraldehyde in PBS for 1 hour at room temperature and then washed three times for 10 mins in PBS. AKGs were dehydrated and rehydrated through graded methanol in PBT to intensify the signal and remove background. AKGs were moved through graded glycerol in PBT. Images were taken with an Olympus SZX-12 dissecting microscope equipped with an Infinity 2 digital camera (Lumenera Corp, Ottawa, ON, CA) using Rincon HD software (Imaging Planet, Goleta, CA).

Immunohistochemistry to Examine Cell Proliferation and Apoptosis

Immunostaining for PCNA was used to assess cell proliferation in the WDs. I also used immunohistochemistry for active caspase-3 to assess apoptosis in the WDs. Frozen sections from each treatment group and stage were removed from -80°C and allowed to air-dry overnight. For proliferating cell nuclear antigen (PCNA) and active caspase-3 immunohistochemistry, nonspecific binding was blocked by incubating slides overnight at 4°C in blocking solution (3% goat serum, 0.1% Triton X-100, 1% bovine serum albumin in phosphate-buffered saline). The slides were removed from the blocking solution and incubated with the primary antibodies diluted in block solution overnight at 4°C. The anti-PCNA primary antibody (Biolegend, San Diego, CA) and the anti-active caspase-3 antibody (Promega Corp, Madison, WI) were diluted 1:250. To ensure specific staining, a negative control slide incubated with block solution instead of the primary antibody was also processed in parallel. Slides were washed three times for 5 min each at room temperature in Dulbecco's PBS. Endogenous peroxidases were quenched by placing slides in 0.3% H_2O_2 in methanol for 15 mins at room temperature. Sections were incubated with the appropriate secondary antibody, either biotinylated goat anti-rabbit secondary antibody for caspase-3 or biotinylated goat anti-mouse for PCNA,

diluted 1:250 in 2% normal goat serum and incubated overnight at 4°C. After washes in

PBS, the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was applied to the

slides and incubated for 30 mins at room temp. Antibody localization was determined by

applying freshly prepared 3,3-diaminobenzidine with nickel (DAB; Vector Laboratories,

Burlingame, CA) to each slide for 5 min and then rinsed in tap water to stop the reaction.

Slides were counterstained with methyl green, dried in ascending alcohol percentages,

cleared in xylene, and coverslipped using a xylene based mounting media. Cellular sites

expressing the PCNA or active caspace-3 were observed and photographed using an

Olympus BX-51 microscope using Rincon HD software (Imaging Planet). Slides from

each stage and treatment group were processed in parallel for both PCNA and active

caspase-3 to allow for reproducibility and accuracy when comparing groups. PCNA-

positive and active caspase-3 positive cells were counted in the epithelium of the WDs as

described below.

TUNEL Assay for Apoptosis

I used terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate

nick end labeling (TUNEL) to assess apoptosis in the WDs. TUNEL assay was

performed according the manufacturer's instructions (Roche Applied Sciences,

Indianapolis, IN) with few modifications. In brief, paraffin-embedded sections

containing the WDs were deparaffinized through xylene, graded ethanol, and washed in

distilled water. Tissue sections were incubated in 10 µg/ml proteinase K in Tris-HCl (pH

7.4) for 30 mins at 37°C. Sections were washed twice in PBS for 10 mins each. We

applied 50 µl of TUNEL reaction mixture containing terminal deoxynucleotidyl

transferase (TdT) and fluorescein-dUTP to each slide and incubated slide in the dark for 2

hours at 37°C. To ensure specific staining, positive and negative control slides were

processed in parallel. The positive control was prepared by treating the slide for 10 mins

with 1500 U/ml Dnase I in 50 mM Tris (pH 7.5), 10 mM MgCl$_2$, 1 mg/ml BSA at room

temperature. The negative control was prepared by omitting TdT from the slide. Slides

were washed three times in PBS for 10 mins each, followed by the addition of 50 µl of

anti-fluorescein with alkaline phosphatase and incubated for 30 mins at 37°C. Slides

were washed three times for 10 mins in PBS and developed using BM Purple (Roche

Applied Science). Slides were coverslipped and photographs were taken from an

Olympus BX-51 microscope equipped with an Infinity 2 digital camera (Lumenera

Corp.) using Rincon HD software (Imaging Planet).

Quantification of PCNA-positive cells using Design-based Stereology

The unbiased quantification of PCNA-positive nuclei was performed using

design-based stereology. Sections from three or four individuals in each treatment group

at each stage were used for counting. PCNA-positive and total cells were counted using

an Olympus BX-51WI microscope with motorized X, Y, and Z stage. Unbiased

quantification of nuclei was performed using the optical fractionator workflow in

StereoInvestigator 9.0 (Microbrightfield Inc., Wiliston, VT).

In each individual, PCNA-positive and total nuclei were counted from 2-5

randomly aligned and systematically selected frames (each measuring 15x15 µm) in

every 20th section using the 40x objective. For PCNA counting, the contour outlined the

epithelium of the WD only and if the efferent ducts were observed in the section, they

were not included in the contour. In brief, the number and location of counting frames

and the counting depth for each section were determined by entering parameters for the

grid size (50 x 50 μm), the thickness of top guard zone (5%) and the optical dissector

height (13 μm). The grid angle was randomly placed on the tracing for each section. The

guard zones were set at 2 μm above and below the counting depth for each section with

regional thickness and variation in section integrity quantified at each count site. The

PCNA-positive nuclei were counted if they were entirely within the 13 μm depth

counting. The StereoInvestigator software calculated the total number of PCNA-positive

cells per WD by utilizing the optical fractionator formula (N=1/ssf.1/asf.1/hsf.ΣQ^-) where

ssf = section sampling fraction (20), asf = area sampling fraction (area sampled/total

Wolffian duct area), hsf = height sampling fraction (counting frame height/section

thickness (20 um)), and ΣQ^- (total particle count). The sampling was optimized for

maximal efficiency, with a final mean coefficient of error (CE) of less than 10% for each

of the two coefficients of error calculations. I used both Gundersen CE, due to the

sampling of three or more sections, as well as the Schmitz-Hof CE as an alternative

means of confidence estimation (Gunderson and Jensen, 1987; Schmitz and Hof, 2000).

Statistical Analysis

I used JMP 5.0.1.2 software for all statistical analyses (SAS Institute, Cary, NC).

Analysis of variance (ANOVA) was used to determine the effects of clutch, treatment,

and developmental stage on cell proliferation in the WDs. Tests of the residuals for each

ANOVA were performed to determine if the data met the assumptions of normality and

homogeneity of variance. The ratio of proliferating cells to total cells was log10

transformed to meet the assumptions of the ANOVA. Given significant non-treatment

effects ($\alpha < 0.05$), I used the Dunn-Sidak method to correct for multiple comparisons

among groups. Given significant effects of treatment, I used the Dunnett's test to correct

for multiple comparisons and to compare the treatment effects to the vehicle control. I used Fisher's exact tests to determine if there was an association between hormone treatments and the morphological development of the gonads. The null hypothesis was that all males should be produced because the embryos were incubated at a male-producing temperature. Sample sizes for experimental groups are shown in each figure.

Results

Androgen Receptor WISH

I used whole mount *in situ* hybridization to determine when the WDs begin to express AR mRNA and if our treatments had effects on AR expression. I did not observe any staining in the WDs before stage 22 (data not shown). In the vehicle treated group, I observed light staining in the WDs at stage 22 and then again at stage 25 of embryonic development (Figure 4.1A and D, respectively). Staining was stronger at the rostral compared to the caudal end of the WDs. Staining was also observed in the WDs at stage 26 in the vehicle treated group, with staining extending more caudal than at stage 25 (Figure 4.1 E). In the DHT and flutamide treated groups, light staining was observed at stage 22 of development and disappeared thereafter (Figure 4.1 F and K, respectively). Staining increased again at stage 25 in the DHT and Flutamide treated groups, with a rostral expression pattern very similar to the vehicle treated group (Figure 4.1 I and N, respectively). Light staining was still observed in the WDs from the DHT treated group at stage 26 (Figure 4.1 J), but disappeared in the WDs from the flutamide treated group (Figure 4.1 N).

Analysis of Cell Proliferation by PCNA Immunohistochemistry and Stereology

I performed immunohistochemistry for PCNA to test whether hormone manipulations had any effect on cell proliferation in the WDs. I observed PCNA staining within the epithelium of WDs in all treatment groups (Figure 4.2). There was not a significant treatment effect on the number of PCNA-positive cells ($F_{(2,23)}$ = 1.59, p = 0.2101) or the volume of the WDs ($F_{(2,23)}$ = 1.15, p = 0.3326). The vehicle-treated embryos did not have significantly more PCNA-positive cells and larger WD volume than the DHT or flutamide-treated embryos (Figure 4.3). Cell proliferation was significantly influenced by embryonic stage ($F_{(6,23)}$ = 2.79, p = 0.0346) with stages 22 and 25 having the highest number of PCNA-positive cells (Figure 4.4). There were no other significant independent variables or interactions within the model.

Although there was no difference in the total number of PCNA-positive cells, I tested whether the ratio of PCNA positive-cells to total cells within the WDs were altered by the hormone treatments. Hormone treatments did not affect the ratio of PCNA positive cells to total cells in the WD ($F_{(2, 23)}$ = 0.38, p = 0.6871), with approximately 6% of the total cells being PCNA-positive in all groups. Although embryonic stage significantly influenced the number of PCNA-positive cells, it did not influence the ratio of positive cells to total cells ($F_{(6,23)}$ = 0.46, p = 0.8306). Clutch and various interactions were not significant.

Analysis of Cell Death by Caspase-3 Immunohistochemistry and TUNEL Assay

To assess whether treatments had any affect on apoptosis in the WDs, I performed TUNEL assays and immunostained for active caspase-3. I observed very diffuse staining surrounding and within the epithelium of WDs in all of the treatment groups. I observed

staining surrounding the MDs for active caspase-3 and the TUNEL assay in all of the vehicle-treated embryos, indicating that MDs were regressing in these embryos (Figure 4.5 A and D, respectively). I did not observe staining surrounding the MDs in many of the DHT- and flutamide-treated embryos (Figure 4.5 B, C, and E). This indicates that the MDs were not regressing in many individuals from these treatment groups.

Assessment of Gonad Morphology

Because Mullerian ducts appeared to be maintained in the DHT group and the Flutamide group, I decided to look more closely at the morphology of developing gonads. Embryos in the vehicle treated groups were developing testes with distinct medullary sex cords and a regressing cortex (Figure 4.6 A, D, G). To my surprise, many of the DHT-treated embryos and a few of the flutamide-treated embryos had gonads with a thickened outer cortex and a disorganized medulla typical of differentiating ovaries (Figure 4.6 B, I, and C, respectively). Clutches varied in their response to DHT and flutamide-treatments. Most DHT treated embryos from clutch 2 developed ovaries, while all embryos from clutch 9 developed testes. A summary of clutch differences in sex ratio is provided in Table 4.1.

Discussion

Studies in mammals indicate that exposure to anti-androgens, like flutamide, can have a major impact on WD stabilization and differentiation. The goal of this study was to determine whether androgens influence WD development in snapping turtle embryos. In contrast to expectations, there were no obvious effects of DHT or flutamide on cell proliferation or cell death in WDs. However, I observed MDs in many of the DHT and flutamide treated embryos, which suggested that DHT and flutamide either inhibited

AMH secretion from Sertoli cells or influenced sex determination. Closer examination of gonads revealed that DHT induced ovary development in embryos incubated at a male-producing temperature. This is the first study to show that DHT can cause sex reversal in turtles incubated at a male-producing temperature. Previous research in the snapping turtle has shown that DHT increases the number of females at a temperature that normally produces a mixed sex ratio (Rhen and Lang, 1994; Rhen and Schroeder et al., 2010). In contrast, DHT has a masculinizing effect at temperatures that produce mixed sex ratios in the red-eared slider turtle (Crews et al., 1989; Wibbels and Crews, 1992; Wibbels et al., 1992). While DHT induced ovary development at male-producing temperatures is a novel finding, it complicates our analysis of DHT and flutamide effects on WD development.

All vehicle-treated embryos developed testes as expected, thus findings in this group can inform us about normal development of WDs in the snapping turtle. It has been suggested that increased AR expression reflects the period when androgens stabilize WDs (Bentevelsen et al., 1994; 1995). I used whole mount *in situ* hybridization to determine when AR mRNA is expressed in developing WDs in the snapping turtle. I detected AR expression in vehicle-treated embryos at stage 22 and again at stages 25 and 26. I observed stronger staining in the rostral portion of the WD than the caudal portion, which supports the hypothesis that WD stabilization occurs in a rostral to caudal direction (Bentevelsen et al., 1995). The timing of AR expression suggests that stabilization of WDs may happen shortly before or after hatching. Although the pattern of androgen secretion from embryonic testes has not been defined in the snapping turtle, testosterone may diffuse through efferent ducts and produce a concentration gradient extending from

the upper to the lower portion of the WD. I did not observe any changes in WD morphology during embryonic development, indicating that differentiation of the epididymus and vas deferens occurs after hatching. The timing of reproductive tract differentiation is delayed in comparison to mice and rats. Stabilization and differentiation of the WDs occurs before birth in these species. However, my findings do support the hypothesis that WD development is a biphasic process (Welsh et al., 2007). This may be due to developmental differences between species because male mice and rats reach reproductive maturity soon after birth, while it takes many years for male snapping turtles to reach maturity. Further studies are necessary to determine when WDs differentiate in snapping turtle males.

Some of the DHT- and flutamide-treated embryos used for *in situ* hybridization were from clutches that produced male hatchlings. Comparisons between vehicle treated males and DHT or flutamide treated males revealed very similar spatial and temporal patterns of AR expression. This suggests that androgens may not regulate AR expression during embryonic development in the snapping turtle.   AR is first expressed in the stroma and then the epithelium of the WDs in mammals. This may be the result of mesenchymal-epithelial cell interactions involving paracrine factors (Cunha et al., 1992). Furthermore, in mice and rats, the critical period for stabilization occurs before regression of the MDs. Complete regression of MDs occurs by stage 25 in the snapping turtle, indicating that AMH expression is occurring between stages 22 and 25 in differentiating testes.

I tested whether hormone manipulations would influence proliferation or apoptosis of WD epithelial cells. Although DHT and flutamide did not influence cell

proliferation in WDs, I did observe an increase in cell proliferation during stages 22 and 25 of development. This finding supports the idea that there may be critical periods for WD development in the snapping turtle. Hormone manipulations did not affect apoptosis in snapping turtle WDs, which is similar to findings in embryonic rats treated with flutamide (Welsh et al., 2006). It is noteworthy that apoptotic staining was not detected in the WDs of DHT-treated embryos that were developing ovaries. This indicates that WDs were not regressing in females, which is consistent with DHT-induced stabilization of WDs in female rats (Bentevelsen et al., 1995). However, it is also important that we did not detect regression of the WDs in flutamide-treated embryos that developed as females. This suggests that WDs do not regress in the snapping turtle until after hatching. It has yet to be determined when WDs regress in female snapping turtles, but in mammals the WDs regress in the female embryo shortly after commitment to ovarian development (Dyche, 1979; Taguchi et al., 1984; Capel, 2000).

The original goal of this study was to determine the role androgens play in stabilization of the WDs in male snapping turtle embryos. However, I unexpectedly found that DHT treatment (and sometimes flutamide treatment) induces ovary development. Thus, I cannot use this model to study molecular aspects of WD stabilization. Instead, the molecular mechanisms underlying the role of androgens in sex determination and ovarian development is a novel question that needs to be examined in more detail. In addition, the observation that there was variation among clutches in androgen responsiveness indicates there may be a genetic basis for this response. Future studies will examine the role of androgens in temperature-dependent sex determination in

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the snapping turtle. In conclusion, androgens may be essential for Wolffian duct

development in the snapping turtle, but I was unable to determine their role in my study.

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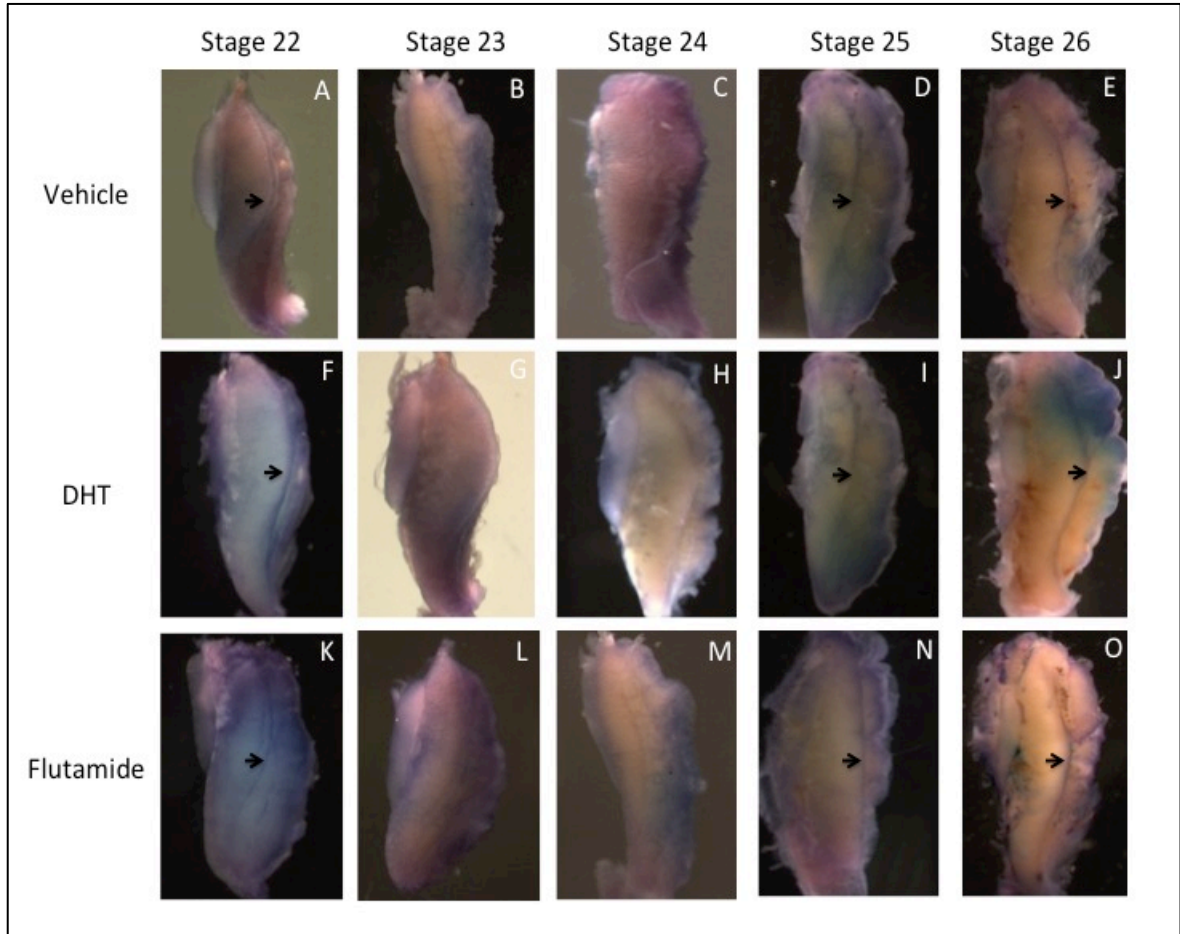
Figure 4.1. Whole mount *in situ* hybridization for androgen receptor mRNA in Wolffian ducts treated with vehicle control (A-E), DHT (F-J), or flutamide (K-O) at stages 22 through 26 of embryonic development. The arrows indicate the staining within the Wolffian ducts.
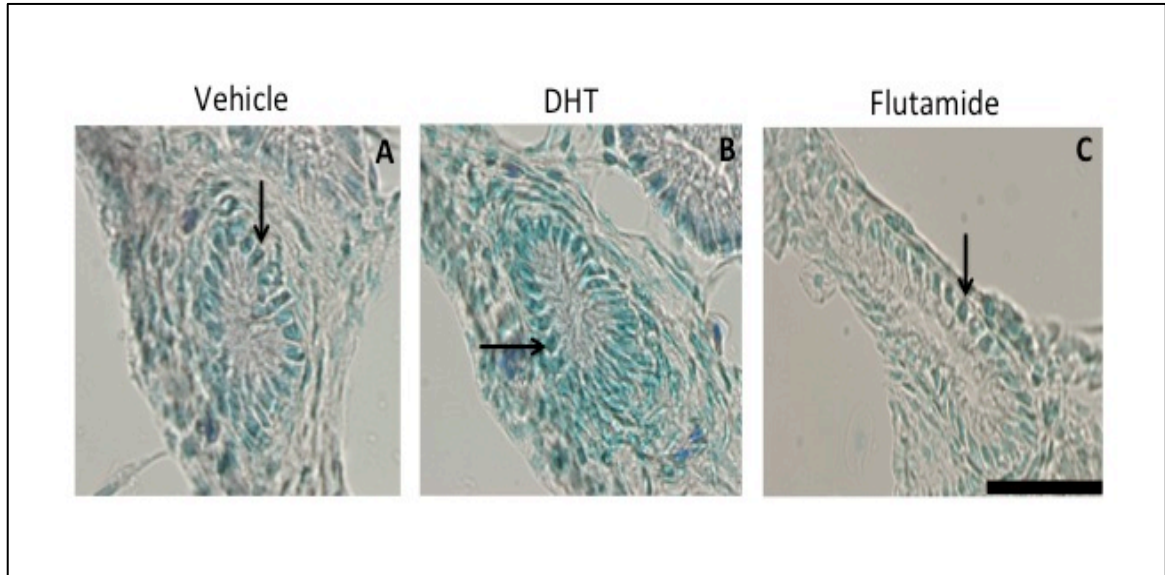
Figure 4.2. Analysis of cell proliferation by immunohistochemistry staining for proliferating cell nuclear antigen (PCNA) in Wolffian ducts from embryos treated with vehicle (A), DHT (B), or flutamide (C). Arrows indicate epithelial cells in the Wolffian ducts labeled for PCNA. Scale bar = 50 µm.
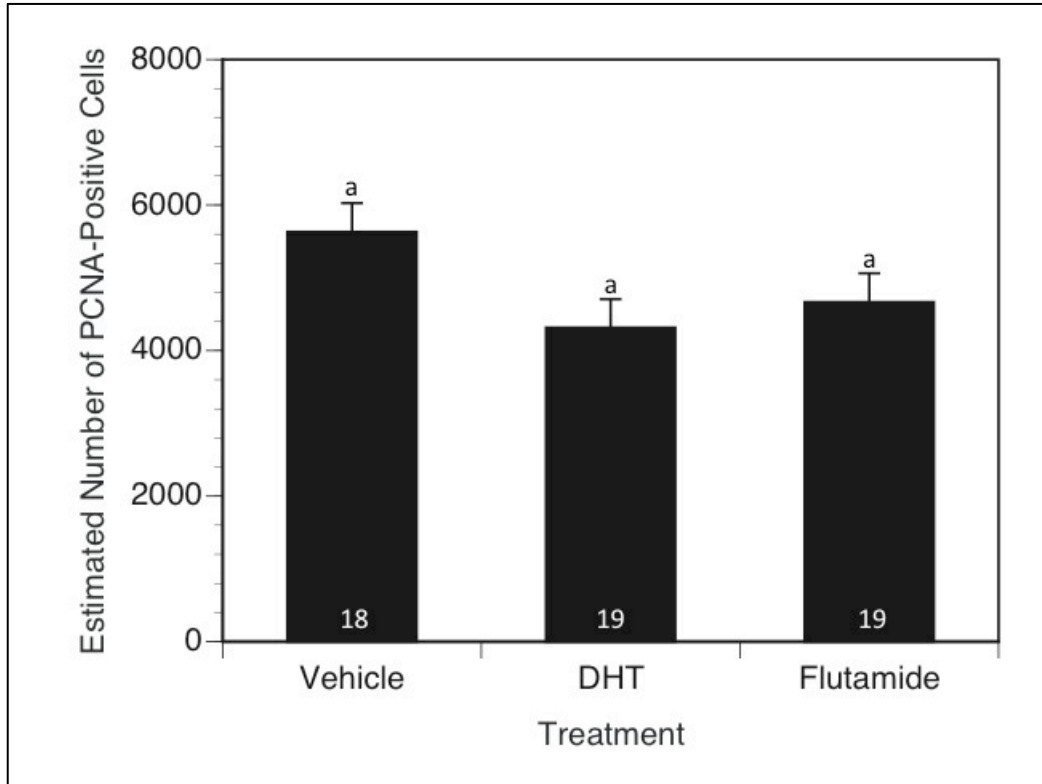
Figure 4.3. Design-based stereological quantification of PCNA-positive cells in Wolffian ducts from embryos treated with vehicle, DHT, or flutamide. Values shown are mean and SEM. Similar letters indicate no significant difference ($p \leq 0.05$) between the vehicle control and the treatment groups based on the Dunnett's post hoc test. Sample sizes for each treatment group are indicated within each column.
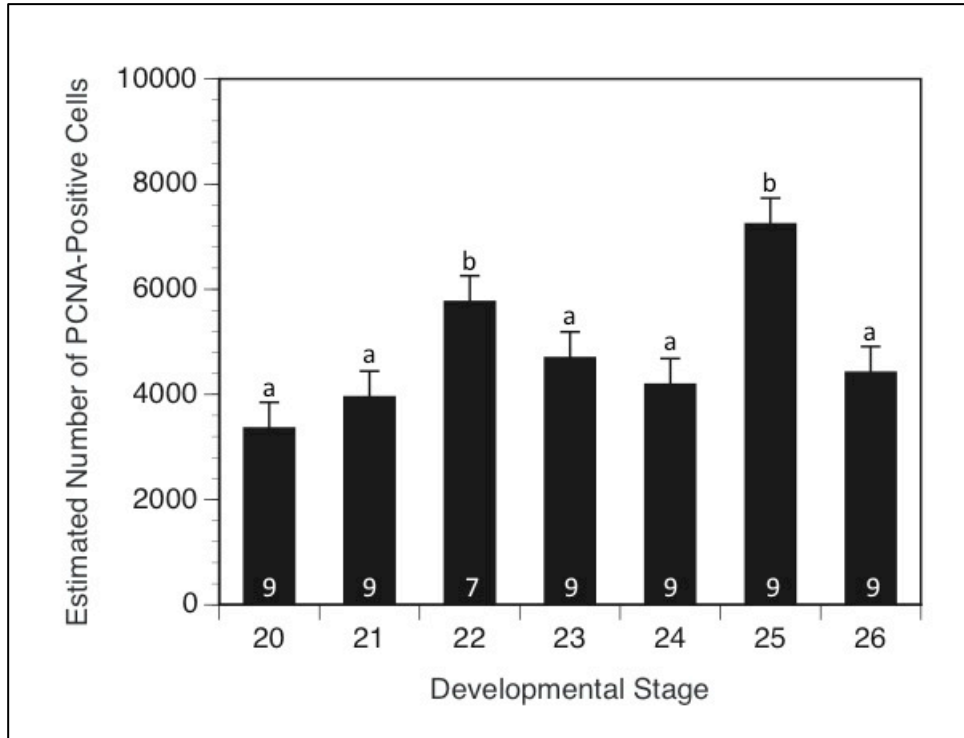
Figure 4.4. Design-based stereological quantification of PCNA-positive cells in Wolffian ducts during developmental stages 20 through 22. Values shown are mean and SEM. Different letters indicate a significant difference ($p \leq 0.05$) between developmental stages based on the Dunn-Sidak post hoc test. Sample sizes for each treatment group are indicated within each column.
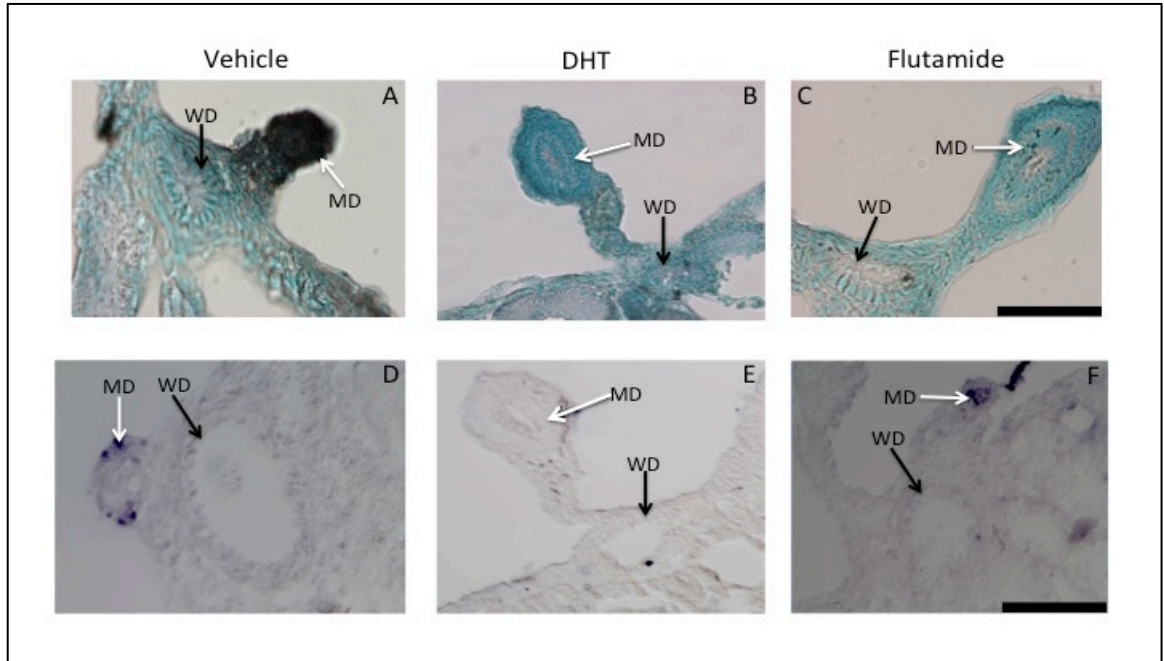
Figure 4.5. Analysis of apoptosis by immunohistochemistry staining for active caspase-3 (A-D) and TUNEL assay (D-F) in Wolffian and Müllerian ducts from embryos treated with vehicle (A, D), DHT (B, E), or flutamide (C, F). White arrows indicate positive staining for caspase-3 (A) or for the TUNEL assay (D and F) in the Müllerian ducts. No positive staining for caspase-3 or TUNEL assay was observed within the Wolffian ducts. MD = Müllerian ducts. WD = Wolffian ducts. Scale bars = 50 μm.
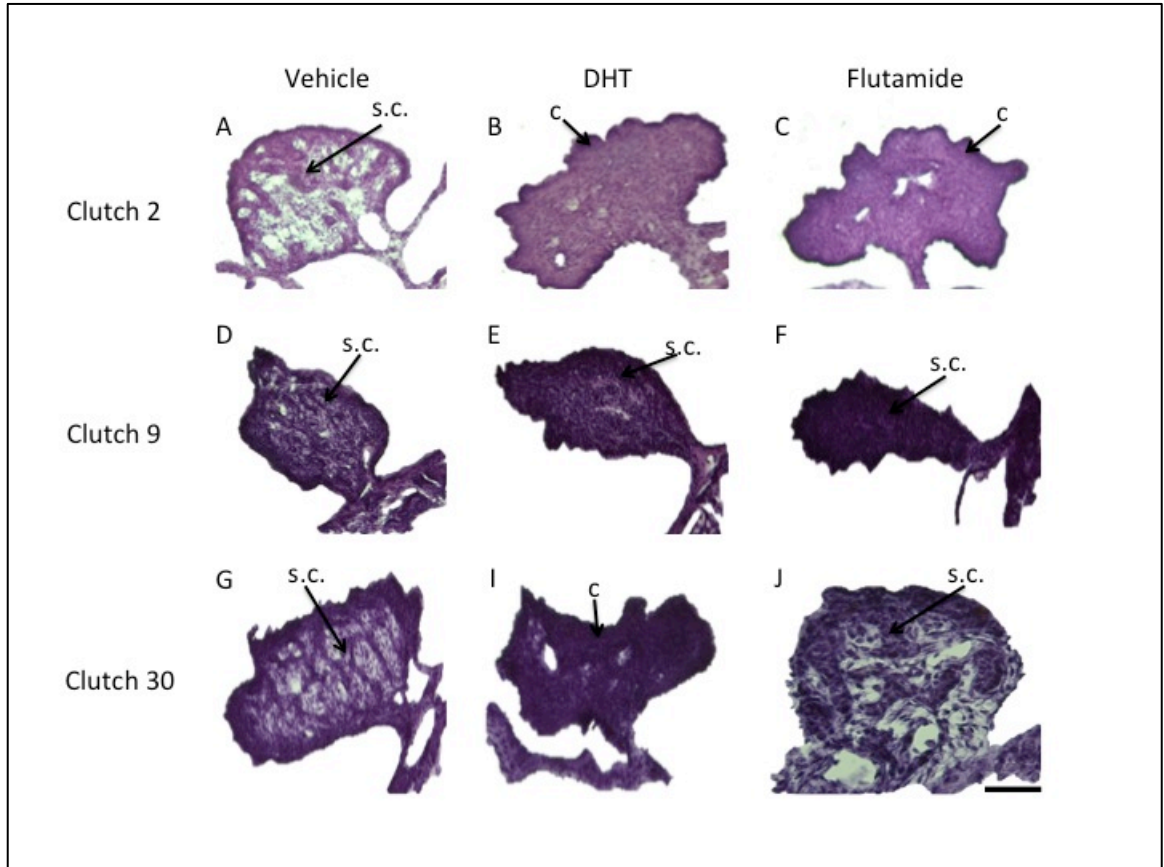
Figure 4.6. Histology of developing gonads from embryos treated with vehicle, DHT, or flutamide from clutches 2 (A-C), 9 (D-F), and 30 (G-J). Embryos treated with vehicle produced testes in all clutches based on the developing medullary sex cords. Embryos treated with DHT produced ovaries in clutches 2 and 30, but not clutch 9, based on the development of the cortex. The embryo treated with flutamide from clutch 2 produced an ovary, but not in the embryos from clutches 9 or 30. s.c. = medullary sex cords. c = cortex. Scale bar = 50 μm.

Table 4.1. Summary of the effects of each treatment on the sex ratios produced (in percent male) in each clutch. The number in parentheses is the total number of embryos sampled from that treatment group. The chi-squared and p-values are based on Fisher's exact test. Bolded p-values indicate significant ($p \leq 0.05$) variation in sex ratio produced by the treatments within each clutch; based on the null hypothesis that all males should be produced due to embryos being incubated at a male-producing temperature.

Clutch	Vehicle	DHT	Flutamide	Chi-squared	p-value
2	100.0 (3)	0.0 (3)	33.33 (3)	8.55	**0.0139**
6	100.0 (3)	33.33 (3)	66.67 (3)	3.92	0.1481
7	100.0 (3)	100.0 (3)	50.0 (2)	2.97	0.2266
9	100.0 (3)	100.0 (3)	100.0 (3)	0.0	1.0000
30	100.0 (5)	0.0 (5)	60.0 (5)	14.00	**0.0009**

CHAPTER V

INFLUENCE OF NON-AROMATIZABLE ANDROGENS AND ANTI-ANDROGENS
ON SEX DETERMINATION IN THE COMMON SNAPPING TURTLE, A REPTILE
WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

Abstract

Sex steroid hormones are involved in sex determination in almost all vertebrates,
including species with temperature-dependent sex determination (TSD). It is well
established that aromatase and estrogens are involved in ovary development. However,
the role of androgens in the ovary remains unclear. There is growing evidence that
androgens and the androgen receptor (AR) may be involved in female sex determination
by regulating aromatase expression. In this study, I use dihydrotestosterone (DHT) and
an AR antagonist (flutamide) to examine the role androgens play in sex determination in
the snapping turtle. I incubated snapping turtle eggs at a male-producing temperature and
treated embryos with DHT or flutamide during the sex-determining period. I examined
expression of ovary-specific genes, testes-specific genes, and genes involved in
steroidogenesis in the developing gonads. Treatments with exogenous DHT or flutamide
had a feminizing effect in many clutches by inducing expression of aromatase and *FoxL2*.
Many genes involved in testis development and steroidogenesis were also influenced by
DHT or flutamide treatments. My findings support the hypothesis that androgens and the

androgen receptor are involved in female sex determination in the common snapping turtle.

Introduction

Sex steroid hormones are critical for sex determination in all vertebrate embryos except eutherian mammals. This is true for reptiles that exhibit temperature-dependent sex determination (TSD), including numerous turtles, lizards, and all crocodilians studied to date (Viets et al., 1993; Lang and Andrews, 1994; Deeming 2004; Ewert et al., 2004; Harlow, 2004). Most of these species lack sex chromosomes. Instead, the temperature of the embryo during the middle third of development determines sex. Administration of exogenous steroid hormones or hormone antagonists to developing embryos can interfere with the temperature signal and reverse the putative sex of embryos (Crews, 1994; Crews et al., 1996; Matsumoto and Crews; 2012). Sensitivity to hormone-induced sex reversal coincides with the temperature sensitive period for sex determination. These findings support a role of steroid hormones in sex determination in TSD reptiles. All of the research to date has implicated estrogens as crucial for ovarian development. Administration of 17ß-estradiol to embryos incubated at male-producing temperatures overrides the temperature signal and leads to ovary development (Raynaud and Pieau, 1985; Gutzke and Bull, 1986; Bull et al., 1988; Dorizzi et al., 1991; Crews et al., 1991; Wibbels et al., 1991; Wibbels et al., 1993; Rhen and Lang, 1994; Crews et al., 1996; Freedberg et al., 2006). While estrogens are clearly involved in ovary formation, the role of androgens in TSD reptiles remains unclear.

Testosterone treatments produce a significant increase in the number of female hatchlings at temperatures that normally produce males or male-biased sex ratios (Wibbels and Crews, 1992; 1995; Rhen and Lang, 1994; Crews and Bergeron, 1994;

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Crews et al., 1995). Aromatase inhibitors block the feminizing effect of testosterone, presumably by preventing its conversion to estrogens (Crews and Bergeron, 1994; Wibbels and Crews, 1994; Rhen and Lang, 1994). Given that aromatase expression and activity are very low at male-producing temperatures, it is not obvious how addition of substrate alone can induce ovary formation.

Researchers have also administered dihydrotestosterone (DHT) to embryos to determine the effect of non-aromatizable androgens on TSD. In the red-eared slider turtle, DHT had a masculinizing effect on embryos incubated at a temperature that normally produces a 1:1 sex ratio. However, DHT did not influence sex determination in red-eared sliders at a temperature that produces exclusively females (Wibbels and Crews, 1992; 1995). Similar results have been observed in the American alligator (Lance and Bogart, 1994). In contrast, DHT had a feminizing effect on snapping turtle embryos incubated at temperatures that produce a 1:1 sex ratio or a female-biased sex ratio (Rhen and Lang, 1994; Rhen and Schroeder, 2010). Furthermore, the anti-androgen flutamide had a feminizing effect in some snapping turtle families, but a masculinizing effect in others (Rhen and Schroeder, 2010). The conflicting effects of DHT and flutamide within and among TSD species indicates that androgen signaling should be studied more closely.

Interestingly, AR expression is higher in gonads at female-producing temperatures than at male-producing temperatures in snapping turtles and red-eared slider turtles, suggesting a role for AR in ovarian development (Rhen et al., 2007; Ramsey and Crews, 2007; 2009). In fact, androgens act via AR to induce aromatase and Foxl2 expression in snapping turtle embryos incubated at a temperature that produce mixed sex

ratios (Rhen and Schroeder, 2010). These findings may help explain the feminizing effect of testosterone at male-producing temperatures: testosterone could enhance its own conversion to estrogens by upregulating aromatase expression. Although an androgen-dependent feed forward mechanism might be involved in ovarian development, sensitivity to exogenous steroids can vary with incubation temperature (Wibbels et al., 1995). Thus, androgens may or may not have any effect on aromatase expression at male-producing temperatures where aromatase expression is very low.

The aim of this study was to determine the effects of DHT and flutamide on sex determination in snapping turtle embryos incubated at an all male-producing temperature. I measured the mRNA expression in gonads after the TSP to determine the effects of hormone treatments on genes involved in ovarian and testicular development. Given that aromatase is involved in steroidogenesis, I examined the effects of our treatments on expression of other genes involved in steroidogenesis (Figure 5.1). I hypothesized that DHT would induce aromatase and FoxL2 expression and ovarian development at a male-producing temperature. I also hypothesized that DHT and flutamide would regulate expression of other genes involved in steroidogenesis.

Materials and Methods

Egg Collection, Incubation, and Hormone Treatments

Animal experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol #0905-1). Eggs were collected within 24 hours of oviposition from 8 snapping turtles nests throughout Minnesota in early June of 2010. Eggs from three snapping turtles nests were also collected in early June of 2011 to replicate the experiment from

2010. Clutch sizes ranged from 29 to 76 eggs. I transported eggs to the animal quarters in the Biology Department at the University of North Dakota. I held eggs at ~20°C for less than one week before clutches were assigned to experimental treatments. Eggs were washed in tepid water, candled for viability and infertile eggs removed. Eggs were placed in containers filled with moist vermiculite and then randomly positioned within foam box incubators as previous described (Rhen and Lang, 1994) and incubated at 26.5°C, which produces males in this population (Rhen and Lang, 1994; Ewert et al., 2005). Prior to hormone treatments, a few eggs from each clutch and treatment group were randomly sampled to determine the developmental stage of the embryos (Yntema, 1968). Eggs were candled again for viability and eggs containing dead embryos were eliminated from the study.

Stage 17 is the middle of the temperature-sensitive period in this species and is considered the stage when embryos are most sensitive to temperature (J. Lang, unpublished data). Hormone manipulations were performed at stage 17 of embryonic development to determine if DHT or flutamide had any influence on sex determination. Treatment groups included one vehicle-treated (ethanol only) group and two hormone groups, either DHT or flutamide. Eggs in the vehicle (control) treatment received a single dose of 5 µl of 95% ethanol. Eggs from the experimental groups received a single 50 µg dose of dihyrotestosterone dissolved in 5 µl 95% ethanol or a single 100 µg dose of flutamide dissolved in 5 µl 95% ethanol. Dosages chosen for each chemical were based on previous studies with turtles (Rhen and Lang, 1994; Rhen and Schroeder, 2010). All solutions were topically applied to the vascularized upper surface of the eggshell as

previously described (Crews et al., 1991). After receiving treatments, all eggs were returned to incubators at a constant male-producing temperature of 26.5°C.

Tissue Collection and Histology

In 2010, one to two eggs from each clutch and treatment group were sampled during embryonic stages 20-26. Eggs were opened and embryos quickly euthanized via decapitation. The adrenal-kidney-gonad (AKG) complex was removed, placed in RNA*later* (Ambion, Austin, TX) and stored at -20°C. The gonads were carefully micro-dissected from the mesonephros, taking care to prevent or remove any kidney tissue from the gonad. Only the micro-dissected gonads were used for RNA isolation and subsequent experiments.

Eggs treated in 2011 were sampled for histology. AKGs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Tissues were washed in PBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin according to standard protocols. AKGs were sectioned at 6 μm and mounted on HistoBond slides (VWR, Radnor, PA). Slides were deparaffinized in xylene, rehydrated in graded ethanol, and washed in PBS. Slides were stained with hematoxylin and eosin by standard protocols and images were taken using an Olympus BX-51 microscope equipped with an Infinity 2 digital camera (Lumenera Corp., Ottawa, ON) using Rincon HD Software (Imaging Planet, Goleta, CA) to observe the morphology of developing gonads in the various treatment groups. We considered development of the cortex and follicles as an indication of ovary development. The observation of sex cords within the medulla as an indication of testis development. I did not include the presence or absence of the Müllerian ducts as an indication of male or female development, because

treatments with DHT or flutamide lead to retention of the Müllerian ducts in snapping

turtle embryos developing testes (Schroeder and Rhen, unpublished data).

RNA Isolation, Dnase Treatment, and cDNA Synthesis

Total RNA was extracted from each pair of gonads using RNAzol RT (Molecular

Research Center, Cincinnati, OH). The RNAzol RT protocol was modified for the small

amount of tissue from a single pair of gonads. I used one-quarter the amount of liquid

recommended by the manufacturer for tissue homogenization, RNA isolation, and

recovery. I added 1 µl of precipitation carrier (Molecular Research Center, Cincinati,

OH) to the homogenate to assist with isolation RNA because the expect yield was less

than 10 µg. I added 15 µl of Rnase-free water to dissolve the RNA pellet. The dissolved

RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop

Technologies, Wilmington, DE). Genomic DNA was removed by DNase treatment

following the RNA extraction to ensure purity of the RNA.

Total RNA (150 ng) from each pair of gonads was reverse transcribed in a 20 µl

reaction using the iScript cDNA Synthesis Kit, which contains a blend of oligo dT and

random hexamers (BioRad, Hercules, CA). I diluted the synthesized cDNA to 0.5 ng

input RNA/µl to include in real-time PCR reactions.

Primer Selection and Quantitative PCR

Primers for quantitative PCR for testis-specific and steroidogenic genes were

developed from Illumina sequences obtained from bipotential gonads for snapping turtles

incubated at male-producing or female-producing temperatures (Rhen et al., unpublished

data). The primers for each gene were developed using Primer Express 2.0 (Life

Technologies, Grand Island, NY). The primers were designed with the following

parameters: length 18-25 base pairs (bp), guanine-cytosine content near 50%, melt temperature ranging from 55 to 60°C, and a short amplicon size (50-150 bp). Sequences and development of primers for *Foxl2*, aromatase, and *Ar* have been previously reported (Rhen et al., 2007). All other primer sequences are listed in Table 5.1. All primers were purchased from Integrated DNA Technologies (Coralville, IA).

Quantitative PCR was used to measure mRNA expression of select genes in the gonads of control and hormone-treated embryos. I evaluated expression of genes involved in ovarian development [aromatase (*Cyp19a1*)**,** *Foxl2,* and *Ar*], testicular development [anti-Müllerian hormone (*Amh*) and anti-Müllerian hormone receptor 2 (*AmhrII*)], and steroidogenic genes [steroidogenic acute regulatory protein (*StAR*)**,** cholesterol side chain cleavage enzyme (*Cyp11a1*), 3ß-hydroxysteroid dehydrogenase (*3ß-Hsd*), *Cyp17a1, 17ß-Hsd*, and steroid-5-α reductase (*Srd5a1*)]. I also measured 18S rRNA in pure gonads. In brief, each reaction contained 5 μl of 2x SsoFast EvaGreen supermix (BioRad, Hercules, CA), 200 nM of each forward and reverse primer, and 2 μl of 0.5 ng/μl diluted cDNA synthesized from gonads isolated from one individual, and water to bring the total reaction to 10 μl. Reactions were run on the CFX 384 Real-Time PCR Detection System (BioRad, Hercules, CA). The thermal profile was 95°C for 30 sec to activate the DNA polymerase followed by 40 cycles of two-step PCR (95°C for 5 sec and 61°C for 10 sec).

Rigorous standard curves across eight orders of magnitude were used to quantify gene expression in absolute terms as described in Rhen et al. (2007). Efficiencies of each real-time PCR reaction was estimated from the slope of our standard curves and ranged from 90-110 % with all slopes having an R^2 > 0.990. Controls lacking reverse

transcriptase or RNA template were prepared to demonstrate no contamination from foreign DNA, RNA, or PCR products. A melting temperature analysis was added at the end of each real-time PCR reaction to verify that a single product was amplified for each gene.

Statistical Analysis

I used JMP 5.0.1.2 software for all statistical analyses (SAS Institute, Cary, NC). I analyzed patterns of gene expression in embryonic gonads using clutch, treatment, and developmental stage as main effects in a three-way analysis of variance (ANOVA). All threshold cycles (Ct) were log10 transformed to meet the assumptions of the ANOVA. Residuals of each ANOVA were assessed for outliers using Cook's distance and outliers were removed if necessary. I used Ct values for 18S rRNA as a covariate to control for potential variation in the quality of input RNA as well as variation in the efficiency of the reverse transcription reaction. Given significant effects of treatment ($\alpha < 0.05$), we used the Dunnett's test to correct for multiple comparisons and to compare the treatment effects to the vehicle control. Sample sizes for experimental groups are shown in each figure.

I used k-means cluster analysis of aromatase and *Foxl2* expression to identify developing ovaries due to the DHT or flutamide treatments. Aromatase and *Foxl2* were chosen because their expression is sexually dimorphic in the developing gonads and both are considered markers for ovarian development (Loffler et al., 2003; Rhen et al., 2007). I then analyzed patterns of gene expression for testicular genes and steroidogenic genes using ANOVA, but included putative sex as a factor in the model. This analysis was performed to determine whether treatment effects were due to regulation of the genes by

androgens and AR or a secondary consequence of gonad differentiation. Assumptions were checked for each ANOVA. When treatment effects were significant in ANOVA, I used the Dunnett's test to compare individual groups.

<div align="center">Results</div>

<div align="center">*Effects of Treatments on Gonad Morphology*</div>

Treatment of embryos with the ethanol vehicle had no effect on sex determination. All hatchlings in this group had normal testes with well-developed seminiferous tubules containing germ cells, as expected for embryos incubated at $26.5^{\circ}C$ (Figure 5.2 A-D). The DHT and flutamide treatments produced similar results in both years. Hormone treatments reversed the sex of some embryos: DHT and flutamide induced ovarian development, as evidenced by a thickened cortex containing numerous follicles with meiotic oocytes (Figure 5.2 E-H, I, K). However, the feminizing effects of DHT and flutamide varied significantly among clutches (Table 5.2).

<div align="center">*Expression of Genes Involved in Ovarian Development*</div>

I measured the expression of *Foxl2*, aromatase, and *Ar* in differentiating gonads from vehicle treated embryos and embryos treated with DHT or flutamide. Expression of aromatase mRNA in embryonic gonads was influenced by hormone treatments, clutch, developmental stage, and the clutch x treatment interaction (Table 5.3). Levels of 18S rRNA were a significant covariate in the model. Aromatase mRNA levels were significantly greater in gonads from DHT-treated embryos compared to vehicle-treated embryos in clutches 2, 4, 6, and 8 (Figure 5.3). Flutamide also induced a significant increase in aromatase mRNA levels in gonads from clutches 2, 4, and 8 compared to the vehicle controls.

Expression of *Foxl2* mRNA in embryonic gonads was significantly influenced by hormone treatments, clutch, and the clutch x treatment interaction (Table 5.3). Levels of 18s rRNA were a significant covariate. Levels of *Foxl2* mRNA were significantly greater in gonads from the DHT-treated embryos compared to vehicle-treated embryos in clutches 2, 6, and 10. In contrast, there was no difference in *Foxl2* mRNA expression between flutamide-treated embryos and vehicle treated controls (Figure 5.4).

Expression of *Ar* mRNA was significantly influenced by hormone treatments and clutch (Table 5.3). Levels of 18S rRNA were a significant covariate. *Ar* mRNA levels were significantly greater in gonads from DHT-treated embryos compared to vehicle-treated embryos in clutches 2, 8, and 10 (Figure 5.5). There was not a difference between levels of *Ar* mRNA in gonads from flutamide- and vehicle-treated embryos in any clutch.

Identification of Putative Females using Cluster Analysis

The observation of greater aromatase and/or *Foxl2* mRNA expression in gonads from the DHT- and flutamide-treated embryos suggested that these genes were involved in ovary development. I performed k-means cluster analysis using aromatase and *Foxl2* mRNA levels as markers for ovarian development to assign a putative sex to each embryo. The predicted sex was then added to statistical models to analyze expression of other genes. Cluster analysis identified 29 embryos as putative females, which were all from the DHT (n = 24) or flutamide (n = 5) groups. Putative females had significantly higher cluster means for mRNA levels (mean $\pm$ SD) for both aromatase and Foxl2 (51.88 $\pm$ 8.54 and 32.86 $\pm$ 4.08, respectively) compared to the putative males (0.73 $\pm$ 5.49 and 0.17 $\pm$ 7.78, respectively). A summary of the sex ratios produced within each clutch from the k-means analysis is presented in Table 5.2.

Expression of Genes Involved in Testicular Development

Given that DHT or flutamide were capable of inducing ovarian development, I analyzed expression of a testis-specific gene, *Amh* and its receptor (*AmhrII*). Expression of *Amh* mRNA was significantly influenced by clutch, treatment, stage of development, the clutch x treatment interaction, and the stage x treatment interaction (Table 5.4). Levels of 18S were a significant covariate. Levels of *Amh* were significantly lower in gonads from DHT-treated embryos in clutches 2, 4, and 6 compared to the vehicle controls, but there was not a significant difference in *Amh* between gonads from flutamide-treated embryos compared to the vehicle controls (Figure 5.6).

To determine if mRNA levels were lower in developing ovaries, we included the putative sex of embryos into the model. Expression of *Amh* mRNA was still influenced by clutch, treatment, developmental stage and the stage x treatment interaction (Table 5.5). Levels of 18S rRNA remained a significant covariate. The clutch x treatment interaction was no longer significant in the model. Expression of *Amh* mRNA was influenced by the putative sex of embryos with levels being 6-fold higher in putative males (33883.7 $\pm$ 2690.4 ag/ng RNA, n = 146) than putative females (2556.3 $\pm$ 8252.1 ag/ng RNA, n = 27).

Expression of *AmhrII* mRNA was significantly influenced by clutch, treatment, stage and the clutch x treatment interaction (Table 5.4). Levels of 18S rRNA were a significant covariate within the model. The stage x treatment interaction did not influence *AmhrII* mRNA expression. Levels of *AmhrII* were significantly higher in gonads from DHT-treated embryos from clutches 2 and 6 compared to the vehicle

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controls, but there was no difference between flutamide-treated embryos and vehicle controls (Figure 5.7).

Including the putative sex into the model, expression of *AmhrII* mRNA was still influenced by clutch, treatment, and stage (Table 5.5). However, the clutch x treatment interaction was no longer significant. Levels of 18S rRNA remained a significant covariate within the model. The stage x treatment interaction did not influence *AmhrII* mRNA expression. The putative sex of embryos significantly influenced *AmhrII* mRNA expression with levels almost double in ovaries (1,344.4 $\pm$ 190.9 ag/ng RNA, n = 28) compared to testes (745.7 $\pm$ 61.5 ag/ng RNA, n = 150).

Expression of Genes Involved in Steroidogenesis

Expression of *StAR* mRNA was significantly influenced by clutch, treatment, developmental stage, clutch x treatment interaction, clutch x stage interaction (Table 5.6). Levels of 18S rRNA were a significant covariate. Expression of *StAR* mRNA was significantly higher in gonads from DHT- and flutamide-treated embryos compared to controls (Figure 5.8 A). Clutches 2, 6, 5, and 10 had significantly higher levels of *StAR* in the DHT-treated group compared to controls. In contrast, *StAR* levels did not differ between flutamide- and vehicle-treated embryos in any clutch (Figure 5.9).

When putative sex was included in the model, expression of *StAR* mRNA was still influenced by clutch, treatment, developmental stage, and the stage x treatment interaction (Table 5.7). The clutch x treatment interaction was no longer significant. Levels of 18S rRNA remained a significant covariate in the model. Expression of *StAR* mRNA differed between the putative sexes with mRNA levels being approximately 6-fold higher in ovaries (12.91 $\pm$ 1.48, n = 27) than testes (2.48 $\pm$ 0.46, n = 151).

Expression of *Cyp11a1* mRNA was significantly influenced by clutch, treatment, and the clutch x treatment interaction (Table 5.6). Levels of 18S rRNA were a significant covariate. Expression of *Cyp11a1* mRNA was significantly higher in gonads from DHT-treated embryos compared to the vehicle-treated controls (Figure 5.8 B). Flutamide had no detectable effect on *Cyp11a1* mRNA expression compared to controls.

When putative sex was included in the model, expression of *Cyp11a1* mRNA was still influenced by clutch and treatment (Table 5.7). Developmental stage and the stage x treatment interaction were still insignificant. The interaction of clutch x treatment was no longer significant. Levels of 18S rRNA remained a significant covariate. The putative sex of embryos influenced expression of *cyp11a1* mRNA with higher levels in ovaries (40.1 ± 9.22 ag/ng RNA, n = 28) than testes (28.6 ± 3.0 ag/ng, n = 151).

Expression of *Cyp17a1* mRNA was significantly influenced by clutch (Table 5.6). In contrast, *Cyp17a1* mRNA levels did not vary among treatment groups (Figure 5.8 C) or any other independent variable. Levels of 18S rRNA were a significant covariate.

When I included putative sex into the model, *Cyp17a1* mRNA expression was still influenced by clutch and levels of 18S rRNA remained a significant covariate in the model (Table 5.7). The putative sex of embryos did not influence *Cyp17a1* mRNA expression. Expression of *Cyp17a1* mRNA was very similar in ovaries (1542.5 ± 513.8 ag/ng RNA, n = 27) and testes (1423.4 ± 161.1, n = 147).

Expression of *3ß-Hsd* mRNA was significantly influenced by clutch, treatment, and developmental stage (Table 5.6). Levels of 18S rRNA were a significant covariate within the model. There were no significant interactions in the model. Levels of *3ß-Hsd*

were significantly higher in gonads from embryos treated with DHT or flutamide compared to the vehicle controls (Figure 5.8 D).

By including the putative sex into the model, expression of *3ß-Hsd* was still influenced by clutch, treatment, and developmental stage (Table 5.7). Levels of 18S rRNA remained a significant covariate in the model. Putative sex did not significantly influence *3ß-Hsd* mRNA expression with similar levels in ovaries (753.9 $\pm$ 239.8 ag/ng RNA, n = 29) and testes (1239.5 $\pm$ 77.2 ag/ng RNA, n = 149).

Expression of *17ß-Hsd* mRNA was significantly influenced by clutch, treatment, and the stage x treatment interaction (Table 5.6). Levels of 18S rRNA were a significant covariate within the model. Levels of *17ß-Hsd* expression were significantly higher in gonads from embryos treated with DHT compared to the vehicle controls (Figure 5.8 E). Flutamide did not influence expression of *17ß-Hsd* compared to controls.

When putative sex was included in the model, expression of *17ß-Hsd* mRNA was still significantly influenced by clutch, treatment, and the stage x treatment interaction (Table 5.7). Levels of 18S rRNA remained a significant covariate within the model. Putative sex did not influence *17ß-Hsd* mRNA expression with similar levels in ovaries (54.6 $\pm$ 8.83 ag/ng RNA, n = 29) and testes (49.0 $\pm$ 2.8 ag/ng RNA, n = 147).

Expression of *Srd5a1* mRNA was significantly influenced by clutch, treatment and developmental stage (Table 5.6). Levels of 18S rRNA were a significant covariate within the model. Levels of *Srd5a1* were significantly higher in gonads from embryos treated with DHT and flutamide compared to vehicle controls (Figure 5.8 F). There were no interactions between independent variables in the model.

Incorporating putative sex into the model, expression of *Srd5a1* was still influenced by clutch and developmental stage (Table 5.7). Levels of 18S rRNA were still a significant covariate in the model. Hormone treatment was no longer significant in the model. Interactions in the model were not significant. Putative sex significantly influenced the expression of *Srd5a1* with levels being higher in ovaries (413.8 ± 59.2 ag/ng RNA, n = 28) than testes (227.5 ± 18.3 ag/ng RNA, n = 153).

Discussion

The involvement of sex steroid hormones in sex determination has been extensively studied in fish, amphibians, and reptiles (Pieau et al., 1994; Ramsey and Crews, 2009). Particular steroids are implicated in sex determination based on their effectiveness of causing complete sex reversal. Administration of DHT or flutamide to snapping turtle embryos incubated at the pivotal temperature had a feminizing effect, suggesting androgens and the androgen receptor are involved in ovary development in the snapping turtle (Rhen and Schroeder, 2010). In this study, I determined whether DHT or flutamide would sex-reverse snapping turtle embryos incubated at an all male-producing temperature.

I found DHT had a feminizing effect in all but one clutch. The response to DHT varied dramatically among clutches: while one clutch produced all males, another clutch produced all females. Flutamide had a feminizing effect on two of eight clutches, supporting the notion that flutamide acts as a partial *Ar* agonist in the snapping turtle. These observations are consistent with previous reports in the snapping turtle (Rhen and Lang, 1994; Rhen and Schroeder, 2010). Variation in the number of females among clutches suggests the existence of genetic variation in at least one component of the

androgen signaling pathway. Studies have shown that genetic variation in the coding sequence of *Ar* influences receptor activity and sensitivity, which leads to various phenotypes in mammals (Chamberlain et al., 1994; Choong et al., 1996; Giovannucci et al., 1997; Mifsud et al., 2000; Westberg et al., 2001; Dejager et al., 2002; Ibanez et al., 2003). Identification of genetic variants in snapping turtle *Ar* or androgen response elements in ovary-specific genes would provide insights into how androgens induce ovary development.

Production of ovotestes have been observed in some turtle species when treated with high levels of sex steroids or aromatase inhibitors and exogenous androgens at mixed sex ratios, but not from treatments with exogenous androgens at all male-producing temperatures (Wibbels et al., 1992; Crews and Bergeron, 1994; Wibbels and Crews, 1995). I did not observe ovotestes in my histological analyses of the gonads, making it unlikely in the gonads used for analyzing mRNA expression.

Feminizing effects were also observed at the molecular level: DHT and flutamide treatments increased aromatase mRNA levels in gonads from embryos at a male-producing temperature. *Foxl2* expression was induced in embryos treated with DHT, but not flutamide. My current findings provide additional support for the hypothesis that androgens and *Ar* are in a feed forward loop that includes aromatase and *Foxl2* (Rhen and Schroeder 2010). Aromatase converts endogenous testosterone into estrogens, which are key regulators of ovarian differentiation in TSD reptiles (Pieau et al., 1994, 1995, 2004; Lance, 1997, 2009). This is the first study demonstrating that DHT can override the effects of male-producing temperatures in the snapping turtle.

I examined expression of *Amh* and its receptor, because *Amh* is expressed in Sertoli cells in the developing testes and *Amh* represses aromatase expression in gonads of birds, reptiles, and mammals (Vigier et al., 1989; di Clemente et al., 1992). As expected, DHT treatments reduced *Amh* expression in clutches that produced mostly females. Levels of *Amh* expression in gonads from DHT-treated embryos were still relatively high in clutch 2, which produced all females. Flutamide treatment had no detectable effect on *Amh* mRNA levels, presumably because mostly males were produced. Additionally, *AmhrII* expression levels were significantly higher in clutches producing mostly females from the DHT-treatment.

I therefore tested for differences in *Amh* expression between putative females and males. Putative sex had a large effect on *Amh* expression, with females having significantly lower *Amh* expression than males, yet *Amh* mRNA expression was still observed in developing females. *Amh* is expressed at low levels in the cortex in developing ovaries of chickens, fish, and mammals, but not in alligators or red-eared slider turtles (Munsterberg and Lovell-Badge, 1991; Carre-Eusebe et al., 1996; Oreal et al., 1998; Smith et al., 1999; Western et al., 1999; Yoshinaga et al., 2004; Shoemaker et al., 2007; Ijiri et al., 2008). Although *Amh* expression decreased in putative females, we observed an increase in *AmhrII* expression. Low levels of *Amh* may be playing a functional role in differentiation of the ovary, because *Amh* is known to be involved in folliculogenesis in the ovary by preventing the recruitment of primordial follicles into the growing pool (Durlinger et al., 2002).

I analyzed expression of other genes that encode steroidogenic enzymes. Treatments with DHT or flutamide significantly influenced expression of all

steroidogenic genes studied except *Cyp17a1*. However, when putative sex was included in the statistical model, the variance explained by the treatments decreased, and for some genes, there was no longer a treatment effect. These results suggest that differences in expression of many of the steroidogenic genes may be due to gonadal sex and not a result of gene regulation by AR.

I observed significant differences in *StAR* and *Cyp11a1* expression among the treatment groups and between putative females and males. *StAR* mediates the rate-limiting step in steroidogenesis by delivering cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where *Cyp11a1* resides. *StAR* levels were higher in gonads from the DHT and flutamide treatments and developing ovaries than testes. Previous studies in the snapping turtle revealed higher *StAR* mRNA expression in bipotential gonads from a female-producing temperature compared to a male-producing temperature, but not until the end of the temperature sensitive period (T. Rhen, unpublished results). Aromatase mRNA expression becomes sexually dimorphic during the temperature sensitive period (Rhen et al., 2007). Estrogens induce a rapid increase in *StAR* protein levels in the ovaries of rabbits, but the effect of estrogens on *StAR* mRNA is unknown (Townson et al., 1996). Androgens and *Ar* may be indirectly influencing *StAR* expression by increasing aromatase expression and estrogen production.

Although androgens and *Ar* have yet to be implicated in ovarian development in other reptilian species with TSD, there is increasing evidence that androgens may be an evolutionarily conserved mechanism in ovarian development. *Ar* is expressed at higher levels in the developing ovary than testes in chickens and reptiles (Katoh, et al., 2006; Ramsey and Crews, 2007; 2009). In contrast, administration of DHT had a masculinizing

effect on red-eared slider embryos incubated at a mixed sex ratio temperature. This suggests the role of androgens in ovarian development may not be conserved among turtles. Furthermore, the role of androgens in ovarian development may be species-specific. For example, administration of DHT to channel and blue catfish has been reported to increase the production of females (Davis et al., 1992), while DHT has a masculinizing effect in many other fish species (Chiasson and Bentley, 2007; Krisfalusi and Cloud, 1999). Davis et al. (1992) reported that the feminizing effect of DHT was influenced by the timing and dose of administration. Crews et al. (1996) reported effects from DHT only during the TSP and a dose response to masculinize red-eared slider turtles. These results suggest a dosage-dependence effect of androgens to produce females and may explain variation in results between turtle species.

The appropriate level of androgens may be critical for normal ovarian development. Exposure to high levels of androgens during mammalian embryonic development is associated with polycystic ovarian syndrome (PCOS; Norman et al., 2007). On the other hand, low levels of circulating androgens or lack of AR function leads to premature ovarian failure (POF; Shiina et al., 2005). PCOS is characterized by an increase in the number of developing follicles compared to normal ovaries (Webber et al., 2003; Steckler et al., 2005). POF is characterized by amenorrhoea and early loss of ovarian function (Goswami and Conway, 2005). Previous studies in snapping turtles revealed a similar phenotype: ovaries from individuals treated with DHT during embryogenesis had increased numbers of developing follicles (Rhen et al., unpublished results). My data supports the hypothesis that genetic variation in androgen receptor sensitivity or activity may be associated with hyper-androgenism in the ovary (Legro et

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al., 1998). Furthermore, *Amh*, *AmhrII,* and many of the genes encoding steroidogenic enzymes have been implicated in the development of PCOS (Prapas et al, 2009; Pellatt et al., 2010; Luense et al., 2011). The expression of these genes in the developing ovary from DHT-treated snapping turtle embryos may lead to the characteristic ovarian phenotypes of PCOS, but the molecular mechanisms underlying PCOS during embryonic development are not well understood. Further analyses of these genes in control and DHT-induced ovaries are warranted.

In sum, my findings strongly support the hypothesis that androgens and AR play a role in ovarian development in the common snapping turtle, presumably by inducing aromatase and leading to the conversion of aromatizable androgens into estrogens. Moreover, AR appears to be involved in regulating steroidogenic genes, *StAR* and *Cyp11a1***,** which are necessary for steroid hormone production. Although androgens appear important in ovarian development, too much or too little androgen production in females leads to disease phenotypes, suggesting that the production of androgens and estrogens is highly regulated during development of normal ovaries.

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Table 5.1. List of primer pairs used for quantitative real-time PCR.

Target	Primer Sequence	Tm (°C)
Amh	For 5' – CATTCTGCTCTCCTGTCTCTG – 3'	54.8
	Rev 5' – ACGTCCTCTTGAAAAGCCAG – 3'	55.2
AmhrII	For 5' – TGAAGCAGGCTGACATCTACTCCT – 3'	59.5
	Rev 5' – TCGTAGGCCAGCTGGAAGGGA – 3'	62.6
StAR	For 5' - TCTTCCGAATGGAGACGGTG - 3'	56.7
	Rev 5' – CATAGAAGAGCTGGTCCACGG - 3'	57.3
Cyp11a1	For 5' – CTCTAAGACCTGGCGGGACC - 3'	59.5
	Rev 5' – TGCCTGAAAATCACATCCCA - 3'	54.6
Cyp17a1	For 5' – CCCCGTCCCTCCTTTGAC - 3'	58.0
	Rev 5' – AATAATTCAGCGCCTCGCTCT - 3'	56.8
3ß-Hsd	For 5' – GAGATCCGAACCCTGGACAA - 3'	56.6
	Rev 5' – AAAATTCCTGCGTGCCTCAT - 3'	55.3
17ß-Hsd	For 5' – CTATCTACGGGAGACCCGGG - 3'	58.5
	Rev 5' – CCCACGAGACTGGAAATGTTG – 3'	56.0
Srd5a1	For 5' – TTCTGGGTGGCTGAAAGGTC-3'	57.4
	Rev 5' – AGCCACGCTGCAAAACCTAC – 3'	58.5

Table 5.2. A summary of the sex ratios produced (in percent male) for each treatment group within each clutch from the k-means cluster analysis. The number in parentheses represents the number of individuals from each treatment group.

Clutch	Vehicle	DHT	Flutamide
2	100 (7)	0.0 (8)	57.1 (7)
3	100 (5)	83.3 (6)	100 (6)
4	100 (7)	71.4 (7)	100 (5)
5	100 (8)	77.8 (9)	100 (9)
6	100 (9)	12.5 (8)	75.0 (8)
8	100 (7)	85.7 (7)	100 (7)
9	100 (12)	100 (11)	100 (10)
10	100 (7)	62.5 (8)	100 (8)

Table 5.3. ANOVA model of the influence of clutch, treatment, and stage on the log10 transformed mRNA expression of genes involved in ovarian development. 18S rRNA was included as a covariate within the model. Significant terms in the model at $p \leq 0.05$ are bolded.

Dependent Variable	Source	Type III Sum of Squares	df	F	p
Aromatase	**Clutch**	**27.65**	**7**	**7.683**	**<0.0001**
	Treatment	**30.11**	**2**	**29.280**	**<0.0001**
	Stage	**11.48**	**6**	**3.722**	**0.0018**
	Treatment x Clutch	**26.07**	**14**	**3.622**	**<0.0001**
	Treatment x Stage	5.47	12	0.887	0.5617
	18S rRNA	**11.71**	**1**	**22.776**	**<0.0001**
	Error	70.43	137		
Foxl2	**Clutch**	**78.32**	**7**	**17.187**	**<0.0001**
	Treatment	**44.16**	**2**	**33.918**	**<0.0001**
	Stage	2.08	6	0.533	0.7823
	Treatment x Clutch	**28.89**	**14**	**3.169**	**0.0002**
	Treatment x Stage	5.96	12	0.764	0.6868
	18S rRNA	**10.99**	**1**	**16.878**	**<0.0001**
	Error	89.19	137		
Ar	**Clutch**	**2.73**	**7**	**2.342**	**0.0274**
	Treatment	**4.06**	**2**	**12.169**	**<0.0001**
	Stage	1.35	6	1.352	0.2384
	Treatment x Clutch	2.76	14	1.184	0.2944
	Treatment x Stage	2.35	12	1.173	0.3085
	18S rRNA	**4.82**	**1**	**28.872**	**<0.0001**
	Error	22.84	137		

Table 5.4. ANOVA model of the influence of clutch, treatment, and stage on the log10 transformed mRNA expression of genes involved in testicular development. 18S rRNA was included as a covariate within the model. Significant terms in the model at p ≤ 0.05 are bolded.

Dependent Variable	Source	Type III Sum of Squares	df	F	p
	Clutch	**12.56**	**7**	**4.200**	**0.0003**
	Treatment	**21.69**	**2**	**25.381**	**<0.0001**
Amh	**Stage**	**8.94**	**6**	**3.488**	**0.0032**
	Treatment x Clutch	**17.03**	**14**	**2.847**	**0.0009**
	Treatment x Stage	**11.07**	**12**	**2.159**	**0.0174**
	18S rRNA	**7.61**	**1**	**17.821**	**<0.0001**
	Error	54.69	128		
	Clutch	**30.84**	**7**	**43.625**	**<0.0001**
	Treatment	**4.22**	**2**	**20.875**	**<0.0001**
AmhrII	**Stage**	**3.48**	**6**	**5.745**	**<0.0001**
	Treatment x Clutch	**3.80**	**14**	**2.690**	**0.0017**
	Treatment x Stage	0.62	12	0.511	0.9045
	18S rRNA	**2.03**	**1**	**20.123**	**<0.0001**
	Error	13.43	133		

Table 5.5 ANOVA model of the influence of clutch, treatment, stage, and putative sex on the log10 transformed mRNA expression of genes involved in testicular development. 18S rRNA was included as a covariate within the model. Significant terms in the model at $p \leq 0.05$ are bolded.

Dependent Variable	Source	Type III Sum of Squares	df	F	p
Amh	**Clutch**	**11.98**	**7**	**5.907**	**<0.0001**
	Treatment	**3.14**	**2**	**5.427**	**0.0055**
	Stage	**11.04**	**6**	**6.355**	**<0.0001**
	Treatment x Clutch	5.38	14	1.327	0.2005
	Treatment x Stage	**6.80**	**12**	**1.957**	**0.0335**
	Putative Sex	**17.92**	**1**	**61.870**	**<0.0001**
	18S rRNA	**9.41**	**1**	**32.480**	**<0.0001**
	Error	36.78	127		
AmhrII	**Clutch**	**19.18**	**7**	**30.310**	**<0.0001**
	Treatment	**0.86**	**2**	**4.735**	**0.0103**
	Stage	**3.89**	**6**	**7.163**	**<0.0001**
	Treatment x Clutch	1.34	14	1.060	0.3992
	Treatment x Stage	0.63	12	0.584	0.8521
	Putative Sex	**1.50**	**1**	**16.578**	**<0.0001**
	18S rRNA	**1.63**	**1**	**18.129**	**<0.0001**
	Error	11.93	132		

Table 5.6. ANOVA model of the influence of clutch, treatment, and stage on the log10 transformed mRNA expression of genes involved in steroidogenesis. 18S rRNA was included as a covariate within the model. Significant terms in the model at p ≤ 0.05 are bolded.

Dependent Variable	Source	Type III Sum of Squares	df	F	p
StAR	**Clutch**	**7.89**	**7**	**11.265**	**<0.0001**
	Treatment	**12.35**	**2**	**61.711**	**<0.0001**
	Stage	**3.25**	**6**	**5.420**	**<0.0001**
	Treatment x Clutch	**3.33**	**14**	**2.376**	**0.0056**
	Treatment x Stage	**2.35**	**12**	**1.960**	**0.0330**
	18S rRNA	**2.60**	**1**	**26.011**	**<0.0001**
	Error	13.00	130		
Cyp11a1	**Clutch**	**33.50**	**7**	**16.538**	**<0.0001**
	Treatment	**6.55**	**2**	**11.314**	**<0.0001**
	Stage	2.71	6	1.561	0.1636
	Treatment x Clutch	**8.68**	**14**	**2.143**	**0.0133**
	Treatment x Stage	4.80	12	1.383	0.1819
	18S rRNA	**11.01**	**1**	**38.072**	**<0.0001**
	Error	70.43	132		
Cyp17a1	**Clutch**	**40.08**	**7**	**27.158**	**<0.0001**
	Treatment	0.62	2	1.461	0.2357
	Stage	0.50	6	0.392	0.8830
	Treatment x Clutch	2.43	14	0.822	0.6440
	Treatment x Stage	2.26	12	0.894	0.5549
	18S rRNA	**4.58**	**1**	**21.741**	**<0.0001**
	Error	27.41	130		
3β-Hsd	**Clutch**	**11.50**	**7**	**18.103**	**<0.0001**
	Treatment	**2.73**	**2**	**15.055**	**<0.0001**
	Stage	**2.07**	**6**	**3.807**	**0.0016**
	Treatment x Clutch	1.06	14	0.835	0.6304
	Treatment x Stage	1.49	12	1.366	0.1898
	18S rRNA	**3.08**	**1**	**33.892**	**<0.0001**
	Error	12.16	134		

	Clutch	**10.73**	**7**	**12.480**	**<0.0001**
	Treatment	**1.58**	**2**	**6.411**	**0.0022**
17β-Hsd	Stage	1.18	6	1.599	0.1524
	Treatment x Clutch	1.18	14	0.685	0.7860
	Treatment x Stage	**3.06**	**12**	**2.074**	**0.0228**
	18S rRNA	**3.13**	**1**	**25.519**	**<0.0001**
	Error	16.09	131		
	Clutch	**7.40**	**7**	**6.534**	**<0.0001**
	Treatment	**2.53**	**2**	**7.815**	**0.0006**
Srd5a1	**Stage**	**3.23**	**6**	**3.329**	**0.0043**
	Treatment x Clutch	3.45	14	1.523	0.1105
	Treatment x Stage	1.19	12	0.611	0.8302
	18S rRNA	**3.37**	**1**	**20.853**	**<0.0001**
	Error	22.16	137		

Table 5.7. ANOVA model of the influence of clutch, treatment, stage, and putative sex on the log10 transformed mRNA expression of genes involved in steroidogenesis. 18S rRNA was included as a covariate within the model. Significant terms in the model at $p \leq 0.05$ are bolded.

Dependent Variable	Source	Type III Sum of Squares	df	F	p
StAR	**Clutch**	**2.47**	**7**	**5.008**	**0.0006**
	Treatment	**2.20**	**2**	**15.615**	**<0.0001**
	Stage	**3.18**	**6**	**7.527**	**<0.0001**
	Treatment x Clutch	0.96	14	0.976	0.4815
	Treatment x Stage	**1.86**	**12**	**2.205**	**0.0149**
	Putative Sex	**3.93**	**1**	**55.920**	**<0.0001**
	18S rRNA	**1.59**	**1**	**22.617**	**<0.0001**
	Error	9.07	129		
Cyp11a1	**Clutch**	**34.22**	**7**	**17.506**	**<0.0001**
	Treatment	**2.36**	**2**	**4.229**	**0.0166**
	Stage	2.92	6	1.741	0.1164
	Treatment x Clutch	4.82	14	1.234	0.2583
	Treatment x Stage	4.77	12	1.423	0.1631
	Putative Sex	**1.62**	**1**	**5.801**	**0.0174**
	18S rRNA	**9.71**	**1**	**34.786**	**<0.0001**
	Error	36.58	131		
Cyp17a1	**Clutch**	**40.15**	**7**	**27.148**	**<0.0001**
	Treatment	0.15	2	0.349	0.2357
	Stage	0.48	6	0.376	0.8830
	Treatment x Clutch	1.61	14	0.544	0.6440
	Treatment x Stage	2.27	12	0.895	0.5549
	Putative Sex	0.16	1	0.763	0.3840
	18S rRNA	**4.37**	**1**	**20.702**	**<0.0001**
	Error	27.25	129		
3β-Hsd	**Clutch**	**11.70**	**7**	**17.867**	**<0.0001**
	Treatment	**2.31**	**2**	**12.689**	**<0.0001**
	Stage	**2.07**	**6**	**3.781**	**0.0017**
	Treatment x Clutch	1.07	14	0.835	0.6232
	Treatment x Stage	1.51	12	1.366	0.1842
	Putative Sex	0.03	1	0.370	0.5440
	18S rRNA	**3.08**	**1**	**33.892**	**<0.0001**
	Error	12.12	133		

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	Clutch	**10.71**	**7**	**12.491**	**<0.0001**
	Treatment	**0.83**	**2**	**3.392**	**0.0367**
17β-Hsd	Stage	1.15	6	1.566	0.1621
	Treatment x Clutch	0.94	14	0.549	0.8994
	Treatment x Stage	**3.03**	**12**	**2.064**	**0.0236**
	Putative Sex	0.16	1	1.339	0.2494
	18S rRNA	**2.93**	**1**	**23.917**	**<0.0001**
	Error	15.93	130		
	Clutch	**4.41**	**7**	**4.094**	**0.0004**
	Treatment	0.74	2	2.414	0.0933
Srd5a1	**Stage**	**3.42**	**6**	**3.708**	**0.0019**
	Treatment x Clutch	2.68	14	1.243	0.2515
	Treatment x Stage	0.81	12	0.439	0.9449
	Putative Sex	**1.25**	**1**	**8.163**	**0.0049**
	18S rRNA	**2.91**	**1**	**18.930**	**<0.0001**
	Error	20.90	136		
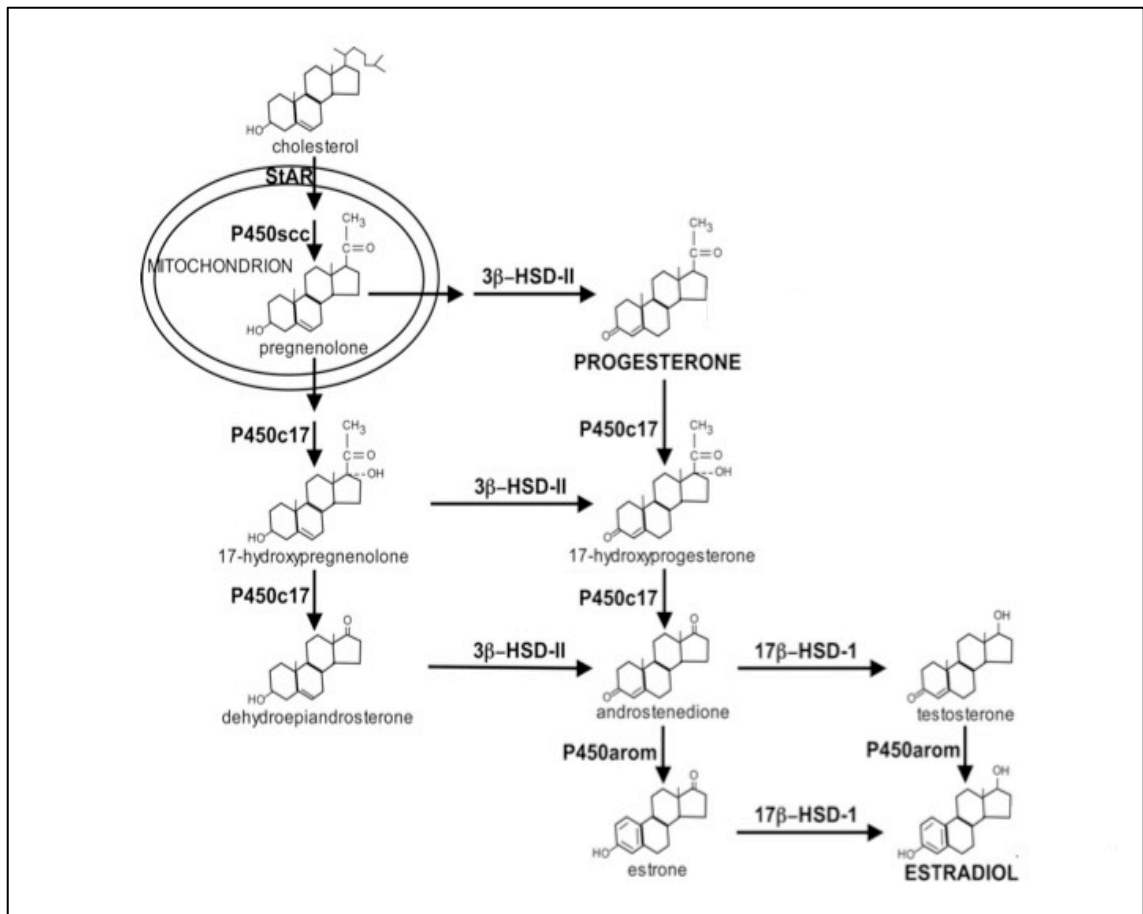
Figure 5.1. Overview of steroidogenesis in the gonads and the genes/proteins involved in sex steroid biosynthesis (adapted from Bulun et al., 2005). We examined the genes that encode the enzymes necessary for the conversion of cholesterol into sex steroid hormones including: Steroidogenic acute regulatory protein (StAR), Cyp11a1 (cholesterol side-chain cleavage; P450scc), Cyp17a1 (cytochrome P450 c17α-hydroxylase, 17, 20-lyase), 3β-hydroxysteroid dehydrogenase (Hsd), 17β-Hsd, and aromatase (cytochrome P450 aromatase, Cyp19a).
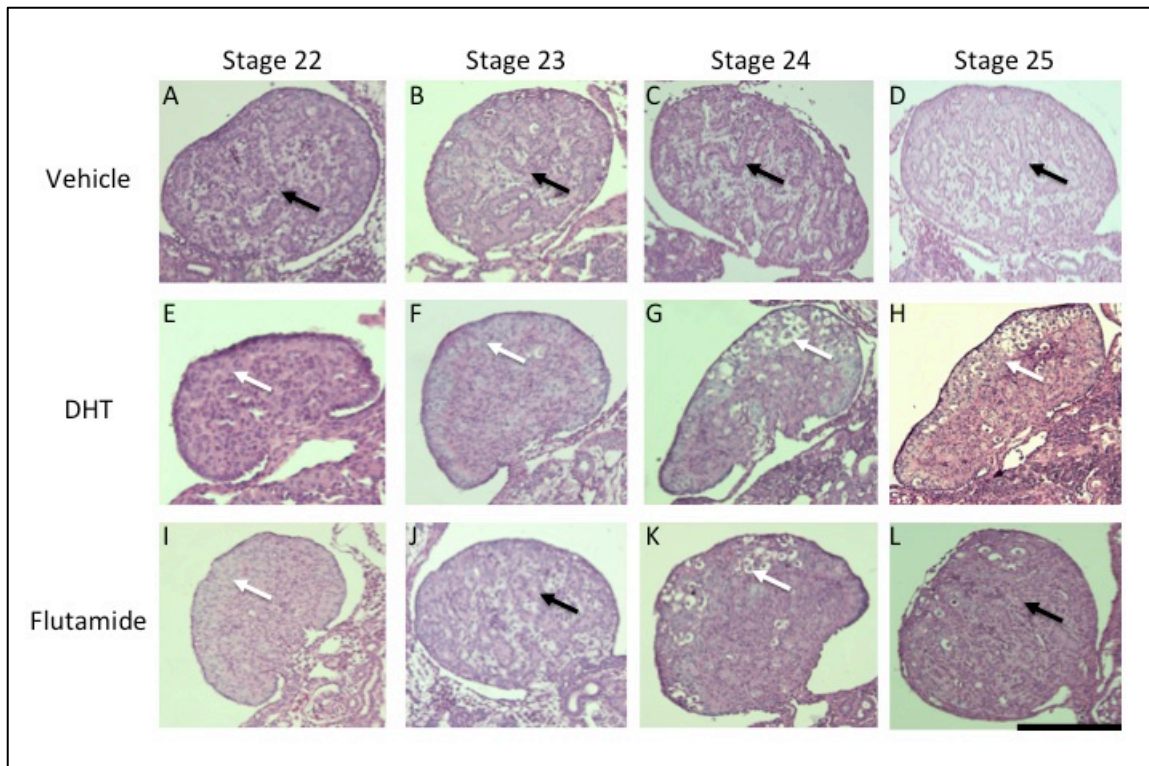
Figure 5.2. Differentiating gonads of snapping turtles during stages 22 through 25 of embryonic development from embryos treated with vehicle control (A-D), DHT (E-H), or flutamide (I-L), at stage 17 of development. Each gonad shown is from a single individual from the same clutch. The vehicle-treated embryos all showed developing medullary sex cords (black arrows) indicating testis development. All DHT-treated embryos from this clutch showed development of the cortex and developing follicles (white arrows) indicating ovarian development. Flutamide-treated embryos showed development of the cortex in some individuals (I, K) and development of medullary cords in the others (J, K). Scale bar = 50 μm.
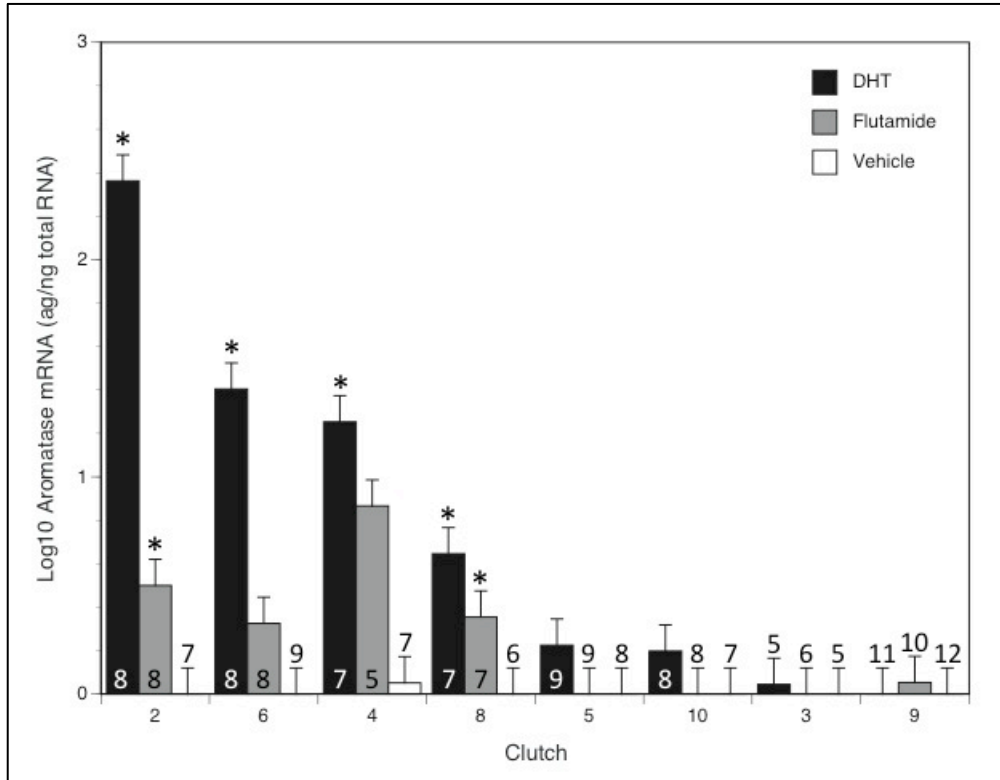
Figure 5.3. Log10 transformed aromatase expression in gonads from 8 clutches for snapping turtle embryos treated with either DHT, flutamide, or vehicle. Aromatase mRNA are least square means ($\pm$ 1 SE) for each clutch and treatment. Sample sizes for each treatment and clutch are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) between the treatments and vehicle control from the Dunnett's post hoc test.
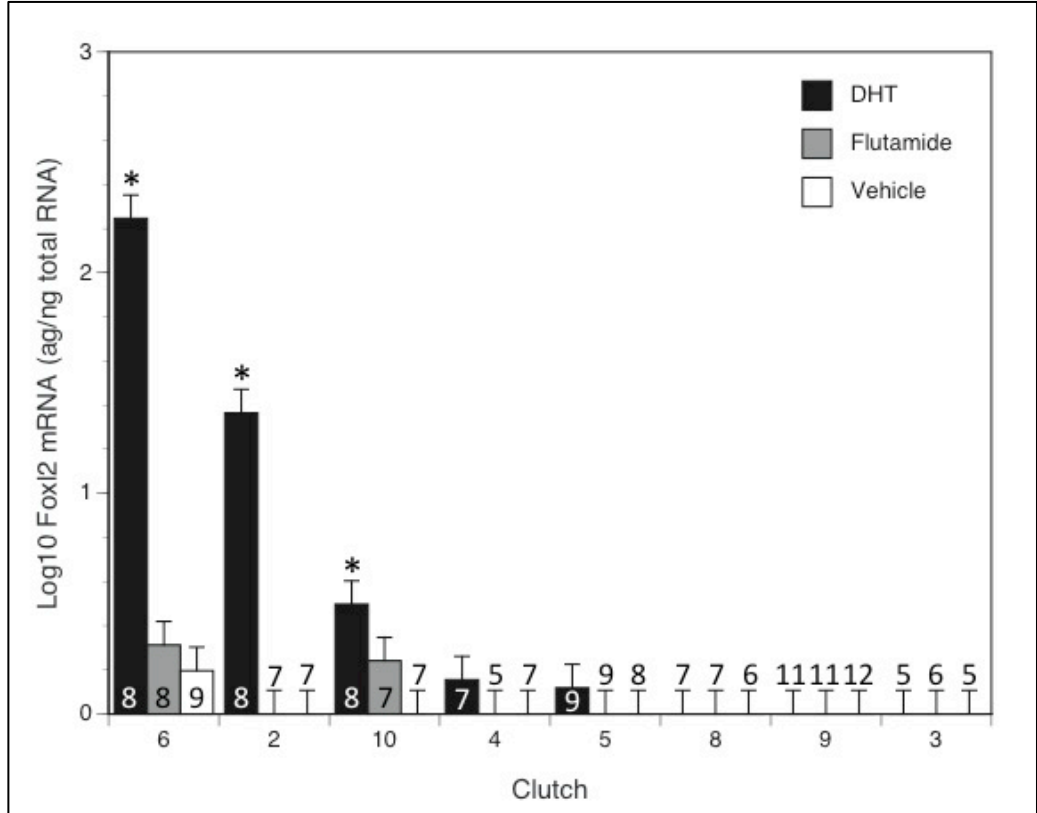
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Figure 5.4. Log10 transformed *Foxl2* expression in gonads from 8 clutches for snapping turtle embryos treated with either DHT, flutamide, or vehicle. *Foxl2* mRNA are least square means ($\pm$ 1 SE) for each clutch and treatment. Sample sizes for each treatment and clutch are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) between the treatments and vehicle control from the Dunnett's post hoc test.
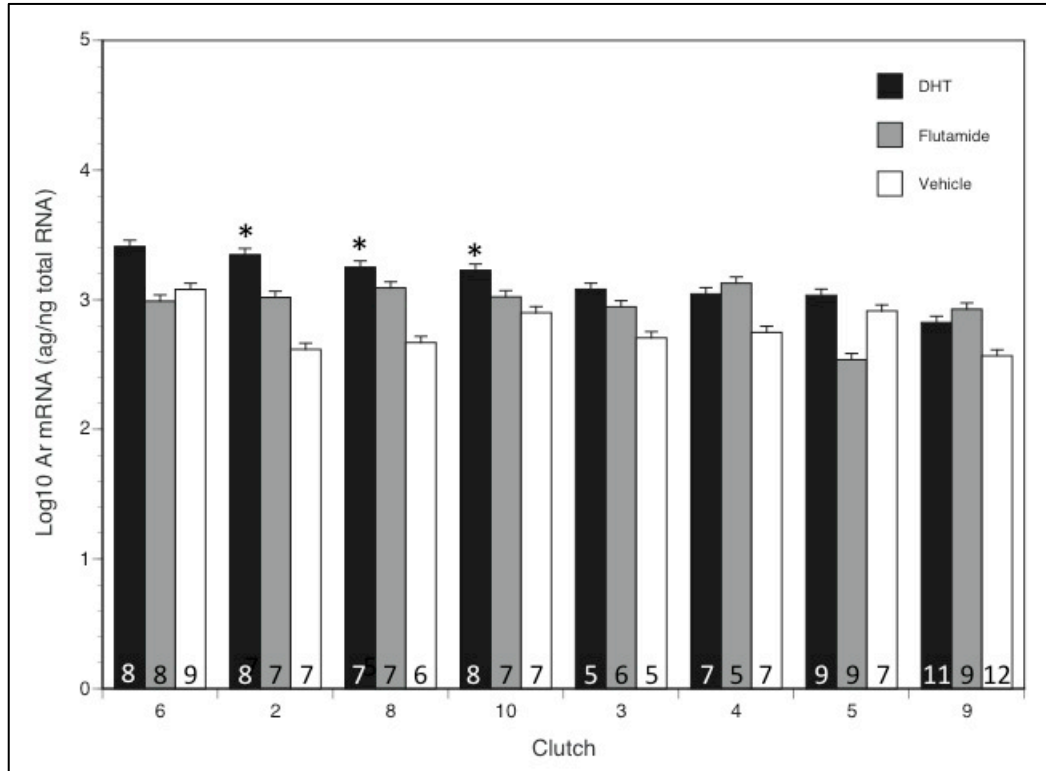
Figure 5.5. Log10 transformed *Ar* expression in gonads from 8 clutches for snapping turtle embryos treated with either DHT, flutamide, or vehicle. *Ar* mRNA are least square means ($\pm$ 1 SE) for each clutch and treatment. Sample sizes for each treatment and clutch are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) between the treatments and vehicle control from the Dunnett's post hoc test.

Figure 5.6. Log10 transformed *Amh* expression in gonads from 8 clutches for snapping turtle embryos treated with either DHT, flutamide, or vehicle. *Amh* mRNA are least square means ($\pm$ 1 SE) for each clutch and treatment. Sample sizes for each treatment and clutch are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) between the treatments and the vehicle control from the Dunnett's post hoc test.
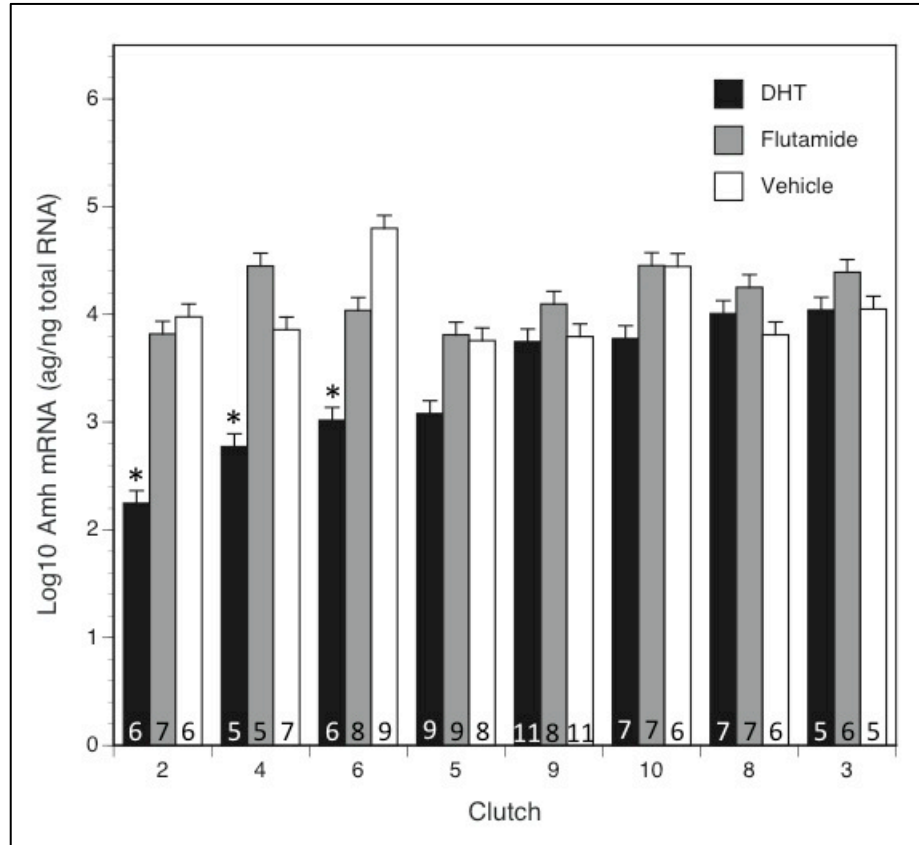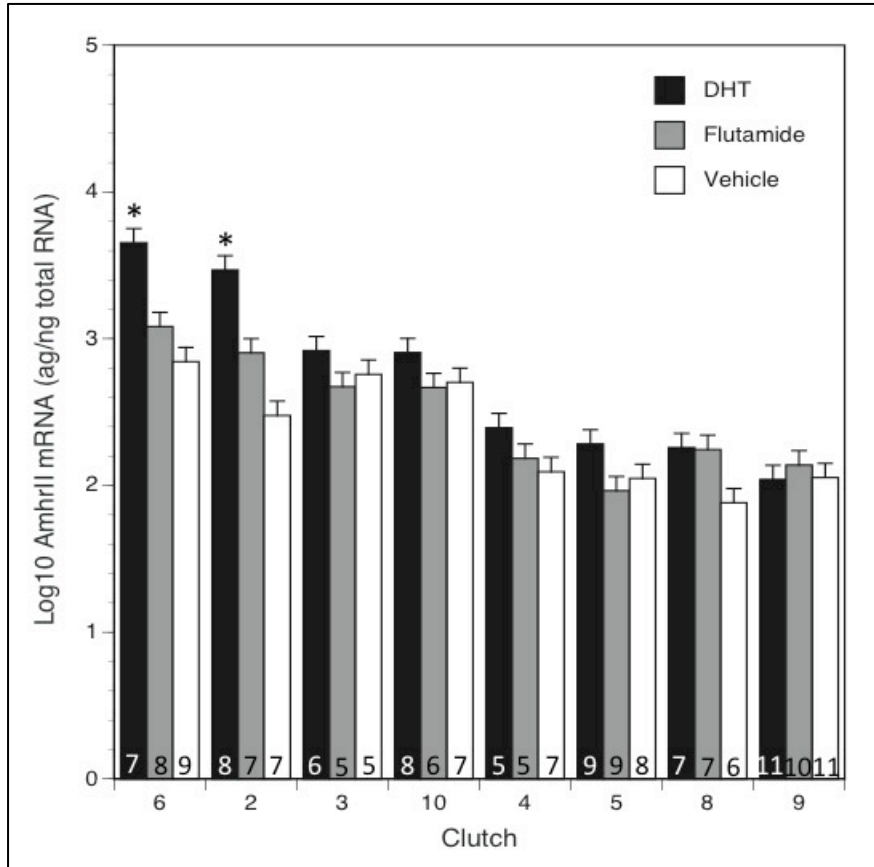
Figure 5.7. Log10 transformed *AmhrII* expression in gonads from 8 clutches for snapping turtle embryos treated with either DHT, flutamide, or vehicle. *AmhrII* mRNA are least square means ($\pm$ 1 SE) for each clutch and treatment. Sample sizes for each treatment and clutch are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) between the treatments and the vehicle control from the Dunnett's post hoc test.

Figure 5.8. Log10 transformed expression of steroidogenic genes StAR (A), Cyp11a1 (B), Cyp17a1 (C), *3ß-Hsd* (D), *17ß-Hsd* (E), *Srd5a1* (D) in gonads from snapping turtle embryos treated with either DHT, flutamide, or vehicle. mRNA levels are least square means ($\pm$ 1 SE) for each treatment from the model that did not include the putative sex. Sample sizes for each treatment are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) between the treatments and the vehicle control from the Dunnett's post hoc test.
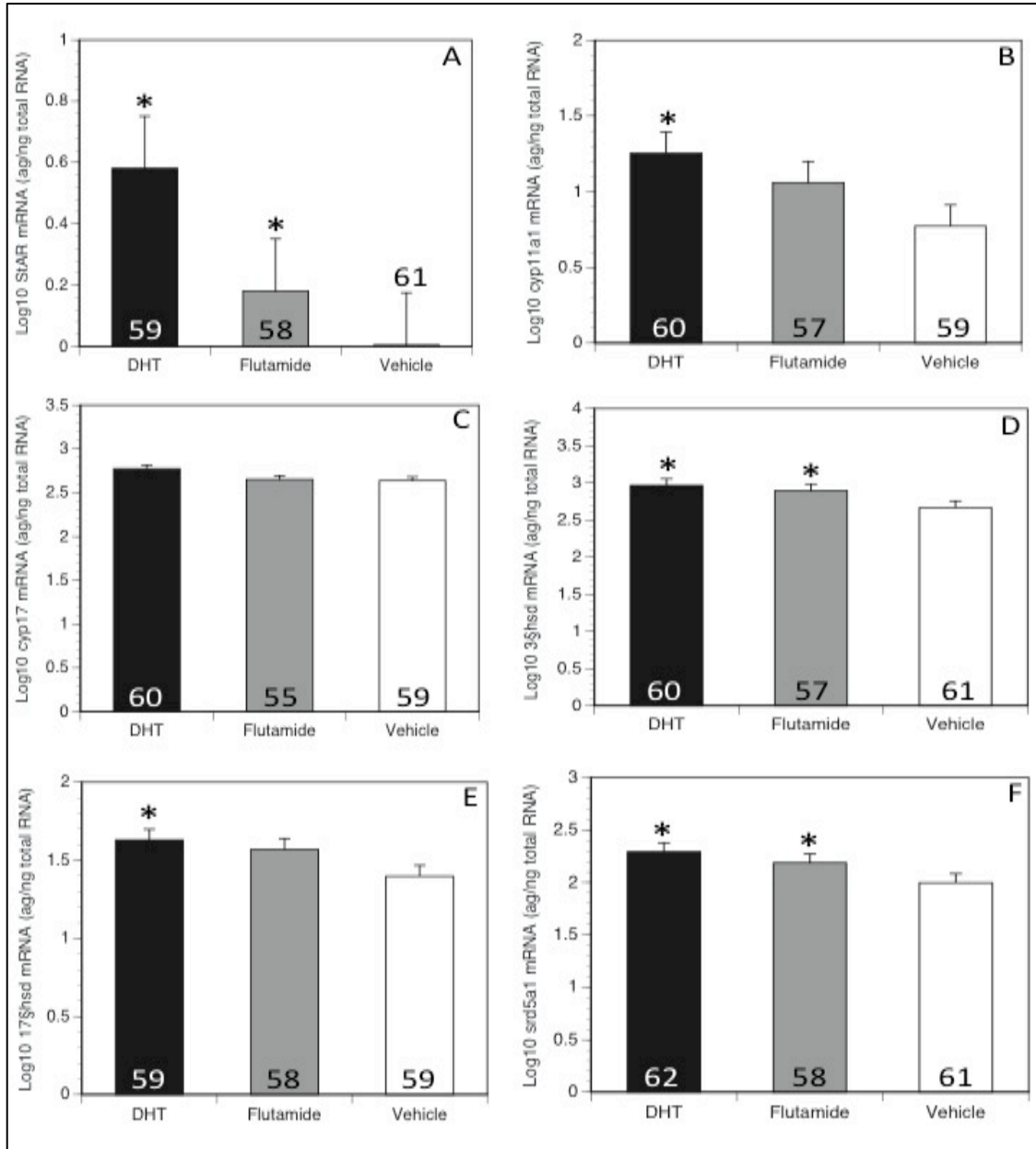
Figure 5.9. Log10 transformed *StAR* expression in gonads from 8 clutches for snapping turtle embryos treated with either DHT, flutamide, or vehicle. *StAR* mRNA are least square means ($\pm$ 1 SE) for each clutch and treatment. Sample sizes for each treatment and clutch are presented in each column or above the error bars. Asterisks indicate a significant difference ($p \leq 0.05$) from between the treatments and the vehicle control from the Dunnett's post hoc test.
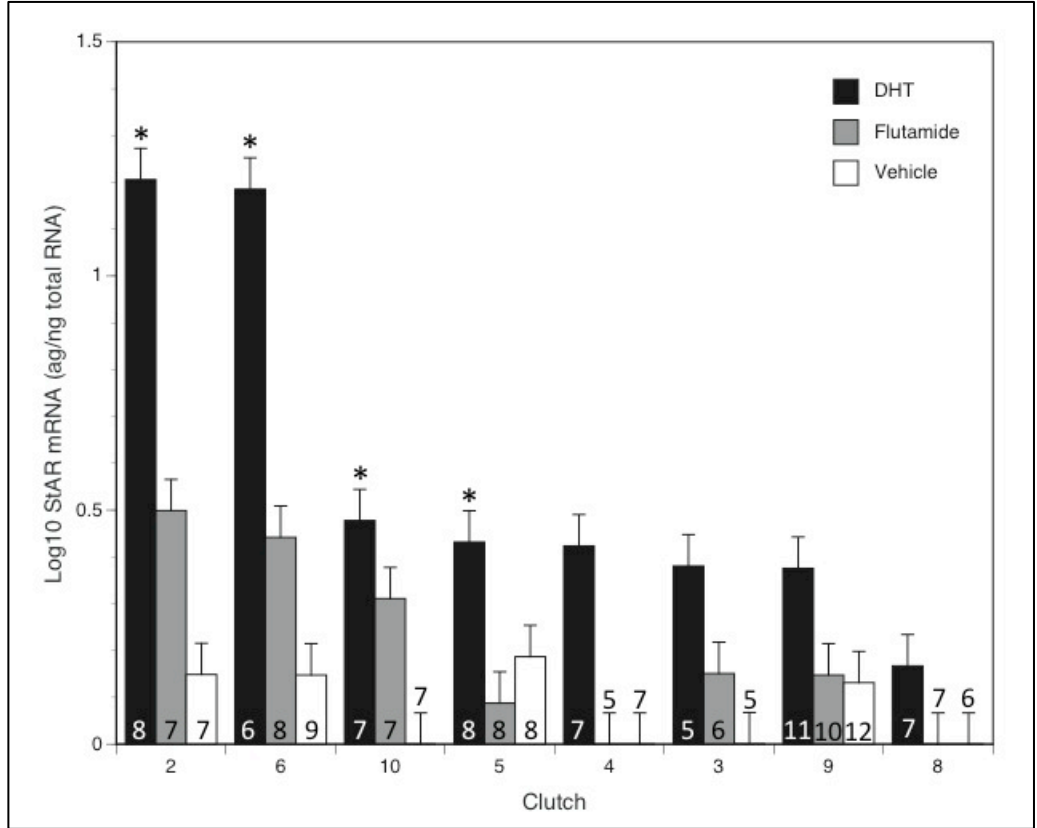
CHAPTER VI

EPILOGUE

Summary of Research Findings

The genetic, physiological, and molecular mechanisms underlying temperature-dependent sex determination (TSD) are not well understood. Numerous studies have focused on cloning orthologous genes known to be important for sex determination in mammals, but little research has focused on the cloning and identification of unique genes involved in TSD.   Also, sex steroid hormones are known to be involved in sex determination or maintenance of the gonads of all vertebrate species. Estrogens are critical for ovarian development, but the role of androgens in sex determination remains unclear. The research described here had two main objectives 1) Identify unique, thermo-sensitive, genes involved in TSD and 2) Determine the role of androgens in sex determination and differentiation in a TSD species.

The goal of the study in the second chapter was to identify a candidate gene involved in TSD. I identified the cold inducible RNA-binding protein (Cirbp) as a strong candidate of TSD in the snapping turtle. Cirbp was strongly induced at a high female-producing temperature, but not at a low female-producing temperature. This suggests that Cirbp is involved in the commitment to ovarian development. Cirbp has been cloned in other reptile species with TSD, but this is the first study to examine its role in sex determination (Kohno et al., 2010; Chojnowski and Braun 2012). Furthermore, I

detected higher Cirbp protein expression in the developing ovaries than testes. I also observed differences in localization of Cirbp protein during sex determination and gonad differentiation, suggesting different roles of Cirbp in sex determination and differentiation.

Genetic association studies are commonly used to provide genotype-phenotype relationships. The study described in chapter III identified a novel association between Cirbp expression and TSD. Next-generation sequencing allowed relatively quick identification of SNPs. I observed allelic specific expression and differences in allele frequencies between turtle embryos from northern and southern Minnesota, suggesting genetic adaptation to local thermal regime. I also found significant genetic associations between Cirbp genotype, Cirbp expression and sexual phenotype in a study that produced mixed sex ratios. These results provided the strongest evidence to date for a TSD gene.

The role of androgens and androgen signaling in development of the male reproductive tract is not well known in any species. The experiments in Chapter IV were developed to address the role of androgens and anti-androgens on the morphology of the developing male reproductive tracts. Unfortunately, the treatments with DHT or flutamide were sufficient to produce females, despite the embryos being incubated at an all-male producing temperature. The development of females by my treatments confounds the results of this study. Although this study provided little information about the mechanisms of androgen action on development of the male reproductive tracts, it provided a new question about the role of androgens in sex determination in the snapping turtle.

There is a growing hypothesis that androgens are important in ovarian development in species where aromatase is a key regulator (Ramsey and Crews; 2009). Our results from Chapter IV, suggest that this is an acceptable hypothesis in the snapping turtle. I found that DHT and flutamide were sufficient to produce females, despite individuals being incubated at an all-male producing temperature. It appears that androgens and AR are likely involved in the regulation of aromatase, presumably to convert endogenous testosterone into estrogens needed to induce ovarian development. I also observed variation in the number of females produced within each clutch treated with DHT or flutamide. This observation suggests the existence of genetic variation in at least one component of the androgen signaling pathway.

Future Studies and Hypotheses

Although I found a significant association between Cirbp allelic expression and sex ratios produced in snapping turtles families, these results do not prove causation. Future studies need to be conducted to determine a functional role of Cirbp in TSD. In mice and rats, the development of a gene knockout provides the functional role for a candidate gene involved in developmental processes, like sex determination. Gene knockouts are not plausible in snapping turtles, but with new technologies, like short interfering RNAs (siRNA), we can knockdown gene expression to determine the function of a candidate gene. We are developing an organ culture system to knockdown expression of Cirbp mRNA using siRNA in bipotential gonads. I hypothesize that knockdown of Cirbp mRNA in gonads incubated at female-producing temperatures will lead to up-regulation of testis-specific genes, and ultimately, the development of testes. Also, these studies did not provide any information about where Cirbp lies in the gene

regulatory network for TSD.   We are performing genetic studies that will examine F1

generations from snapping turtles from different populations that will provide insights

into the possible regulation of Cirbp and its role in the gene regulatory cascade.

My results provide strong evidence for a role of androgens in ovarian

development. These results do not provide causal information of how androgens and AR

are signaling to induce ovary formation. We previously suggested that AR is able to

regulate aromatase and FoxL2 directly (Rhen et al., 2007). Future studies will need to

examine if androgen response elements (AREs) occur within these female-specific genes.

The genetic variation in androgen signaling that I observed presents some important

questions.   For example, 1) Is there a dose-dependent response for androgens to induce

ovarian development? 2) Is the observed variation due to genetic differences in AR or

AREs in female-specific genes? 3) If variation is due to AR, is it because of differences

in AR activity, sensitivity, or combination of the two?

What it All Means (Practical Application of This Work)

The development of male or female organs and secondary sex characteristics

shapes every aspect of our biology. Understanding the underlying molecular processes

involved in this process leads to a better understanding of the sex differences, which lead

to differential life histories, selection pressures and interactions. Also, understanding the

molecular mechanisms underlying sex determination has implications for reproductive

and general health and disease. Perturbations in gene expression during sexual

development can lead to fertility problems and have effects individual fitness.   In species

with TSD, the identification and characterization of required thermo-sensitive genes can

explain the evolutionary potential and adaptability of these organisms to changing

environments. This is important for species with TSD, because global climate change could lead to the development of skewed sex ratios in already threatened species.

Sex steroid hormones and their receptors are crucial for development and/or the maintenance of the gonads in all vertebrate species. Mutations in sex hormone receptors or exposure to high or low levels of hormones can lead to disease phenotypes (Sahay et al., 2002). Understanding how androgens function and elicit their responses in the gonads is essential for fertility in both males and females. In females, exposure to high levels of androgens during development can lead to polycystic ovarian syndrome (PCOS), while insufficient androgen levels leads to premature ovarian failure (Walters et al., 2008). Our previous studies treating turtle embryos with androgens showed ovarian phenotypes similar to PCOS (Rhen, unpublished results). This observation supports conserved role of sex steroid hormones needed for proper ovarian development. The snapping turtle may function as a model for exploring the genetic and molecular mechanisms associated with PCOS. The examination of genetic variation in androgen responsiveness in snapping turtles may also provide a basis for variation in hyperandrogenism observed in humans with PCOS (Escobar-Morreale et al., 2005).

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