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Hypothalamus And Pituitary Gland Development In The Common Snapping Turtle, Chelydra Serpentina, And Disruption With Atrazine Exposure

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HYPOTHALAMUS AND PITUITARY GLAND DEVELOPMENT IN THE COMMON SNAPPING TURTLE, *CHELYDRA SERPENTINA*, AND DISRUPTION WITH ATRAZINE EXPOSURE

by

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This dissertation, submitted by Kathryn Lee Gruchalla Russart 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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PERMISSION

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Kathryn Lee Gruchalla Russart
July 15, 2016

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ABSTRACT

Neural control of the endocrine system originates in the hypothalamus in all vertebrates. The hypothalamus signals directly to the pituitary gland to maintain homeostasis by releasing hormones directly into the bloodstream. Hormones travel to peripheral endocrine organs, and regulate widespread activities including growth, metabolism, stress response, circadian rhythms, water balance, and reproduction. Many of these functions have sex-specific aspects, and therefore sexual dimorphism in morphology and function of the hypothalamus is present. Sex-specific genetic background and morphology is established during pre- and peri-natal development, during which sexually dimorphic cellular differentiation occurs and hormonal profiles are set. Disruption of development can therefore have widespread sex-specific effects. A thorough understanding of underlying molecular regulation of sex-specific development is therefore important for understanding disease and dysfunction. Here, we present four studies investigating the sex-specific development of the hypothalamus and pituitary gland in the common snapping turtle, a species with temperature-dependent sex determination. First, we tracked morphological development of the pituitary gland from its origin early in embryogenesis through hatching. Development is a highly conserved process between vertebrate species. Next, we

analyzed sex differences in gene expression within the hypothalamus and pituitary gland in embryonic and hatchling turtles. We specifically analyze expression of genes involved in reproductive neuroendocrine function, and also use RNA-seq to identify all differentially expressed genes between sexes and development to further understanding of the underlying mechanisms of development and function. We identified overrepresented functional categories of the differentially expressed gene sets between sexes, and identify protein modification as a potential mechanism underlying sexual dimorphism. Last, we analyzed changes in neuroendocrine gene expression following embryonic exposure to the widely used herbicide atrazine, an endocrine disrupting chemical. Our results indicate an acute, embryonic exposure to atrazine can have persistent changes in expression of genes involved in stress response and reproduction in both the hypothalamus and pituitary gland. This dissertation adds to the body of literature on comparative neuroendocrine development, provides background information for numerous future experiments, and identifies the snapping turtle as another species with potential impacts from atrazine exposure.

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CHAPTER I

INTRODUCTION

Industrial and agricultural practices have dramatically advanced in the past century, meeting the increasing demands of a growing population and greatly improving human lives. However, these improvements require the use of chemicals, which increases the risk of environmental contamination. Some chemicals have unforeseen negative side-effects in addition to their intended function. Endocrine disrupting chemicals (EDCs) are one example of a class of contaminants with unforeseen effects in humans and wildlife. EDCs are any chemical that interferes in normal endocrine function. The gonadal, thyroid, and adrenal axes are the most common areas of interference. EDCs include agricultural, industrial, and household products, and are widely distributed in the environment. Phthalates, bisphenol A (BPA), and DDT are a few commonly recognized EDCs (reviewed in Diamanti-Kandarakis et al., 2009). Such chemicals have been found to disrupt the neuroendocrine system in vertebrates at each taxonomic level, and therefore understanding the risks of exposure is of high importance.

EDCs can interfere with sexual differentiation in gonads. For example, Berg et al. (2009) found that exposure of chicken embryos to BPA can disrupt proper testes formation by increasing cortex thickness and decreasing testicular cord production. The brain also undergoes sexual differentiation and displays several sex-specific regions. EDC exposure can alter sex-specific reproductive behavior, which indicates disruption of

sex-specific brain development. Consequences of abnormal sexual differentiation of the brain can be life-long, and include decreased fertility, due to abnormal reproductive behavior and physiology. Furthermore, many diseases and disorders are biased toward one sex, and have been linked to abnormalities in sexual differentiation of the brain, including schizophrenia, autism, and mood disorders.

Atrazine, a commonly-used herbicide, is linked to abnormal reproductive development and sex-specific mating behavior. Atrazine is applied to agricultural fields in April and May, just before egg-laying season for the common snapping turtle; therefore snapping turtle embryos are susceptible to exposure during development. Here, we investigate the effects of atrazine on sexual differentiation in the hypothalamus and pituitary gland of the snapping turtle. Very little work has been done previously on the development of these regions in the snapping turtle, and therefore we first establish normal morphologic and molecular characteristics in the developing hypothalamus and pituitary gland in the snapping turtle.

The hypothalamus is the major brain region involved in regulating the endocrine system. It is located in the diencephalon at the base of the brain and surrounds the third ventricle. The hypothalamus relays hormonal and neuropeptide signals to the pituitary gland, located just below the hypothalamus. The pituitary gland is the master endocrine gland, and responds to hypothalamic signals by synthesizing and secreting hormones directly into the bloodstream, which carries pituitary hormones to peripheral endocrine organs. The endocrine system maintains homeostatic function throughout the body,

including regulation of reproduction, stress responses, water balance, metabolism, growth, and circadian rhythms.

The hypothalamus is composed of several distinct regions, or nuclei, composed of densely-packed neurons sharing similar function. The number of nuclei and nomenclature varies from species to species. In humans the hypothalamus is divided into an anterior, tuberal, and posterior region. The anterior region is composed of the preoptic, suprachiasmatic, supraoptic, anterior, and paraventricular nuclei. The tuberal region contains the dorsomedial, ventromedial, arcuate, and periventricular nuclei, and the posterior region is composed of the posterior and mammillary nuclei. Each nucleus contains neurons with a distinct function which signal to other brain regions and the pituitary gland. The preoptic and arcuate nuclei contain parvocellular neurons which secrete gonadotropin-releasing hormone (GnRH); the periventricular nucleus secretes somatostatin and thyrotropin-releasing hormone, the arcuate nucleus secretes growth hormone-releasing hormone (GHRH) and prolactin releasing hormone (PRLH), and the paraventricular nucleus secretes corticotropin-releasing hormone into the portal system of the median eminence. The supraoptic and paraventricular nuclei contain magnocellular neurons, which send their axons through the infundibulum into the pituitary gland, releasing oxytocin (OXY), and antidiuretic hormone (ADH) from axon terminals directly into the bloodstream.

The pituitary gland is composed of three major regions: the anterior, intermediate, and posterior lobes. The portal system leads to the anterior lobe (adenohypophysis), and contains five major cell types: gonadotropes, thyrotropes, lactotropes, corticotropes, and

somatotropes. In response to hypothalamic signals, these cell types synthesize and secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH), prolactin (PRL), adrenocorticotropic hormone (ACTH), and growth hormone (GH) respectively, which travel through the bloodstream to peripheral endocrine organs. The intermediate lobe synthesizes and secretes only one hormone, melanocyte-stimulating hormone (MSH), which controls melatonin production from the pineal gland and regulates circadian rhythms. The posterior lobe (neurohypophysis) does not synthesize hormones, but instead stores and secretes hormones from hypothalamic magnocellular axon terminals. Antidiuretic hormone (also known as vasopressin, arginine vasopressin, or vasotocin) and oxytocin are released into the bloodstream, affecting water balance and parturition, respectively.

Major regulation of the hypothalamus-pituitary-gonad (HPG) axis of the endocrine system begins with kisspeptin (Kiss1) neurons in the preoptic and arcuate nuclei, which signal via kisspeptin receptor (Kiss1R) on GnRH parvocellular neurons. GnRH is released into the portal system of the median eminence and binds to GnRH receptors (GnRHR) on gonadotropes. LH and FSH are released into the bloodstream, traveling to the gonads where they bind to their respective receptors. In females LH induces production of sex hormones, including estradiol, while FSH stimulates the maturation of ovarian follicles. In males LH induces production of androgens and FSH stimulates spermatogenesis. Androgens and estrogens have negative feedback effects on GnRH neurons and gonadotropes via their receptors, androgen receptor (AR), estrogen

receptor 1 (ESR1), and estrogen receptor 2 (ESR2). Precise control of the HPG is important for proper reproductive function and fertility throughout life.

The hypothalamus displays sex-specific organization (Phoenix et al, 1959; Aste et al, 2010; Buedefield et al, 2015). The preoptic area is a highly conserved region of the brain, and is important for sex-specific mating behavior and gonadotropin secretion. A second region of the brain that exhibits sexual dimorphism is the anteroventral region of the periventricular nucleus, which is involved in initiating the preovulatory surge of LH in female mammals (Bleirer et al, 1982). Sexual dimorphism is established during pre- and post-natal development through a surge in steroid hormones from the gonads. Male-specific organization is established through local conversion of testosterone into estradiol by the enzyme aromatase (Aste et al, 2010). Sexual dimorphism within the pituitary gland is not structural, but rather functional as a result of differences in signaling patterns from the hypothalamus. Therefore, proper establishment of sexual dimorphism within the hypothalamus during development is imperative for normal HPG function throughout life.

Steroid hormones from the gonads establish secondary sex characteristics throughout the organism, including the brain. Therefore, proper gonadal differentiation is also critical for HPG function. Disruption of steroid signaling during development can have lifelong effects on fertility. Alterations in gonadal differentiation, and consequently HPG organization can occur through many mechanisms, from gene mutation to developmental exposure to steroid hormones or other EDCs.

EDCs can interfere in steroid signaling and disrupt proper organization and function of the HPG. EDCs can disrupt steroid signaling by acting as agonists or antagonists of steroid receptors, interfering in steroid production, or disrupting metabolism of steroids, and disruption can interfere with function and proper development.

Here, we focus on neuroendocrine development in the common snapping turtle, *Chelydra serpentina*. The snapping turtle is a species with a wide range, found throughout the United States east of the Rocky Mountains. The snapping turtle is a species with temperature-dependent sex determination (Janzen, 1992; Rhen and Lang, 1994), which allows 100% confidence in the sex of the animal by incubating eggs at set temperatures: 26.5ºC produces males, and 31ºC produces females. Snapping turtles produce large clutches, averaging 45 eggs per clutch, which allows one to control for genetic variation. Furthermore, we have established that gonadal sex is determined between stages 13 and 20 of development (Rhen et al, 2015), and therefore sexual differentiation of the brain can be predicted to occur after stage 20.

Although development of the hypothalamus and pituitary gland has not been previously studied in the snapping turtle, development, morphology, and function of these tissues are highly conserved among vertebrates studied to date (Medina, 2008; Dominguez et al, 2010; Moreno and Gonzalez, 2011; Morales-Delgado et al, 2014; Moreno et al, 2012). Functional similarities in specific hypothalamic regions include sex-specific regulation of reproductive behavior, which is controlled by the preoptic area and

ventromedial hypothalamus (Crews and Silver, 1985; Crews et al, 1990; Kingston and Crews, 1994; Kendrick et al, 1995; Wade and Crews, 1991; Kendrick et al, 1995).

This is the first investigation into development of the hypothalamus and pituitary gland of the snapping turtle. These studies will lay the foundation for understanding neuroendocrine development and function in the snapping turtle and further our understanding of the HPG axis in a species with temperature-dependent sex determination. This work will be an important contribution to the body of literature on reptilian neuroendocrine development and enable deeper understanding of conservation and evolution of these tissues.

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CHAPTER II

SEXUALLY DIMORPHIC DEVELOPMENT OF THE HYPOTHALAMUS AND PITUITARY GLAND IN THE SNAPPING TURTLE, *CHELYDRA SERPENTINA*

Abstract

The hypothalamus signals through the pituitary gland in order to regulate the endocrine system and maintain homeostasis in vertebrates. Sex-specific development is highly conserved among vertebrates, but remains understudied in reptile species. Here, we provide an in-depth morphological analysis of pituitary gland development in the common snapping turtle. We track adeno- and neurohypophysial development from its origins early in embryogenesis through four months after hatching. We also analyzed sex differences in expression of neuroendocrine genes involved in reproduction in both the hypothalamus and pituitary gland during embryonic development, at hatch, and six months after hatching. We identified sex differences as early as stage 20 of development, and dimorphisms are dynamic throughout development. This study adds to the body of literature on comparative neuroendocrine development, and provides background for future experiments in the snapping turtle.

Introduction

The hypothalamus is the main region of neural control for the endocrine system in vertebrates, regulating homeostatic functions such as hunger, circadian rhythms, water balance, growth, stress response, and reproduction. Traditional morphology considers the

hypothalamus part of the diencephalon, but the more recent Prosomeric Model, based on early molecular profiles, holds an alternative view in which the hypothalamus is part of the secondary prosencephalon, a region just rostral to the diencephalon (reviewed in Dominguez et al., 2015). However, many studies contradict the Prosomeric Model, and further work is required to elucidate the exact model of hypothalamic development. Regardless, it is located below the thalamus, and surrounds the third ventricle. It is an anatomically complex region with specific, but ill-defined nuclei; specific nuclear nomenclature varies from species to species. Furthermore, it is located at the sharp flexure of the longitudinal axis of the brain, making hypothalamic development difficult to compare between species.

Hypothalamic nuclei are composed of densely packed neurons, all involved in a similar function. While the exact nomenclature is variable among species, the mouse has a well-characterized atlas describing specific nuclei (Shimogori et al., 2010). Furthermore, different nuclei are present/absent in the hypothalamus depending on species. The reptile hypothalamus, while simpler in overall nuclear structure, retains similar physiological properties and molecular markers of development (Moreno and Gonzalez, 2011; Moreno et al., 2012; Morales-Delgado et al., 2014).

The hypothalamus relays neural and hormonal signals to the pituitary gland, which secretes hormones into the bloodstream, affecting peripheral endocrine glands. The pituitary gland is directly connected to the hypothalamus by the infundibulum, a structure containing the portal vasculature as well as magnocellular neuron axons. The pituitary gland is composed of two regions differing substantially in their developmental origins

and physiology. The anterior pituitary gland, or adenohypophysis, originates from oral ectoderm, and responds to hormonal signals transported from the hypothalamus through the portal vasculature. Neurosecretory neurons in the hypothalamus secrete hypothalamic-releasing hormones into the hypophysial portal system to the anterior lobe, stimulating the release of tropic hormones, including follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, adrenocorticotropic hormone, growth hormone, and prolactin. The posterior lobe, or neurohypophysis, originates from the ventral floor of neural ectoderm, and receives neural signals from the hypothalamus. Neurosecretory magnocellular neurons, whose cell bodies are located in the hypothalamus, send axons through the infundibulum. Action potentials travel through the axons into the posterior lobe to the axon terminals, where oxytocin and vasopressin are released.

The hypothalamus and pituitary gland develop concomitantly, with discrete and interactive molecular signals controlling the development of both structures. Molecular regulation has been studied in a wide variety of species, extensively in mouse and rat, to a lesser extent in chicken and zebrafish, and minimally in reptile species and amphibians (Szarek et al., 2010). Hypothalamus and pituitary development is a highly conserved process in all vertebrates studied to date, with many similarities across vertebrate phyla in control, regulation, and morphology.

Initial anterior-posterior patterning is established by gradient expression of Wingless family (Wnt) signaling proteins prior to E8.5 in mice (Chatterjee and Li, 2012). Sonic Hedgehog (Shh) expression is vital for dorso-ventral patterning and is necessary

for initial induction of the hypothalamus (Mathieu et al., 2002). Following initial establishment of the hypothalamic anlagen, regional restriction of transcription factors is established to form distinct neural cell types which form specific nuclei (Kapsimali et al., 2004; Lee et al., 2006). Cell proliferation, migration, and differentiation are all important mechanisms establishing regionality (Acampora et al., 1999; Wang and Lufkin, 2000). Parvocellular and magnocellular neurons are established at this time through the expression of specific transcription factors, and, in mice, parvocellular neurons in the arcuate (ARC) and ventromedial (VMN) nuclei begin forming at E10.5 (Wang and Lufkin, 2000). Magnocellular neurons are located in the paraventricular (PVN) and the supraoptic (SPN) nuclei, and project axons to the posterior lobe of the pituitary gland. Magnocellular neuronal populations are established beginning at E10.5 in mice (Nakai et al., 1995).

Several studies and reviews of pituitary gland development in mammalian species exist. An overview of morphological development and cellular differentiation of the pituitary gland in human was described by Ikeda et al. (1988) and numerous studies have been published (reviewed in Kelberman et al., 2009). Several papers describe overall morphological development of either the adenohypophysis, or the entire pituitary gland in mouse (Reyes et al., 2008; Rizzoti and Lovell-Badge, 2005). Fewer studies have been conducted on non-mammalian species. Morphological and cellular differentiation of the adenohypophysis was described in chicken and the Japanese quail (Reyes et al., 2008), but few studies describe development of the entire pituitary gland. While studies in amphibians (Raquet and Exbrayat, 2007) and teleosts (Chapman et al., 2005) are

available, hypophysis development is quite different than in amniotes. Studies of pituitary gland development in reptiles are sparse. Ferrandino and Grimaldi (2004) described cellular differentiation in the adenohypophysis in the skink, and Ferrandino et al. (2004) described hypophysial development in the lizard *Anguis fragilis*. One study of the major morphological events in pituitary gland development in a turtle species was published by Pearson et al. (1983); however this study examined hypothalamus and pituitary gland development in the loggerhead sea turtle. Therefore, this is the first description of pituitary gland development in the snapping turtle, and will serve to determine similarity to other turtle species previously described.

The anterior lobe begins formation as an invagination of the oral ectoderm. The initial invagination forms a structure known as Rathke's pouch, first described by Rathke in 1838, and is the primordial anterior lobe. Rathke's pouch continues to invaginate, eventually contacting the posterior lobe and pinching off from the oral ectoderm. Conversely, the posterior lobe forms as an evagination of the diencephalon and therefore is neural in origin. Due to the differences in origin, developmental regulation of the two lobes involves distinct events, but inductive signals from the ventral diencephalon are imperative for formation (Kawamura and Kikuvama, 1992; Zhao et al., 2010).

Appropriate hypothalamic and pituitary gland development is imperative for proper function and survival due to the widespread homeostatic effects in an organism. Null mutations in regionally expressed transcription factors impact hypothalamic development, and many animals do not complete development (Acampora et al., 1999) or die shortly after birth (Hosoya et al., 2001). Failure of neurogenesis within the

hypothalamus results in underdeveloped nuclei (McNay et al., 2006), and can result in numerous abnormalities including decreased fertility (Navarro et al., 2005) and obesity (Goldstone, 2003). Pituitary gland development is likewise important for proper physiological function and survival of the animal. Mutations in genes required for cell proliferation, survival, and differentiation in the anterior lobe result in early death in mice (Szeto et al., 1999; Charles et al., 2005), an underdeveloped anterior lobe (Zhao et al., 2006), and maintenance of undifferentiated cells into adulthood (Chen et al., 2005). Many abnormalities can result from gene mutations affecting the pituitary gland including hypothyroidism (Dattani and Robinson, 2000), failure to complete puberty or precocious puberty (Dattani and Robinson, 2000), and combined pituitary hormone deficiency, a syndrome affecting the secretion of several tropic hormones, which has widespread effects (Cogan et al., 1998; Holl et al., 1997). Posterior lobe formation is also critical with developmental abnormalities causing lifelong disease such as diabetes insipidus (Maghnie et al., 1992).

Proper hypothalamic development includes formation of sexual dimorphisms. Development of the gonads and sex-specific hormonal profiles directly influences differentiation of the brain. The traditional view is that a testosterone surge in male mammals late in embryonic development programs sexual differentiation (Jost, 1983; McEwen, 1992). Testosterone derivatives are responsible for masculinization and defeminization of the brain: aromatase converts T into estradiol, while 5α-reductase converts T into the more biologically active dihydrotestosterone (DHT) (Aste et al., 2010; Tobet et al., 1986). Hormone manipulation studies show diminished sex differences in

the hypothalamus, supporting this viewpoint (reviewed in Dickerson and Gore, 2007; Robinson, 2006). Zebrafish exposed to the aromatase inhibitor fadrozole show a distinct transcriptomic profile when compared to controls (Villeneuve et al., 2009). Elevated androgens during a critical window of development in mice resulted in an acceleration of GnRH pulsatility in the hypothalamus and concomitant suppression of gonadotropin synthesis and secretion (Gonzalez et al., 2011). However, work in the Zebra Finch suggests a steroid-independent mechanism of sexual differentiation in some parts of the brain (Gahr and Metzdorf, 1999; Schlinger, 1998; Wade and Arnold, 1996). Work in the snapping turtle supports the idea that some sex differences are programmed by hormone-independent mechanisms (Rhen and Lang, 1999), while work in other reptiles shows both hormone-dependent and hormone-independent programming (Beck and Wade, 2009; Jeyasuria and Place; 1998).

The hypothalamus contains sexually dimorphic regions involved in the control and regulation of the reproductive system and growth throughout life. The preoptic area (POA) contains important regions of sexual dimorphism and neurosecretory neurons which signal to the pituitary gland. The POA in rats is larger in males than in females (Gorski et al., 1978) and is known as the sexually dimorphic nucleus of the POA (SDN-POA). This region is conserved in all vertebrates studied to date, and is involved in the regulation of copulatory behavior and gonadotropin secretion, and contains a high density of neurons known to be targets of steroid hormones. Exposure to ethenyl estradiol decreases the volume of the SDN-POA in male rats (Shibutani et al., 2005). Two other regions of sexual dimorphism include the anteroventral periventricular nucleus (AVPV)

which is larger in female mice and rats (Bleier et al., 1982). The AVPV is involved in initiating the preovulatory luteinizing hormone (LH) surge in female mammals through GnRH neurons and innervates neurons of the arcuate nucleus (ARC). The ARC is responsible for prolactin (PRL) secretion, and is also sexually dimorphic, having greater dendritic arborization in females, but more total neurons in males (Leal et al., 1998). Apoptosis is an important mechanism for development of sexual dimorphism within the hypothalamus (Forger et al., 2004), and genes regulating pro- and anti-apoptotic factors display dimorphic expression in males and females as well (Hsu et al., 2000). Furthermore, genes important for regulating the reproductive endocrine axis also show sexually dimorphic expression in rat, such as expression of kisspeptin (Kiss1) and its receptor (Kiss1R) (Cao and Patisaul, 2011). Steroids, and therefore steroid receptors in the hypothalamus play a central role in development of sex differences during embryogenesis (reviewed in Kawata, 1995). Proper development is imperative for sex-appropriate behaviors, including sexual and maternal behavior, aggressive and affiliative behaviors, stress response, and learning, as well as fertility later in life.

Comparative studies using the same molecular markers in different vertebrates, including reptiles, have highlighted conservation in the genoarchitecture of the hypothalamus (Dominguez et al., 2015; Moreno and Gonzalez, 2011; Morales-Delgado et al., 2014). Dominguez et al. (2015) identified a regionally specific molecular profile in the hypothalamus of the fresh-water turtle, *Pseudemys scripta*, and compared vertebrates, finding a high similarity among amniotes in the preoptic region, supraoptoparaventricular region, suprachiasmatic region, tuberal region, and the mammillary region (Dominguez et

al., 2015). Furthermore, the posterior region of the basal hypothalamus in the Reeves Turtle appears to be homologous to the tubero-mammillary region in chicken (Inagaki et al., 1990). While the exact functions of some tropic hormones vary among vertebrate phyla, most hormones are functionally equivalent. Hormones with seemingly mammal-specific functions (i.e. prolactin) are present in reptiles as well.

Functional similarities are also present within specific hypothalamic regions. The preoptic area and anterior hypothalamus are involved in male-typical behavior (Crews et al., 1990; Kingston and Crews, 1994), while the ventromedial hypothalamus is important for female-typical reproductive behaviors in many reptile species (Kendrick et al., 1995; La Vaque and Rodgers, 1975; Wade and Crews, 1991). Furthermore, the anterior hypothalamus-POA, or smaller regions within this area are larger in male whiptale lizards (Crews et al., 1990) and have transient greater cell density in males of the green anole during development (Beck and Wade, 2009). Conversely, the VMH is larger in females (Crews et al., 1990; Wade and Crews, 1992). Unlike in rodents, where E2 masculinizes these regions (Davis and Barfield, 1979), testosterone primarily activates masculinization in green anoles (Winkler and Wade, 1998), whereas both T and E2 facilitate female reproductive behaviors and feminization of the VMH in green anole lizards (Tokarz and Crews, 1980; Beck and Wade, 2009), similar to rodents (Davis et al., 1979).

Sexual dimorphisms in the pituitary gland are not well-documented. However, there are greater numbers of LH-secreting gonadotropes in females during sexual maturity (Chen, 1988). There are few differences in the cellular make-up/morphology of the tropic cells, but more differences in function and regulation of these cells (Oishi et al.,

2005). For instance, by puberty, male rats contain more growth hormone than females (Birge et al., 1966; Childs et al., 1999) and Denef et al. (1973) found greater rates of testosterone metabolism in the pituitary gland of male rats. While well-documented differences between males and females in the timing of luteinizing hormone secretion exist (Ramirez and Mccann, 1962; Dohler and Wuttke, 1973), the molecular mechanisms involved in sexually dimorphic function of gonadotropes remains unclear.

Here, we describe the timing of events in pituitary gland development in the common snapping turtle, *Chelydra serpentina*. We also measure expression of 11 genes that play a regulatory role in the hypothalamic-pituitary-gonad axis, including kisspeptin (Kiss1), kisspeptin receptor (Kiss1R), gonadotropin releasing hormones 1 and 2 (GnRH1 and GnRH2), gonadotropin releasing hormone receptor (GnRHR2b), follicle-stimulating hormone beta subunit (FSHb), luteinizing hormone beta subunit (LHb), androgen receptor (AR), estrogen receptors 1 and 2 (ESR1 and ESR2), and aromatase (Cyp19a1). We tested whether these genes display sexually dimorphic expression during embryogenesis (just after gonadal sex determination), at hatch, and six months after hatch. This is the first investigation into development of the hypothalamus and pituitary gland in the snapping turtle, and establishes baseline information for future studies of neuroendocrine structure and function. This data can also be used for comparative studies of sexual differentiation of the neuroendocrine system.

Methods

Egg Collection

We collected snapping turtle eggs immediately after oviposition from north-central Minnesota and northeastern North Dakota during May and June of 2011and 2012 with permits from the Minnesota Department of Natural Resources and the North Dakota Game and Fish Department. Eggs, embryos, and hatchlings were treated according to protocols approved by the Institutional Animal Care and Use Committee at the University of North Dakota. Eggs were transported to the laboratory, washed with tap water, and numbered to track individual eggs within each clutch. Eggs were placed into plastic containers, covered with moist vermiculite (1 part vermiculite : 1 part water by mass), and placed into incubators. Half of the eggs were incubated at 31^{o}C (female-producing temperature) and half at 26.5^{o}C (male-producing temperature) (Rhen and Lang, 1998; Rhen et al., 2015). Temperature variation within the incubators is minimal (Lang et al., 1989).

Morphological Analysis of Pituitary Gland Development

Embryo Collection

Eggs were candled and embryos sampled to determine developmental stage, using anatomical criteria described by Yntema (1968). Embryos are at stage 1 or 2 at oviposition and stage 26 at hatch. We determined the stage of young embryos (before stage 9) by counting somite number. Little variation within clutch exists for developmental rate at each incubation temperature; therefore sampling of early embryos from one egg determined staging for the entire clutch. We began collecting embryos

when they reached stage 6 of development and continued through hatching. We collected 4 - 6 embryos from each incubation temperature at each stage of development.

Processing of Embryos: Stages 6 - 13

Embryos below stage 14 were left intact with the shell in order to minimize damage to delicate tissue during processing. Embryos were fixed in 4% paraformaldehyde for 24 hours at 4°C. Embryos were removed from the fixative after 24 hours and washed twice in 1X PBS buffer for 10 minutes each. Following washing, embryos were dehydrated through an ethanol gradient (30%, 50%, 70%, 80%, 95%, 100%). Following the last dehydration step, embryos were placed into a fresh tube of 100% ethanol for storage at -20°C. Prior to embedding, embryos were cleared in two washes, 20 minutes each, in Citrasolv. All wash, dehydration, and clearing steps were performed on a shaker at room temperature. Embryos were then removed from the eggshell and soaked in a paraffin bath for one hour at 90ºC (2 times) before embedding in paraffin. Paraffin was allowed to solidify at room temperature overnight.

Processing of Embryos: Stages 14 – 20

The head and mandible were removed from embryos between stages 14 and 20 prior to fixation. Embryos were fixed in 4% paraformaldehyde for 48 hours at 4°C. Samples were removed from fixative after 48 hours, and washed three times in 1X PBS buffer for 10 minutes each. The samples were then dehydrated through graded ethanol, and cleared with Toluene. Samples were placed into two paraffin baths at 90ºC before final embedding in paraffin, which was allowed to solidify at room temperature overnight.

Processing of Embryos: Stages 21 – Hatch

The brain was dissected from embryos of stages 21 through hatching. The pituitary gland is embedded in the hard palette. Therefore, removal of the hard palette pulls the pituitary gland away from the brain. To leave the pituitary gland intact with the hypothalamus, the region of the hard palette surrounding the pituitary gland was left attached to the brain. Brains were fixed in 4% paraformaldehyde for 24 – 36 hours at 4°C and washed three times in 1X PBS buffer for 10 minutes each. The samples were then dehydrated through graded ethanol and cleared with Toluene. Samples were placed in three paraffin baths at 90ºC before embedding in paraffin.

Tissue Sectioning, Staining, and Visualization

Tissues were sectioned on a sagittal plane at 7 µm thickness, placed on VWR VistaVision HistoBond Microscope Slides, and dried overnight. Tissue sections were stained with hematoxylin and eosin for histologic analysis using the following protocol: 100% EtOH for 30 seconds, purified water for 2 minutes, Gill's Hematoxylin for 10 seconds, Scott's tap water for 5 minutes, 70% EtOH for 1 minute, 95% EtOH for 1 minute, Eosin Y in alcohol for 2 minutes, 95% EtOH for 1 minute (2x), 100% EtOH for 1 minute, toluene for 3 minutes (2x). Slides were air dried and cover-slipped. Sections were visualized using an Olympus BX51WI light microscope, and photographs of pituitary gland development were taken using an Olympus CX9000 camera.

Sex Differences in Gene Expression

Tissue Collection and RNA Isolation

Tissues for these studies were the control (untreated) and vehicle-treated (100% ethanol) groups from a larger study described in chapters 3 and 4. Eggs were incubated until stage 20 of development, when they were candled to separate infertile eggs and dead embryos. Treatments were administered to eggs at this time: controls were left untreated, and 5 µl of 100% EtOH was spotted onto the eggshell in the vehicle-treated group. Eggs were then returned to the incubators. After 24 hours, 10 embryos from the male-producing temperature and 10 embryos from the female-producing temperature were euthanized by rapid decapitation. Ten male hatchlings and 10 female hatchlings were also euthanized at 1 week of age. Brains were collected, placed into RNAlater© solution (Ambion), and stored at -80^{o}C. Carapace length, plastron-cloaca distance, and mass were measured for the remaining hatchlings, and each was marked with a unique tag: colored beads were attached with wire through marginal scutes. Marked hatchlings were released into large pools and fed ad libitum for six months, at which time measurements were taken again. Hatchlings were euthanized, brains were collected and placed into RNAlater© for storage at -80^{o}C.

The hypothalamus and pituitary gland were micro-dissected from each brain through the hard palette. Total RNA was isolated from the hypothalami with RNAzol®RT (Molecular Research Center, Inc.). In brief, tissues were homogenized using a Pro200 Homogenizer (Pro Scientific Inc., Oxford, CT) in 200µl RNAzol®RT for 45-60 seconds. DNase/RNase-free water was added to the sample in a 1:1 ratio, and the

first centrifugation step was performed for 15 minutes at 12,000xg at 4°C. The supernatant was removed and placed in a clean 1.5 mL microcentrifuge tube. A subsequent centrifugation was carried out at 20°C. The upper clear phase was added to a clean microcentrifuge tube, leaving phenol in the lower phase. Isopropanol was added to the aqueous supernatant and incubated at room temperature for 15 minutes, followed by centrifugation. The RNA formed a pellet at the bottom of the tube, and the supernatant was discarded. Three washes were performed with 75% ethanol and centrifugation at 8,000xg. The final ethanol was removed, and pellets were dried at 40°C for 2 minutes. The pellets were dissolved in 30 µl of DNase/RNase-free water and stored overnight at -80°C prior to quantification.

Due to the particularly troublesome nature of pituitary gland tissue, total RNA was isolated from the pituitary glands using a different method at each stage. RNA from embryonic tissues was isolated with RNAzol®RT using the same protocol described above, with the following changes. The initial volume of RNAzol®RT used for homogenization was 100 µl and each subsequent step was carried out at room temperature. In addition to DNase/RNase-free water, 0.6ul of Precipitation Carrier was added to each sample following homogenization for better visualization of the RNA pellet. The final RNA pellet was dissolved in 11ul of DNase/RNase-free water and stored overnight at -80°C prior to quantification. RNA samples were checked for contaminating genomic DNA. Those with contaminating DNA were cleaned up using an in-tube DNase treatment and sodium acetate/ethanol precipitation.

Due to sample loss and phenol contamination from incomplete phase separation, a different method was used for RNA isolation from hatchling tissues. Total RNA was isolated using the *Quick-RNA*™ MicroPrep (Zymo Research) according to the manufacturer's protocol. Tissues were homogenized in 100µl Lysis buffer. One hundred percent ethanol was added to the sample in a 1:1 ratio. The mixture was transferred to a Zymo-Spin™ IC Column in a Collection Tube and centrifuged for 30 seconds. The optional on-column treatment with DNase I was included using DNase I supplied with the kit. Four hundred microliters of RNA Prep Buffer was added to the column and centrifuged for 30 seconds. Next, samples were washed with 700ul RNA Wash Buffer and centrifuged for 30 seconds, followed by a wash with 400ul RNA wash Buffer and 2 minutes centrifugation. An additional wash step was performed using 400ul Wash Buffer prior to RNA elution in 11ul water. RNA samples were checked for contaminating genomic DNA by running qPCR prior to reverse transcription. The majority of the samples contained genomic DNA, and therefore required an additional clean-up treatment. This was performed using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems™) with on-column DNase I treatment using an RNase-Free DNase set (Qiagen). Briefly, 25µl extraction buffer was added to each eluted RNA sample and incubated at room temperature for 5 minutes. One hundred microliters of 70% ethanol was added to the homogenate and mixed by inversion. The sample was transferred to the conditioned column and centrifuged at 100g for 2 minutes, followed by 16,000g for 30 seconds. Next, 100ul of wash buffer 1 was added and centrifuged at 8,000g for 1 minute. The flow-through was discarded. A mixture of 10µl DNaseI and 30µl Buffer RDD was

added to each column and incubated at room temperature for 15 minutes. Forty microliters wash buffer I was then added to the column and centrifuged at 8,000g for 15 seconds, followed by a wash with wash buffer 2 and centrifugation at 8,000g for 1 minute. Two more wash steps were performed with 100µl wash buffer 2 and centrifugation at 16,000g for 2 minutes. Flow-through was discarded, and the column was placed into a fresh DNase/RNase-free tube. Eleven microliters of elution buffer was applied directly to the column by gently tapping the filter with the tip and incubated at room temperature for 1 minute. Tubes were then centrifuged at 1,000g for 1 minute, followed by 1 additional minute at 16,000g.

Total RNA from the 6 month old hatchling tissue was isolated using the Arcturus® PicoPure® RNA Isolation Kit as described above with initial homogenization carried out in 100ul extraction buffer. Samples with contaminating genomic DNA following this protocol were cleaned-up with an additional run with the kit.

Reverse Transcription, quantitative PCR, and Statistical Analysis

A total of 50 ng of input RNA was reverse transcribed in a 20 µl reaction using Applied Biosystems' High-Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. Reverse transcription was carried out on a BioRad MyiQ™ Single-Color Real-Time PCR Detection System. The cDNA was diluted to the equivalent of 0.625 ng input RNA/µl for use in qPCR reactions.

We designed PCR primers (Integrated DNA Technologies) to measure expression of 11 genes (Table 2.1) using Primer Express® 2.0 software (ThermoFisher Scientific). Primers were designed using gene sequences from the snapping turtle transcriptome

Table 2.1: Forward and Reverse primer sequences for 11 hypothalamic and pituitary gland genes from the snapping turtle.

Gene Name	Forward Primer Sequence (5' – 3')	Reverse Primer Sequence (5' – 3')
Kisspeptin (*Kiss1*)	TCTTCTCCAATGCTCCGTTTG	TGGTCACCTGTGCGAGG
Kisspeptin Receptor (*Kiss1R*)	TGGGCATCTGGATCTGTTCC	AACCAGTAGCCCTCCGTCAAC
Gonadotropin-releasing Hormone 1 (*GnRH1*)	GTCTGTGGAGATTTGCTTGGC	CAGATTGTCAGCATCCCGCT
Gonadotropin-releasing Hormone 2 (*GnRH2*)	TGGCATGTCAACGACCTCTC	TGCTGAGCTCTCGACAGGTG
Gonadotropin-releasing Hormone Receptor 2b (*GnRHR2b*)	CTCTACACGCCTTCCTTCCG	TCTCCTGCCCCGTAATGACT
Androgen Receptor (*AR*)	TGGGATGGAGATCTTTCACCA	GGAGCAAAGTAAAGCATCCGG
Estrogen Receptor 1 (*ESR1*)	AACCAGTGCACCATCGACAAG	GGTCTTTTCGGATCCCACCTT
Estrogen Receptor 2 (*ESR2*)	TGCACACACACTTCGAGGTCA	TGTATCGGTTTGTTCTGAG
Aromatase (*CYP19A1*)	TCTGGTCCAGGTCTCGTGC	GGTCTTTCGTTGATTCAACGC
Follicle-Stimulating Hormone subunit beta (*FSHb*)	TCACCATAGCAGTGGAGAAGGA	GTATCCAGAGCACCAAGTGGC
Leutinizing Hormone subunit beta (*LHb*)	GGGTACTGCCAGACCAAGGA	AGCGCGTTCTTGTACACCG

(paper in preparation). We prepared standard curves for each gene as described in Rhen et al. (2007). Purified PCR products for each gene were added to reaction tubes in the following amounts: 2,000,000 attograms (ag=10^{-18} g)/tube, 200,000 ag/tube, 20,000 ag/tube, 2,000 ag/tube, 200 ag/tube, 20 ag/tube, 2 ag/tube, and 0.2 ag/tube.

Real-time quantitative PCR was performed using the Bio-Rad CFX 384 Real-Time PCR System (Bio-Rad) with Bio-Rad SsoFast™ EvaGreen® Supermix according to manufacturer's instructions (Bio-Rad, Hercules, CA). Each 10 µl PCR reaction contained 5 µl of 2x supermix, 0.3 µl of forward and 0.3 µl of reverse primers (0.3 µM final concentration), 2.4 µl of water, and 2 µl of cDNA (input=1.25 ng total RNA). Standard curves were used to estimate the amount of mRNA in attograms cDNA (~mRNA) per 1.25 ng of total RNA from each hypothalamus. Amplification did not occur in wells without cDNA.

We used JMP 11.1.1 software for all statistical analyses (SAS Institute, Cary, NC). We used two-way ANOVA with treatment (untreated or ethanol treated), incubation temperature (sex), and their interaction as effects in the model. In cases where ethanol treatment did not affect gene expression, the control and ethanol (vehicle) groups were collapsed into one group for calculating means for each temperature (or sex). The ethanol-treated group differed significantly from the control group for some genes and in this case we did not collapse the groups. Instead, we only report means for the male and female control groups. A Tukey's post hoc test was used to compare groups with a significant interaction effect.

Results

Pituitary Development in the Snapping Turtle

A total of 65 embryos were successfully sectioned and stained to show pituitary gland morphology on a midsagittal plane. These samples encompass stages 6 – hatch, as well as two samples from 4-5 month old hatchlings. For our purposes, stage 6 is referenced as day 0 of incubation, and stage 26 corresponds to the day of hatching. Events during pituitary gland morphogenesis are shown in reference to developmental stage (Figure 2.1).

Invagination of the Oral Ectoderm

Initial events of adenohypophysis development occur very early in development. The oral ectoderm does not invaginate until after stage 6 (Figure 2.2b). Initial invagination of the oral ectoderm is clear at late stage 6 or stage 7, with some clutch to clutch variation (Figure 2.2c – e). Invagination of the oral ectoderm continues through stage 15. The oral ectoderm remains separated from the neural ectoderm and does not thicken until after stage 15 (Figure 2.3).

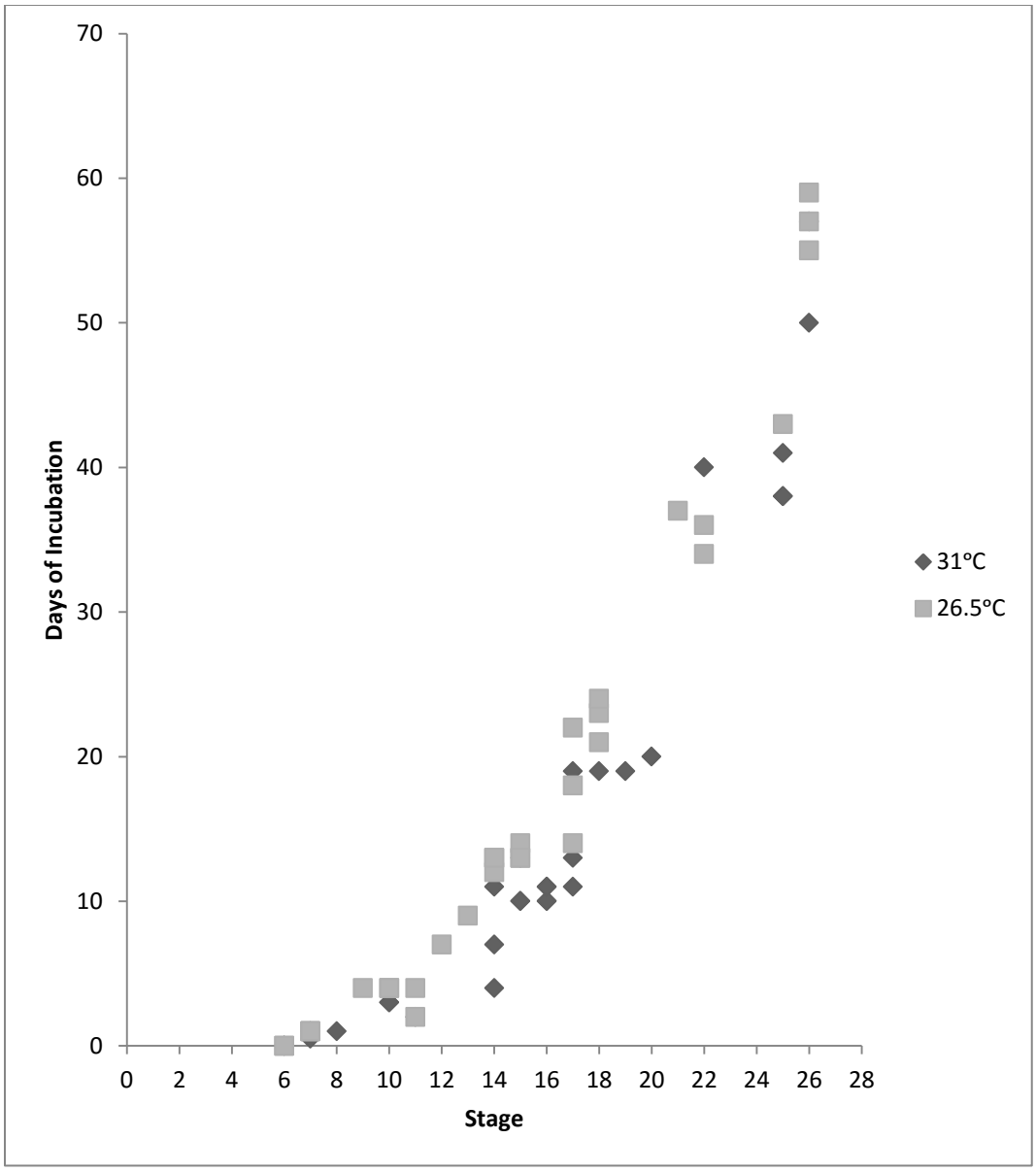


Figure 2.1: Embryonic and hatchling samples collected for morphological analysis of pituitary gland development in the snapping turtle. Individuals were collected throughout development based on staging in Yntema (1968). Stage 6 was assigned as day 1, and subsequent days of incubation are indicated for eggs incubated at both male (26.5ºC) - and female (31 ºC) - producing temperatures.

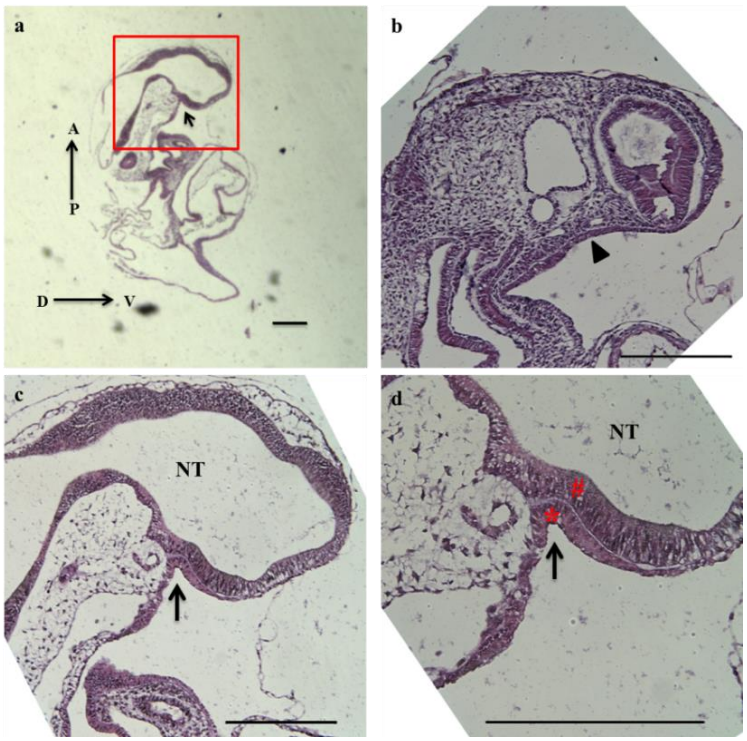


Figure 2.2: Invagination of the oral ectoderm starts at late stage 6/stage 7. a) Stage 7 embryo at 2x magnification. Orientation is indicated by arrows. The red box indicates the approximate location of figures b-d. Oral ectoderm invagination is indicated by the arrow. b) Head region of a stage 6 embryo at 10x magnification. The oral ectoderm is indicated with an arrow head. Scale bar = __. c) Head region of a stage 7 embryo at 10x magnification. Initial invagination of the oral ectoderm is indicated with an arrow. d) The same embryo shown in c at 20x magnification. A red asterisk marks the oral ectoderm invagination and the red pound symbol indicates the neural ectoderm, A = anterior, P = posterior, D = dorsal, V = ventral, NT = neural tube, scale bars = 250 µm.

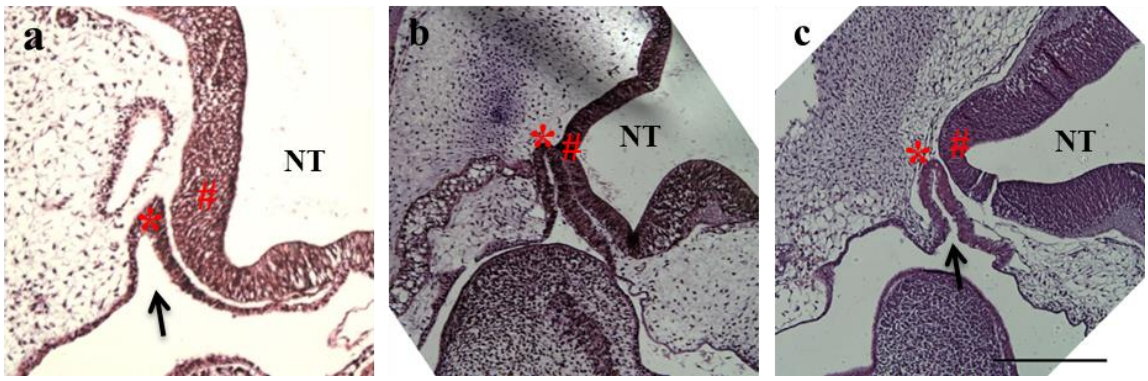


Figure 2.3 Invagination of the oral ectoderm forms Rathke's pouch, the primordial adenohypophysis. Late stage 9 (a), stage 13 (b), and stage 14 (c) embryos at 10x magnification. Arrow = invagination, asterisk = oral ectoderm, pound sign = neural ectoderm, NT = neural tube, Scale bar = 250 µm.

Cellular Proliferation and Folding

Many changes in adenohypophysial structure occur between stages 15 and 18. At stage 15, the oral ectoderm begins to pinch in, separating Rathke's pouch as a distinct structure (Figure 2.4a and b). Intricate folding of the tissue begins, but the structure remains separate from the neuroectoderm (Figure 2.4b). By stage 16, the layered structure of Rathke's pouch has completely separated from the oral ectoderm boundary (Figure 2.4c-d). The adenohypophysial structure grows in size through stage 18, presumably through cell proliferation and continued folding of the tissue (Figure 2.4e-f). The developing adenohypophysis is in close contact with the neuroectoerm, but evidence of neurohypophysial development is lacking at stage 18. Cell proliferation of neural tissue fills in the neural tube from the superior/rostral region, but the third ventricle remains expanded (Figure 2.4f).

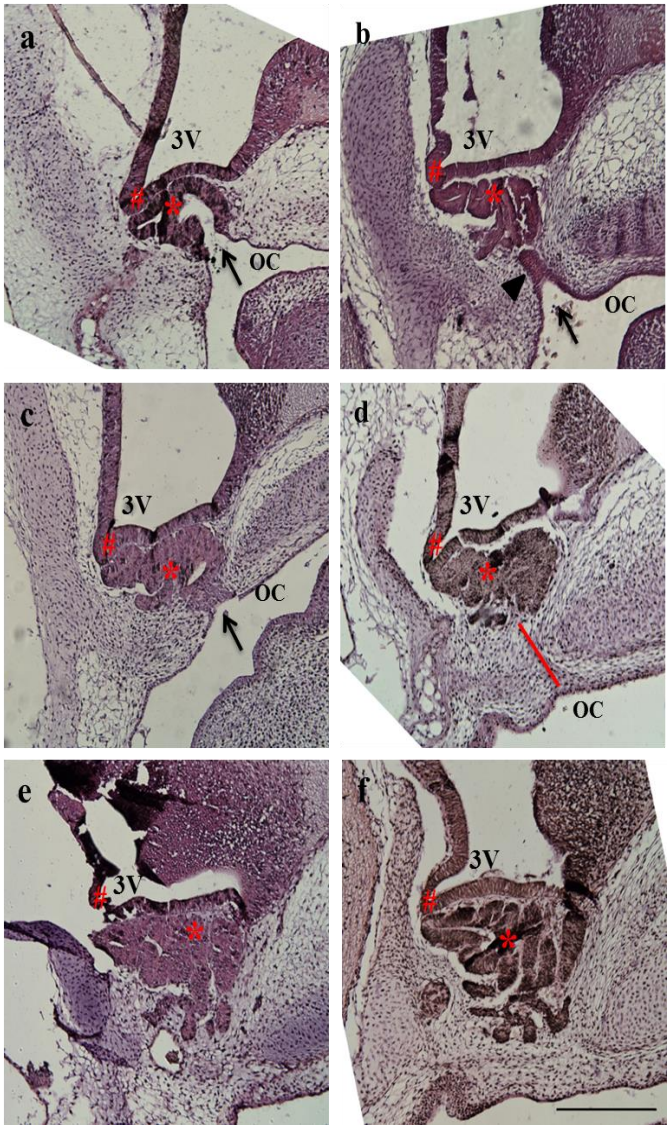


Figure 2.4: Cellular proliferation and folding of Rathke's pouch. Cell proliferation and folding can be detected in Rathke's pouch by stage 15 of embryogenesis (a). Some variation exists in the timing of the separation of Rathke's pouch from the oral ectoderm, shown in (b), another stage 15 embryo. The invagination of the oral ectoderm is noted (arrow), but the oral ectoderm is closing in this embryo (arrow head). Cell proliferation and folding continues at stage 16 (c), and the oral ectoderm separates from Rathke's pouch, and closes (arrow). By late stage 16 (d), the primordial adenohypophysis has separated from the oral ectoderm, with mesenchymal cell proliferation between the oral ectoderm and adenohypophysis (red bar). Cell proliferation and folding continues into stage 17 (e) and 18 (f). The hypothalamus is filling in rostrally, but leaving the third ventricle open. Evidence of the neurohypophysis is not yet evident. OC = oral cavity, 3V = third ventricle, asterisk = presumptive adenohypophysis, pound sign = neural ectoderm, 10x magnification, scale bar = 250 µm.

Adenohypophysial Formation

At stage 19, the adenohypophysial folds are consolidated into one primary structure (Figure 2.5a). The structure in Figure 2.5a has been pulled away from its normal plane as an artifact of sectioning, allowing for visualization of the infundibulum, and the thin connection between the adenohypophysis and the hypothalamus. At stage 21, the adenohypophysis rests within the sella turcica (Figure 2.5b), which thickens through the rest of embryonic development (Figure 2.5d, e, i). The infundibulum thickens slightly at stage 24 (Figure 2.5d), but an expanded neurohypophysis is not evident even 4 months after hatching (Figure 2.5i). A high level of vascularization is evident within the adenohypophysis after stage 21 (Figure 2.5b).

Overall developmental rate is slower at lower incubation temperatures as is the rate of pituitary gland development. In other words, pituitary development is tightly correlated with development of other morphological traits that are used for staging embryos. Furthermore, the events of pituitary gland development display only slight variation among individuals.

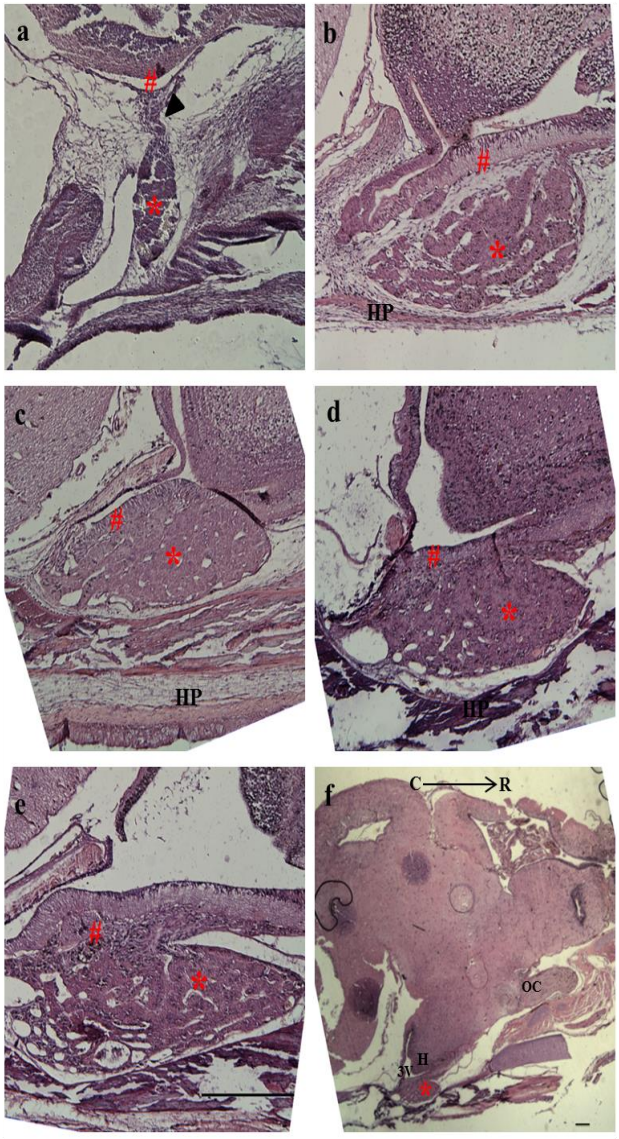


Figure 2.5: Formation of the pituitary gland. Panels a – e show pituitary gland maturation at 10x magnification. At early stage 20, the structure of the adenohypophysis can be detected. The connection with the hypothalamus by the infundibulum is clearly shown in panel a), where the hard palette has been slightly pulled away from the brain, lengthening the adenohypophysis on a vertical axis. The infundibulum is shown with an arrow head. By late stage 21 (b), the adenohypophysis is a discrete structure in contact with the thickening neural ectoderm, forming the infundibulum (pound sign). Pituitary gland maturation continues through stage 25 (c), at hatching (d), and continues through 4 months after hatch (e). The neurohypophysis of the snapping turtle never forms a discrete lobe but is more of an expansion of the neural ectoderm, contacting the adenohypophysis at the superior border (pound sign). Panel f is a hatchling brain at 2x magnification indicating orientation. Asterisk = adenohypophysis, HP = hard palette, H = hypothalamus, OC = optic chiasm, scale bars = 250 µm.

Sex Differences in Gene Expression

Sex Differences in Gene Expression in the Hypothalamus of Turtle Embryos at Stage 20

Two-way ANOVA revealed sexually dimorphic expression of *AR, CYP19A1, ESR1, and Kiss1R* in the hypothalamus of late stage 20 embryos, while expression of *ESR2, GnRH1, GnRH2,* and *Kiss1* did not differ between the sexes (Table 2.2). Expression of *AR, CYP19A1, ESR1,* and *Kiss1R* was significantly higher in the hypothalami of animals incubated at the female-producing temperature (Table 2.3). The only gene affected by ethanol treatment was *ESR1* (Table 2.2). Ethanol treatment significantly decreased expression of *ESR1* in both males and females (Figure 2.6). There was no detectable interaction between sex and ethanol treatment for any gene (Table 2.2).

Sex Differences in Gene Expression in the Hypothalamus of One-week Old Turtles

Two-way ANOVA revealed sex differences in expression of *CYP19A1, ESR1, ESR2, GnRH2,* and *Kiss1R* in snapping turtle hatchlings at one week of age, while expression of *AR, GnRH1,* and *Kiss1* were not differentially expressed between the sexes (Table 2.4). Expression of *CYP19A1, ESR1,* and *GnRH2* was higher in males, while expression of *ESR2* and *Kiss1R* was higher in females (Table 2.5). Ethanol treatment altered expression of *Kiss1* and *Kiss1R* in hatchlings. Ethanol decreased expression of both of these genes in males and females (Figures 2.7 and 2.8). The interaction between sex and ethanol treatment was not significant for any gene (Table 2.4).

Table 2.2: Two-way ANOVA testing for ethanol treatment and sex effects on gene expression in the hypothalamus of snapping turtles at stage 20 of embryonic development. We measured expression of 8 genes involved in reproduction. Significant effects are denoted with an asterisk (*, $p < 0.05$).

Gene	**Effects**	**df**	**F-value**	**p-value**
AR	Treatment	1	2.922	0.098
	Sex	1	7.064	0.013*
	Treatment x Sex	1	0.186	0.669
CYP19A1	Treatment	1	1.678	0.206
	Sex	1	5.674	0.024*
	Treatment x Sex	1	0.449	0.508
ESR1	Treatment	1	5.024	0.033*
	Sex	1	7.926	0.009*
	Treatment x Sex	1	0.048	0.828
ESR2	Treatment	1	0.861	0.361
	Sex	1	2.816	0.104
	Treatment x Sex	1	0.183	0.672
GnRH1	Treatment	1	0.012	0.913
	Sex	1	1.763	0.195
	Treatment x Sex	1	0.028	0.868
GnRH2	Treatment	1	0.692	0.509
	Sex	1	2.059	0.162
	Treatment x Sex	1	1.179	0.286
Kiss1	Treatment	1	0.171	0.682
	Sex	1	2.916	0.098
	Treatment x Sex	1	0.559	0.461
Kiss1R	Treatment	1	0.845	0.366
	Sex	1	4.253	0.048*
	Treatment x Sex	1	0.476	0.496

Table 2.3: Gene expression in male and female snapping turtle hypothalami at stage 20 of embryonic development. Genes presented here showed a significant sex difference as determined by two-way ANOVA.

Gene	Sex	Least Square Means	Standard Error
AR	Female	921.572	850.295 to 992.849
	Male	670.059	600.835 to 739.283
CYP19A1	Female	699.598	626.796 to 772.400
	Male	469.358	398.654 to 540.062
ESR1	Female	9.723	8.925 to 10.592
	Male	6.959	6.355 to 7.621
Kiss1R	Female	83.120	75.390 to 90.984
	Male	61.308	53.444 to 69.172

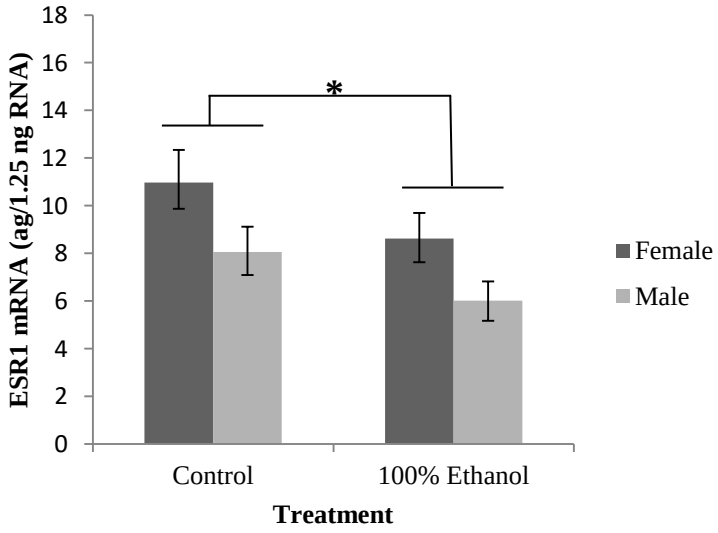


Figure 2.6: Two-way ANOVA shows that ethanol treatment decreased expression of *ESR1* in the hypothalamus of females and males at late stage 20 of embryonic development. C = control, V = vehicle (100% ethanol), n=7-9/group.

Table 2.4: Two-way ANOVA results testing for ethanol treatment and sex effects on gene expression in the hypothalamus of hatchling snapping turtles at one week of age. We measured expression of 8 genes involved in reproduction. Significant effects are denoted with an asterisk (*, $p < 0.05$).

Gene	**Effects**	**df**	**F-value**	**p-value**
AR	Treatment	1	1.635	0.210
	Sex	1	0.098	0.756
	Treatment x Sex	1	2.990	0.093
CYP19A1	Treatment	1	0.787	0.382
	Sex	1	6.638	0.015*
	Treatment x Sex	1	3.747	0.062
ESR1	Treatment	1	0.396	0.534
	Sex	1	4.402	0.044*
	Treatment x Sex	1	0.120	0.732
ESR2	Treatment	1	3.149	0.086
	Sex	1	4.558	0.041*
	Treatment x Sex	1	0.774	0.386
GnRH1	Treatment	1	0.633	0.432
	Sex	1	0.087	0.770
	Treatment x Sex	1	0.048	0.828
GnRH2	Treatment	1	3.932	0.056
	Sex	1	4.730	0.037*
	Treatment x Sex	1	0.813	0.374
Kiss1	Treatment	1	4.426	0.043*
	Sex	1	2.306	0.139
	Treatment x Sex	1	1.933	0.174
Kiss1R	Treatment	1	8.491	0.007*
	Sex	1	8.775	0.006*
	Treatment x Sex	1	0.482	0.493

Table 2.5: Gene expression in male and female snapping turtle hypothalami at one week of age. Genes presented here showed a significant sex difference as determined by two-way ANOVA.

Gene	Sex	Least Square Means	Standard Error
CYP19A1	Female	3965.484	3490.457 to 4440.511
	Male	5690.928	5218.842 to 6163.014
ESR1	Female	17.103	15.000 to 19.206
	Male	23.251	21.210 to 25.292
ESR2	Female	2143.641	1887.358 to 2434.725
	Male	1461.204	1287.525 to 1658.312
GnRH2	Female	1121.103	909.184 to 1333.022
	Male	1780.919	1563.831 to 1998.007
Kiss1R	Female	1689.125	1521.839 to 1874.799
	Male	1085.405	975.426 to 1207.785

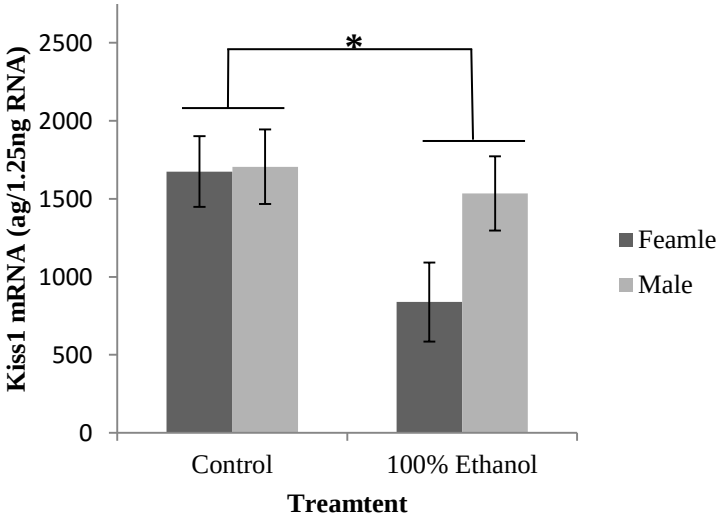


Figure 2.7: Two-way ANOVA shows that ethanol decreased expression of *Kiss1* in the hypothalamus of one-week old hatchlings. C = control, V = vehicle (100% ethanol), n=7-9/group.

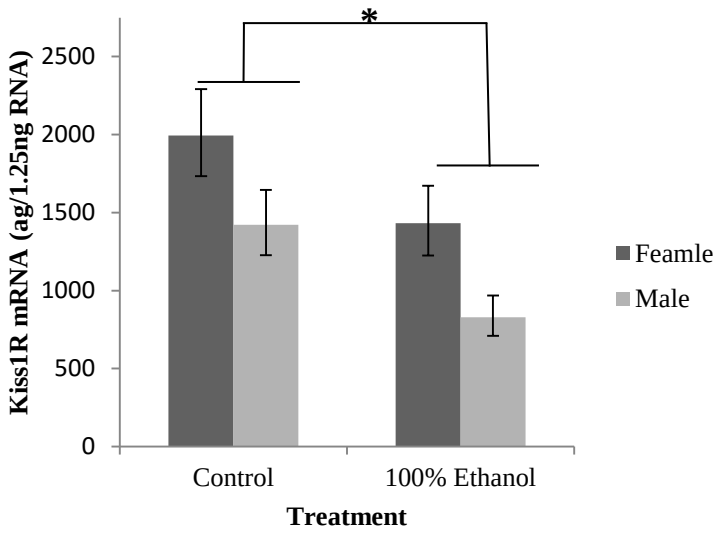


Figure 2.8: Two-way ANOVA shows that ethanol treatment decreased expression of *Kiss1R* in the hypothalamus in one-week old hatchlings. C = control, V = vehicle (100% ethanol), n=7-9/group.

Sex Differences in Gene Expression in the Hypothalamus of Six-Month Old Turtles

Two-way ANOVA showed that expression of *ESR1*, *ESR2*, and *Kiss1R* differ between males and females at six months of age. In contrast, *AR*, *CYP19A1*, *GnRH1*, *GnRH2* expression did not differ between the sexes (Table 2.6). Two-way ANOVA revealed a significant interaction between sex and ethanol treatment for *Kiss1*: a Tukey's post hoc test showed expression of *Kiss1* was decreased by ethanol treatment in males only (Figure 2.9). I therefore used a one-way ANOVA to compare males to females within the control group. After excluding the ethanol treated groups, expression of Kiss1 still differed significantly between males and females ($F_{1,14}$=10.575, p=0.007). Expression of *ESR1*, *ESR2*, and *Kiss1R* was higher in females than in males, but *Kiss1* expression was higher in males than females (Table 2.7).

Table 2.6: Two-way ANOVA results testing for ethanol treatment and sex effects on gene expression in the hypothalamus of snapping turtles at six months of age. We measured expression of 8 genes involved in reproduction. Significant effects are denoted with an asterisk (*, $p < 0.05$).

Gene	Effects	df	F-value	p-value
AR	Treatment	1	0.120	0.732
	Sex	1	1.876	0.183
	Treatment x Sex	1	0.917	0.347
CYP19A1	Treatment	1	0.725	0.403
	Sex	1	2.813	0.106
	Treatment x Sex	1	1.799	0.192
ESR1	Treatment	1	0.171	0.683
	Sex	1	9.054	0.006*
	Treatment x Sex	1	0.418	0.524
ESR2	Treatment	1	0.150	0.702
	Sex	1	11.576	0.002*
	Treatment x Sex	1	0.096	0.759
GnRH1	Treatment	1	0.429	0.518
	Sex	1	0.038	0.846
	Treatment x Sex	1	0.004	0.952
GnRH2	Treatment	1	0.756	0.393
	Sex	1	1.604	0.217
	Treatment x Sex	1	2.995	0.096
Kiss1	Treatment	1	5.949	0.022*
	Sex	1	3.770	0.064
	Treatment x Sex	1	14.631	0.001*
Kiss1R	Treatment	1	0.003	0.961
	Sex	1	7.942	0.009*
	Treatment x Sex	1	0.392	0.537

Table 2.7: Gene expression in male and female snapping turtle hypothalami at six months of age. Genes presented here showed a significant difference between sexes as determined by a two-way ANOVA.

Gene	Sex	Least Square Means	Standard Error
ESR1	Female	102.235	93.331 to 111.139
	Male	65.330	56.895 to 73.765
ESR2	Female	2200.977	2031.933 to 2370.021
	Male	1424.939	1271.815 to 1578.063
Kiss1	Female	3785.740	2331.990 to 5239.490
	Male	10471.391	9017.641 to 11925.141
Kiss1R	Female	4466.798	3877.292 to 5145.932
	Male	2607.807	2294.017 to 2964.519

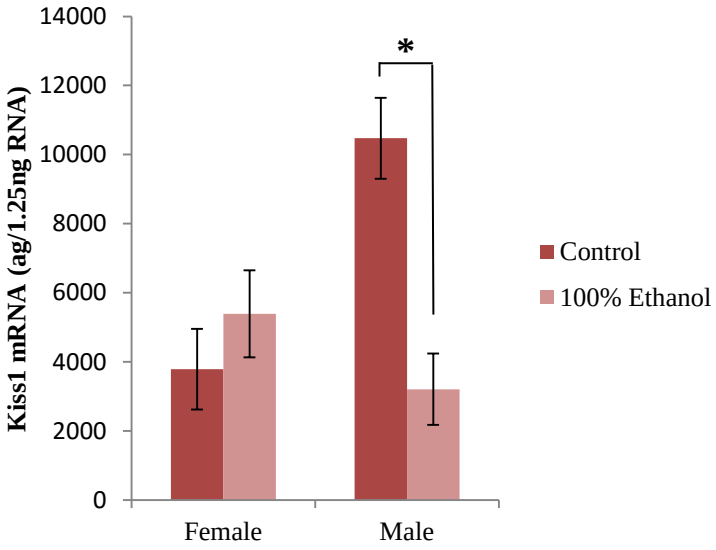


Figure 2.9: Two-way ANOVA shows a significant interaction between ethanol treatment and sex. Ethanol decreased expression of *Kiss1* in males 6 months after hatching (*), but did not affect females. C = control, V = vehicle (100% ethanol), n=7-9/group.

Sex Differences in Gene Expression in the Pituitary Gland of Turtle Embryos at Stage 20

Fewer genes were differentially expressed in the pituitary gland than in the hypothalamus. At embryonic stage 20, *AR*, *CGA*, *CYP19A1*, *ESR1*, *ESR2*, *FSHb*, and *GnRhR2b* were not differentially expressed between sexes. The only gene showing differential expression was *LHb* (Table 2.8). Females (17.9 ± 4.9) had significantly higher expression than males (8.3 ± 2.2). Ethanol treatment only influenced expression of one gene: *FSHb* expression was higher in the ethanol treated group compared to the control group (Figure 2.10).

Table 2.8: Two-way ANOVA results testing for ethanol treatment and sex effects on gene expression in the pituitary gland of snapping turtles at stage 20 of embryonic development. We measured expression of 8 genes involved in reproduction. Significant effects are denoted with an asterisk (*, $p < 0.05$).

Gene	**Effects**	**df**	**F-value**	**p-value**
AR	Treatment	1	0.002	0.965
	Sex	1	0.008	0.929
	Treatment x Sex	1	2.943	0.098
CGA	Treatment	1	0.884	0.369
	Sex	1	0.075	0.786
	Treatment x Sex	1	1.395	0.248
CYP19A1	Treatment	1	1.228	0.278
	Sex	1	0.002	0.963
	Treatment x Sex	1	3.140	0.088
ESR1	Treatment	1	0.410	0.528
	Sex	1	0.118	0.734
	Treatment x Sex	1	0.954	0.338
ESR2	Treatment	1	0.047	0.829
	Sex	1	0.034	0.855
	Treatment x Sex	1	0.936	0.342
FSHb	Treatment	1	6.072	0.021*
	Sex	1	0.866	0.361
	Treatment x Sex	1	0.000	0.997
GnRHR2b	Treatment	1	0.008	0.931
	Sex	1	0.381	0.543
	Treatment x Sex	1	0.321	0.576
LHb	Treatment	1	3.635	0.067
	Sex	1	8.497	0.007*
	Treatment x Sex	1	0.310	0.582

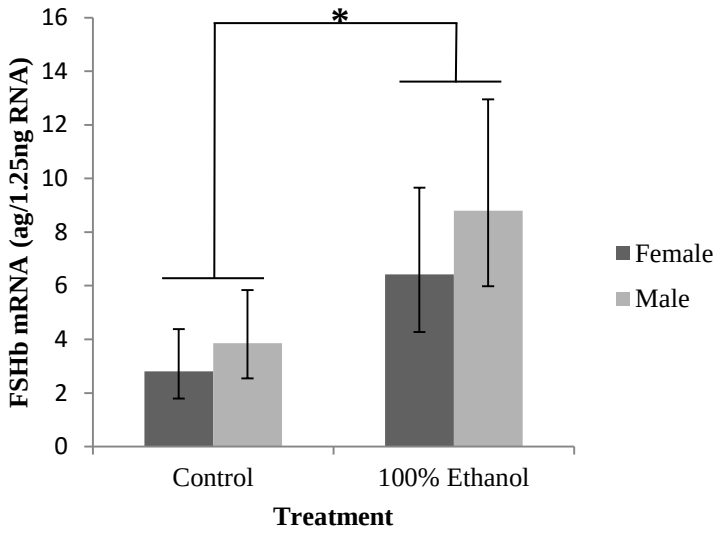


Figure 2.10: Two-way ANOVA shows that ethanol treatment increased expression of *FSHb* in the pituitary gland of males and females at late stage 20 of embryonic development. Two-way ANOVA shows increased expression when male and female means are combined. C = control, V = vehicle (100% ethanol), n=7-9/group.

Sex Differences in Gene Expression in the Pituitary Gland of One-Week Old Turtles

There were several genes that displayed sexually dimorphic expression in pituitaries of one week–old hatchlings. Expression of *CGA, CYP19A1, ESR1, ESR2, FSHb, GnRHR2b,* and *LHb* differed between males and females, while *AR* was the only gene that did not differ between the sexes (Table 2.9). Expression of these genes was higher in males than in females (Table 2.10). Ethanol exposure did not have an effect on expression of any of the genes tested in the pituitary glands of hatchlings.

Table 2.9: Two-way ANOVA results testing ethanol treatment and sex effects on gene expression in the pituitary gland in snapping turtles at one week of age. We measured expression of 8 genes involved in reproduction. Significant effects are denoted with an asterisk (*, $p < 0.05$).

Gene	**Effects**	**df**	**F-value**	**p-value**
AR	Treatment	1	2.155	0.154
	Sex	1	2.636	0.116
	Treatment x Sex	1	0.302	0.587
CGA	Treatment	1	0.300	0.589
	Sex	1	7.902	0.010*
	Treatment x Sex	1	1.085	0.308
CYP19A1	Treatment	1	0.417	0.524
	Sex	1	16.661	<0.001*
	Treatment x Sex	1	0.964	0.336
ESR1	Treatment	1	0.828	0.371
	Sex	1	16.140	<0.001*
	Treatment x Sex	1	1.952	0.174
ESR2	Treatment	1	2.996	0.095
	Sex	1	13.304	0.001*
	Treatment x Sex	1	3.431	0.075
FSHb	Treatment	1	0.004	0.950
	Sex	1	13.711	0.001*
	Treatment x Sex	1	1.073	0.309
GnRHR2b	Treatment	1	0.546	0.467
	Sex	1	11.272	0.003*
	Treatment x Sex	1	2.692	0.114
LHb	Treatment	1	0.000	0.989
	Sex	1	40.769	<0.001*
	Treatment x Sex	1	1.489	0.234

Table 2.10: Gene expression in male and female snapping turtle pituitary glands at one week of age. Genes presented here showed a significant difference between sexes as determined by a two-way ANOVA.

Gene	**Sex**	**Least Square Means**	**Standard Error**
CGA	Female	49555.710	39278.984 to 62521.178
	Male	123394.6670	94663.738 to 160845.587
CYP19A1	Female	4782.608	3818.489 to 5990.154
	Male	17257.219	13349.415 to 22308.962
ESR1	Female	22.421	22.421 to 39.716
	Male	138.098	100.266 to 190.204
ESR2	Female	124.206	100.076 to 148.336
	Male	242.261	215.792 to 268.730
FSHb	Female	8.900	6.807 to 11.637
	Male	33.703	25.117 to 45.225
GnRHR2b	Female	5.738	4.580 to 7.191
	Male	15.874	12.461 to 20.222
LHb	Female	122.438	95.929 to 156.273
	Male	1017.737	772.614 to 1340.630

Sex Differences in Gene Expression in the Pituitary Gland of Six-Month Old Turtles

Two-way ANOVA did not reveal any treatment, sex, or interaction effects in pituitary glands after six months (Table 2.11), based on the genes we examined.

Table 2.11: Two-way ANOVA results testing for ethanol treatment and sex effects on gene expression in pituitary glands in snapping turtles at six months of age. We measured expression of 8 genes involved in reproduction. Significant effects are denoted with an asterisk (*, $p < 0.05$).

Gene	Effects	df	F-value	p-value
AR	Treatment	1	1.012	0.329
	Sex	1	0.934	0.347
	Treatment x Sex	1	4.286	0.054
CGA	Treatment	1	3.422	0.080
	Sex	1	0.704	0.412
	Treatment x Sex	1	1.448	0.244
CYP19A1	Treatment	1	1.945	0.181
	Sex	1	2.778	0.114
	Treatment x Sex	1	0.001	0.977
ESR1	Treatment	1	0.418	0.527
	Sex	1	4.271	0.054
	Treatment x Sex	1	1.043	0.321
ESR2	Treatment	1	0.001	0.977
	Sex	1	0.091	0.767
	Treatment x Sex	1	0.129	0.724
FSHb	Treatment	1	0.015	0.905
	Sex	1	0.801	0.383
	Treatment x Sex	1	0.051	0.824
GnRHR2b	Treatment	1	0.361	0.561
	Sex	1	1.923	0.196
	Treatment x Sex	1	4.182	0.068
LHb	Treatment	1	0.752	0.397
	Sex	1	2.882	0.106
	Treatment x Sex	1	0.742	0.400

Discussion

Major morphological events during pituitary gland development are highly conserved among vertebrates. Adenohypophysis development begins with invagination of the oral ectoderm. This event starts 4-7 days after oviposition in the snapping turtle (depending on incubation temperature) at late stage 6 or early stage 7 when embryos have 8 – 10 pairs of somites (Yntema, 1968). In the sea turtle, *Caretta caretta*, initiation of pituitary development occurs at stage 7 (Pearson et al., 1983; Mahmoud et al., 1973) and

5 days after oviposition in the Japanese Soft-shelled turtle (Saga and Yamaki, 2006). Reyes et al. (2007) report invagination of the oral ectoderm at E.4 in chicken and quail, and Hammond (1974) reports the first appearance of Rathke's pouch at stage 12, (14 – 15 somites) in chickens. In mice Sano and Sasaki (1969) report this event at E.12, much later in development than in birds and reptiles, but Kerr (1946) reports the initial appearance of Rathke's pouch at E.9, in a 16-somite embryo.

Invagination continues, deepening Rathke's pouch, without thickening or pinching of the membrane, through stage 15 (days 10 – 14 after oviposition) in the snapping turtle. These events occur through approximately stage 11 in the sea turtle (Pearson et al., 1983; Mahmoud et al., 1973). The sea turtle study used staging described for *Chrysemys picta* (Mahmoud et al., 1973) and does not correspond to staging in the snapping turtle, complicating comparison of events. However, stage 11 in the sea turtle occurs 21 days after oviposition, therefore corresponding approximately with stage 12 in the snapping turtle.

In some species, the adenohypophysis originates from two independent origins: Rathke's pouch, as well as Sessile's pouch, a structure with endodermic origins and joins Rathke's pouch to form the rostral part of the adenohypophysis (Gorbman, 1983; Hall and Hughes, 1985; Reyes et al., 2007). However, there is no evidence for participation of Sessile's pouch in formation of the adenohypophysis in the snapping turtle.

Extensive cellular proliferation, mesenchymal infiltration, and folding of Rathke's pouch occur between stages 15 and 18 in the snapping turtle. The initial pinching of the oral ectoderm occurs at stage 11 in the sea turtle and extensive cell proliferation is

evident prior to stage 18 (Pearson et al., 1983). Adenohypophysial structure within the sea turtle at stage 18 appears to correlate with events occurring at stage 16 in the snapping turtle. However, Yntema staging of the snapping turtle and Mahmaud's staging of the western painted turtle show that stage 18 is anatomically similar to stage 19 in the snapping turtle. Therefore, the cellular proliferation/folding stage of adenohypophysial development appears to occur slightly earlier in the snapping turtle in relation to anatomical staging. These same events are present 22 days after oviposition in the Japanese soft-shelled turtle (Saga and Yamaki, 2006), and between E.6 and E.10 in chicken and quail (Reyes et al., 2007), with early proliferation and the pinching of the oral ectoderm (stage 15 in the snapping turtle) occurring at E.6 while E.10 is similar to stage 18 in the snapping turtle. In mouse initial thickening of the ectodermal wall and closure of Rathke's pouch begins at E.11, and proliferation and folding continues through E.15 (Kerr, 1946; Sano and Sasaki, 1969).

Cellular differentiation begins at this stage of pituitary development in other species. Tracking cellular differentiation is outside the scope of this study; however it likely begins at this time as well. The order of cell type differentiation varies from species to species, with ACTH-immunoreactive (ir) cells differentiating first in quail, sea turtle, golden hamster, and rat (Reyes et al., 2007; Saga et al., 1996; Watanabe and Daikoku, 1979), PRL-ir cells in the Clouded salamander (Saga and Oota, 1990), and either ACTH-ir or LH-ir in chicken (Reyes et al., 2007; Gasc and Sar, 1981). A cytodifferentiation study in the snapping turtle would elucidate the events of adenohypophysial development further.

Cell proliferation also occurs in the neural tube during stages 15 – 18, filling in the neural tube and leaving only the third ventricle. However, the hypothalamus is not yet developed. At stage 15, the neural ectoderm begins to evaginate to form the infundibulum, establishing the primordial neurohypophysis. Initiation of neurohypophysial development occurs at about E.9 in the mouse (Kerr, 1946), much later in overall development than in the snapping turtle. The adenohypophysis makes contact with the neuroectoderm at this time.

Distinct adenohypophysial structure is evident at stage 19 with a compacted mesenchyme and vasculature, and overall compaction of morphological structure continues through hatching in the snapping turtle. In the sea turtle, these events begin between stages 17 and 18 (Pearson et al., 1983), and by 40 days after oviposition, 5 – 7 days before hatching in the Japanese soft-shell turtle (Saga and Yamaki, 2006). While these events are not clearly defined for chicken or quail, it occurs sometime after E.10 and the adenohypophysis has distinct structure by E.19 (Reyes et al., 2007). In mouse, compaction of the structure occurs by E.16 – E.17, and by birth the structure is similar to a mature pituitary gland (Sano and Sasaki, 1969).

Adenohypophysial maturation continues beyond hatching in the snapping turtle, and a change in overall morphology occurs between one week and 4 months of age in the snapping turtle. Similar events are seen in hatchling sea turtles, while Saga and Yamaki (2006) report nearly the same shape of the adenohypophysis 5 – 7 days before hatching as seen in adults of Japanese soft-shell turtles. Furthermore, all cell types are

differentiated by this time in the Japanese soft-shell turtle, whereas cell differentiation continues post-hatching in chicken and quail (Reyes et al., 2007).

The distinct neurohypophysial structure seen in mammals never forms in the snapping turtle, similar to other turtle and bird species; however, the inferior portion of the neuroectoderm, below the third ventricle undergoes an increase in overall thickness at stage 21, forming the median eminence, pars tuberalis, pars intermedia, and neurohypophysis. This structure is similar to the neurohypophysis in chicken, quail, sea turtle, and the Japanese soft-shell turtle. The neurohypophysis is well developed by hatch and changes little through the first 4 months of life. A more enlarged, lobular neurohypophysis is present in mouse and humans (Kerr, 1946; Ikeda et al., 1988).

Overall, the process of pituitary gland development is fairly well conserved. There is slight variation in timing of events, and while the mammalian pituitary gland is complete by birth, the pituitary gland continues to mature and differentiate after hatch in turtles and birds. The neurohypophysis arises from the infundibulum in all species, but shows the most significant difference in final morphology.

The hypothalamus signals to the pituitary gland to regulate the endocrine system. Sex differences are present within the hypothalamus of many adult vertebrates and include differences in hypothalamic nuclear volumes, cell densities within specific nuclei, and production of hormones that regulate release of pituitary hormones. Sex differences in circulating steroid hormones, local testosterone aromatization into estradiol, and some hormone-independent mechanisms induce sexual differentiation of hypothalamic nuclei. Prominent sexual dimorphisms include differences in periodicity of the gonadotropin

releasing hormone pulse generator and LH production. These differences are thought to originate during a critical embryonic and post-natal period of development in mammals. Therefore, we examined gene expression in the hypothalamus during embryogenesis, at hatch, and six months after hatch to determine when sex differences develop in the snapping turtle.

We found sex differences in gene expression at stage 20 of embryonic development, just after gonadal sex has been determined. Embryos at 31ºC, a female-producing temperature, had higher expression of *AR*, *Cyp19A1*, *ESR1*, and *Kiss1R* than did embryos at 26.5^{o}C, a male-producing temperature. Steroid hormones are involved in the establishment of sex differences in the brain/hypothalamus in other species so differences in expression of steroid receptors might be expected in the snapping turtle.

The sex difference in *AR* expression in embryos did not persist in one-week old or 4-month old turtles. Expression of AR is not evident at E.18 in mice (Kanaya et al., 2014). However, AR is present at P.4, and is sexually dimorphic with higher expression in males at P.5 and P.25 (Brock et al., 2015). AR expression was not sexually dimorphic during the critical period for sexual differentiation in the fetal lamb (Reddy et al., 2014). Therefore, AR follows a different pattern based on species, and is not thought to play a large role in sexual differentiation of the brain.

Aromatase, which is encoded by *Cyp19A1*, is a critical enzyme involved in sexual differentiation of the brain. Local aromatization of testosterone occurs within the brain and is linked to masculinization of specific brain regions (Kanaya et al., 2014). *Cyp19A1* shows higher expression in females at stage 20, but the pattern is reversed in one-week

old hatchlings, with higher expression in males. Expression is no longer sexually dimorphic at 6-months of age. Aromatase activity is highest around birth in rats and mice (Tobet et al., 1985; Beyer et al., 1993), with a decrease following birth; therefore it is likely to exert most of its effects at this time. *Cyp19A1* is expressed in murine hypothalamus at E.15, but a sex difference was not detected (Karolczak et al., 1998). However, aromatase expression fluctuated after birth, especially in males. Sex differences were observed at birth and on P.15, with higher levels in males (Karolczak et al., 1998).

Local aromatization of testosterone leads to increased levels of estradiol, and brain differentiation is linked with estradiol activity, specifically with binding to ESR1 (Kanaya et al., 2014). *ESR1* expression was higher in female snapping turtle embryos than in males. This pattern of sexual dimorphism was reversed in hatchlings, with males exhibiting higher expression than females. Differential expression of *ESR1* shifts back to being female biased at 6-months of age. ESR2 is less known for its role in the brain, but we detected higher expression in hatchling females.

We detected a significant sex difference in expression of *GnRH2* at hatch, but the dimorphism was transient. We are not aware of sex differences in *GnRH2* expression during embryogenesis or the post-natal period in other species. However, it is well-known that the GnRH pulse generator differs between males and females at reproductive maturity in mammals (Carmel et al., 1976; Clarke and Cummins, 1982). Kisspeptin and its receptor modulate the activity of GnRH neurons in other species. Furthermore, kisspeptin and kisspeptin receptor are implicated in the male-specific testosterone surge

in rodents during embryonic development (Clarson and Herbison, 2016). We did not find expression differences in *Kiss1* until 6 months after hatching. However, *Kiss1R* expression was higher in embryonic and hatchling females. Similar results were found in mice, with regionally-specific higher expression in females postnatally (Brock and Bakker, 2013).

While morphology and cellular composition of the pituitary gland do not differ by sex, hormone production differs in adults. Sex differences in the pituitary gland may not have developmental origins, but rather may be driven by differences in releasing-hormones and release inhibiting hormones produced by the hypothalamus. We identified sex differences in gene expression in the pituitary gland in the same individuals used for analysis of hypothalamic differences.

Fewer sex differences in gene expression are present in pituitary glands as compared with the hypothalamus. Embryos at late stage 20 only showed differential expression of *LHb* in the pituitary, with higher expression in females. It is important to point out the overall expression of *LHb* is extremely low in the snapping turtle. In other species, LHb-producing cells have not fully differentiated at this stage. In the sea turtle, LH-producing cells are present as early as stage 17 in the adenohypophysis and in the pars tuberalis at stage 22, which is near hatching (Pearson et al., 1983). Conversely, LHb-producing cells are not evident in chicken and quail until P.10 (Reyes et al., 2007). Based on our morphological study, the pituitary gland at stage 20 has not taken on a fully developed morphology, and it is very likely the cells have not fully differentiated. Therefore, sex differences may not be biologically relevant at this point.

Many more genes showed sex differences in hatchlings. Expression of *CGA*, *Cyp19A1, ESR1, ESR2, GnRHR2b,* and *LHb* was higher in males than in females. The majority of cellular differentiation occurs during late embryogenesis and the pituitary gland is still undergoing morphological changes after hatching. Thus, we would expect more expression differences shortly after hatching when different cell types were differentiating. There were no longer sex differences in 6-month old turtles. The reproductive axis may be quiescent until sexual maturity, which does not occur until 8 – 10 years of age.

Lastly, 100% ethanol has been used to carry chemicals through the eggshell in studies of temperature-dependent sex determination, but its impact on neural development has not been examined. We therefore analyzed the effects of ethanol on gene expression in the hypothalamus and pituitary gland. We found that exposure to a small amount of ethanol can significantly alter gene expression within the hypothalamus, but not in the pituitary gland. Considering evidence for effects of alcohol on neural development in other species, this is not an entirely surprising outcome. However, evidence for reproductive effects of ethanol is lacking. Our results suggest that alterations in reproduction with embryonic alcohol exposure should be studied. It will be important in future studies to compare the effects of chemical treatments with ethanol-treated groups when studying effects in the brain.

CHAPTER III

HIGH-THROUGHPUT IDENTIFICATION OF DEVELOPMENTAL AND SEXUAL DIMORPHISM WITHIN THE HYPOTHALAMUS AND PITUITARY GLAND IN THE SNAPPING TURTLE

Abstract

The hypothalamus regulates the endocrine system by signaling through the pituitary gland to peripheral endocrine organs. Specific hypothalamic nuclei display sexual dimorphisms which allow for sex-specific reproductive control and behavior. Sex-specific genetic background is established during pre- and peri-natal development, regulating sexually dimorphic cellular differentiation and hormonal profiles. Several diseases are sex-biased in etiology and incidence, and therefore understanding underlying sex-based development is important in understanding the origins and causes of disease. Here we use RNA-Seq to identify differentially expressed genes between sexes and development during hypothalamus and pituitary gland development in the common snapping turtle, a species with temperature-dependent sex determination. We also identified overrepresented functional categories in genes that are differentially expressed between sexes. Genes involved in protein regulatory processes were in the top overrepresented functional categories. We also manually identified genes involved in neuroendocrine pathways. We found genes associated with stress response, growth, metabolism, and reproduction differentially expressed between sexes in 4-month old hatchlings. Investigating development within turtles will further understanding of

underlying mechanisms of morphological and functional differences in the hypothalamusand pituitary gland. It could also help elucidate the evolutionary origin of sexual dimorphism in the brain.

Introduction

The hypothalamus is the main regulator of homeostasis and numerous other physiological and behavioral processes essential for survival and reproduction in all vertebrates. A major role of the hypothalamus is the regulation of the endocrine system. Neuroendocrine signals are relayed from the hypothalamus to the pituitary gland, and hormonal signals are released into the bloodstream, circulating to tissues throughout the body. Targets of pituitary signals include the thyroid gland, gonads, liver, and adrenal glands, among others; inducing widespread effects throughout the organism. Sexually dimorphic regions of the hypothalamus are noted in the majority of vertebrates studied to date, and can originate during pre- or perinatal development. Considering sex differences in the incidence of autoimmune, cardiovascular, metabolic, and reproductive disorders (Lissner and Heitmann, 1995; Lynn and Davies, 2007; Sharma and Eghbali, 2014; Chiaroni-Clarke et al., 2016), it is important to understand sex-specific development as a major factor contributing to disease.

The hypothalamus originates from the ventral most region of the anterior diencephalon. Molecular regulation has been partially characterized in a wide variety of vertebrate species including Japanese macaque (Grayson et al., 2006), mouse (Shimogori et al., 2010), frog (Dominguez et al., 2015), and chicken (King and Millar, 1982). While some species differences in overall structure and function within the hypothalamus exist,

comparison reveals high conservation in molecular regulation of development. The hypothalamus follows an "outside-in" pattern of neurogenesis, relying on canonical Wnt signaling for early establishment (Kapsimali et al., 2004). Later development of posterior-ventral hypothalamus is dependent on Nodal signaling, while Hedgehog pathways antagonize posterior-ventral hypothalamus and promote anterior-dorsal hypothalamic fate (Mathieu et al., 2002). Molecular signals from underlying tissues are also required for proper development (Vieira et al., 2010). Following the initial establishment of hypothalamic structure, individual nuclei are formed through specific sets of signaling molecules, differentiating distinct neuronal subtypes (Shimogori et al., 2010). There is a dynamic pattern of gene expression throughout hypothalamic development.

The pituitary gland is composed of two major regions originating from distinct tissue types. The neurohypophysis differentiates from neural ectoderm while the adenohypophysis is from an invagination of the oral ectoderm (Watanabe and Daikoku, 1979; Chapman et al., 2005; Sanchez-Arrones et al., 2015). The first pituitary-specific transcription factor expressed is Hesx1, a transcriptional repressor. Restricted expression of Hesx1 is responsible for formation of Rathke's pouch (Hermesz et al., 1996). Several other signaling pathways dictate development of Rathke's pouch, including sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), and Wnt pathways (de Moraes et al., 2012). Following initial formation of Rathke's pouch, five distinct cell types are differentiated in response to a dorsal-ventral FGF8 gradient and an opposing ventral-dorsal BMP2 gradient. Specific concentrations of FGF8

and BMP2 give rise to activation or repression of distinct transcription factors, which direct cell fate (for review, see de Moraes et al., 2012). Mutations in transcription factors can lead to long-term health effects, and the same mutation can lead to different phenotypes, indicating the importance of precise timing and transcription factor interactions during hypothalamic development. Improper signaling can lead to the development of hypopituitarism, or specific mutations can lead to disorders involving one or more cell types. For instance, a mutation in Prop1 can result in gonadotropin deficiency, whereas a mutation in Pax6 results in fewer lactotropes and somatotropes, resulting in lower levels of prolactin and growth hormone, respectively (Simmons et al., 1990; Bentley et al., 1999).

The neurohypophysis originates from neuroectoderm, through proliferation of medial hypothalamic cells to form the infundibulum. Neurohypophysis development is highly dependent on FGF signaling (Ohuchi et al., 2000; Manning et al., 2006). FGFs are involved in both the origin of the neurohypophysis and guidance of axons from parvocellular and magnocellular neurons through the infundibulum and into the neurohypophysis (Liu et al., 2013). While having distinctive origins, the adenohypophysis and neurohypophysis function together to regulate the endocrine system through release of hormones into the general circulation.

Sexual dimorphism of hypothalamic nuclei has been found in fish, reptiles, mammals, and birds (Gorski, 1984; Panzica et al., 1987; Wade and Crews, 1992). Differences between sexes include volume of nuclei, cell number, connectivity, neurotransmitter phenotype, and molecular signaling. The medial preoptic area-anterior

hypothalamus (MPOA/AH; or sexually dimorphic nucleus of the preoptic area, SDN-POA) is involved in facilitating the display of male-typical copulatory behaviors such as mounting and intromission, and is larger in males than in females of many species (Raisman and Field, 1971; Crews et al., 1990). The ventromedial nucleus (VMN) has been well established in facilitating female-typical receptive behavior (Pfaff and Sakuma, 1979; reviewed in Whittier and Tokarz, 1992). Interestingly, the volume of the VMN is larger in male compared to female rats, but is larger in female whiptail lizards (Gorski et al., 1978; Crews et al., 1990; Wade and Crews, 1991). These volumetric dimorphisms hold true in most species, but in at least one, the leopard gecko, neither the MPOA/AH nor VMN show size differences between sexes (Coomber et al., 1996). Therefore, sexual dimorphism is thought to be involved in sex-typical reproductive behavior in most species, but is not necessarily required.

Expression of several genes differs between the sexes and changes throughout neuroendocrine development. For example, estrogen receptors ESR1 and ESR2 are involved in reproductive neuroendocrine function and sexual differentiation of behavior in mice (Wersinger et al., 1997; Lindzey et al., 1998). Both genes undergo changes in expression patterns throughout the hypothalamus in a sexually dimorphic pattern as development progresses. ESR2 is also dimorphic in rats, with higher expression in the anteroventral periventricular nucleus (AVPV) (Orikasa et al., 2002) and the VMN (Ikeda et al., 2003) of female rats when compared to male rats.

Taking sex differences into account is valuable when considering disease and reactions to drugs. Conclusions based on the study of one sex do not always hold true in

the other sex (Mogil and Chanda, 2005; Cahill, 2006). Susceptibility to disease and the effect of damage can be as much as 2-5 fold greater in one sex. These include higher rates of aging-related neuropsychiatric and learning disorders with developmental origins in males and higher rates of aging-related neurodegenerative diseases and mental health dysfunctions in females (Swaab and Hofman, 1995; Voskuhi, 2011). Disruption of sexual differentiation within the hypothalamus can interrupt lifelong endocrine function, and can lead to abnormal reproductive behavior and function, infertility, depression, cardiovascular disease, and altered stress response later in life (Goldstein et al., 2014; Leon-Olea et al., 2014; Zhang et al., 2014). Therefore, monitoring the origins of sexual dimorphism is imperative in preventing or treating these types of disorders.

The cellular mechanism behind formation of sexually dimorphic nuclei in the hypothalamus is not fully established; however the current idea involves increased apoptosis in one sex. Apoptosis was proposed as the main mechanism over 30 years ago, and subsequent research has supported the idea. The male SDN-POA is larger, and it has been found that both testosterone and estrogen prevent apoptosis in neonatally castrated males. Prevention of programmed cell death is therefore a likely mechanism for the effect of aromatizable androgens on SDN-POA volume in males (Davis et al., 1996; Arnold, 1996). Apoptosis might not be the only mechanism, however, and sex differences in cell migration within the MPOA/AH of mice have also been noted (Henderson et al., 1999). It is likely that both of these mechanisms, along with other, yet to be identified mechanisms, could be involved in creating long-lasting sex differences in hypothalamic nuclei.

While the specific cellular mechanism of action has yet to be fully defined, castration and hormonal manipulation have led to the belief that steroid hormones exert major organizing effects on the brain, as seen through behavior changes following treatment. Male rats castrated during the neonatal period show lordosis behavior when they are treated with estrogen in adulthood (Gerall, 1967; Thomas and Gerall, 1969; Corbier et al., 1983;), while female rats treated with exogenous androgen or estrogen during the neonatal period fail to show lordosis in adulthood (Mullins and Levine, 1968; Pfaff and Zigmond, 1971; Kouki et al., 2005). The classic model for sexual differentiation of the brain begins with the differentiation of the gonads, which release sex steroid hormones into circulation. These hormones then influence differentiation of secondary sexual characteristics, including the brain. More recently, it is becoming clear this might not be the whole picture.

Evidence points to a possible direct role of sex-linked genes on sex differences. The use of Sry transgenic mice has allowed for the partial teasing out of individual roles of chromosomal genes and steroid hormones, and it appears neural and behavioral phenotypes, such as behavior and ovulation, are influenced by perinatal gonadal steroid hormones. However, sex chromosomes appear to influence vasopressin innervation of the lateral septum, aggressive and parenting behaviors, social behaviors, and gene expression (reviewed in McCarthy and Arnold, 2011). Another idea came about through study of the zebra finch song system, which suggests some neural sexual dimorphisms are caused by hormones produced locally within the brain rather than in the gonads (Wade and Arnold, 1996; Wade and Arnold, 2004). Yet another alternative has been found in studying

species with temperature-dependent sex determination (TSD), in which temperature during a critical period of embryogenesis determines sex. In this model, temperature might influence sexual differentiation of the nervous system independently of gonadal steroids (Flores and Crews, 1995; Coomber et al., 1996). Few animal models allow the effective manipulation of sex chromosomes or incubation temperature without also influencing gonadal steroid levels. It is likely a combination of genes, steroid hormones, and the environment which influence sexually dimorphic development in the brain. Identification of genes that display dimorphic expression could provide insight into molecular pathways that are involved in neural differentiation in vertebrates with different sex determination mechanisms. Most brain sex studies have been carried out in lizards, mammals, and birds with genotypic sex determination.

Here we study the common snapping turtle, *Chelydra serpentina*, a species with TSD. Incubation of embryos at 26.5°C produces 100% males, whereas incubation at 31°C produces 100% females. Incubation of eggs at 28.2°C produces a 50:50 sex ratio. Working with a TSD species allows control of the sex of each individual. There could be major differences in development of the sexually dimorphic brain in species with TSD. Investigating this process within turtles could help elucidate the evolutionary origin of sexual dimorphism in the brain.

Performing gene expression studies on turtles can be challenging because genome and transcriptome sequences are not widely available. Having such data provides a wealth of information for hypothesis generation and testing. Here we use RNA-Seq to identify genes that are differentially expressed in snapping turtle hypothalami and

pituitary glands during development and between the sexes. Morphological studies indicate cellularization of the hypothalamus and pituitary gland begins around stage 20 of development. We therefore analyzed embryos at stage 20 of development as well as hatchlings at 4 months of age to capture early developmental events as well as changes that occur later in development.

We also identified functional categories overrepresented in the genes that are differentially expressed between sexes. We manually identified neuroendocrine genes differentially expressed between the four groups to identify neuroendocrine pathways differentiated by sex, and to further understand underlying mechanisms of morphological and functional differences in the hypothalamus and pituitary gland.

Methods

Animal and Tissue Collection

With permits from the Minnesota Department of Natural Resources and the North Dakota Game and Fish Department, we collected snapping turtle eggs from north-central Minnesota and northeastern North Dakota during May and June of 2011. Eggs, embryos, and hatchlings were treated according to protocols approved by the Institutional Animal Care and Use Committee at the University of North Dakota.

Eggs were washed with tap water, numbered to track individual eggs, placed into plastic containers, covered with moist vermiculite (1 part vermiculite : 1 part water by mass), and placed into incubators. Half of the eggs from each clutch were incubated at 31^{o}C (female-producing temperature) and half at 26.5^{o}C (male-producing temperature) (Rhen and Lang, 1994; Rhen et al, 2015). Temperature variation within the incubators is

minimal (Lang et al, 1989). We determined embryonic stages by visualizing anatomical features and comparing with morphological criteria described by Ynteman (1968). Embryos at stage 20 were removed from the egg and euthanized by rapid decapitation. Hatchlings at four months of age were also euthanized by rapid decapitation and the brains were dissected from the skull. Heads/brains were placed in RNAlater© solution immediately after dissection (Ambion, Austin, TX) and stored at -20ºC.

RNA Preparation and Sequencing

The hypothalamus and pituitary gland were micro-dissected together from each individual and total RNA was isolated using RNAzol®RT (Molecular Research Center, Inc.). RNA purity and integrity was assessed by spectrometry, gel electrophoresis, and the Experion^{TM} RNA StdSens Aanlysis (Bio-Rad). Only samples with an RQI above 8.0 were included in subsequent studies. As described above, our experiment was a 2 x 2 factorial design (2 incubation temperatures x 2 developmental stages). Equal amounts of RNA from 5 individuals were pooled as one biological replicate at the embryonic stage. Equal amounts of RNA from 3 individuals were pooled to make one biological replicate at the hatchling stage. Overall, we had 2 biological replicates at each temperature/stage for a total of 8 samples. RNA was shipped on dry ice to the Huntsman Cancer Institute at the University of Utah for cDNA library preparation and sequencing. Eight cDNA libraries were multiplexed and sequenced on a single lane using an Illumina HiSeq2000 next generation sequencer.

Sequence Assembly and Differentially Expressed Gene Detection

Sequence data from the HP was combined with data from embryonic gonads sequenced with Roche 454 (2.8 million reads; 350 bp average read length) and Illumina platforms (156 million reads; 100 bp reads). We also sequenced two cDNA libraries generated from hatchling intestines to include tissues derived from all three embryonic germ layers. Raw reads were filtered for quality and assembled using Newbler and CLC Genomics Workbench. In addition, we used transcriptomes from several other vertebrates as references for mapping and assembling the snapping turtle transcriptome. Assemblies using chicken, turkey, lizard, monotreme, and marsupial reference transcriptomes were combined with *de novo* assemblies to produce a composite assembly. This process was reiterated several times with subsequent mapping of snapping turtle reads to produce a high quality set of contigs. Full-length cNDAs have been assembled for approximately 10,000 unique genes with partial cDNAs for another 7,000 unique genes. An in-depth description of the snapping turtle transcriptome is in preparation (Rhen et al, manuscript in preparation).

We used the newly assembled snapping turtle contigs as the reference for RNA-Seq analysis of our eight HP libraries. CLC Genomics Workbench was used to calculate gene expression values in reads per kilobase of exon model per million mapped reads (RPKM) for each gene (Mortazavi et al., 2008). We tested for differences in gene expression among the four groups (2 sexes x 2 stages) using Baggerly's test on proportions (Baggerly et al., 2003) and a false discovery rate (FDR) set at $p<0.05$ (Benjamini and Hochberg, 1995).

Sequence and Functional Annotation

Contigs that were differentially expressed between incubation temperatures in embryos or between female and male hatchlings were manually annotated using BLAST. Start and stop codons were identified using a BLASTn search against the NCBI non-redundant database and the ExPASy translate tool (http://web.expasy.org/translate/). A much larger number of contigs were differentially expressed between developmental time points, which precluded manual annotation. We therefore assigned gene descriptions using Blast2Go (Conesa et al., 2005) with a Blastn search against the non-redundant database and E-value of $1x10^{-5}$. Comparison of names assigned manually to the automated Blast2Go (B2G) description indicates 94% similarity between methods. Functional annotation of differentially expressed genes in each group was conducted in B2G with an InterProScan annotation, gene ontology mapping, and gene ontology annotation with an E-value cut-off of $1x10^{-6}$. Venny 2.1 (Oliveros, 2007-2015), CLC Genomics Workbench, B2G, and GeneMANIA (Warde-Farley et al., 2010) were used to identify genes in common between treatment groups and overrepresented functional groups in sexually dimorphic gene sets.

Quantitative PCR Validation

To validate the RNA-Seq results, we used RNA samples from the individuals that were pooled for RNA sequencing; RNA from each individual was used as input for quantitative PCR. Reverse transcription was performed with the Applied Biosystems' High-Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. A total of 50 ng of input RNA was reverse transcribed in a 20 µl reaction on a BioRad

MyiQ™ Single-Color Real-Time PCR Detection System. The cDNA was diluted to the equivalent of 0.625 ng input RNA/µl for use in qPCR reactions.

Primers were designed for the following neuroendocrine genes: *CGA, GH, PDYN, POMC, PRL, PRLH,* and *Kiss1R*. Each of these was identified as differentially expressed between developmental time points or between the sexes using RNA-Seq. Quantitative PCR was also carried out for 18S rRNA as a housekeeping gene. We prepared standard curves for each gene as described in Rhen et al. (2007). Purified PCR products were diluted to an initial concentration of 2 picograms/ml, and 10-fold serial dilutions were used to create a series of 8 standards across 8 orders of magnitude. Quantitative PCR was performed using 2 µl of cDNA template in 10 µl reactions with EvaGreen Supermix (BIO-RAD), and run on a CFX384 Real-Time PCR system (Bio-Rad). Amplification did not occur in blanks.

Results

We obtained 19 to 26 million reads (50 bp, unpaired) in 8 libraries for a total of over 172 million reads (Table 3.1). Contigs were assembled with reads from hypothalamus-pituitary gland, gonads, and intestines, and the final assembly comprised 422,185 total contigs (Guo et al., paper in preparation). A total of 87% of the HP reads mapped to the final transcriptome assembly (Table 3.1).

Baggerly's test on proportions revealed that many more genes were differentially expressed between developmental stages when compared with incubation temperature or sex differences (Table 3.2).

Table 3.1: The number of reads from Illumina Sequencing of the hypothalamus and pituitary gland and the percent of reads mapped back to the snapping turtle transcriptome assembly.

Library	Number of Reads	% Mapped Reads
31ºC, Embryo	19,683,000	88
31ºC, Embryo	19,098,624	90
26.5 ºC, Embryo	18,085,213	88
26.5 ºC, Embryo	26,929,894	87
31ºC, Hatchling	22,343,510	88
31ºC, Hatchling	19,488,985	86
26.5 ºC, Hatchling	20,333,523	85
26.5 ºC, Hatchling	26,280,982	85

Table 3.2: Total number of differentially expressed genes (DEGs) between the sexes and between developmental stages. The percentage of sequences with a BLAST hit in the NCBI non-redundant database (E-value < $1x10^{-5}$) and functional annotation with GO terms (E-value < $1x10^{-6}$).

	Total DEGs	Percent with BLAST Hit	Percent with Functional Annotation
Male vs. Female (embryo)	141	51.1	47.9
Male vs. Female (hatch)	195	60.5	53.4
Table 3.2 cont.			
Embryo vs. Hatch (male)	1064	59.3	35.5
Embryo vs. Hatch (female)	2187	59.6	42.1

There were 2,187 differentially expressed genes (DEGs) between embryos and hatchlings at 31ºC (in females), 1,064 DEGs between embryos and hatchlings at 26.5ºC (in males), 141 DEGs between incubation temperatures in embryos, and 195 DEGs between female and male hatchlings. Venny 2.1 was used to determine DEGs in common

between groups. Only 19 genes were sexually dimorphic in both embryos and hatchlings. More genes displayed developmental changes in both sexes: 601 developmental DEGs were shared by females and males (Figure 3.1).

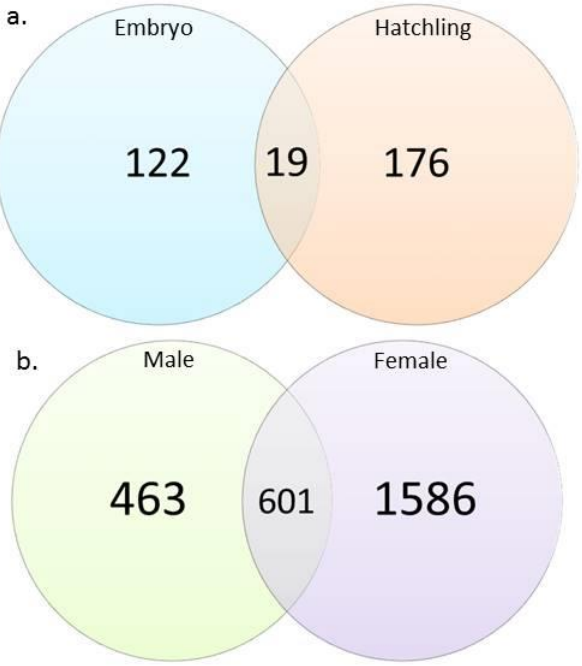


Figure 3.1: Differentially expressed genes between incubation temperatures at stage 20 of development and 4 months after hatching (a). 19 differentially expressed genes are in common between stages. Developmentally differentially expressed genes in males and females (b). 601 differentially expressed genes are in common between sexes.

A heat map of all 2,755 differentially expressed genes between the sexes and developmental time points in the hypothalamus and pituitary gland is shown in Figure 3.2. A dendrogram based on hierarchical clustering shows that gene expression patterns are primarily clustered by developmental stage (embryo or hatchling) and secondarily by sex (Figure 3.2).

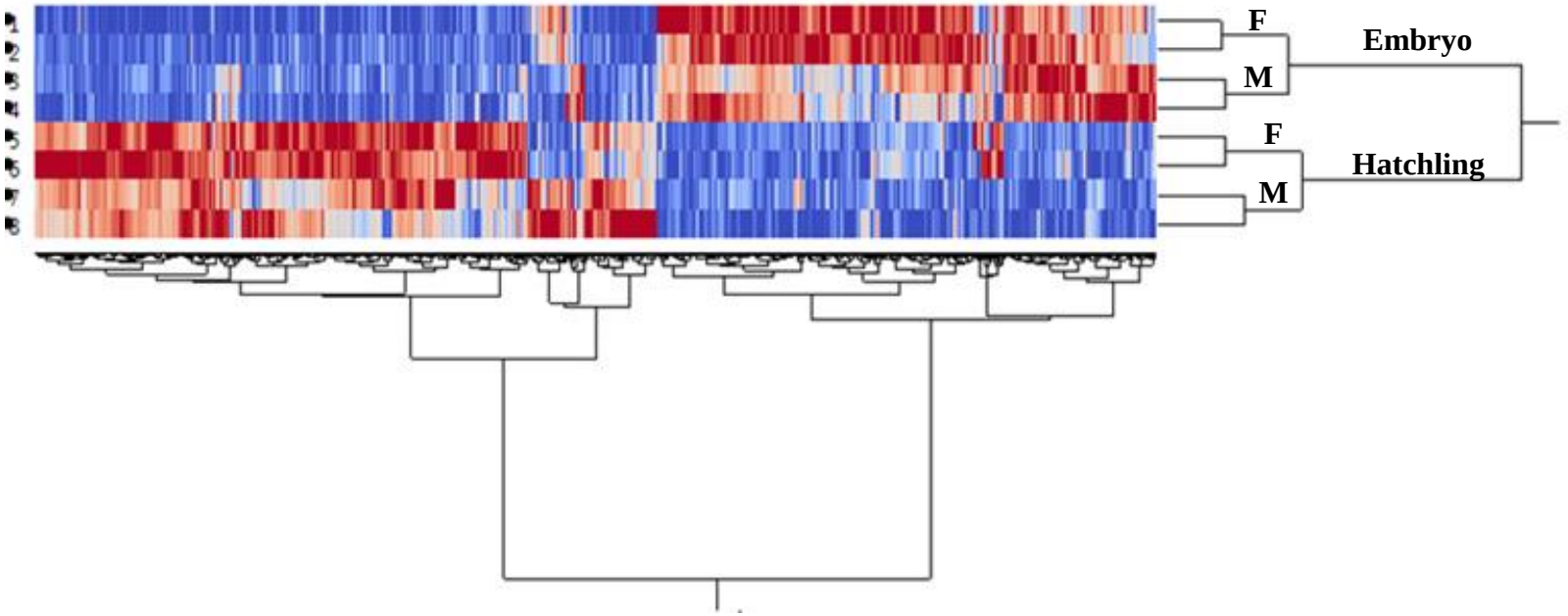


Figure 3.2: Heat map of transcripts that are differentially expressed in the hypothalamus and pituitary gland of snapping turtles, including both sexually dimorphic and developmentally regulated transcripts. Each column represents one of 2,755 differentially expressed genes. Hierarchical clustering was performed using Wards method. The dendrogram on the right shows that expression patterns were primarily clustered by developmental stage and secondarily clustered by sex. (F = Female, M = Male, Blue = Low Expression, Red = High Expression)

Differentially expressed genes for each group were identified with a BLAST hit E-value < $1x10^{-5}$. An average of 57.6% of sequences had a BLAST hit, and they were named accordingly. Gene Ontology (GO) terms were also assigned to each differentially expressed gene, with an average of 44.7% of sequences having a functional annotation (Table 3.2). Gene Ontology terms were assigned to differentially expressed gene sets between stages in males and females (figures 3.3 and 3.4). Sexually dimorphic genes with a BLAST hit were analyzed using GeneMANIA (Warde-Farley et al., 2010) to identify overrepresented functional categories at each stage of development.

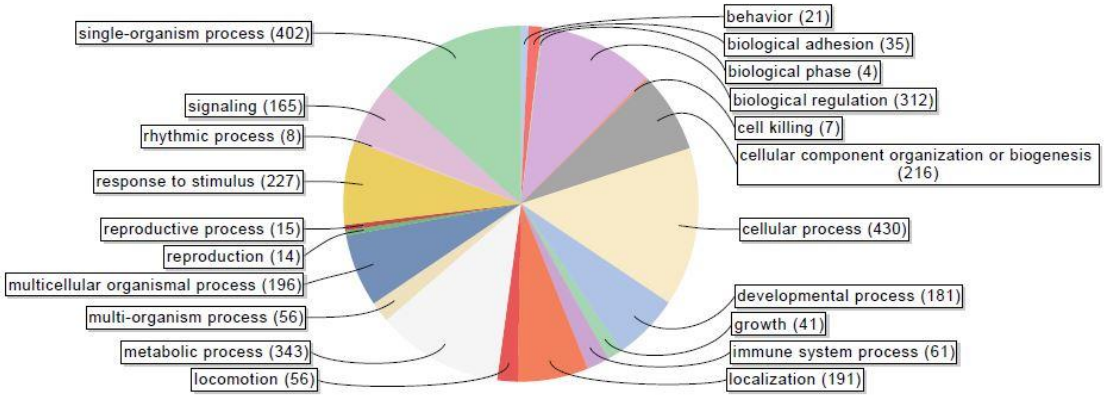


Figure 3.3: GO terms associated with biological processes for developmentally differentially expressed genes between stage 20 and 4 month hatchlings incubated at a male-producing temperature.

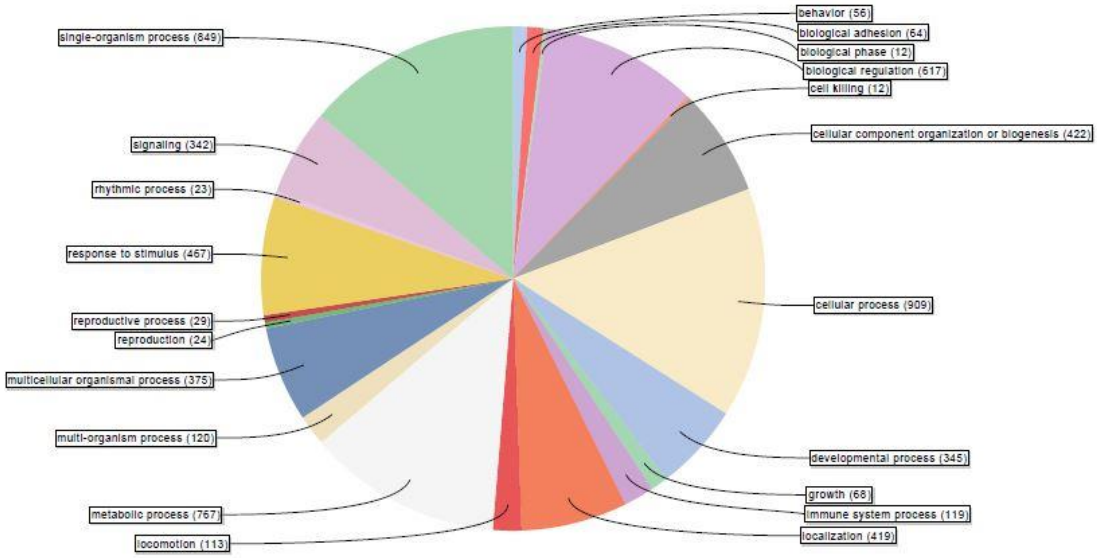
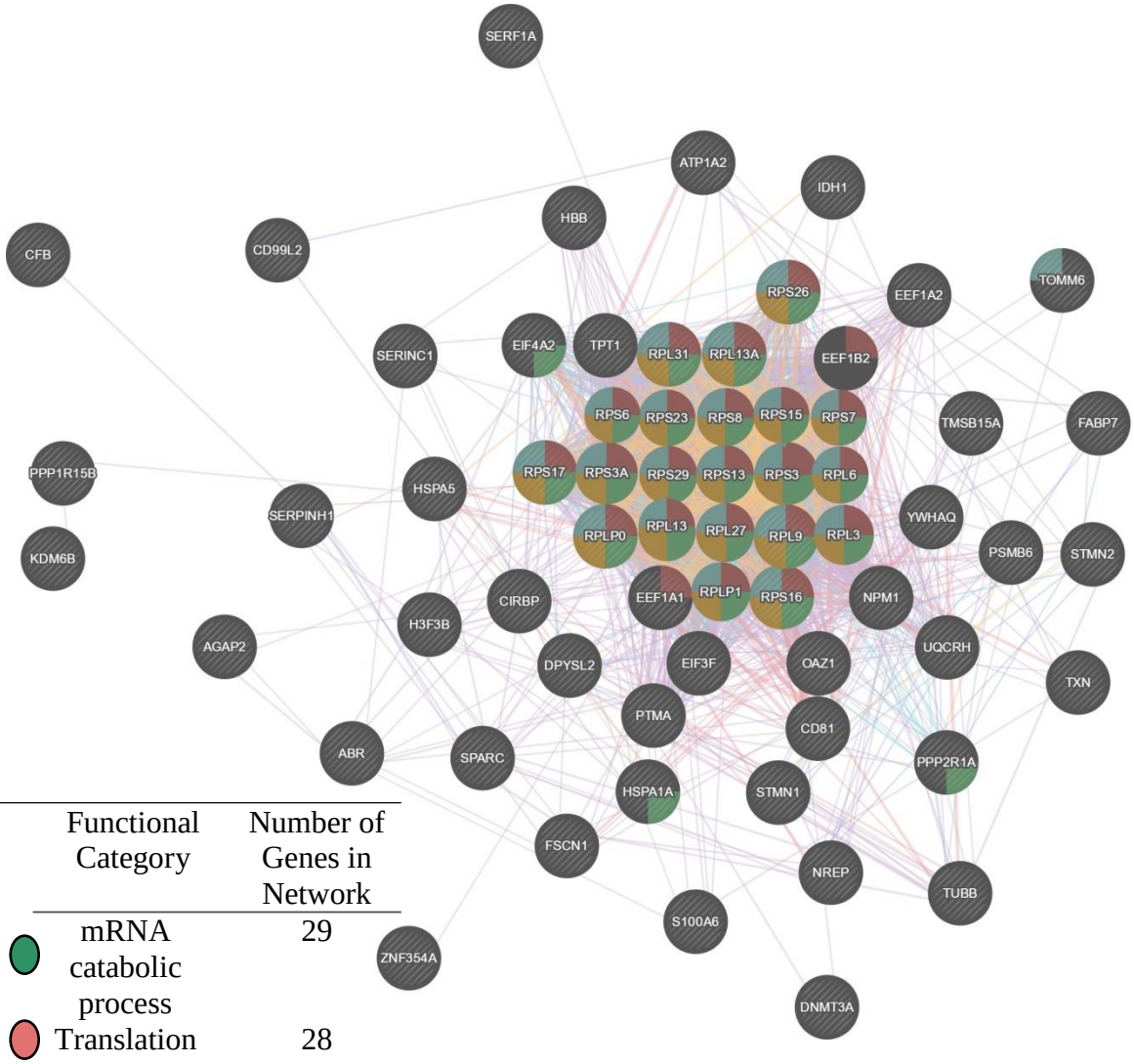


Figure 3.4: GO terms associated with biological processes for developmentally differentially expressed genes between stage 20 embryos and 4-month old hatchlings incubated at a female-producing temperature.

Sexually dimorphic genes in embryos were entered into GeneMANIA and compared with human gene sets. Over 20 functional groups were identified as overrepresented, but many of the categories in GeneMANIA are redundant. We therefore consolidated these groups into major functional categories. At stage 20, sexually dimorphic genes fell into 4 categories: mRNA catabolic process, translation, protein targeting, and ribosomal organization (Figure 3.5). Each category was nearly equally represented. Over 30 functional groups were identified for sexually dimorphic genes in hatchlings, which were consolidated into 7 major functional categories. The top three categories were translation, ribosomal organization, and protein targeting. The other four categories were represented by fewer genes in the network, and include matrix organization, receptor signaling pathway, glucose metabolic processing, and hormone metabolic process (Figure 3.6).

We were particularly interested in genes that play a role in neuroendocrine control and function; we manually searched the differentially expressed gene lists for such genes. We identified 11 neuroendocrine genes that were differentially expressed either between sexes or developmental stages (Table 3.3). Sex differences were not observed at stage 20, but *CGA*, *CHGA*, *PDYN*, *POMC*, *PRLH*, and *SST* were all expressed at a higher level in females than in males at 4 months of age. Males showed developmental changes in expression of *CGA, CHGA, GH, KISS1, PDYN, POMC, PRLH*, and *SST*, each having higher expression in hatchlings compared with embryos. Hatchling females had higher expression of *CGA, CHGA, KISS1, KISS1R, PDYN, POMC, PRL, PRLH, SST*, and *TSHβ* than embryonic females.



	Functional Category	Number of Genes in Network
●	mRNA catabolic process	29
●	Translation	28
●	Protein Targeting	27
●	Ribosomal Organization	26

Figure 3.5: Networks of genes that are sexually dimorphic in hypothalami and pituitary glands of embryos and hatchlings (GeneMANIA.org). Each functional category is highlighted with a different color. Genes in black did not fall into an overrepresented category. Four major functional categories were overrepresented in the sexually dimorphic gene set in embryos.

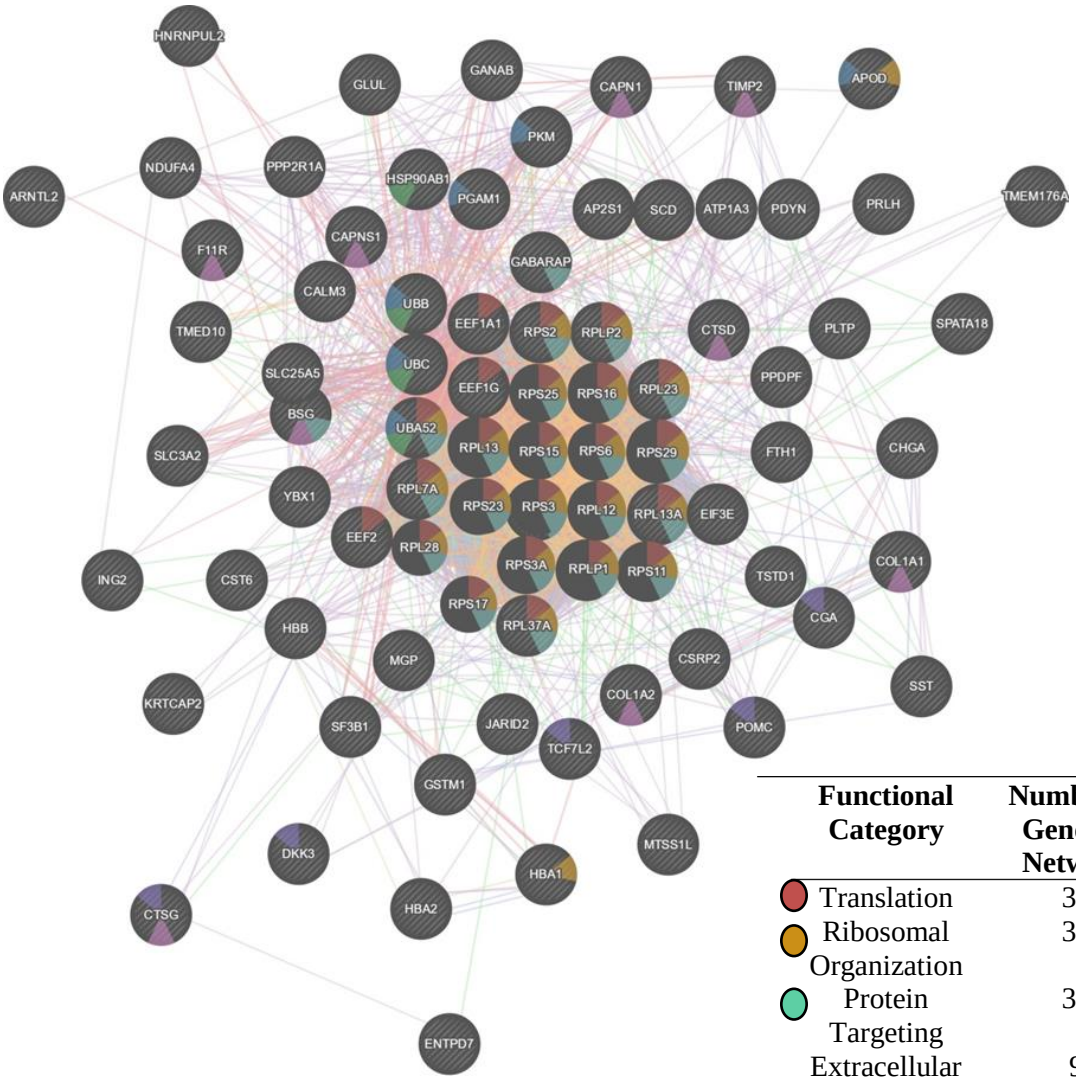


Figure 3.6: Networks of genes that are sexually dimorphic in hypothalami and pituitary glands of hatchlings (GeneMANIA.org). Each functional category is highlighted with a different color. Genes in black did not fall into an overrepresented category. Seven major categories were overrepresented in the sexually dimorphic gene set in hatchlings.

Functional Category	Number of Genes in Network
Translation	34
Ribosomal Organization	33
Protein Targeting	33
Extracellular Matrix Organization	9
Receptor Signaling Pathway	4
Glucose Metabolic Processing	6
Hormone Metabolic Process	5

Table 3.3: Differentially expressed genes in hypothalamus the pituitary glands of male and female snapping turtles at stage 20 of development and 4 months after hatching. Each gene is involved in neuroendocrine function. Fold change is based on expression values in RPKM.

	Differentially Expressed Neuroendocrine Genes	Expression Difference (Fold Change)
Female vs Male (Embryo)	None	
Female vs Male (Hatch)	*CGA*	3.0
	CHGA	2.1
	PDYN	1.7
	POMC	1.8
	PRLH	1.8
	SST	1.7
Hatch vs Embryo (Female)	*CGA*	13.7
	CHGA	4.2
	Kiss1	101.8
	Kiss1R	13.4
	PDYN	4.5
	POMC	22.9
	PRL	238.5
	PRLH	10.3
	SST	4.0
	TSHβ	40.7
Hatch vs Embryo (Male)	*CGA*	7.5
	CHGA	2.8
	GH	183.3
	Kiss1	58.6
	PDYN	2.6
	POMC	17.5
	PRLH	6.3
	SST	2.6

Quantitative PCR Validation

We analyzed expression of 7 genes using qPCR to validate our RNA-Seq results, including *CGA, PDYN, PRLH, GH, Kiss1R, PRL,* and *POMC.* All of these genes showed a significant developmental change, with higher expression in hatchlings, though this was not differentiated by sex. Expression of PRL was extremely low in embryos and did not amplify. Thus, PRL expression could not be statistically compared between embryos and 4-month old hatchlings. The only gene showing a significant sex difference was Kiss1R ($F_{1,1} = 4.06$, $p = 0.04$). Quantitative PCR analysis shows higher expression of Kiss1R in males (Figure 3.7). However, this gene did not show a sex difference in the RNA-Seq data.

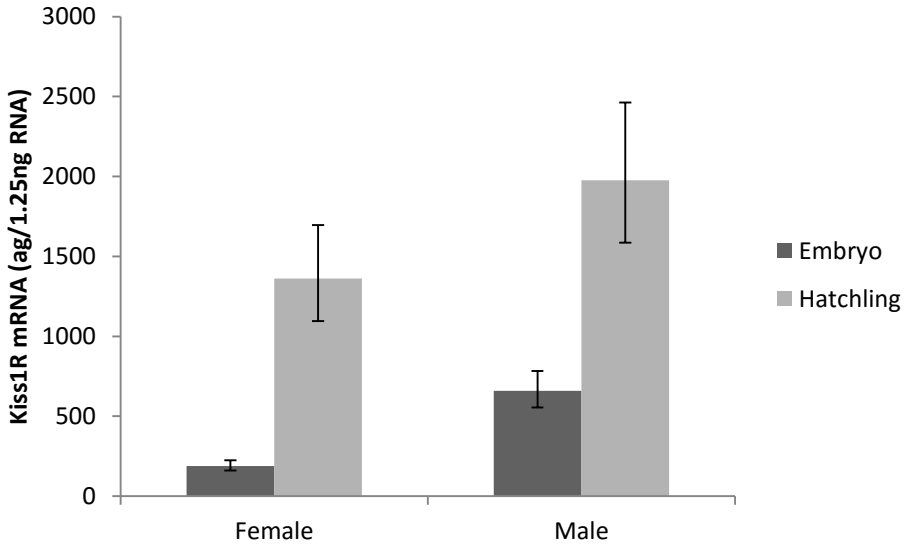


Figure 3.7: Gene expression in male and female hypothalami and pituitary glands from stage 20embryos and 4-month old hatchlings. Two-way ANOVA revealed a significant sex difference in embryos and 4-month old individuals ($F_{1,1}=4.60$, $p = 0.04$).

Discussion

In this study we analyzed developmental and sex-based differences in gene expression in the hypothalamus and pituitary gland by comparing transcriptome-level

differences in expression. We found that 1) many genes are differentially expressed between embryos and hatchlings; 2) transcripts of genes involved in neuroendocrine function are abundant in hatchlings, but mostly absent in embryos at stage 20 of development; 3) few genes are differentially expressed between the sexes even though there are morphological differences, and physiology and behavior regulated by the hypothalamus.

Identifying sex differences in gene expression in the developing hypothalamus is important for understanding physiological and behavioral differences in reproductive function. Furthermore, several diseases are sex-biased, including depression, anxiety disorders, and autism (Woods et al., 2003; Kajantie and Phillips, 2006; Asarian and Langhans, 2010), and genetic differences likely underlie the sex difference in disease etiology and incidence.

Developmental differences in gene expression were numerous in both males and females. Assignment of functional categories revealed the same GO terms for biological process in both males and females, therefore similar processes are changing between embryos and hatchlings in both males and females. Many functional categories include genes involved in basic cellular processes and function such as cell signaling, localization, cell adhesion, and cell death. Our manual search of genes that change during development identified several genes involved in neuroendocrine function, such as response to stimulus, growth, reproductive process, and reproduction. Ten genes were identified for females, while eight were identified for males. Seven of these genes were differentially expressed in both males and females. Females showed higher expression of

Kiss1R, *PRL*, and *TSHβ* in hatchlings, while males showed higher expression of *GH* in hatchlings. *Kiss1R* and *PRL* play a reproductive role in the hypothalamus, while *GH* has differing functions when expressed in the hypothalamus or pituitary gland.

Approximately equal numbers of genes are expressed at a higher level in embryos vs. hatchlings or vice versa. In contrast, all neuroendocrine genes are expressed at higher levels in hatchlings. As seen in our morphological study (chapter 1) and shown in the green sea turtle and Japanese soft-shelled turtle, cellular differentiation within the pituitary gland begins around stage 20 (Pearson et al., 1983; Saga and Yamaki, 2006). These observations suggest that different pituitary cell-types have not yet differentiated in embryos, but have differentiated by 4 months after hatching. This is likely true for the hypothalamus because distinct nuclei are not obvious in embryos at stage 20.

It was somewhat surprising that few genes were differentially expressed between incubation temperatures in embryos or between female and male hatchlings at 4-months of age. Sex differences in morphology and physiology of the hypothalamus of many other vertebrates led us to hypothesize that molecular differences would be numerous. Yet, previous studies in mice also report few differences in gene expression between the sexes during development and in adulthood (Shimogori et al., 2010; Mozhui et al., 2012). Mozhui et al. (2012) suggest such a small number of transcriptional differences act on physiology and behavior by a combination of cumulative differences in transcripts. They also found that global network structure is very similar between males and females, and the establishment of sex-dependent hypothalamic gene networks is dependent on the differential expression of just a few transcripts. We suggest an alternative hypothesis

based on the overrepresented functional categories present in both embryo and hatchling hypothalami. Genes involved in translation and protein processing were the top overrepresented functional categories in embryos and hatchlings, suggesting that sex differences in morphology and physiology are established primarily at the protein level rather than the transcript level.

We identified several genes known to play a role in neuroendocrine function among the differentially expressed genes in embryos and hatchlings. These genes were not differentially expressed between the sexes in stage 20 embryos. In contrast, 6 genes involved in stress response, growth, and reproduction were all expressed at a higher level in four-month old females. For example, *SST* inhibits growth in vertebrates, which matches a previous report that growth rate is reduced in hatchlings from a female-producing temperature (Rhen and Lang, 1994). *POMC* and *PDYN* are both involved in stress responses, acting as endogenous opioids and decreasing activity of the stress pathway in response to increased levels of circulating cortisol (Petraglia et al, 1986; Rushen et al, 1993). *PDYN* and *POMC* also influence the reproductive endocrine axis. Kisspeptin neurons are inhibited by dynorphin, the protein encoded by the PDYN gene (Navarro et al, 2009; Wakabayashi et al., 2010). Dynorphin is currently a target of translational research to moderate LH pulsatility in pathologies like hypogonadotropic hypogonadism and polycystic ovary syndrome (Skorupskaite et al., 2014). We found *PDYN* was expressed at a higher level in females. Interestingly, the role of dynorphin as a reproductive regulator is better established in women than in men (Rance, 2009).

Incubation temperature has been found to impact several physiological and behavioral traits in snapping turtles independent of its effect on sex determination, including growth physiology, thermoregulatory behavior, and metabolic rate (Rhen and Lang, 1994; O'Steen and Janzen, 1999; Rhen and Lang, 1999). These characteristics are known to be regulated by the hypothalamus and pituitary gland in other vertebrates, suggesting that incubation temperature may directly program hypothalamic development. While this is an important caveat, the underlying differences between the sexes presented here are important to identify regardless of the underlying mechanism.

Here we provide a wealth of hypothesis-generating information for the development of sex differences in the neuroendocrine system. Identification of differentially expressed genes will provide insight into the mechanisms necessary for formation and sexual differentiation of the hypothalamus and pituitary gland in the snapping turtle. This data can also be used for comparative studies to determine whether there are conserved mechanisms for neuroendocrine development. Identification of genes that display sexually dimorphic expression will help us better understand differences in endocrine function between the sexes.

This study provides baseline information for further research on sexual differentiation in the common snapping turtle. Availability of an annotated transcriptome will allow generation of a wide range of molecular resources specific to the snapping turtle, including PCR primers, DNA and RNA probes, and antibodies for protein studies. Some of the sex differences in gene expression reported here provide a plausible

mechanistic explanation for previously reported differences in growth and physiology in the snapping turtle.

CHAPTER IV

ATRAZINE ALTERS EXPRESSION OF REPRODUCTIVE AND STRESS GENES IN THE DEVELOPING HYPOTHALAMUS OF THE COMMON SNAPPING TURTLE, *CHELYDRA SERPENTINA*

Abstract

Atrazine is an herbicide used to control broadleaf grasses and a suspected endocrine disrupting chemical. Snapping turtles lay eggs between late May and early June, which could lead to atrazine exposure via field runoff. Our goal was to determine whether a single exposure to 2ppb or 40ppb atrazine during embryogenesis could induce short- and long-term changes in gene expression within the hypothalamus of snapping turtles. We treated eggs with atrazine following sex determination and measured gene expression within the hypothalamus. We selected genes *a priori* for their role in the hypothalamus-pituitary-gonad or the hypothalamus-pituitary-adrenal axes of the endocrine system. We did not identify any changes in gene expression 24-hours after treatment. However, at hatching *AR*, *Kiss1R*, and *POMC* expression was upregulated in both sexes, while expression of *CYP19A1* and *PDYN* was increased in females. Six months after hatching, *CYP19A1* and *PRLH* expression was increased in animals treated with 2ppb atrazine. Our study shows persistent changes in hypothalamic gene expression due to low-dose embryonic exposure to the herbicide atrazine with significant effects in

both the HPG and HPA axes. Effects reported here appear to be conserved among vertebrates.

Introduction

Endocrine disrupting chemicals (EDCs) are any environmental chemical that alters endocrine function. EDCs are found in plasticizers, pharmaceuticals, pesticides, and personal care products, and can mimic endogenous hormones, alter hormone homeostasis, and disrupt hormone synthesis, transport, and metabolism (reviewed in Diamanti-Kandarakis et al., 2009). Actions of EDCs on hormone homeostasis often occur at extremely low dose exposures, and therefore do not always follow classic dose-response toxicological principles (Diamanti-Kandarakis et al., 2009). Therefore, traditional maximum contaminant levels for ground and surface waters might be inappropriate for handling EDC contamination.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is an herbicide used to control broadleaf and grassy weeds, mainly in corn. Atrazine (ATR) is one of the most widely used pesticides in the country, and can be found in approximately 70% of surface streams and groundwater (Bexfield, 2008; Arlos et al., 2014). While ATR does not bioaccumulate (McMullin et al., 2003; Ross et al., 2009), prolonged persistence in soil and the environment results in chronic exposure in animals and humans. The European Union banned the use of ATR in 2003 due to widespread environmental contamination (European Commission Health and Consumer Protection Directorate-General, 2003).ATR is found in ground and surface waters at concentrations averaging 20ppb; however, it has been found in excess of 100ppb in some agricultural areas

(Blanchard and Lerch, 2000). These levels are drastically above the U.S. EPA's set maximum contaminant level of 3 ppb (USEPA, 2009), but evidence shows endocrine disrupting effects at very low dose exposures (Neuman-Lee and Janzen, 2011). The U.S. EPA began a reevaluation of the safety of ATR in 2009, and according to a 2013 update, is considering whether new restrictions are necessary to protect environmental and public health (USEPA, 2013).

Acute and chronic exposure to ATR during development and adulthood can produce adverse effects. Adult exposure to ATR is linked to development of some cancers (Huang et al., 2015; Schroeder et al., 2001; Albanito et al., 2015), can disrupt the dopaminergic system in the central nervous system (Coban and Filipov, 2007; Lin et al., 2013; Zhang et al., 2015), can impair fetal growth and development (Ochoa-Acuna et al., 2009), impacts the immune system (Karrow et al., 2005; Thueson et al., 2015), and has various effects in the reproductive system (Cragin et al., 2011; Davis et al., 2011). Many of these negative effects are due to disruption of the endocrine system. ATR is shown to activate the Hypothalamus-Pituitary-Adrenal (HPA) axis of the endocrine system (Fraites et al., 2009), and is implicated in alterations of the Hypothalamus-Pituitary-Gonad (HPG) axis. A wide range of exposure levels have been linked to abnormal testis development across vertebrate taxa including decreases in testis mass, tubule size and spermatogenesis, loss of Sertoli cells, and development of testicular oocytes (Hayes et al., 2010; Stoker et al., 2008; Victor-Costa et al., 2010). While the mechanism of action is not well-understood, one hypothesis is ATR upregulates aromatase expression, therefore

increasing the amount of testosterone converted into estrogen (Laville et al., 2006). In this model, the estrogenic properties of ATR are indirect.

Steroid hormones produced by the gonads induce changes in gene expression within the hypothalamus during development, resulting in morphological and functional differences between sexes (Phoenix et al., 1959; Aste et al., 2010; Buedefeld et al., 2015). The hypothalamus in turn regulates a wide variety of homoeostatic mechanisms, including water balance, growth, body temperature, stress response, and reproduction. In a simplistic description of the HPG axis, regulation occurs in a negative-feedback mechanism beginning with Kisspeptin (Kiss1) neurons, which signal to gonadotropin-releasing hormone (GnRH) neurons within the hypothalamus, which in turn release GnRH to the pituitary gland. GnRH stimulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotropes in the pituitary gland, which are then released into the bloodstream. LH and FSH receptors are found in the gonads, and stimulate production of gonadal steroid hormones, including testosterone and estradiol. Testosterone and estradiol travel through the bloodstream, where they bind to receptors throughout the body, including the hypothalamus, in order to decrease activity of both GnRH neurons and gonadotropes. In several vertebrate species, testosterone derivatives (estradiol and/or dihydrotestosterone) are responsible for masculinization and defeminization of the brain (Aste et al., 2010). The cellular structure and function required for this tightly controlled signaling mechanism is established during pre-and postnatal development, and is imperative to fertility later in life. Any disruption to this process could have life-long effects on reproductive success. Considering the evidence

for ATR effects on gonads and the tight regulation of the HPG, ATR is likely to impact the hypothalamus as well.

Atrazine disruption of the HP axis and gonadal sex hormone signaling is supported by considerable evidence. One study indicates exposure to ATR in adulthood can disrupt the GnRH pulse generator necessary for proper release of LH and FSH (Foradori et al., 2009(1); Foradori et al., 2009 (2)). Disruption of the HPG could also arise from ATR's impact on neurotransmitters such as serotonin and dopamine since these feed into the HPG axis (Wirbisky et al., 2015; Ribeiro et al., 2015). While adulthood exposures can have negative impacts, the precise events during development of the HPG axis are especially vulnerable to disruption. According to the Developmental Origins of Health and Disease hypothesis, alterations in the epigenetic landscape by the environment during development can have long-term effects on adult phenotypes (Feuer et al., 2014; Barker and Osmond, 1986; Barker, 2007). Therefore, organisms have increased vulnerability to environmental contaminants during embryonic development, and exposures can cause long-lasting defects. It has been demonstrated that exposure to ATR during development impairs fetal survivorship and growth (Ochoa-Acuna et al., 2009; Chen et al., 2015), decreases fertility, delays puberty, and reduces testosterone levels in males (Swan, 2006; Fraites et al., 2011), and delays mammary gland development and alters estrous cycles in female rodents (Davis et al., 2011; Rayner et al., 2005).

The common snapping turtle (*Chelydra serpentina*), is a reptile species with a wide range, found nearly everywhere east of the Rocky Mountains. The snapping turtle is

a species with temperature-dependent sex determination (Janzen, 1992; Rhen and Lang, 1994), which allows for 100% confidence in the sex of the animal by incubating eggs at a set temperature, even prior to morphological changes in the gonads. By incubating eggs at 26.5°C or 31°C, 100% of the embryos will develop into males or females respectively. The snapping turtle lays clutches with an average of 45 eggs, but some can be as large as 90 eggs (unpublished data). In northern regions, female snapping turtles lay eggs in late May or early June. Because ATR is applied to fields in April and May, nests are susceptible to exposure via run-off. Therefore, they are a useful species for investigating the effects of environmental toxicants.

We were interested in whether an acute developmental exposure to ATR could induce short-term and long-term changes in gene expression within the hypothalamus of the snapping turtle. We collected freshly laid snapping turtle eggs from the wild, and incubated them at male- and female-producing temperatures. We treated eggs with ATR after sex had been determined in order to eliminate the potential impact of treatment on sex determination. We collected tissue 24 hours after treatment, at hatch, and 6 months after hatch and measured expression of genes using RT-qPCR. Genes were selected *a priori* based on their critical involvement in the regulation of the HPG axis, and include Kiss1, kisspeptin receptor (Kiss1R), gonadotropin-releasing hormone 1 and 2 (GnRH1 and GnRH2), prolactin releasing hormone (PRLH), estrogen receptors 1 and 2 (ESR1 and ESR2), androgen receptor (AR), and aromatase (CYP19A1). We also examined stress and growth pathways in the HPG, measuring expression of prodynorphin (PDYN), proopiomelanocortin (POMC), and somatostatin (SST).

Atrazine and estradiol did not alter gene expression in the hypothalamus 24 hours after treatment. However, at hatch (approximately 35 days after treatment), we found the low dose of atrazine increased expression of Kiss1R, AR, and POMC in both males and females. The low dose of atrazine also increased expression of PDYN and CYP19A1, but only in females. The only gene affected by the high dose of atrazine was CYP19A1, and again, expression was only increased in females. After 6 months, the only two genes with altered expression due to treatment were CYP19A1 and PRLH. However, the trend of the low dose of atrazine increasing expression was present in several other genes, but did not show up as significant, most likely due to the lower sample size in the vehicle-treated control groups. Based on this study, we found that a single exposure to a low dose of atrazine results in alterations in gene expression of reproductive and stress-related genes in the hypothalamus of the snapping turtle.

Methods

Egg Collection and Treatment

Snapping turtle eggs, embryos, and hatchlings were treated according to protocols approved by the Institutional Animal Care and Use Committee at the University of North Dakota. We collected eggs immediately after oviposition from north-central Minnesota and northeastern North Dakota during May and June of 2012, with permits from the Minnesota Department of Natural Resources and the North Dakota Game and Fish Department. Eggs were transported to the Biology Department at the University of North Dakota, washed with tap water, and numbered to track individual eggs within each clutch. Eggs were then placed into plastic containers, covered with moist vermiculite (1

part vermiculite: 1 part water by mass), and placed into incubators; half at 31°C (female-producing temperature), and half at 26.5°C (male-producing temperature). Spatial and temporal variation in temperature within incubators is minimal: the coefficient of variation over time is approximately 1%, while maximal spatial deviation is <0.2°C (Lang et al., 1989).

Eggs were incubated until stage 20 of development (Yntema, 1968), a time point directly after gonadal sex has been determined (Rhen et al., 2015). Eggs were candled to separate infertile eggs and dead embryos. Treatments were then administered to eggs containing viable embryos. Estradiol-17β and ATR were dissolved in 100% ethanol and administered in a 5 µl bolus spotted on the eggshell using a Hamilton Microliter syringe. Seven to nine eggs per group received the following treatments: no treatment (control, C), 100% ethanol (vehicle, V), estradiol-17β (0.5 µg/egg, E2, United States Biochemical Corporation), a low dose of ATR, or a high dose of ATR (Atrazine PESTANAL®, Sigma-Aldrich, 98.8% purity). We aimed for a low dose of 2 ppb and a high dose of 40 ppb based on levels found in the environment (USGS, 2003). We administered 0.02 µg and 0.4 µg of ATR for the low and high doses respectively. Average egg volume is 10 mL; therefore, the maximum concentration within the egg would be approximately 2 ppb or 40 ppb, respectively. Transmission of small molecules through the eggshell is not 100% (de Solla and Martin, 2011); however, analysis of transmission is outside the scope of this experiment. Eggs were placed back into their original incubators until tissues were collected.

Tissue Collection and RNA Isolation

Twenty-four hours following treatment, 10 embryos from each treatment group were removed from their egg and euthanized by rapid decapitation. Brains were collected, placed into RNAlater© solution (Ambion, Austin, TX), and stored at -20°C. The remaining eggs were allowed to hatch. Within one week, brains were collected from 10 hatchling turtles from each treatment group and placed into RNAlater© for long-term storage at -20°C. Carapace length, plastron-cloaca distance, and mass were measured for the remaining hatchlings, and each was marked with a unique tag: colored beads were attached with wire through marginal scutes. Marked hatchlings were then released into pools and fed *ad libitum* for six months, at which time measurements were again taken, hatchlings euthanized, and brains were collected and placed into RNAlater© for storage at -20°C.

The hypothalamus was micro-dissected from each brain. Total RNA was isolated from the hypothalami with RNAzol®RT (Molecular Research Center, Inc.). In brief, tissues were homogenized using a Pro200 Homogenizer (Pro Scientific Inc., Oxford, CT) in 200ul RNAzol®RT for 45-60 seconds. DNase/RNase-free water was added to the sample in a 1:1 ratio, and the first centrifugation step was performed for 15 minutes at 12,000xg at 4°C. The supernatant was removed and placed in a clean 1.5 mL microcentrifuge tube. A subsequent centrifugation was carried out at 20°C. The upper clear phase was added to a clean microcentrifuge tube, leaving phenol in the lower phase. Isopropanol was added to the aqueous supernatant and incubated at room temperature for 15 minutes, followed by centrifugation. The RNA formed a pellet at the bottom of the

tube, and the supernatant was discarded. Three washes were performed with 75% ethanol and centrifugation at 8,000xg. The final ethanol was removed, and pellets were dried at 40°C for 2 minutes. The pellets were redissolved in 30 µl of DNase/RNase-free water and stored overnight at -80°C prior to quantification.

RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer. RNA integrity was examined via formaldehyde-agarose gel electrophoresis. All RNA samples were checked for contaminating genomic DNA by running a qPCR prior to reverse transcription. Samples with evidence of genomic DNA contamination were cleaned up with an in-tube DNase treatment and sodium acetate precipitation of pure RNA.

Reverse Transcription and quantitative PCR

A total of 50 ng of input RNA was reverse transcribed in a 20 µl reaction using Applied Biosystems' High-Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. Reverse transcription was carried out on a BioRad MyiQ™ Single-Color Real-Time PCR Detection System. The cDNA was diluted to the equivalent of 1.25 ng input RNA/µl for use in qPCR reactions.

We designed PCR primers (Integrated DNA Technologies) to measure expression of 12 genes (Table 4.1) using Primer Express® 2.0 software (ThermoFisher Scientific). Primers were designed using gene sequences from the snapping turtle transcriptome (Rhen et al. paper in preparation). We prepared standard curves for each gene as described in Rhen et al. (2007). Purified PCR products for each gene were added to reaction tubes in the following amounts: 2,000,000 attograms (ag=10^{-18} g)/tube, 200,000

Table 4.1: Forward and Reverse primer sequences for 12 hypothalamic genes from the snapping turtle.

Gene Name	Forward Primer Sequence (5' – 3')	Reverse Primer Sequence (5' – 3')
Kisspeptin (Kiss1)	TCTTCTCCAATGCTCCGTTTG	TGGTCACCTGTGCGAGG
Kisspeptin Receptor (Kiss1R)	TGGGCATCTGGATCTGTTCC	AACCAGTAGCCCTCCGTCAAC
Gonadotropin-releasing Hormone 1 (GnRH1)	GTCTGTGGAGATTTGCTTGGC	CAGATTGTCAGCATCCCGCT
Gonadotropin-releasing Hormone 2 (GnRH2)	TGGCATGTCAACGACCTCTC	TGCTGAGCTCTCGACAGGTG
Prolactin Releasing Hormone (PRLH)	GCCAGCGCAGATCGTTTAAC	CCACGTCCCACATACCAGAAC
Androgen Receptor (AR)	TGGGATGGAGATCTTTCACCA	GGAGCAAAGTAAAGCATCCGG
Estrogen Receptor 1 (ESR1)	AACCAGTGCACCATCGACAAG	GGTCTTTTCGGATCCCACCTT
Estrogen Receptor 2 (ESR2)	TGCACACACACTTCGAGGTCA	TGTATCGGTTTGTTCTGAG
Aromatase (CYP19A1)	TCTGGTCCAGGTCTCGTGC	GGTCTTTCGTTGATTCAACGC
Prodynorphin (PDYN)	ATGGAGTGGCAGGTTCTGGT	GAGCACTGGGCTGCACAGT
Proopiomelanocortin (POMC)	GCCGAGTCACCTGTGTACCC	GGCTCATGACGTACTTCCGG
Somatostatin (SST)	GCAGCAGGGAGCAGACCA	ATGTCCATCCCATCCAGGAG

ag/tube, 20,000 ag/tube, 2,000 ag/tube, 200 ag/tube, 20 ag/tube, 2 ag/tube, and 0.2 ag/tube.

Real-time quantitative PCR was performed using the Bio-Rad CFX 384 Real-Time PCR System (Bio-Rad) with Bio-Rad SsoFast^{TM} EvaGreen® Supermix according to manufacturer's instructions (Bio-Rad, Hercules, CA). Each 10 µl PCR reaction contained 5 µl of 2x supermix, 0.3 µl of forward and 0.3 µl of reverse primers (0.3 µM final concentration), 2.4 µl of water, and 2 µl of cDNA (input=1.25 ng total RNA). Standard curves were used to estimate the amount of mRNA in attograms cDNA (=mRNA) per 1.25 ng of total RNA from each hypothalamus. Amplification did not occur in wells without cDNA.

Statistical Analysis

We used JMP 11.1.1 software for all statistical analyses (SAS Institute, Cary, NC). We used two-way ANOVA to analyze E2 and ATR effects on gene expression: incubation temperature (or sex), chemical treatment, and the interaction between these variables were main effects in the model. We only compared specific treatment groups when main effects or their interaction were significant ($p<0.05$). We decided *a priori* to make seven comparisons: vehicle treated males and females were compared to test for sex differences (1 contrast), each chemical treatment group was then compared to the vehicle group of the same sex to test for sex-specific treatment effects (3 contrasts for each sex). The Dunn-Sidak method was used to correct for multiple comparisons. The nominal significance level was calculated as $\alpha'=1-(1-\alpha)^{1/k}$, where k is the number of comparisons for an experiment wise $\alpha=0.05$. Sample sizes are shown in each figure.

Results

Gene Expression in Turtle Embryos

As expected, minimal effects were seen 24 hours after treatment. Two-way ANOVA did not reveal any differences in expression of CYP19A1, AR, ESR1, Kiss1R, GnRH1, GnRH2, SST, POMC, or PDYN (Table 4.2). Expression of ESR2, Kiss1, and PRLH differed significantly between sexes (Table 4.2). Females had higher expression of ESR2 than males. In contrast, males had higher expression of Kiss1and PRLH compared to females (Table 4.3). Chemical treatments did not immediately alter gene expression and there were no significant interactions.

Table 4.2: Summary of 2-way ANOVA (Type III Sums of Squares) for treatment and incubation temperature (sex) effects and their interaction on the expression of 12 reproductive, growth, and stress associated genes in embryonic snapping turtles 24 hours after treatment with estrogen or atrazine. Embryos were at between stages 20 and 21 of development, according to anatomical staging first characterized by Yntema, 1968. Differences were considered significant with $p<0.05$, and are indicated with *.

Gene	Effects	DF	F-value	p-value
AR	Treatment	3	0.6080	0.6124
	Sex	1	3.4037	0.0700
	Treatment x Sex	3	1.7805	0.1605
CYP19A1	Treatment	3	0.8345	0.4803
	Sex	1	0.2223	0.6389
	Treatment x Sex	3	0.5724	0.6354
ESR1	Treatment	3	0.1810	0.9089
	Sex	1	0.1009	0.7518
	Treatment x Sex	3	1.1447	0.3383
ESR2	Treatment	3	0.8254	0.4850
	Sex	1	7.1576	0.0096*
	Treatment x Sex	3	0.2904	0.8322
GnRH1	Treatment	3	0.7055	0.5524
	Sex	1	1.0836	0.3020
	Treatment x Sex	3	0.4681	0.7056
GnRH2	Treatment	3	1.0128	0.3933
	Sex	1	0.0454	0.8319
	Treatment x Sex	3	0.3606	0.7817
Kiss1	Treatment	3	0.7986	0.4994
	Sex	1	10.0828	0.0023*
	Treatment x Sex	3	2.7044	0.0531
Kiss1R	Treatment	3	1.7993	0.1568
	Sex	1	0.7126	0.4019
	Treatment x Sex	3	0.6955	0.5584
PDYN	Treatment	3	0.8590	0.4675
	Sex	1	1.1214	0.2939
	Treatment x Sex	3	0.7238	0.5418
POMC	Treatment	3	0.9413	0.4264
	Sex	1	0.0360	0.8501
	Treatment x Sex	3	0.7297	0.5383
PRLH	Treatment	3	1.0268	0.3874
	Sex	1	6.7247	0.0120*
	Treatment x Sex	3	0.6744	0.5712
SST	Treatment	3	0.6259	0.6010
	Sex	1	3.5129	0.0657
	Treatment x Sex	3	0.3393	0.7969

Table 4.3: Gene expression in male and female hypothalami during late stage 20 of development. Genes presented here showed a significant difference between sexes (as a result of incubation at male- and female-producing temperatures). Analysis was performed using a two-way ANOVA with incubation temperature and treatment as variables.

Gene	Sex	Least Square Mean (ag/1.25 ng total RNA)	Standard Error
ESR2	Female	620.502	578.199 to 662.805
	Male	466.669	423.905 to 509.433
Kiss1	Female	32.161	27.272 to 37.869
	Male	65.310	55.325 to 77.101
PRLH	Female	150.799	134.680 to 168.848
	Male	224.177	199.923 to 251.373

Gene Expression in Hatchling Turtles

There were no sex differences or treatment effects in expression of Kiss1, GnRH1, ESR1, PRLH, and SST in hatchling hypothalami (Table 4.4). However, expression of GnRH2 and ESR2 differed between the sexes. Expression of GnRH2 was significantly higher in males than in females. In contrast, ESR2 expression was significantly higher in females than in males (Table 4.5). E2 and ATR had no effect on GnRH2 or ESR2 expression.

Expression of AR and POMC did not differ between the sexes, but was affected by at least one chemical treatment. The low ATR treatment significantly increased expression of both genes compared to the vehicle-treated controls (Figure 4.1 and Figure 4.2) in both sexes. Kiss1R expression was significantly higher in females than in males (Table 4.5), and the low ATR treatment significantly increased expression of Kiss1R when compared to vehicle-treated controls (Figure 4.3) in both sexes.

Table 4.4: Summary of two-way ANOVA results, including treatment (estradiol, low dose atrazine, and high dose atrazine) and sex (male or female) effects and their interaction in snapping turtle hatchling hypothalami. Expression of 12 genes involved in reproduction, growth, or stress response were tested in hypothalami collected within 1 week of hatching. Significant effects were determined as those with $p<0.05$ and are denoted with an asterisk (*).

Gene	Effects	df	F-value	p-value
AR	Treatment	3	5.4797	0.0021*
	Sex	1	3.8905	0.0532
	Treatment x Sex	3	0.3560	0.7850
CYP19A1	Treatment	3	2.4628	0.0711
	Sex	1	3.8255	0.0551
	Treatment x Sex	3	3.3033	0.0262*
ESR1	Treatment	3	2.4328	0.0737
	Sex	1	1.5901	0.2122
	Treatment x Sex	3	0.3974	0.7554
ESR2	Treatment	3	2.2707	0.0895
	Sex	1	13.5140	0.0005*
	Treatment x Sex	3	1.3323	0.2723
GnRH1	Treatment	3	2.4863	0.0695
	Sex	1	1.0997	0.2987
	Treatment x Sex	3	0.4055	0.7496
GnRH2	Treatment	3	0.9867	0.4056
	Sex	1	6.0978	0.0166*
	Treatment x Sex	3	1.0848	0.3629
Kiss1	Treatment	3	2.1549	0.1025
	Sex	1	3.5468	0.0644
	Treatment x Sex	3	1.2662	0.2939
Kiss1R	Treatment	3	3.5622	0.0196*
	Sex	1	11.2075	0.0014*
	Treatment x Sex	3	0.2272	0.8771
PDYN	Treatment	3	5.2438	0.0028*
	Sex	1	4.7624	0.0331*
	Treatment x Sex	3	3.7053	0.0164*
POMC	Treatment	3	2.9309	0.0406*
	Sex	1	0.0066	0.9353
	Treatment x Sex	3	1.0071	0.3958
PRLH	Treatment	3	2.6873	0.0546
	Sex	1	0.0002	0.9888
	Treatment x Sex	3	0.1116	0.9530
SST	Treatment	3	1.1210	0.3481
	Sex	1	2.9804	0.0896
	Treatment x Sex	3	1.0037	0.3978

Table 4.5: Gene expression in male and female snapping turtle hypothalami at hatch. Genes presented here showed a significant difference between sexes (as a result of incubation at male- and female-producing temperatures). Analysis was performed using a two-way ANOVA with incubation temperature and treatment as effects and their interaction effect.

Gene	Sex	Least Square Means	Standard Error
ESR2	Female	2465.085	2313.556 to 2616.613
	Male	1688.645	1541.509 to 1835.781
GnRH2	Female	920.770	829.729 to 1011.811
	Male	1411.982	1322.290 to 1501.674
Kiss1R	Female	1946.316	1801.446 to 2091.186
	Male	1250.709	1101.758 to 1399.660

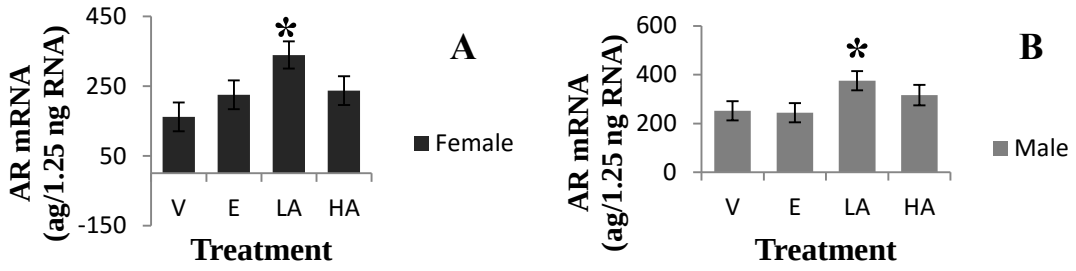


Figure 4.1: AR expression in the female (A) and male (B) snapping turtle hypothalamus within 1 week of hatching. Input RNA was equivalent to 1.25ng for each qPCR reaction. The low atrazine treatment significantly increased expression of AR in both males and females (two-way ANOVA, $F_{3,67}$=5.4797, p=0.0021). The results of a Dunn-Sidak post hoc shows an increase in AR expression when compared to vehicle-treated controls in both males and females (p=0. 0004). Groups significantly different from the control group are indicated by an asterisk (*).V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

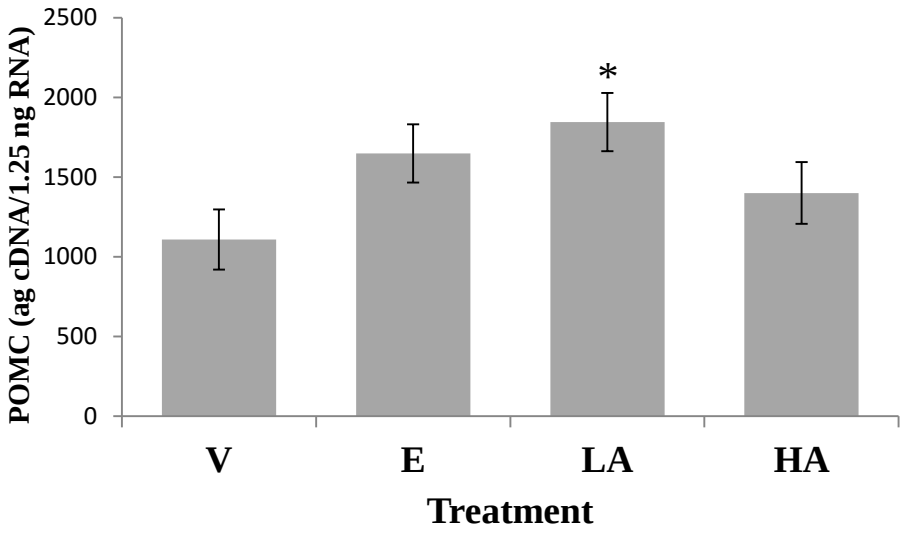


Figure 4.2: POMC expression in the snapping turtle hypothalamus within 1 week of hatching. Male and female means are combined since there is not a difference in their expressions. Input RNA was equivalent to 1.25ng for each qPCR reaction. The low atrazine treatment significantly increased expression of POMC (two-way ANOVA, $F_{3,68}$=2.9309, p=0.0406). The results of a Dunn-Sidak post hoc shows an increase in POMC expression when compared to vehicle-treated controls (p=0.0067). Groups significantly different from the control group are indicated by an asterisk (*).V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

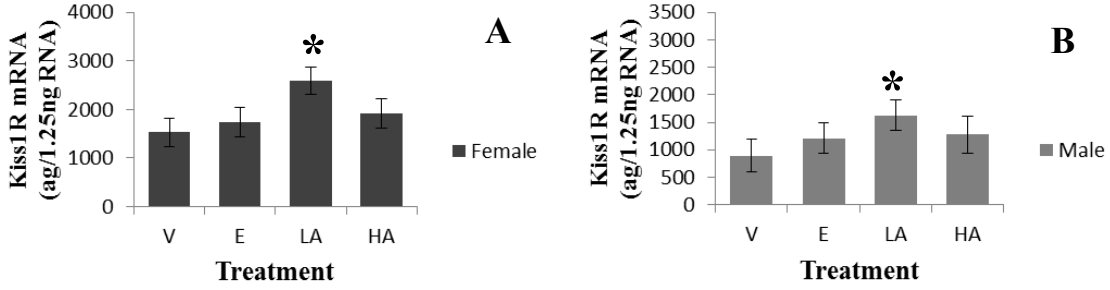


Figure 4.3: Kiss1R expression in the female (A) and male (B) snapping turtle hypothalamus within 1 week of hatching. Input RNA was equivalent to 1.25ng for each qPCR reaction. The low atrazine treatment significantly increased expression of Kiss1R in both males and females (two-way ANOVA, $F_{3,64}$=3.5622, p=0.0196). The results of a Dunn-Sidak post hoc show an increase in Kiss1R expression when compared to vehicle-treated controls in both males and females (p=0.0026). Groups significantly different from the control group are indicated by an asterisk (*). V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

The treatment by sex interaction was significant for CYP19A1 expression. Both low and high ATR doses increased CYP19A1 expression in females (Figure 4.4). CYP19A1 expression was not altered by E2, nor did the ATR treatments modify expression in males. Sex, chemical treatment, and their interaction all influenced PDYN expression. The post hoc test revealed the low dose ATR increased PDYN expression compared to the vehicle-treated controls, but only in females (Figure 4.5).

Gene Expression in 6-Month Old Turtles

Significant sex differences were detected in the majority of genes, including Kiss1R, GnRH1, GnRH2, ESR1, ESR2, AR, CYP19A1, PDYN, PRLH, and POMC (Table 4.6). Females had higher expression for each (Table 4.7). In contrast, the overall chemical treatment effect was only significant for CYP19A1 and PRLH expression (Table 4.6). However, the Dunn-Sidak post hoc test was unable to detect which groups were significantly different. This may have been due to lower statistical power to detect treatment effects because three vehicle-treated females died before six months of age. The p-value for the comparison of low dose ATR and vehicle-treated controls approached significance ($p=0.02$ and $p=0.0184$ for CYP19A1 and PRLH respectively, $\alpha \leq 0.017$ for significance after correction for multiple comparisons). Upon further investigation of each group, this difference was most evident in females (Figure 4.6 and Figure 4.7). Many of the genes showed the same pattern of expression changes observed in hatchlings, with the low dose of ATR increasing expression in females. Expression of Kiss1 and SST was not affected by sex, treatment, or the interaction between sex and treatment at 6 months.

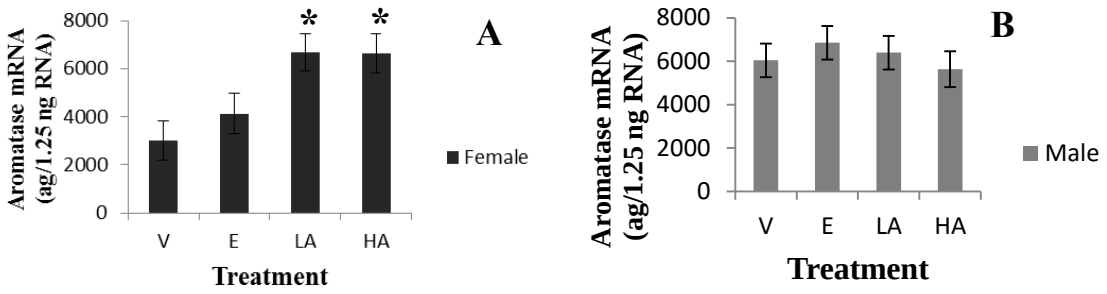


Figure 4.4: CYP19A1 expression in the female (A) and male (B) snapping turtle hypothalamus within 1 week of hatching. Input RNA was equivalent to 1.25ng for each qPCR reaction. The low and high atrazine treatments significantly increased expression of CYP19A1 in females (two-way ANOVA, $F_{3,67}$=3.3033, p=0262). The results of a Dunn-Sidak post hoc shows an increase in CYP19A1 expression when compared to vehicle-treated controls in females (LA, p=0. 0018; HA, p=0.0028), but none of the treatments altered expression in males. Groups significantly different from the control group are indicated by an asterisk (*).V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

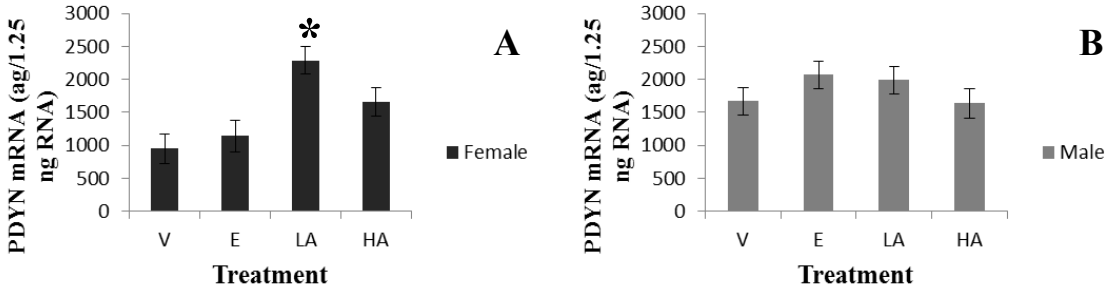


Figure 4.5: PDYN expression in the female (A) and male (B) snapping turtle hypothalamus within 1 week of hatching. Input RNA was equivalent to 1.25ng for each qPCR reaction. The low atrazine treatment significantly increased expression of PDYN in females (two-way ANOVA, $F_{3,66}$=3.7053, p=0.0164). The results of a Dunn-Sidak post hoc shows an increase in PDYN expression when compared to vehicle-treated controls in females (p<0.0001), but none of the treatments altered expression in males. Groups significantly different from the control group are indicated by an asterisk (*).V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

Table 4.6: Summary of two-way ANOVA results, including treatment (estradiol, low dose atrazine, and high dose atrazine) and sex (male or female) effects and their interaction in snapping turtle hatchling hypothalami. Expression of 12 genes involved in reproduction, growth, or stress response were tested in hypothalami collected 6 months after hatching. Significant effects were determined as those with $p<0.05$ and are denoted with and asterisk (*).

Gene	Effects	df	F-value	p-value
AR	Treatment	3	2.1130	0.1069
	Sex	1	14.6783	0.0003*
	Treatment x Sex	3	2.3811	0.0774
CYP19A1	Treatment	3	2.7521	0.0495*
	Sex	1	22.7228	<0.0001*
	Treatment x Sex	3	2.4100	0.0748
ESR1	Treatment	3	2.3141	0.0839
	Sex	1	8.6432	0.0045*
	Treatment x Sex	3	1.6745	0.1810
ESR2	Treatment	3	1.7333	0.1687
	Sex	1	17.2280	<0.0001*
	Treatment x Sex	3	1.4340	0.2408
GnRH1	Treatment	3	1.4011	0.2503
	Sex	1	6.5896	0.0125*
	Treatment x Sex	3	2.2241	0.0935
GnRH2	Treatment	3	1.6343	0.1899
	Sex	1	5.1158	0.0270*
	Treatment x Sex	3	0.2208	0.8816
Kiss1	Treatment	3	1.6341	0.1899
	Sex	1	0.1868	0.6670
	Treatment x Sex	3	0.3364	0.7991
Kiss1R	Treatment	3	2.4399	0.0721
	Sex	1	21.6955	<0.0001*
	Treatment x Sex	3	0.4698	0.7044
PDYN	Treatment	3	1.4297	0.2420
	Sex	1	6.4589	0.0134*
	Treatment x Sex	3	0.4617	0.7100
POMC	Treatment	3	1.0422	0.3799
	Sex	1	19.6516	<0.0001*
	Treatment x Sex	3	1.2147	0.3115
PRLH	Treatment	3	3.0872	0.0331*
	Sex	1	4.1092	0.0467*
	Treatment x Sex	3	0.9587	0.4176
SST	Treatment	3	1.7304	0.1693
	Sex	1	0.1391	0.7104
	Treatment x Sex	3	0.9741	0.4103

Table 4.7: Gene expression in male and female snapping turtle hypothalami during 6 months after hatching. Genes presented here showed a significant difference between sexes. Analysis was performed using a two-way ANOVA with incubation temperature and treatment as effects and their interaction effect.

Gene	**Sex**	**Least Square Means**	**Standard Error**
AR	Female	1116.054	1053.67 to 1178.438
	Male	790.050	732.182 to 847.918
CYP19A1	Female	10842.099	11494.298 to 11494.298
	Male	6601.556	5996.568 to 7206.544
ESR1	Female	114.511	106.605 to 122.417
	Male	82.807	75.473 to 90.141
ESR2	Female	2233.618	2110.684 to 2356.552
	Male	1537.632	1423.597 to 1651.667
GnRH1	Female	53.660	48.841 to 58.479
	Male	36.787	32.317 to 41.257
GnRH2	Female	1040.181	904.227 to 1176.135
	Male	1528.728	1384.999 to 1672.457
Kiss1R	Female	4795.025	4421.137 to 5200.532
	Male	2862.829	2655.160 to 3086.741
PDYN	Female	3457.069	3280.988 to 3633.150
	Male	2846.687	2683.352 to 3010.022
PRLH	Female	1844.877	1691.436 to 1998.318
	Male	1420.619	1278.283 to 1562.953
POMC	Female	2736.487	2560.494 to 2912.480
	Male	1664.049	1498.061 to 1830.037

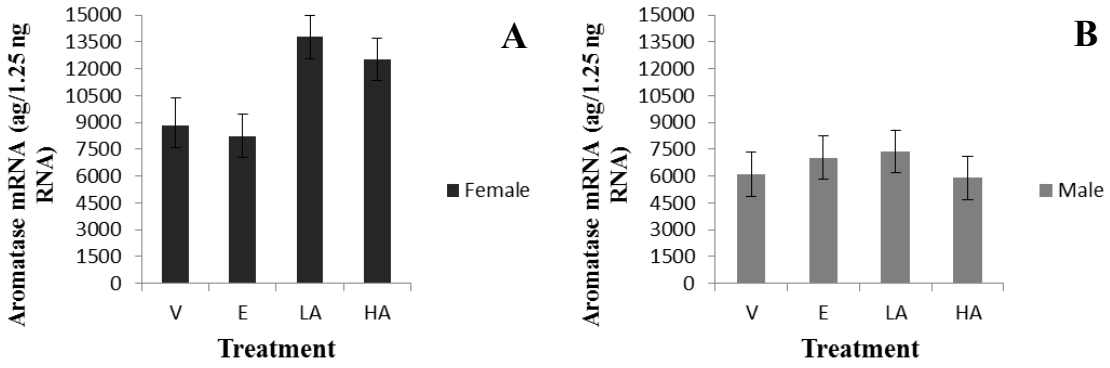


Figure 4.6: CYP19A1 expression in the snapping turtle hypothalamus 6 months after hatching. Input RNA was equivalent to 1.25ng for each qPCR reaction. The two-way ANOVA shows a significant treatment effect ($F_{3,73}$=2.7521, p=0.0495), with male and female means combined. The Dunn-Sidak post hoc was unable to detect which group(s) was significantly different from the vehicle-treated control, however, the comparison between the low dose of atrazine and the vehicle group shows a p-value closest to our set α<0.017 at p=0.020. V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

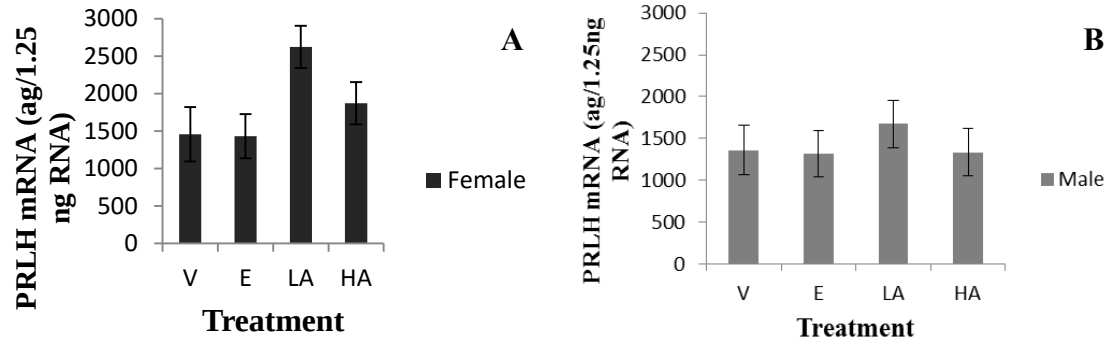


Figure 4.7: PRLH expression in the snapping turtle hypothalamus 6 months after hatching. Input RNA was equivalent to 1.25ng for each qPCR reaction. The two-way ANOVA shows a significant treatment effect ($F_{3,73}$=3.0872, p=0.0331), with male and female means combined. The Dunn-Sidak post hoc was unable to detect which group(s) was significantly different from the vehicle-treated control, however, the comparison between the low dose of atrazine and the vehicle group shows a nearly significant p-value, closest to our set α<0.017 of p=0.0187. V=vehicle (100% ethanol), E=Estradiol (0.5 µg), LA=Low Atrazine (2ppb), and HA=high atrazine (40ppb).

Discussion

ATR exposure has been shown to have endocrine disrupting effects in several vertebrate species. Numerous studies have indicated effects within reproductive organs in both males and females (Hayes et al., 2002; Qin et al., 2015) as well as altered steroidogenesis (Victor-Costa et al., 2010). While limited studies have been conducted in reptiles, a few indicate similar effects (Stoker et al., 2008; Neuman-Lee and Janzen, 2011), such as altered steroidogenesis in alligators that were exposed to ATR (Crain et al., 1997). Disruption in steroidogenesis and gonad differentiation led to investigation of endocrine-disrupting effects within the HPG axis. Exposure to ATR disrupts GnRH pulsatility (Foradori et al., 2009, 2013) and the preovulatory surge of LH in female rats (Cooper et al., 2000; Foradori et al., 2009, 2011). Qin et al. (2015) noted changes in expression of hypothalamic and pituitary genes involved in regulation of the HPG axis including GnRH, PRL, LH, and FSH in female quail.

Many studies of ATR exposure in adults exist, but studies involving the lasting effects of embryonic exposure to ATR are limited. One study indicates female zebrafish exposed to ATR during embryogenesis displayed alterations in serotonin metabolites as well as transcriptomic alterations in adulthood (Wirbisky et al., 2015). ATR induced changes in the HPG axis due to embryonic exposure could have negative impacts on fertility later in life.

Our goal was to test whether a single exposure to ATR during embryonic development would cause persistent changes in gene expression within the hypothalamus of snapping turtles. We chose a time directly following gonadal sex determination to

eliminate confounding effects that could stem from complete sex reversal. ATR did not have an immediate effect on gene expression (within 24 hours) in turtle embryos. However, ATR exposure did cause long-lasting changes up to 6 months after hatching.

Unexpectedly, the estradiol treatment did not cause any detectable changes in hypothalamic gene expression. Circulating estradiol and estradiol treatment have been shown to induce changes in gene expression within the hypothalamus in other species. Up-regulation of ESR2 is seen in the paraventricular nucleus of the hypothalamus during proestrous in rats when circulating levels of estradiol are high (Isgor et al., 2003). Kiss1 mRNA expression is also known to be altered by E2. Expression in Kiss1 neurons within the ARC is decreased, while expression is increased in Kiss1 neurons of the AVPV in response to E2 (Ohkura et al., 2009). Therefore, estrogen effects could potentially be obscured by opposing changes within different populations of Kiss1 neurons. While we would expect E2 treatment to impact gene expression within the hypothalamus, there are several possible reasons we did not see any changes. Changes might be seen at the level of a single population of neurons, but diminished by including the entire hypothalamus. E2 might have induced changes later than 24 hours after treatment with recovery of normal expression levels by hatching. The dose we selected was based on its effects on gonad differentiation, and this dose might not have been appropriate to induce hypothalamic changes. Further investigation will reveal which of these is correct.

Some sex differences in gene expression were observed in embryos at the end of the sex determining period. Females had higher ESR2 expression than males, while Kiss1 and PRLH were more highly expressed in males, indicating brain differentiation has

begun by stage 20 of development. None of the genes examined were affected 24 hours after ATR treatment, indicating that more time is needed for ATR to penetrate the egg/embryo and induce transcriptional changes. It would be interesting to repeat this study and sample at several intervals to clarify the time course for ATR induced changes in gene expression.

ATR exposure was shown to have dimorphic regulatory effects on SST in male and female mice (Giusi et al., 2006). Also, Fakhouri et al. (2010) showed that ATR exposure resulted in lowered expression of growth hormone (GH) within pituitary cells from male rats. We therefore chose to measure SST expression, however, we did not see any alterations in SST due to ATR at any time point of our study.

Of the 9 genes we examined, only 2 were not altered by either low or high dose ATR treatments. Neuroendocrine control of the HPG originates through kisspeptin neuronal action in conjunction with its receptor on GnRH neurons. The pulsatile release of GnRH is altered by ATR treatment (Foradori et al., 2013), and is implicated as the cause of decreased luteinizing hormone release from the pituitary gland following ATR treatment (Foradori et al., 2009; Qin et al., 2015). While Goldman et al. (2013) found Kiss1 expression to be upregulated by a single dose of ATR after 1 hour of treatment in the AVPV, this effect was absent after 4 days. We found Kiss1 expression unchanged due to either dose of ATR at both hatch and 6 months. However, expression of Kiss1R was significantly upregulated in both male and female hatchlings following a single treatment with 2ppb ATR. While expression was not significantly upregulated after 6 months, the trend persisted ($p<0.10$). Substantial variation exists between individuals; therefore a

larger sample size might allow detection of significant expression differences at 6 months. If Kiss1R expression remains altered, this could be a point of interference between ATR and GnRH pulsatility at sexual maturity.

Neither GnRH1 nor GnRH2 was altered by ATR treatment. In female quail exposed to 50 and 250mg/kg ATR, GnRH expression was decreased (Qin et al., 2015). However, Foradori et al. (2013) determined GnRH expression was not altered by ATR treatment in female rats, and is not the source of modifications in GnRH pulsatility. Differences between studies could arise from differences in treatment timing or dosage, or simply due to species differences. These studies were conducted in adults, so new studies would be required to elucidate effects of ATR treatment during embryonic development.

Kiss1 and GnRH activity is directly or indirectly regulated by steroid hormones. Therefore, we examined expression of ESR1, ESR2, and AR to identify potential points of alteration. AR expression was upregulated in both males and females following 2ppb ATR exposure. This trend was also observed in 6-month old females although it was not statistically significant ($p<0.1$). To our knowledge, few studies have reported effects on AR expression. However, many studies have shown ATR effects on circulating androgens. Effects are variable, with increasing or decreasing production of androgens depending on the cell type, concentration and duration of ATR exposure (Trentacoste et al., 2001; Pogrmic-Majkic et al., 2010). Kucka et al. (2012) showed that shorter duration exposures increased production of androgens in cultured rat Leydig cells. It is possible

that embryonic exposure to ATR could alter circulating androgen levels and indirectly alter expression of AR in the hypothalamus in our study.

Many EDCs have been shown to alter expression of ERs, and ATR is suspected to have estrogenic-like effects. Thus we expected alterations in ER expression. However, we did not find significant changes in expression of either ESR1 or ESR2. Identifying transcriptional changes within individual populations of neurons might identify smaller-scale alterations in expression.

Aromatase has frequently been identified as a potential point of endocrine disruption. Upregulation of CYP19A1 activity following ATR exposure has been seen in human adrenocortical carcinoma H295R cells (Fan et al., 2007), immortalized testis cells from the green sea turtle (Keller et al., 2004), testis and ovaries (both in vivo and in vitro) from rodents (Abarikwu et al., 2011; Jin et al., 2013), and in bullfrog tadpoles (Gunderson et al., 2011). However, Hecker et al. (2005) did not find changes in aromatase activity or gene expression in testes of African clawed frogs after a 36-day exposure to 1, 25, or 250 µg/L ATR. Overall, shorter duration exposures at lower doses appear to upregulate CYP19A1 compared with long-durations and higher doses. Our study fits this pattern. We saw a significant increase in CYP19A1 expression at hatch and after 6 months, and our 'high dose' of ATR is actually a low dose compared with prior studies. Increases in circulating androgens could induce upregulation of CYP19A1, thus it will be important to measure androgen levels in snapping turtles following ATR treatment.

We found that ATR significantly increased PRLH expression after 6 months in both males and females. Though the post hoc test could not detect which group was significantly different from vehicle controls, this effect appears to be due to the low dose of ATR (2ppb). At this time, there are no other studies identifying ATR effects on PRLH expression. However, Qin et al. (2015) show an increase in expression and serum levels of PRL following a 45 day exposure to 50 and 250 mg/kg ATR in quail. Fakhouri et al. (2010) found an increase in PRL mRNA expression in cultured rat pituitary cells following exposure to 1.0 ppb or 1.0 ppm ATR. Conversely, ATR treatment in rats either had no effect or suppressed the prolactin surge, depending on the strain of rat, the concentration of ATR, or the duration of exposure (Cooper et al., 2000). While this result is contrary to other studies, Cooper et al. concluded the effect on PRL is mediated by the hypothalamus and is not a direct effect on the pituitary itself. Our finding implicates PRLH as a potential point of ATR action.

The HPG axis interacts with the HP-adrenal (HPA) axis. At high levels, cortisol caused a decrease in LH pulse frequency and an increase in amplitude, and is linked to the inhibition of GnRH release (Stackpole et al., 2006). Furthermore, exposure to ATR resulted in activation of the HPA with an increase in ACTH, corticosterone, and adrenal progesterone in adult female rodents (Pruett et al., 2003; Laws et al., 2009; Fraites et al., 2009). Foradori et al. (2011) linked the effects of ATR on the LH pulse generator to alterations in adrenal hormone secretion, and Goldman et al. (2013) found ATR-induced changes in adrenal hormones increased the expression of Kiss1 in the AVPV.

We examined two genes, PDYN and POMC, involved in the regulation of the HPA (reviewed in Brann and Mahesh, 1991; Pechnick, 1993; Dobson and Smith, 2000). Both PDYN and POMC were significantly upregulated at hatch in the 2ppb treatment group, but the change did not persist to 6 months. POMC was upregulated in both males and females, while PDYN was only upregulated in females. Both genes encode multiple protein products, including the endogenous opioids dynorphin (PDYN) and β-endorphin (POMC). Endogenous opioids are thought to inhibit the HPA in response to stress (Rushen et al., 1993). Evidence indicates endogenous opioid peptides also play a major inhibitory role in regulating GnRH pulse frequency and inhibit LH pulse frequency (reviewed in Kalra, 1993). Therefore, alterations in the HPG due to ATR exposure could at least in part arise from effects on these two genes.

Dynorphin is linked to alterations in LH release from the anterior pituitary gland in response to stress (Petraglia et al., 1986). Therefore a possible mechanism for the impact of ATR on GnRH pulsatility and reduction of LH could be through an increase in stress hormones from the adrenal gland, leading to increases in PDYN expression within the hypothalamus, and increasing the inhibitory action on Kiss1 and/or GnRH neurons.

While POMC encodes β-endorphin, another end product is ACTH within the pituitary gland. Many more studies have investigated the impact of increased adrenal hormones on POMC within the pituitary gland. Eberwine and Roberts (1984) found an increase in glucocorticoid levels to have an inhibitory effect on POMC. However, hypothalamic POMC inhibits corticotropin releasing hormone (CRH). Neuron specific knockout of POMC leads to elevated CRH and stimulation of coricotrophs within the

pituitary gland (Smart et al., 2007). Furthermore, stress increases the levels of β-endorphin in mammals and birds (Johnston and Negro-Vilar, 1986; Barna et al., 1998). Therefore, it is possible ATR acts through an increase in adrenal hormones to increase POMC expression within the hypothalamus. Further work is needed to determine whether ATR has direct effects on dynorphin and β-endorphin or indirect effects via adrenal hormones.

A comprehensive look at the effects of ATR on reproduction in the snapping turtle will require identifying changes in the pituitary gland, gonads, and circulating hormones following exposure. Furthermore, it will be important to identify the physiological impact of ATR exposure on reproduction and long-term survival. This, however, would require a lengthy experiment, rearing atrazine-exposed turtles to 8-12 years of age (sexual maturity).

We determined a single dose of ATR administered after sex determination is capable of producing persistent alterations in hypothalamic gene expression. In this study, the 2 ppb dose of ATR consistently produced a greater effect on gene expression compared with the higher dose of 40 ppb, highlighting the importance of low-dose exposures when monitoring EDCs. This study provides new data demonstrating the effects of ATR in a reptile species, and is the first of its kind in the common snapping turtle, a species with great potential for exposure in the wild via runoff. The results presented here support studies conducted in other species and strengthen hypotheses of aromatase upregulation, greater effects with low-dose exposures, and the potential for

multiple indirect mechanisms of disruption including aromatase upregulation and through the HPA.

CHAPTER V

EFFECTS OF ATRAZINE ON EXPRESSION OF REPRODUCTIVE AND STRESS GENES IN THE PITUITARY GLAND OF THE SNAPPING TURTLE, *CHELYDRA SERPENTINA*

Abstract:

Atrazine is a suspected endocrine disrupting chemical, and is used widely to control broadleaf grasses. Our goal was to determine whether a single exposure to 2ppb or 40ppb atrazine could induce short- and long-term changes in gene expression within the pituitary gland of the common snapping turtle. Atrazine is applied to fields in April and May, just prior to the snapping turtle nesting season. Therefore eggs can conceivably be exposed to atrazine via field runoff. We selected genes *a priori* for their role in the hypothalamus-pituitary-gonad or HP-adrenal axis of the endocrine system. Just 24-hours after treatments, expression of *PDYN* and *PRL* were altered in treated groups. At hatch *PDYN* expression remained altered. After six months, *PDYN* expression was no longer altered, but *ESR1* and *CGA* were altered by treatment with atrazine. Our results show embryonic exposure to atrazine can alter gene expression in the pituitary gland, with alterations occurring through six months after hatching.

Introduction

This study was conducted in parallel with chapter 3, and therefore much of the background will be similar. However, this study was conducted in the pituitary gland, and

therefore additional background presented here will be specific for this tissue. The hypothalamus signals directly to the pituitary gland via neuropeptide and hormonal cues.

Receptors for chemical signals are present in pituicytes, which release hormones directly into the bloodstream in response to receptor activation. Hormones then travel to peripheral tissues throughout the body, regulating endocrine functions including growth, development, stress responses, and reproduction.

Endocrine disrupting chemicals (EDCs), defined in chapter 3, can directly impact the pituitary gland, influencing transcription, translation, and secretion of hormone precursors and hormones. Due to the pituitary gland's broad function, disruption of its normal pattern of development can have widespread detrimental effects. Additionally, effects at other levels of endocrine axes, such as the hypothalamus, can indirectly alter pituitary gland function. Luteinizing hormone (LH) levels were decreased in goldfish exposed to Di-(2-ethylhexyl)-phthalate (Golshan et al. 2015), a chemical used in the production of polyvinyl chloride in plastics. However, LH levels increased in pre-pubertal Wistar rats when exposed to bisphenol A (BPA) (Gamez et al. 2015), another chemical found in plastics. Exposure to pentachlorophenol, a chemical with several uses including wood preservation, decreased mRNA expression of thyroid-stimulating hormone β-subunit in the zebrafish brain (Yu et al. 2014). Exposure to the heavy metal cadmium used in many industrial applications, increased prolactin synthesis and secretion in cultured anterior pituitary cells from female Wistar rats (Ronchetti et al. 2013). Effects of chemical exposure can be widespread, affecting many factors involved in reproduction, growth, and stress responsiveness.

The widely used herbicide atrazine (ATR) is a suspected EDC. Initially, reports were of demasculinizing effects in testes. Exposure to ATR induces atrophy of testes in adult rats (Victor-Costa et al. 2010) and 10% of *Xenopus laevis* exposed to ATR underwent complete feminization, while a decrease in testis size was noted for many other exposed frogs (Hayes et al. 2010). Sprague-Dawley rats exposed to ATR showed disordered and irregular seminiferous tubule epithelium, had a decreased number of spermatozoa, and increased numbers of abnormal spermatozoa (Song et al. 2014). However, with further study, ATR has been found to affect other endocrine functions as well, including growth, stress pathways, and neuroendocrine function. The presence of ATR in drinking water was correlated with impaired fetal growth and development in humans (Ochoa-Acuna et al. 2009). Furthermore, exposure to ATR activates the hypothalamus-pituitary-adrenal axis, increasing corticosterone and adrenal progesterone in adult female rodents (Fraites et al. 2009). Neuroendocrine effects include an increase in Kiss1 within the AVPV (Goldman et al. 2013), inhibition of GnRH pulsatility (Foradori et al. 2009), and increased Kiss1R within the hypothalamus (Chapter 3).

Atrazine has also been linked to changes in the pituitary gland and its hormones in other species. Growth hormone (GH) expression was decreased in pituitary cells from male rats exposed to ATR, while prolactin (PRL) mRNA was increased (Fakhouri et al., 2010). Levels of ACTH were increased in adult female rodents following ATR exposure (Laws et al. 2009). Qin et al. (2015) noted changes in both expression and serum levels of PRL, LH, and FSH in female quail after developmental exposure to ATR, and Song et al. (2014) also saw increased levels of FSH and LH in Male Sprague-Dawley rats. Due to

the complexity of endocrine regulation, it remains unknown how exactly ATR exerts its effect; whether it acts directly at each level of regulation, or interference at one level influences the entire system. However, Fakhouri et al. (2010) reported that ATR binds directly to growth hormone releasing hormone receptor and therefore increases expression of GH directly.

We measured expression of 12 pituitary gland genes involved in the stress, growth, and reproductive axes of the endocrine system following developmental exposure to ATR. Atrazine or 17β-estradiol treatments were administered at stage 20 of embryonic development, after gonadal sex has been determined. At stage 20, the pituitary gland is just consolidating into one distinct structure, and the adenohypophysis is undergoing vascularization. The neurohypophysis is forming through an increase in thickness of the infundibulum by cellular proliferation. By hatching, the pituitary gland has taken on a distinct structural form below the hypothalamus, remaining in contact with the hypothalamus via the infundibulum. The pituitary gland undergoes slight morphological change even after hatching, up to at least 4 months of age, indicating post-hatching alterations in cellular composition. Selected genes include gonadotropin releasing hormone receptor (*GnRHR2b*), chorionic gonadotropin subunit alpha (*CGA*) Luteinizing Hormone beta subunit (*LHβ*), follicle-stimulating hormone beta subunit (*FSHβ*), estrogen receptor 1 (*ESR1*), estrogen receptor 2 (*ESR2*), androgen receptor (*AR*), aromatase (*CYP19A1*), growth hormone (*GH*), prolactin *(PRL*), proopiomelanocortin (*POMC*), and prodynorphin (*PDYN*). Measurement of the selected genes continues our study of the total neuroendocrine effects of developmental exposure to ATR.

Methods

Pituitary glands were micro-dissected from the same animals that were used to study hypothalamic gene expression in chapter 3. Therefore, chapter 3 can be referred to for an in-depth explanation of egg collection, treatment protocols, and tissue collection. In brief, eggs were collected from the wild and incubated at constant male- and female-producing temperatures in the lab. At stage 20, directly following gonadal sex determination, one of four treatments was administered to the eggshell in 5µl volumes: 100% ethanol (vehicle), 0.5 µg 17β-estradiol in 100% ethanol, 2ppb ATR in 100% ethanol, and 40ppb ATR in 100% ethanol. Eggs were then place back into the incubators. Tissues were collected 24 hours after treatment, at hatch, and 6 months after hatch.

RNA Isolation

Pituitary glands were microdissected after removing the hard palate (Figures 5.1 and 5.2). Due to the particularly troublesome nature of these tissues, total RNA was isolated from the pituitary glands using a different method at each stage. RNA from the tissues collected twenty-four hours after treatment was isolated with RNAzol®RT using the same protocol described in chapter 3, with the following changes. The initial volume of RNAzol®RT used for homogenization was 100 µl and each subsequent step was carried out at room temperature. In addition to DNase/RNase-free water, 0.6µl of Precipitation Carrier was added to each sample following homogenization to allow better visualization of the RNA pellet following centrifugation. The final RNA pellet was dissolved in 11µl of DNase/RNase-free water and stored overnight at -80^{o}C prior to quantification. RNA samples were checked for contaminating genomic DNA. Those with

contaminating DNA were cleaned up with an in-tube DNase treatment and ethanol/sodium acetate precipitation of pure RNA.

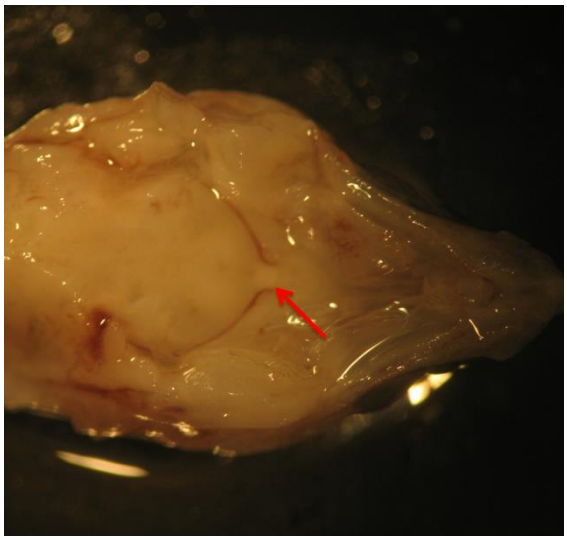


Figure 5.1: Image of the snapping turtle pituitary gland as seen through the hard palate. Pituitary gland (red arrow) can be seen between the two hypophysial arteries. The caudal region of the palate is on the left side of the picture, while the rostral region is to the right

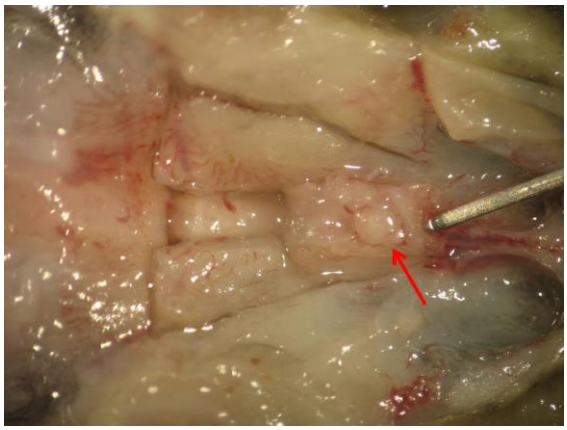


Figure 5.2: The pituitary gland in the snapping turtle. The hard palate was cut away around the pituitary gland and pulled back. The pituitary gland remained embedded in the hard palate (red arrow). The caudal region of the palate is on the left side of the picture, while the rostral region is to the right

Due to sample loss and phenol contamination from incomplete phase separation, a different method was used for total RNA isolation from the hatchling tissues. Total RNA was isolated using the *Quick-RNA*™ MicroPrep (Zymo Research) according to manufacturer's protocol. Tissues were homogenized in 100µl Lysis buffer. One hundred percent ethanol was added to the sample in a 1:1 ratio. The mixture was transferred to a Zymo-Spin™ IC Column in a Collection Tube and centrifuged for 30 seconds. The optional on-column treatment with DNase I was included using DNase I supplied with the kit. Four hundred microliters of RNA Prep Buffer was added to the column and centrifuged for 30 seconds. Next, samples were washed with 700ul RNA Wash Buffer and centrifuged for 30 seconds, followed by a wash with 400ul RNA wash Buffer and 2 minutes of centrifugation. An additional wash step was performed using 400ul Wash Buffer prior to RNA elution in 11ul water. RNA samples were checked for contaminating genomic DNA by running a qPCR prior to reverse transcription.

All of the samples contained some genomic DNA, and therefore required an additional clean-up treatment. This was performed using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems™) with on-column DNase I treatment using an RNase-Free DNase set (Qiagen). Briefly, 25ul extraction buffer was added to each eluted RNA sample and incubated at room temperature for 5 minutes. One hundred microliters of 70% ethanol was added to the homogenate and mixed by inverting. The sample was transferred to the conditioned column and centrifuged at 100g for 2 minutes, followed by 16,000g for 30 seconds. Next, 100ul of wash buffer I was added and centrifuged at 8,000g for 1 minute. The flow-through was discarded. A mixture of 10ul DNaseI and

30ul Buffer RDD was added to each column and incubated at room temperature for 15 minutes. Forty microliters wash buffer I was then added to the column and centrifuged at 8,000g for 15 seconds, followed by a wash with wash buffer 2 and centrifugation at 8,000g for 1 minute. Two more wash steps were performed with 100ul wash buffer 2 and centrifugation at 16,000g for 2 minutes. Flow-through was discarded, and the column was placed into a fresh DNase/RNase-free tube. Eleven microliters of elution buffer was applied directly to the column by gently tapping the filter with the tip and incubated at room temperature for 1 minute. Tubes were then centrifuged at 1,000g for 1 minute, followed by 1 additional minute at 16,000g.

Total RNA from the 6 month old hatchling tissue was isolated using the Arcturus® PicoPure® RNA Isolation Kit as described above with initial homogenization carried out in 100ul extraction buffer. Samples with contaminating genomic DNA following this protocol were cleaned-up with an additional run with the kit.

Reverse Transcription, quantitative PCR and Statistical Analysis

Reverse transcription and quantitative PCR was carried out using the same protocol as described in chapter 3. We designed primers for 8 additional genes (Table 5.1) in addition to the primers used for *ESR1*, *ESR2*, *AR*, and *CYP19A1* from chapter 3. Expression was very low for PRL and GnRHR2b, and therefore the total input cDNA was increased to the equivalent of 4.125 ng input RNA in hatchling samples.

Statistical analysis was carried out in JMP 11.1.1 software as described in chapter 3. We used 18S rRNA expression as a covariate to control for possible variation in quantity of input RNA. We also included a blocking factor of whether or not the sample

underwent an additional genomic DNA clean-up step. The control group was compared with the vehicle group. If these groups did not differ, they were combined into a larger control group for comparison to E2 and ATR treatments. If there was a significant difference between the control and vehicle-treated group, the E2 and ATR treated groups were compared to the vehicle-treated group. Sample sizes for each group are shown in each figure.

Table 5.1: Forward and Reverse primer sequences for 6 pituitary gland genes from the snapping turtle.

Gene Name	*Forward Primer Sequence (5' -3')*	*Reverse Primer Sequence (5' – 3')*
Gonadotropin Releasing Hormone Receptor 2b (GnRHR2b)	*CTCTACACGCCTTCCTTCCG*	*TCTCCTGCCCCGTAATGACT*
Follicle-Stimulating Hormone subunit beta (FSHβ)	*TCACCATAGCAGTGGAGAAGGA*	*GTATCCAGAGCACCAAGTGGC*
Luteinizing Hormone subunit beta (LHβ)	*GGGTACTGCCAGACCAAGGA*	*AGCGCGTTCTTGTACACCG*
Growth Hormone (GH)	*CTACGGCCTGTTGTCCTGCT*	*GCGCCTGCACTTCATCAGTT*
Chorionic Gonadotropin subunit alpha (CGA)	*GGGTTGTCCAGAATGCAAGC*	*TCCTGTGCACTGGTAAATGGG*
Prolactin (PRL)	*CTGCGGAGTGTGAGGAACAA*	*TTCTGGTGCTTGGGTTCAGAC*

Results

Gene Expression in Turtle Embryos

At late stage 20 of development, 24-hours after treatments were applied, two-way ANOVA did not reveal any treatment, incubation temperature, or interaction effects in expression of *ESR1*, *ESR2*, *CGA, FSHβ, AR, GnRHR2b*, or *POMC* (Table 5.2). However, *LHβ, GH, Cyp19a1, PDYN*, and *PRL* were differentially expressed between incubation temperatures (Table 5.2), with females having higher expression for each gene (Table 5.3).

Chemical treatments influenced *PDYN* and *PRL* expression (Table 5.2). PDYN expression was significantly lower in embryonic pituitary glands from the 2ppb ATR group when compared with vehicle-treated controls (Figure 5.3). Expression of *PRL* was not altered in the group treated with 2ppb ATR, but was decreased by both E2 and 40ppb ATR treatments (Figure 5.4).

Table 5.2: Summary of 2-way ANOVA (Type III Sums of Squares) for treatment and incubation temperature (sex) effects and their interaction on the expression of 12 reproductive, growth, and stress associated genes in pituitary glands of snapping turtle embryos 24 hours after treatment with 17β-estradiol or atrazine. Embryos were at late stage 20 of development, according to anatomical staging by Yntema (1968). Differences were considered significant with $p<0.05$, and are indicated with *.

Gene	Effects	DF	F-value	p-value
AR	Treatment	3	0.053	0.984
	Sex	1	0.002	0.967
	Treatment x Sex	3	1.721	0.170
CGA	Treatment	3	0.373	0.773
	Sex	1	0.050	0.823
	Treatment x Sex	3	0.431	0.731
Cyp19A1	Treatment	3	0.877	0.459
	Sex	1	5.469	0.023*
	Treatment x Sex	3	0.336	0.799
ESR1	Treatment	3	0.061	0.980
	Sex	1	0.452	0.504
	Treatment x Sex	3	0.373	0.773
ESR2	Treatment	3	1.142	0.338
	Sex	1	0.601	0.441
	Treatment x Sex	3	0.240	0.868
FSHβ	Treatment	3	1.829	0.153
	Sex	1	0.464	0.499
	Treatment x Sex	3	0.571	0.636
GH	Treatment	3	0.421	0.739
	Sex	1	4.689	0.034*
	Treatment x Sex	3	0.403	0.752
GnRHR2b	Treatment	3	0.778	0.511
	Sex	1	0.251	0.618
	Treatment x Sex	3	0.499	0.684
LHβ	Treatment	3	1.191	0.319
	Sex	1	5.645	0.020*
	Treatment x Sex	3	0.773	0.513
PDYN	Treatment	3	2.850	0.046*
	Sex	1	7.702	0.008*
	Treatment x Sex	3	0.377	0.770
POMC	Treatment	3	0.121	0.947
	Sex	1	0.604	0.439
	Treatment x Sex	3	0.338	0.798
PRL	Treatment	3	9.628	<0.001*
	Sex	1	4.311	0.041*
	Treatment x Sex	3	0.176	0.913

Table 5.3: Gene expression in male and female pituitary glands during late stage 20 of development. Genes presented here showed a significant difference between sexes (as a result of incubation at male- and female-producing temperatures). Analysis was performed using a two-way ANOVA with incubation temperature and treatment as variables.

Gene	Sex	Least Square Mean (ag/1.25 ng total RNA)	Standard Error
Cyp19A1	Female	325.221	257.863 to 410.174
	Male	187.176	147.392 to 237.699
GH	Female	747.117	590.116 to 945.889
	Male	433.165	338.110 to 554.946
LHβ	Female	16.948	13.574 to 21.161
	Male	9.653	7.645 to 12.188
PDYN	Female	5.083	3.728 to 6.931
	Male	2.155	1.577 to 2.943
PRL	Female	4.946	4.661 to 5.232
	Male	4.313	4.0132 to 4.613

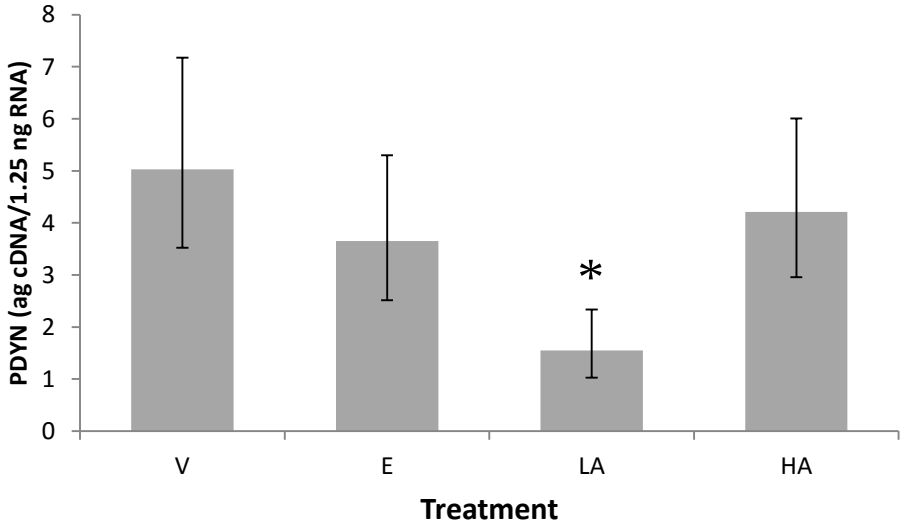


Figure 5.3: *PDYN* expression in the pituitary gland of snapping turtle embryos at late stage 20. Input RNA was equivalent to 1.25 ng for each qPCR reaction. Groups significantly different from the vehicle-treated control group based on a Dunn-Sidak correction for multiple comparisons are indicated by an asterisk (*). V = vehicle (100% ethanol), E = Estradiol (0.5 µg), LA = Low Atrazine (2ppb), and HA = High Atrazine (40ppb).

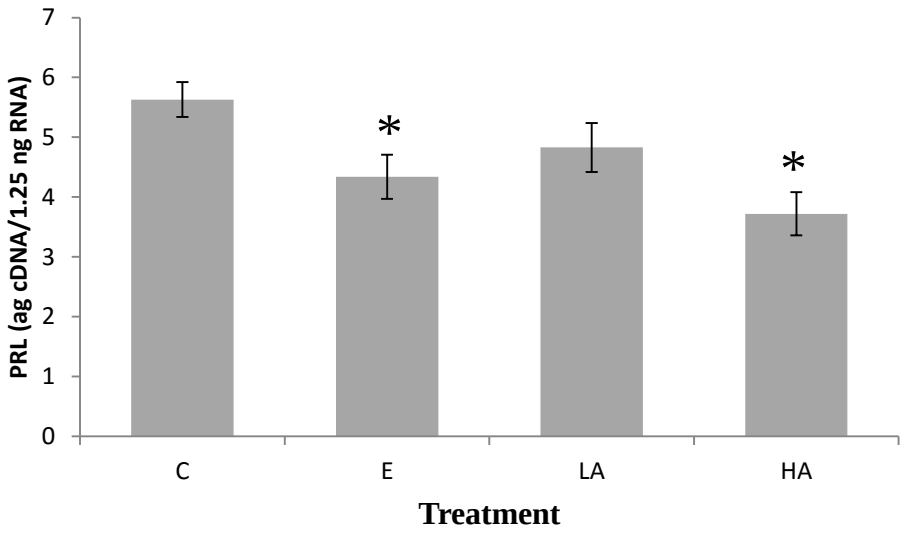


Figure 5.4: *PRL* expression in the pituitary gland of snapping turtle embryos at late stage 20. Input RNA was equivalent to 1.25ng for each qPCR reaction. Groups significantly different from the control group based on a Dunn-Sidak correction for multiple comparisons are indicated by an asterisk (*). C = untreated and vehicle-treated controls (100% ethanol), E = Estradiol (0.5 µg), LA = Low Atrazine (2ppb), and HA = High Atrazine (40ppb).

Gene Expression in Hatchling Turtles

At hatching, expression of *PRL* was not significantly different between treatments or incubation temperatures, and there was no detectable interaction effect (Table 5.4). Nearly all other genes showed differential expression between sexes (Table 5.4). Expression of *AR, CGA, Cyp19A1, ESR1, ESR2, FSHβ, GH, GnRHR2b, LHβ*, and *POMC* was higher in males (Table 5.5). The temperature by treatment interaction effect was significant for expression of *PDYN* (Table 5.4). However, only the vehicle-treated control groups were differentially expressed between the sexes using the Dunn-Sidak post hoc test (Figure 5.5).

Table 5.4: Summary of 2-way ANOVA (Type III Sums of Squares) for treatment and incubation temperature (sex) effects and their interaction on the expression of 12 reproductive, growth, and stress associated genes in pituitary glands of hatchling snapping turtles. Embryos were treated with 17β-estradiol or atrazine at late stage 20 of development. Differences were considered significant with $p<0.05$, and are indicated with *.

Gene	Effects	DF	F-value	p-value
AR	Treatment	3	1.449	0.235
	Sex	1	11.734	0.001*
	Treatment x Sex	3	0.800	0.498
CGA	Treatment	3	1.499	0.222
	Sex	1	12.653	0.001*
	Treatment x Sex	3	0.991	0.402
Cyp19A1	Treatment	3	2.198	0.095
	Sex	1	34.243	<0.001*
	Treatment x Sex	3	0.884	0.453
ESR1	Treatment	3	1.900	0.137
	Sex	1	61.176	<0.001*
	Treatment x Sex	3	1.318	0.275
ESR2	Treatment	3	0.753	0.524
	Sex	1	30.122	<0.001*
	Treatment x Sex	3	0.409	0.747
FSHβ	Treatment	3	0.131	0.941
	Sex	1	17.486	<0.001*
	Treatment x Sex	3	0.683	0.565
GH	Treatment	3	1.462	0.232
	Sex	1	44.921	<0.001*
	Treatment x Sex	3	0.994	0.401
GnRHR2b	Treatment	3	0.748	0.527
	Sex	1	36.111	<0.001*
	Treatment x Sex	3	0.369	0.776
LHβ	Treatment	3	0.238	0.870
	Sex	1	46.454	<0.001*
	Treatment x Sex	3	1.003	0.396
PDYN	Treatment	3	2.418	0.076
	Sex	1	11.848	0.001*
	Treatment x Sex	3	3.476	0.022*
POMC	Treatment	3	1.856	0.144
	Sex	1	57.963	<0.001*
	Treatment x Sex	3	1.282	0.287
PRL	Treatment	3	2.068	0.116
	Sex	1	0.187	0.667
	Treatment x Sex	3	1.126	0.347

Table 5.5: Gene expression in male and female pituitary glands within 1 week of hatching. Genes presented here showed a significant difference between sexes (as a result of incubation at male- and female-producing temperatures). Analysis was performed using a two-way ANOVA with incubation temperature and treatment as variables.

Gene	Sex	Least Square Mean (ag/1.25 ng total RNA)	Standard Error
AR	Female	1,696.798	1,449.252 to 1,986.628
	Male	3,265.006	2,791.285 to 3,819.125
CGA	Female	63,632.795	54,591.837 to 74,171.027
	Male	123,361.233	105,902.226 to 143,698.525
Cyp19A1	Female	5,991.877	5,068.725 to 7,083.161
	Male	19,622.991	16,616.218 to 23,173.852
ESR1	Female	30.931	25.973 to 36.837
	Male	159.392	134.271 to 189.213
ESR2	Female	95.739	83.584 to 109.662
	Male	236.164	206.346 to 270.291
FSHβ	Female	8.722	7.159 to 10.624
	Male	23.814	19.590 to 28.949
GH	Female	18,332.429	15,017.306 to 22,379.376
	Male	92,620.133	75,961.134 to 112,932.609
GnRHR2b	Female	5.188	4.172 to 6.451
	Male	24.396	19.928 to 229.865
LHβ	Female	157.660	130.218 to 190.884
	Male	764.802	632.402 to 924.921
POMC	Female	54,382.116	45,196.183 to 65,435.050
	Male	299,706.181	249,355.040 to 360,224.502

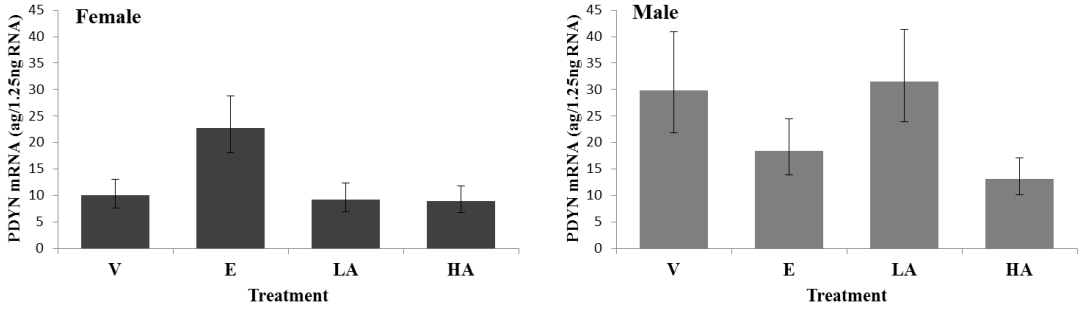


Figure 5.5: PDYN expression in the pituitary gland of hatchling snapping turtles. Input RNA was equivalent to 1.25ng for each qPCR reaction. Although there was a significant interaction between sex and chemical treatment, the Dunn-Sidak correction for post hoc comparisons was unable to detect which group(s) was significantly different from the vehicle-treated control. V = vehicle (100% ethanol), E = Estradiol (0.5 µg), LA = Low Atrazine (2ppb), and HA = High Atrazine (40ppb).

Gene Expression in Six-month Old Hatchling Turtles

Six months after hatching, two-way ANOVA did not reveal any treatment, incubation temperature, or interaction effects for expression of *AR, Cyp19A1, ESR2, FSHβ, GH, LHβ, PDYN*, or *POMC* (Table 5.6). The only gene showing a significant difference in expression between incubation temperatures was *PRL* (Table 5.6), with males having significantly higher expression (10.0 ± 1.8 ag/1.25 ng total RNA) than females (6.5 ± 0.7 ag/1.25 ng total RNA). Chemical treatments had a significant effect on expression of *ESR1, GnRHR2b,* and *CGA* (Table 5.6). *ESR1* expression was significantly decreased in females treated with 40 ppb ATR (Figure 5.6). However, the post hoc test did not reveal significant differences between treatment groups and the control group for *GnRHR2b* (Figure 5.7). However, it is evident that the E2 treated group is significantly different from the group treated with 40ppb ATR (Figure 5.7). Pituitary glands from hatchlings treated with 40ppb ATR had significantly lower expression of *CGA* when compared with vehicle-treated controls (Figure 5.8).

Table 5.6: Summary of 2-way ANOVA (Type III Sums of Squares) for treatment and incubation temperature (sex) effects and their interaction on the expression of 12 reproductive, growth, and stress associated genes in pituitary glands of six-month old hatchling snapping turtles. Turtles were treated with 17β-estradiol or atrazine at late stage 20 of embryonic development. Differences were considered significant with $p<0.05$, and are indicated with *.

Gene	Effects	DF	F-value	p-value
AR	Treatment	3	0.267	0.849
	Sex	1	0.794	0.377
	Treatment x Sex	3	2.017	0.123
CGA	Treatment	3	3.073	0.035*
	Sex	1	1.655	0.204
	Treatment x Sex	3	1.300	0.285
Cyp19A1	Treatment	3	2.126	0.106
	Sex	1	0.018	0.893
	Treatment x Sex	3	1.690	0.178
ESR1	Treatment	3	1.437	0.240
	Sex	1	1.250	0.268
	Treatment x Sex	3	7.076	<0.001*
ESR2	Treatment	3	0.343	0.794
	Sex	1	0.209	0.650
	Treatment x Sex	3	1.626	0.192
FSHβ	Treatment	3	1.061	0.372
	Sex	1	0.021	0.885
	Treatment x Sex	3	0.570	0.637
GH	Treatment	3	0.653	0.585
	Sex	1	0.237	0.629
	Treatment x Sex	3	2.035	0.120
GnRHR2b	Treatment	3	3.225	0.030*
	Sex	1	3.109	0.084
	Treatment x Sex	3	0.370	0.775
LHβ	Treatment	3	1.494	0.224
	Sex	1	1.840	0.180
	Treatment x Sex	3	1.467	0.232
PDYN	Treatment	3	2.103	0.108
	Sex	1	0.012	0.913
	Treatment x Sex	3	1.293	0.284
POMC	Treatment	3	2.085	0.113
	Sex	1	1.550	0.219
	Treatment x Sex	3	0.539	0.658
PRL	Treatment	3	2.156	0.104
	Sex	1	5.945	0.018*
	Treatment x Sex	3	0.681	0.568

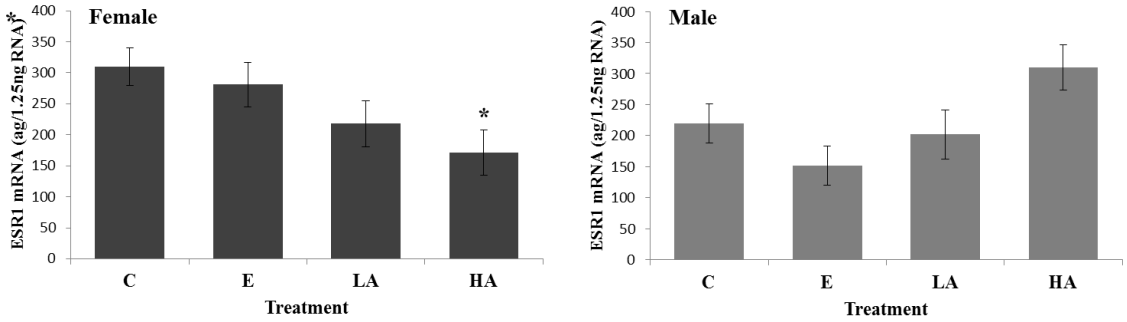


Figure 5.6: *ESR1* expression in the pituitary gland in 6-month old hatchling snapping turtles. Input RNA was equivalent to 1.25ng for each qPCR reaction. Groups significantly different from the control group based on a Dunn-Sidak correction for multiple comparisons are indicated by an asterisk (*). C = untreated controls and vehicle-treated controls (100% ethanol), E = Estradiol (0.5 µg), LA = Low Atrazine (2ppb), and HA = High Atrazine (40ppb).

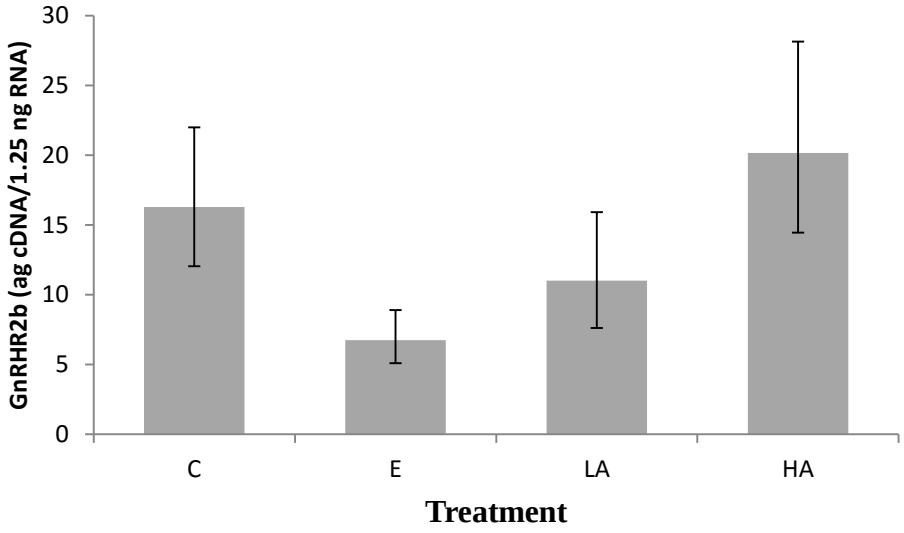


Figure 5.7: *GnRHR2b* expression in the pituitary gland of 6-month old hatchling snapping turtles. Input RNA was equivalent to 1.25ng for each qPCR reaction. Although there was a significant effect of chemical treatment, the Dunn-Sidak correction for post hoc comparisons was unable to detect which group(s) was significantly different from the vehicle-treated control. C = untreated controls and vehicle-treated controls (100% ethanol), E = Estradiol (0.5 µg), LA = Low Atrazine (2ppb), and HA = High Atrazine (40ppb).

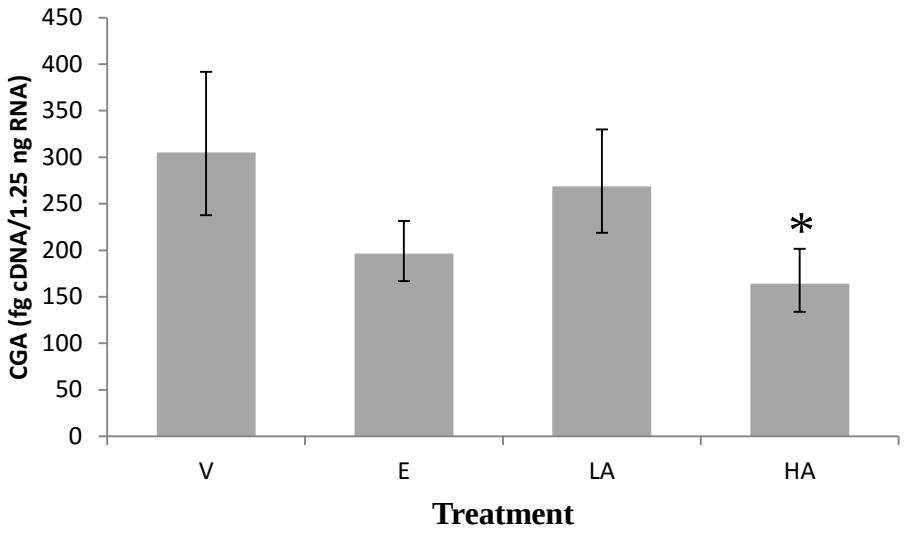


Figure 5.8: *CGA* expression in the pituitary gland of 6-month old hatchling snapping turtles. Input RNA was equivalent to 1.25ng for each qPCR reaction. Groups significantly different from the vehicle-treated group based on a Dunn-Sidak correction for multiple comparisons are indicated by an asterisk (*). V = vehicle (100% ethanol), E = Estradiol (0.5 µg), LA = Low Atrazine (2ppb), and HA = High Atrazine (40ppb).

Discussion

While there is increasing evidence for gonadal and neuronal effects from atrazine (ATR) exposure, studies of effects in the pituitary gland remain limited. Pituitary glands from rats exposed to ATR during embryogenesis were heavier (Rayner et al, 2005). Exposure to ATR at varying concentrations and durations are shown to impact LH serum levels and gene expression (Foradori et al, 2011; Qin et al, 2015), but studies show both increases and decreases. Previous studies in quail also show alterations in expression of FSH and PRL following exposure during post-hatching ovarian development (Qin et al, 2015). Overall, we saw fewer and less consistent effects on gene expression in the pituitary gland compared to the hypothalamus

Gene expression is extremely variable in the pituitary gland, differing up to four orders of magnitude between genes. For example, expression of *PRL* is extremely low at

all three developmental stages, with mean expression of 13.9 ag/1.25 ng RNA at 6 months, while expression of *CGA* increases with developmental stage, with mean expression of 300,600 ag/1.25 ng RNA at 6 months. Further investigation of cellular composition within the pituitary gland at these young ages, as well as gene expression quantification in older turtles, would shed light on whether this difference is due to differences in transcription or the total number of each cell type.

Surprisingly, changes in gene expression were evident in the pituitary gland just 24 hours after treatment. While expression of *PRL* is extremely low, it was altered by treatment with both E2 and 40ppb ATR in males and females. Both treatments significantly decreased expression of *PRL*. Here, a higher dose of ATR had a similar effect on expression as treatment with E2, though ATR caused a greater decrease in expression (Figure 5.4). Prolactin has a broad range of functions, from regulating aspects of reproduction, reproductive behavior, reproductive development, lactation, and inducing maternal behavior, to migratory behavior, maintenance of water and electrolyte balance, growth, metabolism, and immunoregulation (Bole-Feysot et al, 1998). While prolactin's function is better understood in mammals, it plays an important role in reproduction in reptiles as well (Bachelot and Binart, 2007). Our work in the hypothalamus revealed an increase in *PRLH* at hatch and 6 months due to treatment with 2 ppb ATR. However, *PRL* was not altered in hatchling or 6-month old pituitary glands but was decreased 24 hours after treatment. Previous studies have shown variable results of ATR treatment on *PRL* expression. Kucka et al (2012) report a stimulatory effect on PRL release, whereas Cooper et al (2000) showed suppression of the PRL surge or no

effect. Long-term exposure to high levels of ATR in quail induced an increase in PRL expression (Qin et al, 2015). Therefore, it is likely effects of ATR treatment on PRL expression depend on the age of the animal, duration and dose of the treatment, and species. Furthermore, since the physiological effects of PRL are so widespread, it is difficult to determine the potential consequences of changes in PRL expression.

Expression of *PDYN* is also very low in embryonic pituitary glands, and was decreased 24 hours after treatment with 2ppb ATR in both sexes. PDYN is a precursor molecule for endogenous opioid peptides that inhibit the hypothalamus-pituitary-adrenal (HPA) axis in response to stress (Rushen et al, 1993). Mice lacking PDYN display increased anxiolytic behaviors (Wittmann et al, 2009). Previous evidence of adrenal activation from ATR treatment is clear. Corticosterone levels were increased within 20 minutes of ATR treatment, and remained elevated over 12 hours following treatment in Wistar rats (Foradori et al, 2011). Therefore, short-term effects on PDYN expression within the H-P are likely, but the precise effects on PDYN within the pituitary gland have not been previously studied.

The low dose of ATR effect did not persist to hatch. While the interaction effect was significant for *PDYN* expression at hatch, the post hoc test did not reveal the source of the treatment effect. Expression is significantly different between sexes, with higher expression in males, but treatment effects were not significant. However, two treatments did show a marginally significant effect: the E2 treatment group in females had higher expression than controls, and the high ATR treatment group in males had lower expression of *PDYN* than controls. Estradiol and ATR had converse impacts and were

different between sexes, thus, ATR does not have an 'estrogenic' effect on *PDYN* expression at hatch. Furthermore, while expression of *PDYN* was significantly increased by treatment with 2 ppb ATR at hatch in the hypothalamus, this treatment did not alter expression in the pituitary gland. Therefore, we cannot accurately predict the effects of treatment on the pituitary gland based solely on effects seen in the hypothalamus, and different treatments have vastly different effects depending on tissue type and age.

Treatment effects on expression of HPG genes were only seen in 6-month old hatchlings. Expression of *ESR1* was decreased in females treated with 40 ppb ATR when compared with control females, while *ESR1* expression was marginally increased in males treated with 40ppb ATR. Therefore, treatment with the high dose of ATR had opposing effects on *ESR1* expression in males and females. Studies largely point to estrogen-like effects of ATR exposure, and *ESR1* downregulation could be a result of increased circulating estrogens. In vivo and in vitro studies show that treatment with estradiol has inhibitory effects on ESR1 in rats (Schreihofer et al., 2000), though these effects were seen shortly after treatment. However, estrogenic effects on gene expression do not seem to be consistent for all genes tested.

While expression of *GnRH1* and *GnRH2* in the hypothalamus was not altered by chemical treatments, there was a significant treatment effect on expression of *GnRHR2b* in the pituitary gland. The post hoc test did not reveal a significant difference between any of the three treatments when compared with controls, but expression was marginally lower in the E2-treated group, while ATR treatment did not impact expression. GnRH receptors are found on gonadotropes, cells which synthesize and release LH and FSH. In

adult ewes, treatment with estradiol produces a rapid increase in GnRH receptor mRNA (Turzillo et al, 1998) and a similar effect is seen in adult female rats (Quinones-Jenab et al 1996). Conversely, in a gonadotrope-derived cell line, estradiol reduced GnRHR number (McArdle et al, 1992) Estrogens have an organizational effect on gonadotropes, and in zebrafish, exposure during development caused a decrease in FSH cells (Golan et al, 2014). Results vary, and it is important to note differences in the timing of the treatments (embryos vs. adults) and potential species differences. A Tukey's post hoc test revealed a significant difference between the high dose ATR group and the E2-treated group (i.e., *GnRHR2b* expression was significantly lower). Therefore, ATR did not have an estrogenic effect on gene expression.

Lastly, expression of *CGA* was decreased in males and females treated with a high dose of ATR. *CGA* is the alpha subunit for FSH, LH, and TSH, and therefore expression changes could impact not only the HPG axis, but also thyroid signaling. We did not test expression of TSHβ, but it would be interesting to determine if ATR influences thyroid signaling at these younger ages. However, previous studies have revealed minimal ATR effects on TSH and other markers of thyroid function (Stoker et al, 2000, 2002; Laws et al, 2000). Many studies show a consistent inhibitory effect of ATR on LH. Foradori et al (2011) show that high doses administered over 4 days suppress LH release in adult rats. Goldman et al (2013) also report attenuation of the LH surge following ATR exposure. Quail also have lower LH expression following ATR treatment (Qin et al, 2015). While our study does not show an effect of treatment on *LHβ* expression, the decrease in *CGA* expression could potentially indicate a decrease in overall LH serum levels. Furthermore,

it is unlikely that we would observe a direct effect at this early, non-reproductive stage of development.

There is another important consideration for the hatchling samples. All of the genes we examined, except *PRL*, were expressed at a significantly higher level in males than in females. This sex difference was not observed in embryos or 6-month old turtles. Therefore it prompted further inquiry into whether this was an accurate result, or if there were inaccuracies in the samples themselves. We had to complete a second full RNA isolation in order to remove contaminating gDNA, decreasing the total concentration of RNA. However, we included whether the RNA underwent a second round of isolation as a factor in our analysis, and it did not change the results. Furthermore, the quantification of the 18S rRNA housekeeping gene did not show a difference between incubation temperatures, which would be expected if the quantification difference was actually due to inaccurate input RNA concentrations. Therefore, we are confident in our results.

Overall, ATR treatment altered gene expression in the pituitary gland in some genes and the effects did not show a consistent pattern between sexes. While effects seen in gonads have been described as 'estrogenic', effects of ATR treatment in the pituitary gland do not follow the same pattern as E2 effects. We found that E2 treatments had little effect on gene expression in the hypothalamus. In contrast, E2 induced significant changes in pituitary gland gene expression. Furthermore, ATR and E2 are likely to have a direct impact on the pituitary gland because effects cannot be predicted by gene expression alterations in the hypothalamus. When comparing the current work and previous studies, ATR exposure seems to induce larger effects within the gonads and

hypothalamus versus the pituitary gland in embryos. Interference in HPG development likely occurs more at the ‘H’ and ‘G’ levels, with less severe effects in the pituitary gland at early stages of development.

CHAPTER VI

EPILOGUE

Here, we establish a basic understanding of neuroendocrine development in the common snapping turtle. Morphological development of the pituitary gland is highly conserved among vertebrates and morphogenesis in the snapping turtle is quite similar to development in other species. The adenohypophysis has oral ectoderm origins, while the neurohypophysis is of neural origin. Development is initiated early in development, and continues post-hatching. A high level of conservation in developmental processes exists from reptiles, to birds and mammals, highlighting the importance of this gland for survival and reproduction.

We also established sex differences in developmental gene expression within both the hypothalamus and pituitary gland. Genes involved in neuroendocrine function are not sexually dimorphic until hatching, and the number of dimorphic genes decreases by six months after hatching. Sexually dimorphic genes are not numerous during development, and many are functionally involved in translational processes. This could indicate the importance of protein-level dimorphism in establishing overall sexually dimorphic structure, and is an important area of future study.

Last, we identified the effects of an endocrine-disrupting herbicide, atrazine, on gene expression in both the hypothalamus and the pituitary gland. Alterations are more prevalent in the hypothalamus, where atrazine at a low dose, 2ppb, has a significant effect

on expression of certain neuroendocrine genes. Furthermore, alterations are more numerous in females. Changes in the pituitary gland occur, but the effects are variable. Our results show the snapping turtle is another species vulnerable to the impacts of atrazine, and show atrazine can impact a wide variety of taxa.

These studies begin the investigation of neuroendocrine development in the snapping turtle. A morphologic description of hypothalamus development, and identification of specific hypothalamic nuclei will further understanding of normal neuroendocrine development. Investigation of proteome-level sex differences in the hypothalamus and pituitary gland will help to clarify the underlying mechanisms in sexual differentiation. Further investigation into the effects of atrazine is also needed to solidify whether atrazine is in fact having endocrine-disrupting effects. Studies including multiple exposures as well as the timing of initial changes in gene expression following exposure are needed. Furthermore, measuring hormone levels in the blood will be important to determine true endocrine disrupting effects. Ultimately, rearing exposed embryos through hatching to reproductive maturity will be important to identify any physiological consequences of embryonic exposure to atrazine.

This work will be an important contribution to the body of literature on comparative neuroendocrine development, and will contribute to future studies of endocrine disruption. It provides further evidence of the endocrine-disrupting potential of atrazine, and suggests the need for regulatory changes in atrazine use and environmental monitoring of EDCs.

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