EVOLUTIONARY PLASTICITY IN THE PLEIOTROPIC REGULATION OF SEXUALLY DIMORPHIC TRAITS IN GEKKOTAN LIZARDS

by

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ABSTRACT OF THE DISSERTATION

Evolutionary Plasticity in the Pleiotropic Regulation of

Sexually Dimorphic Traits in Gekkotan Lizards

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Sexually dimorphic traits evolve due to selection for reproductive advantage, and their expression is often functionally correlated by the pleiotropic effects of gonadal steroids (e.g., testosterone, T, in males). When T mediates the correlated expression of traits, then how can changes in individual traits evolve? The main objective of this dissertation was to investigate whether circulating T or androgen receptors in the brain underlie sex- and species-differences in sexually dimorphic trait expression in a comparative study of three species of gekkotan lizards (*Goniurosaurus lichtenfelderi*, *Coleonyx elegans*, and *Paroedura picta*). I hypothesized sex differences in trait expression are due to differences in circulating T, and that the absence of various traits in *C. elegans* and *P. picta* are due to a specific alteration in the androgen signaling system rather than a reduction in T. I conducted surgical manipulations to alter levels of T in adult males and females of each species. Testosterone-sensitive traits included courtship, copulatory mounting, and aggressive behaviors, secretions from precloacal pores, enlargement of the hemipenes and head width. Elevated T in males was almost always the primary mediator of sex differences in trait expression within species. Some, but not

all, of the male-typical traits were induced in adult females, suggesting the neural or physiological substrates underlying certain traits are permanently differentiated between the sexes prior to adulthood. Traits absent from the phenotype of a species, such as courtship in *C. elegans* and *P. picta*, cannot be induced by exogenous T. I hypothesized differences in behavioral sensitivity to T would be due to differences in androgen receptors in brain regions associated with control of reproductive behaviors. Immunohistochemistry revealed *P. picta* had increased abundance of androgen receptor immunoreactivity (AR-ir) in the preoptic area and ventromedial hypothalamus relative to *G. lichtenfelderi*. Thus, the abundance of AR-ir does not reflect the expression of courtship and aggressive behaviors in these species. Although circulating T or AR-ir do not explain interspecific differences in trait expression, results indicate that correlated traits are not constrained by the pleiotropic effects of T but targeted changes in sensitivity to T allows evolutionary diversification of trait expression.

DEDICATION

To all the teachers in my life,

who have set me on this path to accomplish my goals.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
MCAST	Castrated Male Treatment Group
MCON	Control Male Treatment Group
DHT	5α-Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
E_2	17β-Estradiol
mRNA	Messenger Ribonucleic Acid
PCA	Principal Components Analysis
PBSG	Phosphate-buffered Saline with Gelatin
RIA	Radioimmunoassay
SEM	Standard Error of the Mean
SVL	Snout-Vent Length
T	Testosterone
MTEST	Testosterone-replaced Male Treatment Group
FTEST	Testosterone-supplemented Female Treatment Group
FCON	Control Female Treatment Group
FOVX	Ovariectomized Female Treatment Group
IHC	Immunohistochemistry
AR	Androgen Receptor
AR-ir	Androgen Receptor Immunoreactivity

CHAPTER 1

INTRODUCTION

Questions regarding the evolution of sexually dimorphic traits

Reproduction is the culminating act in an individual's life, ensuring one's genes will be represented in future generations. Thus, selection favoring successful reproduction has led to the development of fine-tuned mechanisms – including social signals – to coordinate physiology and behaviors of both sexes (Andersson 1994; Crews 1997; Adkins-Regan 2005). As males and females play functionally different roles in reproduction, sexual selection for traits that confer reproductive advantage within a sex has contributed to the evolution of sex-specific traits (i.e., sexually dimorphic traits; Darwin 1871; Andersson 1994). Whereas evolutionary ecologists have made great strides in documenting and understanding sexual selection since the time of Darwin (Carothers 1984; Shine 1989; Basolo 1990; Preziosi and Fairbairn 1997; Stamps et al. 1997; Blanckenhorn 2005; Morris et al. 2005; Chaine and Lyon 2008), proximate mechanisms (e.g., endocrine, genetic, molecular) that give rise to suites of sexually dimorphic traits and enable the evolution of diverse patterns in trait expression among closely-related species are still not fully understood (Badyaev 2002; Williams and Carol 2009).

Sexual dimorphism arises when traits are expressed only in one sex (sex-specific expression) or are expressed asymmetrically in both sexes (sex-biased expression; Badyaev 2002; Ellengren and Parsch 2007). As males and females share most of their genomes and express many of the same traits, sex-specific modifiers such as gonadal

hormones provide a mechanism allowing differential regulation of shared genes and thus enable the expression of sexually dimorphic traits (Badyaev 2002; Ellegren and Parsch 2007). Hormones are pleiotropic signaling molecules, meaning that they affect multiple target sites simultaneously, coordinating developmental and physiological processes. The pleiotropic actions of hormones suggest endocrine mechanisms create patterns of correlations in the expression of dimorphic traits (Stearns 1989; Hau 2007).

The occurrence of androgenic hormonal pleiotropy – where androgens mediate the expression of suites of male-specific or male-biased traits – is widespread among vertebrates (reviewed in Adkins-Regan 2005). This phenomenon raises questions regarding the role of this mechanism in the evolution of suites of correlated traits in light of the diversity in sexually dimorphic phenotypes observed even among closely-related species (Hau 2007; McGlothlin and Ketterson 2008; Adkins-Regan 2012). If a shared proximate mechanism, such as control by gonadal androgens, mediates the expression of functionally related suites of traits, then how is it possible for the expression of one or more traits to change or even be lost from a conserved suite? What type of changes in the androgen control mechanism could enable the dissociation of a trait? Does selection act on the level of circulating hormones to change trait expression (i.e., evolutionary constraint hypothesis, see Fig. 1.1; Hau 2007; McGlothlin and Ketterson 2008)? Or, does selection act on the linkages between components of the endocrine signaling system, and thus allow correlation among traits to be loosened or broken (i.e., evolutionary potential hypothesis, see Fig. 1.1; Hau 2007; McGlothlin and Ketterson 2008)? If so, which components underlie the diversity in suites of sexually dimorphic traits observed in nature? These questions are addressed in the present dissertation.

Hormonal pleiotropy and the androgen control module

Sex steroids are primarily produced by the gonads, from which they are secreted into general circulation and transported throughout an organism. Steroids affect many processes simultaneously (i.e., hormonal pleiotropy), which often manifest as suites of correlated morphological, physiological, and behavioral traits (Finch and Rose 1995; Badyaev 2002). Therefore, hormonal effects are evident in multiple and diverse aspects of an organism's phenotype (Finch and Rose 1995; Hau 2007; Ball and Balthazart 2008). Hormonal control systems are complex, involving multiple components – circulating hormone, binding proteins, metabolizing enzymes, specific receptors – which can elicit multiple types of responses. Furthermore, gonadal hormone systems are subject to hierarchical regulation by the hypothalamus, which integrates cues from the internal and external environment to modulate the system (Sherwood et al. 2005; Hill et al. 2008: Adkins-Regan 2008). Hormone control systems are genetically complex because they involve several organs and components that are coded by different genes, which provide the substrate for evolution (West-Eberhard 2003).

The signaling system between a hormone and its physiological responses involves multiple components, which I have labeled the androgen control module. Steroid hormones are hydrophobic molecules which circulate in plasma predominantly attached to binding proteins (Södergard et al. 1982; Petra 1991). Only the free fraction of unbound hormones are available to bind to receptors in target cells (Sherwood et al. 2005; Hill et al. 2008). Testosterone (T) can be enzymatically metabolized by 5α-reductase into 5α-dihydrotestosterone (DHT). Testosterone and DHT are ligands that bind to androgen receptors (AR) (Heemers and Tindall 2009). Testosterone can also be

enzymatically aromatized into estradiol (E_2), thus exerting effect through estrogen receptors (ER) (Sherwood et al. 2005).

Physiological responses to steroid hormones in target tissues primarily involve modification of gene transcription. Sex steroids from circulation diffuse into the cell and bind to intracellular steroid receptors. After the hormone-receptor complex forms, it translocates to the nucleus. The complex then binds to the hormone response elements located in the promoter region (i.e., upstream) of steroid-regulated genes, which functions to modify transcription in association with other transcription factors (e.g., co-activators and/or co-repressors) and enzymes required for gene transcription (Gobinet et al. 2002; Charlier and Balthazart 2005; Tetel et al. 2009; Heemers and Tindall 2009). Androgenic versus estrogenic actions regulate transcription of different sets of genes (e.g., Pasqualini et al. 2009; Van Nas et al. 2009; Xu et al. 2012). Even the androgens T and DHT may regulate transcription of different sets of genes (Hsiao et al. 2000). Furthermore, the genes being regulated by a single sex steroid can differ in tissue-specific and sex-specific manners (Sullivan et al. 2009; Xu et al. 2012). Additionally, sex steroids not only exert effects by modifying gene transcription, but E_2 also induces rapid, short-term cellular changes by binding membrane-bound molecules such as ion channels and receptors associated with second messenger signaling pathways (Brinton and Nilsen 2001; Lange 2007; Sarkey et al. 2008).

Studies in diverse vertebrate taxa have reported many similarities in the components of the androgen control module, including the conserved distribution of AR in the brain (Kah et al. 1993; Moga et al. 2000; Gahr 2001; Rhen and Crews 2001; Rosen et al. 2002; reviewed in Guerriero 2009). However, several differences in various

components of the endocrine signaling system have been reported between sexes (Ho et al. 1987; Feng et al. 2010a; Cohen and Wade 2011) and species (Shaw and Kennedy 2002; Hews et al. 2012), as well as temporally through ontogeny (Meek et al. 1997). These reports suggest that each component of the androgen control module has the potential to change as organisms evolve (Hau 2007; McGlothlin and Ketterson 2008). The goal of this dissertation is to investigate which components of the androgen control module may have changed in species of gecko lizards that have evolved different patterns of sexually dimorphic trait expression.

Gecko lizard model system

The role of sex steroids in the expression of sexually dimorphic morphology, physiology, behavior and brain function has been extensively studied in vertebrates, revealing general principles that are taxonomically conserved but also strikingly diverse (Adkins-Regan 2005; Adkins-Regan 2012). To test hypotheses regarding the proximate causation of evolutionary differences in the sexually dimorphic phenotypes, an ideal model system would have 1) well-supported phylogenetic relationships among species, 2) the expression of a suite of sexual dimorphic traits, with 3) various combinations of traits that have been evolutionarily lost (Harvey and Pagel 1991; Gittleman et al. 1996; Martins 2000). Furthermore, 4) these species would need to be readily accessible for experimental studies. This dissertation investigates the androgenic control of sexually dimorphic morphological, physiological, and behavioral traits in a comparative study of gekkotan lizards in the families Eublepharidae and Gekkonidae. Here, I address the question of how the components of the endocrine mechanisms that underlie suites of

traits may have changed in species that evolved differences in sexually dimorphic trait expression.

Gekkotan lizards provide a suitable model system for testing hypotheses regarding proximate regulation of sexually dimorphic traits. The infraorder Gekkota is a relatively basal lineage of squamate reptiles that diverged approximately 165-225 million years ago (Kluge 1987; Vitt et al. 2003; Vidal and Hedges 2005; Jonniaux and Kumazawa 2008) from the sister group which gave rise to Serpentes, Anguimorpha and Iguania (Wiens et al. 2012; but see Losos et al. 2012; Fig. 1.2). Gekkotan lizards include approximately 1135 species in 108 genera (Han et al. 2004; reviewed by Jackman et al. 2008), and as a group are characterized by nocturnality and nasal olfaction that detects small, volatile molecules, characteristics which separate geckos from other lineages within squamate reptiles (Dial and Schwenk 1996; Vitt et al. 2003).

Gekkotan lizards are known for their diversity in sexually dimorphic traits, which include sex differences in body size, coloration, aggressive and courtship behaviors, and even vocalization, which is very rare in lizards (Marcellini 1977). Lizards of the gekkotan family Eublepharidae, known as eyelid geckos, have a well-supported phylogeny in which most species express a suite of sexually dimorphic features: namely, male-larger head and body size, aggression, courtship and functional precloacal pores, all of which are hypothesized to be ancestral traits (Kratochvíl and Frynta 2002; Fig. 1.3). In lizards, male-larger body and head size along with the expression of combative behaviors is often attributed to intrasexual selection for access to female mates (Howard 1979; Arnold 1983; Vitt and Cooper 1985; Hasagawa 2003; McCoy et al. 2003; Husak et al. 2009). Courtship displays, which in eublepharid species involve rapid vibrations of the

distal portion of the tail prior to physical contact, stimulate receptivity in females and are thought to be favored by intersexual selection (i.e., female mate choice; Crews et al. 1998). Therefore, the suite of sexually dimorphic traits in eublepharid geckos likely originated as a result of sexual selection in the common ancestor (Kratochvíl and Frynta 2007). For the comparison with a gekkotan species that is not characterized by many sexually dimorphic traits, *Paroedura picta* (family Gekkonidae) exhibit male-larger body size but lack the presence of precloacal pores and sex differences in head width. Behaviorally, males of *P. picta* express a low level of aggression, and precopulatory courtship is absent. Therefore, gekkotan lizards are characterized by a range of sexually dimorphic phenotypes, making this group particularly suitable for examining the proximate regulation underlying suites of traits, specifically with the aim of uncovering what component of the androgen control module differs among these species.

Previous studies in lizards, including the gekkotan species *Eublepharis macularius*, have demonstrated that the expression of sexually dimorphic behaviors, including courtship and aggression in males, requires concurrently elevated levels of androgens (Lindzey and Crews 1986; Moore 1987, 1988; Rhen and Crews 1999, 2000; Smith and John-Alder 1999; Tokarz et al. 2002; Weiss and Moore 2004; Kabelik et al. 2006; reviewed by Woolley et al. 2004). Elevated levels of androgens in male lizards are also required for the expression of sexually dimorphic morphological and physiological traits, including growth (Cox et al. 2005; Cox and John-Alder 2005; Cox et al. 2009) and the secretory functions of precloacal and femoral pores (Chiu et al. 1975; Ferguson 1985; Lindzey and Crews 1993; Hews and Moore 1995; Crews et al. 1998; Rhen et al. 2005;

Martin and Lopez 2011). The data suggest androgens mediate sexually-selected suites of traits which together facilitate reproductive success.

However, in some extant eublepharids, one or more of the traits from a conserved suite have been lost through evolution. For example, the ancestral suite of sexually dimorphic traits is expressed in *Goniurosaurus lichtenfelderi* (GL in Fig. 1.3) but is absent in *G. splendens* (GS). The loss of all traits from the suite in *G. splendens* suggests that androgenic control may be constrained to evolve as a self-contained module, which is either active or inactive depending on the presence of T (i.e., evolutionary constraint hypothesis; Hau 2007; McGlothlin and Ketterson 2008). The presence versus absence of this suite of traits could be caused by evolutionary change in circulating T levels between species, which predicts males of species where traits have been evolutionarily lost have low levels of T that are unable to induce trait expression. To test this hypothesis, I will compare the effect of circulating T levels on trait expression in *G. lichtenfelderi*, which express multiple sexually dimorphic traits, with *P. picta*, which express only low levels of aggression and no courtship behavior. Similar to *G. lichtenfelderi*, *Coleonyx brevis* (CB in Fig. 1.3) and *C. variegatus* (CV) also express the ancestral suite of traits for eublepharids. In contrast, males of *C. elegans* (CE in Fig. 1.3) express all ancestral traits except tail vibration courtship behavior. The evolutionarily loss of only one trait from the suite in this species suggests the change in the androgen control module may not be due to a change in the level of T, but a change in sensitivity to T in the physiological structures (e.g., brain, motor neurons, tail muscle) underlying tail vibration courtship behavior. Similar to *C. elegans*, the proximate regulation of the few sexually dimorphic behavioral traits present in *P. picta* may not be explained simply by the level of

circulating T, but rather the sensitivity to T in the brain regions mediating aggression and sexual behaviors. To test the hypothesis that differences in behavioral trait expression among gecko species is due to differences in androgen receptors (or other components of cellular sensitivity), I will compare androgen receptor immunoreactivity in the brains of *G. lichtenfelderi* and *P. picta*. Therefore, a comparative study of gekkotan lizards could be used as a phylogenetically controlled test of how the androgenic control of sexually dimorphic trait expression differs among species with different patterns of trait expression.

Androgen-mediated suites of traits in male vertebrates

Studies on the regulation of male-limited traits in all classes of vertebrate have revealed some general principles regarding temporal relations between gonadal steroids and trait expression. Early investigators dichotomized this temporal variability in terms of the organizational/activational paradigm with regard to ontogeny (Phoenix et al. 1959; Goy 1966), and in terms of associated versus dissociated reproductive patterns with regard to seasonal cycles (Crews 1984). The organizational/activational paradigm postulates that exposure to gonadal steroids during early stages of development causes permanent differentiation (i.e., organization) of male- versus female-typical physiological substrates and neural circuits, whereby subsequent exposure at the appropriate ontogenetic stage (and/or season) permits the expression (i.e., activation) of sex-specific physiological and behavioral traits within the proper social context (Phoenix et al. 1959; reviewed in Adkins-Regan 2005; Arnold 2009). In reproductively mature animals, the mating season either 1) coincides with elevated androgen levels in males that coordinate the expression of reproductive behaviors and gametogenesis (i.e., associated reproductive

pattern), or 2) males display reproductive behaviors when androgen levels are low and spermatogenesis occurs during the non-breeding season when androgen levels are high (i.e., dissociated reproductive pattern; Crews 1984; reviewed in Crews 1999).

Across vertebrate taxa, gonadal androgens mediate the expression of male-limited behavioral, physiological, and morphological traits, which together facilitate reproductive success (Adkins-Regan 2005). Many vertebrates are characterized by the associated reproductive pattern whereby the seasonal rise in circulating androgens in males correlates with the expression of a suite of sexually dimorphic traits. The suite of sexually dimorphic traits often includes sex-limited morphological traits (e.g., coloration, weaponry, and ornaments), physiological processes (e.g., production of oils or waxy substances, Ebling 1974), and behaviors such as territory defense, aggression and courtship (Andersson 1994; Badyaev 2004; Adkins-Regan 2005). These male traits serve as signals to potential female mates and potential male rivals.

Morphology: targets for steroid hormones

In all classes of vertebrates, androgens exert organizational and activational effects on several morphological traits, some of which are conserved targets, whereas others are species-specific traits. Skeletal muscle tissue is a conserved target for androgen actions (Staub and DeBeer 1997; Herbst and Bhasin 2004). Sex steroids often organize permanent sex differences by altering proliferation or cell death early in the development of a tissue, leading to sex differences in cell number (hyperplasia) and cell size (hypertrophy; reviewed in Emerson 2000). Activational effects of androgens induce hypertrophy of muscles that have a functional role in courtship and copulation (e.g., Thomas and Licht 1993; Blackburn and Bernardo 1998; Sidor and Blackburn 1998;

Holmes and Wade 2004; Feng et al. 2010b; Fuxjager et al. 2012). Androgens also exert effects in diverse morphological target tissues that are lineage-specific or species-specific (reviewed in Folstad and Karter 1992; Andersson 1994), fangs in amphibians (Emerson 2000), red face coloration in mandrills (Setchell et al. 2008), and beak color in a bird (McGraw et al 2006).

Social behavior in the brain: targets for steroid hormones

Actions of sex steroids in the brain increase the likelihood of the expression of social behaviors necessary for successful reproduction. Newman (1999) defined a hypothetical network of neural circuitry in the mammalian brain that mediates social behaviors, including aggression, parental care, mating, sexual behaviors and communication. This neural network comprises multiple limbic brain nuclei, including the lateral septum, medial extended amygdala, medial preoptic area, anterior hypothalamus, ventromedial and ventrolateral hypothalamus, midbrain periaqueductal gray, and tegmentum (Newman 1999). The amygdala processes input from the olfactory system and is critical for behavioral motivation (i.e., arousal and interest in a conspecific animal; reviewed in Hull and Dominguez 2007). The medial preoptic area (POA) receives sensory input from the medial amygdala and the bed nucleus of the stria terminalis and sends output to the hypothalamic, midbrain, and brain stem nuclei that regulate autonomic and somatomotor patterns to execute copulatory reflexes for the performance of sexual behavior (reviewed in Hull and Dominguez 2007). The ventromedial hypothalamus (VMH) is critical for the expression of sexually-receptive behaviors in females (e.g., Flanagan-Cato 2011; reviewed in Kelly et al. 1999), but is also is a part of the hypothalamic ‘aggression area’ (Kruk 1991; Kruk et al. 1983).

Experimental studies have also determined that the neural circuitry identified as the social behavior network is sensitive to steroid hormones and contains high concentrations of androgen receptors (ARs; Simerly et al. 1990; reviewed in DeVries and Simerly 2002; Cunningham et al. 2012).

Although the social behavior network was originally characterized in studies of mammals, homologous regions have been found in birds (Gahr 2001), fish (Maruska et al. 2012) and reptiles (Crews 2003; Hews and Quinn 2003; Woolley et al. 2004). In reptiles, the anterior hypothalamus (AH), preoptic area (POA) and the external nucleus of the amygdala (AME, also known as the ventromedial nucleus of the amygdala, see Rosen et al. 2002) are critical for male sexual behavior (Morgentaler and Crews 1978; Wheeler and Crews 1978; Greenberg et al. 1984; Friedman and Crews 1985; Kingston and Crews 1994). The AME receives direct projections from the olfactory system (Lanuza and Halpern 1997, 1998) and relays sensory information to the VMH (Bruce and Neary 1995; Martinez-Marcos et al. 1999). The VMH mediates female receptivity (e.g., Kendrick et al. 1995; reviewed by Whittier and Tokarz 1992) as well as aggression in males (Kabelik et al. 2008). Also similar to the mammalian findings, these brain regions in reptiles contain androgen receptors (Moga et al. 2000; Rhen and Crews 2001; Rosen et al. 2002; Hews et al. 2012) and are sensitive to steroid hormones (Morgentaler and Crews 1978; Rozendaal and Crews 1989; reviewed in Crews et al. 2009).

Androgens organize and activate neural substrates in male vertebrates

Neuroanatomical studies have shown that sex steroids organize neural substrates through cellular and molecular processes leading to the development of sex differences in cell number, connectivity between nuclei (i.e., clusters of neurons), dendritic spine

density and neuronal excitability, as well as neurotransmitter systems (Amateau and McCarthy 2002a,b; Simerly 2002; Arnold 2009; McCarthy et al. 2009; McCarthy 2010; Semaan and Kauffman 2010). During the neonatal period in male mammals, gonadal secretions of T are metabolized into E_2, which binds to estrogen receptors (ERs) in the brain to regulate major developmental processes leading to permanent sex differences in structure (reviewed in McCarthy 2010). The existence of sexually dimorphic brain nuclei was first discovered in rats (Gorski et al. 1978, 1980) and has also been shown in reptiles (reviewed in Crews et al. 2009). In the lizard *Cnemidophorus inornatus*, androgens increase the size of the POA and decrease the size of the VMH (Crews et al. 1990; Wade et al. 1993).

Although early studies indicated that organizational effects of sex steroids occur only during brief critical periods in early ontogeny (e.g., embryonic, neonatal; Feder 1967; Resko et al. 1968; Orcutt 1971; Deviche and Balthazart 1976), more recent studies indicate neural change continues through sexual maturation and into adulthood (e.g., Matsumoto et al. 1988; Nottebohm 1981; Woolley 2007; reviewed in Arnold 2009; Simerly 2002; Romeo 2003). For example, studies on the hamster *Mesocricetus auratus* have demonstrated that sex steroids are required during puberty for the development of typical sexual and social behaviors in adult animals (Schultz et al. 2004; Sisk and Zehr 2005; Schultz and Sisk 2006) and neuronal changes in the medial amygdala and POA occur during this time (Zehr et al. 2006; Schultz et al. 2009). Finding that steroids act to alter neural circuits throughout life, rather than strictly during an early critical period supports the idea that the organization/activation paradigm functions along a continuum

rather than discrete points in ontogeny (Arnold and Breedlove 1985; Romeo 2002; Arnold 2009; Schulz et al. 2009; McCarthy et al. 2009).

In vertebrates that are characterized by an associated reproductive pattern, contemporaneously elevated levels of circulating androgens are required for the seasonal activation of male reproductive behaviors including aggression, territoriality, courtship, and copulation (e.g., Tokarz 1986; Moore and Marler 1987; Moore 1988; Marler et al. 1995; Woolley et al. 2004; Fusani 2008). Activation of behaviors by androgens involves physiological responses within hormone-sensitive regions of the brain or may also be affected in part through modulatory effects on other neurotransmitter systems (e.g., Kabelik et al. 2006; reviewed in Rubinow and Schmidt 1996).

However, separate and perhaps overlapping brain regions mediate different androgen-dependent behaviors or even different components of male reproductive behaviors (Cunningham et al. 2012). For example, a recent neuroscience study determined that distinct neural pathways regulate the appetitive versus consummatory aspects of male sexual behavior in the hamster *M. auratus* (Been and Petrulis 2012). Classical ethologists made a distinction between appetitive and consummatory phases of sexual behavior (Lorenz 1950; Tinbergen 1951; Beach 1956); the former involves searching for and approaching a potential mate as well as most courtship behaviors, whereas the latter phase involves physical contact between the sexes culminating in copulation and the transfer of gametes. Although the appropriate expression of both phases of male reproductive behavior requires a functional POA, several studies including the recent report by Bean and Petrulis (2012) and references therein provide evidence to suggest that different neural pathways between brain nuclei or even different

subnuclei within the POA (e.g., Balthazart and Ball 2007) may regulate separate phases of reproductive behavior. Thus, future studies will be needed to delineate how androgens act on these neural pathways or subnuclei to mediate appetitive versus consummatory behaviors, or in modern terminology, courtship versus copulatory behaviors.

Specific aims of this dissertation

Hypothesis 1: The expression of sexually dimorphic traits is due to sex differences in circulating testosterone (T).

Aim 1: To test this hypothesis, I conducted surgical manipulations to alter levels of circulating testosterone in males and females of three gekkotan species that differ in the expression of sexually dimorphic morphology, physiology and behaviors. Specifically, *G. lichtenfelderi* expresses the ancestral suite of sexually dimorphic traits for eublepharids, *C. elegans* lost only the display of courtship behavior from that ancestral suite, and *P. picta* expresses sexually dimorphic body size and aggression but no courtship. I predicted that exogenous T activates sexually dimorphic traits in males of all species (i.e., shared proximate mechanism).

Hypothesis 2: The differences between sexes and among species in the sensitivity to T for the expression of sexually dimorphic traits are due to differences in androgen receptor distribution or abundance in the brain, specifically in regions associated with control of reproductive behaviors.

Aim 2: To test this hypothesis, I conducted an immunohistochemical investigation of androgen receptor immunoreactivity in brains collected from experimental individuals of *G. lichtenfelderi* and *P. picta*. I predicted that males of *G. lichtenfelderi*, with

aggressive and courtship behaviors, have more AR than females and also relative to males of *P. picta*.

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Figures

Figure 1.1. Schematic diagram of two physiological scenarios representing two hypotheses on the linkage between testosterone (T) and androgen-mediated traits in male vertebrates: evolutionary constraint versus evolutionary potential (see Hau 2007; McGlothlin and Ketterson 2008). Boxes indicate units on which selection may act.

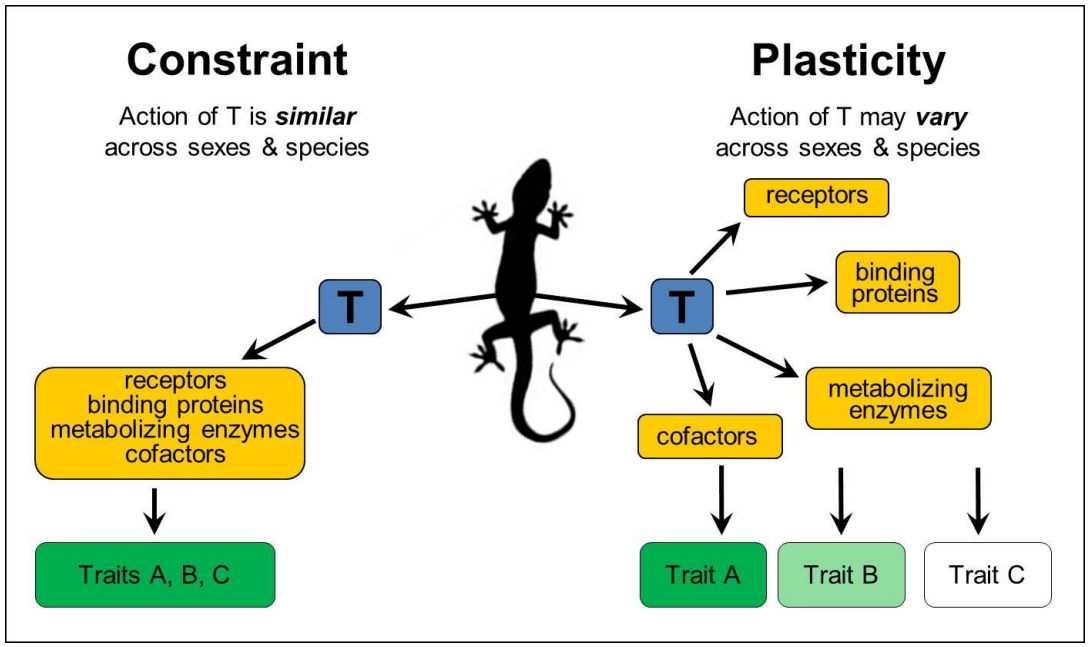


Figure 1.2. Phylogeny of squamate reptiles based on molecular data, adapted from Wiens et al. 2012. Note that phylogeny based on morphologial characteristic contrasts with this version (see Loso et al. 2012).

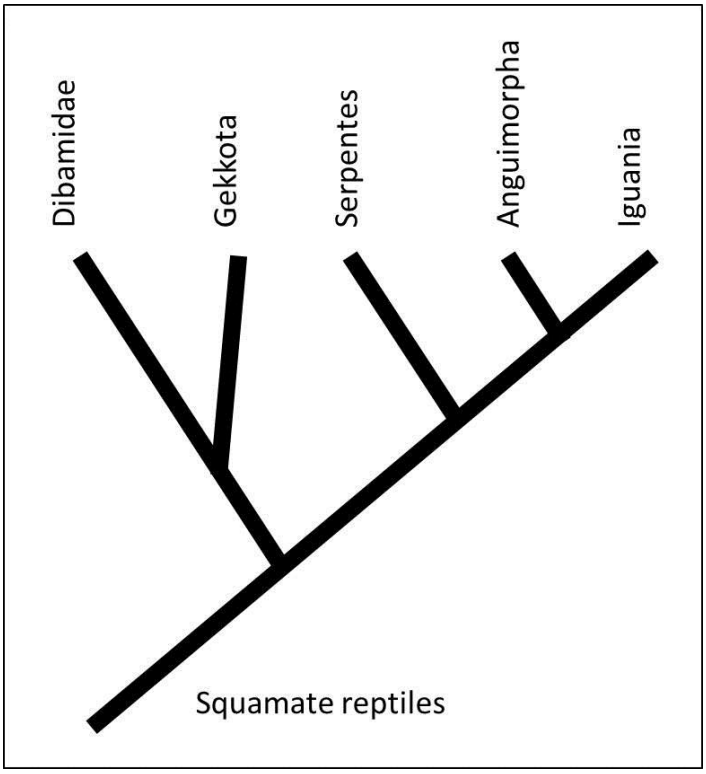
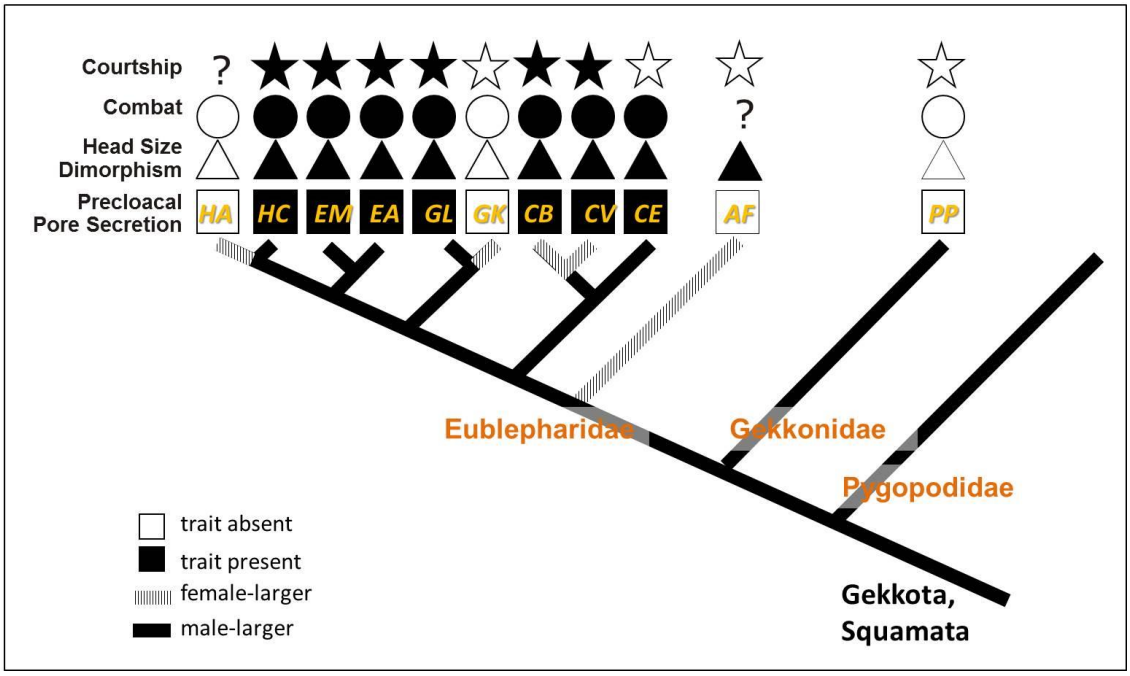


Figure 1.3. Abbreviated phylogeny of gekkotan lizards with historical analysis of sexually dimorphic traits in the family Eublepharidae. Pattern of line branching indicates evolutionary relationships. Eublepharid species names are as abbreviated: HA = *Holodactylus africanus*, HC = *Hemitheconyx caudicinctus*, EM = *Eublepharis macularius*, EA = *Eubelpharis angramainyu*, GL = *Goniurosaurus lichtenfelderi*, GS = *Goniurosaurus splendens*, CB = *Coleonyx brevis*, CV = Coleonyx variegatus, CE = *Coleonyx elegans*, AF = *Alueroscalebotes felinus*. Although family Gekkonidae is very specious, only one species (PP = *Paroedura picta*) is included in this diagram (adapted from Kratochvíl and Frynta 2002).



CHAPTER 2

EFFECTS OF TESTOSTERONE ON THE EXPRESSION OF SEXUALLY DIMORPHIC TRAITS IN THE YUCATAN BANDED GECKO (EUBLEPHARIDAE: *COLEONYX ELEGANS*)

Abstract

Lizards from the family Eublepharidae (eyelid geckos) have a well-supported phylogeny and exhibit a suite of sexually dimorphic morphological, physiological and behavioral traits, which are expressed in males. The full suite of traits, including male-larger head and body size, functional precloacal pores, aggressive behavior between males, and both the courtship and copulatory phases of sexual behavior, is hypothesized to be ancestral, but one or more traits have been evolutionarily lost in some species. In *Coleonyx elegans*, for example, males fail to display courtship behavior but express all of the other traits in the ancestral suite. To investigate the underlying mechanism of trait expression and the selective loss of courtship, I studied the control of sexually dimorphic traits by testosterone (T) in males and females of *C. elegans*. I hypothesized that sex differences in trait expression would be due to differences in circulating levels of T, and that the selective loss of courtship from the sexual repertoire would be due to a specific alteration in the endocrine control module rather than a general reduction in the availability of T. Experiments included 3 groups of males (sham-operated intact control, surgically castrated, castrated with T replacement) and 2 groups of females (intact control, T supplemented) conducted over an 11-week period in the laboratory. In males, T activated precloacal pores and aggressive behavior, but sexual behavior was not

affected by castration or replacement of T; courtship was not induced, and copulatory behavior was not altered. In females, T induced the expression of male-typical copulatory behavior, precloacal pore activity, and enlargement of head width, but failed to activate aggressive behavior and, as in males, did not induce courtship. These data indicate that the absence of courtship from the sexual repertoire of *C. elegans* must involve a selective loss of sensitivity to T. Furthermore, sexual dimorphism in the expression of traits is due in most cases to sex differences in the availability of T. However, females appear to have evolved a selective loss of sensitivity to T in the neural substrate responsible for aggression. Thus, the endocrine control module that regulates the correlated suite of male-typical traits in eublepharids is largely the same in *C. elegans* and *E. macularius*, but the inability of T to induce courtship in both sexes and aggression in females of *C. elegans* indicates that specific components of the control module have the potential to evolve independently.

Introduction

Gonadal hormones and their metabolites coordinate the expression of suites of sexually dimorphic morphological, physiological, and behavioral traits in all classes of vertebrates, including reptiles. These functionally correlated traits may be constrained from independent evolutionary changes because of their common proximate regulatory mechanism (i.e., evolutionary constraint hypothesis, Hau 2007; McGlothlin and Ketterson 2008). Alternatively, components of the common regulatory pathway may not be tightly linked and may have the ability to evolve independently, allowing evolutionary change in the expression of one or more traits within a suite (i.e., evolutionary potential

hypothesis, Hau 2007; McGlothlin and Ketterson 2008). Lineages characterized by interspecific differences within suites of correlated traits are especially suitable to investigations how alterations in the hormonal control module could allow loosening or severance of the linkage between functionally related traits. Eublepharid gecko lizards (Eublepharidae; eyelid geckos) provide an advantageous model system to investigate this question. The accepted phylogenetic hypothesis of eublepharids is well-supported, and most species exhibit a suite of correlated sexually dimorphic traits that are hypothesized to be ancestral (Kratochvíl and Frynta 2007). Compared to females, males of most species exhibit larger head and body size, secretions from precloacal pores, precopulatory courtship, and aggressive behaviors. Interestingly, through the course of evolution, some of these traits have been lost in some species, including *Coleonyx elegans* (Yucatan banded gecko). Species with selective losses from the ancestral suite of traits allow investigations on how changes in the proximate mechanisms may have enabled the dissociation of one or more traits from a conserved suite.

Males of *Coleonyx elegans* express most of the ancestral sexually dimorphic traits, but they do not exhibit precopulatory courtship behavior. In contrast, males of most eublepharid species express precopulatory courtship that involves rapid, audible tail vibrations and deposition of secretions from precloacal pores onto the substrate (Gutzke and Crews 1988; Brillet 1993; Fig. 2.1). In *C. elegans*, male sexual behavior is reduced to the consummatory (or copulatory) phase, which includes biting the female's neck (i.e., body grip) prior to mounting her for copulation (Kratochvíl and Frynta 2007; Fig. 2.1). Hormonal control of sexually dimorphic traits including mating behaviors has been studied in one eublepharid species, *Eublepharis macularius* (Leopard Gecko), which

displays the ancestral behavioral repertoire including courtship prior to the copulatory phase. Surgical castration and hormone replacement in *E. macularius* demonstrated that sexually dimorphic traits including courtship behavior are dependent on elevated androgen concentrations typical of mature males (Rhen and Crews 1999; Rhen et al. 2005).

To investigate the underlying mechanism of trait expression and the selective loss of courtship, I studied the regulation of sexually dimorphic traits by testosterone (T) in males and females of *C. elegans*. I hypothesized that sex differences in trait expression would be due to differences in circulating levels of T, and that the selective loss of courtship from the sexual behavior repertoire would be due to a specific alteration in the androgen control module rather than a general reduction in the availability of T. The hypothesis on interspecific differences predicts castration reduces trait expression and T-replacement restores it in males, except courtship is not sensitive to T and cannot be induced by exogenous T. The hypothesis on intersexual differences predicts that sexually dimorphic traits can be induced by T-supplementation in females.

Material and methods

Animals

Coloenyx elegans are small, nocturnal ground-dwelling lizards that live in leaf litter of tropical forests in the Yucatan peninsula (Dial and Grismer 1992; Luja et al. 2008). Eggs from the captive breeding colony in the laboratory of Lukáš Kratochvíl at Charles University were collected within 24 h of oviposition. Eggs were placed in individual cups filled with moist vermiculite and incubated at 28°C. Upon hatching,

animals were individually housed with a substrate of coconut husk, a water dish, and a shelter. The animal facility was maintained on a 12L:12D photoperiod with lights off at 1700 h, ambient temperature set at 26°C and relative humidity of approximately 60%. Animals were fed crickets dusted with calcium powder (Roboran, Univit, Czech Republic) three times per week. Drinking water was supplemented with vitamins A, D_3 and E once every two weeks. Animals were treated in accordance with research protocols approved by the Czech Central Commission for Animal Protection (protocol #18847/2003-1020) and the Rutgers University Animal Care and Facilities Committee (protocol #01-019).

Coleonyx elegans reach sexual maturity at approximately one year of age (Holfert and Seufer 2005). With the approval from the U.S. Fish and Wildlife Service, animals between the ages of 14 and 18 months were transported to the laboratory at Rutgers University, where they were individually housed in plastic cages (59 x 43 x 46 cm) and maintained as described above.

Surgical and hormone treatments

Lizards of each sex were ranked by snout-vent length (SVL, mm) and were sequentially assigned to different groups, resulting in very similar size distributions among groups ($F_{4,49}$=1.58, p=0.20). Males were assigned to one of three treatment groups: sham-operated intact control (MCON: n=11, mean SVL=71.2 ± 1.4 mm), surgically castrated (MCAST: n=11, 70.7 ± 1.3 mm), and castrated with T replacement (MTEST: n=11, 70.4 ± 1.2 mm). Females were assigned to one of two treatment groups: intact control (FCON: n=11, 73.4 ± 1.0 mm) and intact with T supplement (FTEST: n=11, 73.4 ± 0.8 mm). Prior to surgery, the reproductive status of females was

ascertained by visual inspection of follicles through the abdominal wall (see Rhen et al. 2000). For surgery, animals were anaesthetized with an intramuscular injection of ketamine (Vetus Animal Health, MFA Inc., Columbia, MO, USA; 130 μg/g body mass). In males, the testes were exposed via a medial ventral incision. Bilateral castration (orchiectomy) was done on both MCAST and MTEST groups by ligating each spermatic cord with surgical silk, then ablating each testis. For control males (MCON) and all females (FCON and FTEST), "sham" surgeries were performed, in which ventral incisions were made to expose and manipulate the testes or ovaries while leaving the gonads completely intact. Empty implants (MCAST, MCON, FCON) or T implants (MTEST, FTEST) were then inserted into the coelomic cavity, and the incision was closed with Nexaband® surgical glue (Closure Medical Corporation, Raleigh, NC, USA). Tonic-release T implants were constructed as described in Cox et al. (2005). Briefly, implants consisted of Silastic® tubing (Dow Corning, 0.058'' i.d., 0.077'' o.d.) containing 300 μg of crystalline T. Total length of each implant was 4 mm. Crystalline T was contained in the lumen of about 1.5 mm in length. Animals were allowed five weeks to recover prior to the start of behavioral trials. Hormone treatments were verified by assaying plasma T levels after completion of all experimental procedures (see below).

Behavioral trials

Behavioral trials were begun five weeks after surgical manipulations and were completed over the course of five weeks. First, sexual behavior of experimental animals was assessed in response to the introduction of a stimulus animal of the opposite sex. In these trials, control males (MCON) and females (FCON) were used as the stimulus animals. Sequential trials involving the same stimulus animal were separated by a

minimum of 48 hours. Second, aggressive behavior was assessed in response to opponents of the same sex. In these trials, behavioral responses of members of all treatment groups were assessed in response to experimental animals of all treatments of the same sex. All behavioral trials and subsequent analyses were conducted without the observer having knowledge of experimental treatments. *Coleonyx elegans* is nocturnally active, thus all behavioral trials were conducted between 1730 and 2030 h under dim red light. Trials were monitored in real time from a second room via closed circuit television to reduce disturbance to the animals. Each trial was video-recorded (Sony Handycam® DCR SR42) and subsequently analyzed for the occurrence and duration of observed behaviors using JWatcher™ Video software (Dan Blumstein's Laboratory, UCLA and The Animal Behavior Laboratory, Macquaric University, Sydney).

Sexual behavior trials

To minimize the effect of novelty and maximize the likelihood of sexual responsiveness, each male was presented with a stimulus female in his home cage (Crews et al. 1978; Sakata et al. 2002b). Five minutes prior to the start of the trial, the water dish and shelter were removed from the home cage of the male. Subsequently, a stimulus female was introduced into the male's home cage and remained there for 15 minutes or until mounting position was achieved. The dyads were interrupted at this point to prevent intromission and reduce the potential for ovulation in the stimulus female, allowing them to remain receptive in subsequent trials (Rhen et al. 2000). Sexual behavior of experimental males was recorded, including body grip and mount (see Table 2.1).

Agonistic behavior trials

One week after the completion of sexual behavior trials (seven weeks post-treatment), same-sex trials were begun to assess the behavior of each experimental animal in all treatment groups in response to opponents of all treatment groups of the same sex. Males of *C. elegans* are highly aggressive toward conspecific males. Trials were conducted in a neutral arena (glass aquarium, 77 x 32 x 31 cm) to eliminate the potential for residency biases. Each experimental male experienced a total of three trials with a male opponent, one from each of the three different treatment groups (i.e., MCON, MCAST, and MTEST). The order of pairing in trials was randomized. At the start of a trial, two males were placed into the neutral arena on opposite sides of an opaque central divider. After five minutes, the divider was lifted via a remote pulley system, and behaviors were observed in each male for a maximum of 15 minutes. Trials were terminated early when mounting occurred and when behaviors appeared to be highly stressful (i.e., repeated fleeing by one individual) or potentially injurious. The neutral arena was cleaned with 70% ethanol between trials. Behavioral responses of each experimental female were similarly assessed during trials with opponents of both female treatment groups (i.e., FCON, FTEST) following the same protocol described for males.

Digital recordings were viewed and analyzed for the occurrence and duration of behavioral responses of each experimental animal in the dyads. Informative behaviors are listed in Table 2.1. Note that "agonistic" behavior refers to all behaviors associated with the contest or struggle between two animals, including the threat (i.e., high posture), attack, flee, approach, and retreat (King 1973). The role of aggressor versus defender often alternates between two individuals in a dyad during agonistic interactions (King

1973). The term "aggression" refers to the initiation and attack phase of the agonistic encounter (King 1973).

Morphological measurements

Body mass (g), snout-vent length (SVL, mm), head width (mm), and hemipenes width (nearest mm) were measured in each animal prior to surgical manipulations and after completion of behavioral trials, 11 weeks after surgical treatments. At these two time points, digital images of the dorsal and ventral surfaces of each lizard were made with a desktop digital scanner. In this species, precloacal pores are present on the ventral surface anterior to the cloacal vent, and scanned images were used to quantify the number of active pores. The presence of waxy secretions was used as the indicator of pore activity.

Radioimmunoassay (RIA)

After completion of all behavioral trials (11 weeks after surgeries), blood samples were collected in heparinized microhematocrit capillary tubes from trunk and neck wounds following rapid decapitation. Whole blood was held on ice for a maximum of 4 hours and then centrifuged at 1500 rpm for 10 min at 4°C. Plasma was stored in plastic microfuge tubes at -20°C until assayed for T. Radioimmunoassay (RIA) for plasma T followed the methods of Smith and John-Alder (1999). Samples were extracted twice in diethyl-ether, dried under a stream of ultra-filtered air, and reconstituted in phosphate-buffered saline with gelatin (PBSG, pH 7.4). Reconstituted samples were assayed with ^{3}H-T (Perkin Elmer Life and Analytical Sciences Inc., Waltham, MA, USA) and T antiserum developed in rabbits by A. L. Johnson (The University of Notre Dame, Indiana, USA). Recoveries averaged 79%. Samples were processed in a single assay

with a detection limit of 4.8 pg T. Typical intra-assay variation is 6.7% (Smith and John-Alder 1999).

Statistical Analysis

Statistical analyses were conducted on behavioral data from all subjects with successful experimental treatment, which was verified via plasma hormone concentrations or, in one case where the plasma sample was lost from a male in the MTEST group, by the presence of the T implant in the body cavity at termination. All statistical analyses were conducted using SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) or GraphPad Prism (version 5.00; GraphPad Software, San Diego CA USA).

Plasma T levels were analyzed using one-way ANOVA with the Ryan-Einot-Gabriel-Welsch multiple range test for post hoc analysis. The number of active pores was analyzed using a non-parametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons post hoc test, and further comparisons between groups were made using Mann-Whitney U test. Statistical significance was accepted at $\alpha = 0.05$ based on the H-statistic, which is the non-parametric analogue of an F-statistic. One-way ANOVA was used to analyze treatment effects on mass of the hemipenes and snout-vent length. ANCOVA was used to analyze the main effect of treatment on head width, using snout-vent length as the covariate.

For analysis of behavioral trials with a stimulus female, a Fisher exact test or maximum-likelihood χ^2 test was used for comparisons of the presence/absence (i.e., illustrated as percent of trials) of sexual behavior displayed by different treatment groups.

Principal components analysis (PCA) was used to reduce the dimensionality and to help identify informative behavioral traits during trials between two experimental

males. To ensure that no skew in interpretation of the data was created by use of data from both male opponents in each dyad, I performed a repeated subsampling randomization analysis. I verified that the variance in the computed impact of each factor from the PCA was relatively unaffected (i.e., no effect beyond that expected by limiting sample size) by any sub-designation for groups of trials. Therefore, results reported are from the PCA of the full dataset (n=86), regardless of the behavioral role of the male opponents in each dyad. The first three axes of the PCA explain a combined total of 53.5% of variation in the primary data, and the trait loading patterns of these axes were most amenable to interpretation. Thus, only these three axes are reported here. For each of these analyses, the Dunn-Sidak method was used to control for experiment-wise error level at α=0.05. Effects of treatment on PCA axis scores were analyzed using one-way ANOVA with Ryan-Einot-Gabriel-Welsch multiple range post hoc test.

Results

Plasma testosterone

Measurement of T from plasma samples verified the efficacy of experimental manipulations (ANOVA: $F_{4,48}$=80.4, P<0.0001; Fig. 2.2). Plasma T in MCON was high with large variation among individuals (n=11; mean 75.18 ± 25.3 ng/ml; range 9.40-228.40 ng/ml). Plasma T was significantly reduced by surgical castration (MCAST n=11; 1.19 ± 0.06 ng/ml) and restored to levels of controls by T-replacement in 10 of 11 MTEST (n=9 with measured plasma T levels; 41.8 ± 7.98 ng/ml). In females, intact controls had low levels of T (FCON n=11; 1.34 ± 0.22 ng/ml) and T-supplementation significantly increased plasma T in 9 of 11 individuals (FTEST n=9; 22.5 ± 4.9 ng/ml) to

physiological levels of control males. One of 11 MTEST died following surgery and two of 11 FTEST lost their implants at an unknown point during the experiment. Thus these three animals were excluded from all subsequent analyses.

Morphological measurements

The production of waxy secretions from precloacal pores is limited to males in *C. elegans*, and at the end of the 11-week treatment period MCON had an average of 6 active pores while no pores were active in FCON (Mann-Whitney U: U=-3.25, P<0.005; Fig. 2.3A). The number of active precloacal pores was reduced in MCAST (U=2.33, P=0.02) and restored by T-replacement in MTEST compared to MCON (U=-0.28, P=0.78). In females, T-supplementation (FTEST) increased the number of active pores to levels of MCON (Kruskal-Wallis: H=25.9, P< 0.0001).

Testosterone treatment significantly affected the mass of the hemipenes in both males and females (ANOVA: $F_{4,46}$ =280, P< 0.0001; Fig. 2.3B). The mass of the hemipenes was reduced by castration (MCAST) and restored by T-replacement (MTEST) to the size of intact (MCON) males. Among the three groups of males, the width of the tail base, a reflection of the size of the hemipenes, was significantly reduced in MCAST (ANOVA $F_{2,28}$=4.17, P=0.026). The hemipenes were undetectable in intact females (FCON) but significantly increased by T-supplementation (FTEST).

As a species, *C. elegans* is characterized by male-larger head size dimorphism. In this experiment, head width was not significantly larger in males (MCON mean=13.91mm) than in females (FCON mean=13.45mm; two tailed t-test, t=1.99, P=0.061), but was affected by treatment in females (ANCOVA: $F_{4,46}$=4.0, P=0.007; Fig. 2.3C). The comparison between female groups indicates that head width was

significantly larger in T-supplemented (FTEST) than in intact (FCON) females ($F_{1,19}$=11.6, P=0.003), whereas the three male groups were similar in size ($F_{2,28}$=1.56, P=0.228).

As a species, *C. elegans* is characterized by male-larger body size (snout-vent length, SVL). In the present experiment, SVL did not significantly differ between sexes or among treatment groups ($F_{4,46}$=1.10, P=0.368; Fig. 2.3D).

Sexual behavior

During trials between a male and a female of this species, the male rapidly approaches, bites the female usually near the neck (i.e., body grip) and mounts for copulation (Fig. 2.1). Precopulatory displays, including tail vibration and scent-marking are completely absent from the behavioral repertoire of *C. elegans* (also see Golinski et al. 2011; Kratochvíl and Frynta 2007).

Males from all treatment groups were observed to approach, body grip and mount the female introduced into their home cages, and 30 out of 32 trials were terminated to prevent copulation. The proportion of males exhibiting the copulatory behaviors of body grip and mount was not statistically different among treatment groups (maximum likelihood χ^2: P> 0.95; Fig. 2.4). To verify the persistence of sexual behavior observed in MCAST, a few individuals were randomly selected for a second trial with a female conducted 5 weeks after the initial trials (i.e., 10 weeks after castration). Once again, these castrates mounted and attempted to copulate with the female.

Male-typical sexual behavior was very rarely exhibited by control females (FCON), but T supplementation significantly increased the proportion of females exhibiting male-typical sexual behavior toward control females (Fisher exact test:

P=0.0011; Fig. 2.4). Only one of 10 FCON females displayed male-typical body grip, whereas 8 of 9 FTEST females displayed this behavior. Seven of those FTEST females proceeded to mount for copulation, and upon interruption of these trials, it was observed that some FTEST females had everted their hemipenes.

Agonistic behavior

Males of *C. elegans* are highly aggressive toward conspecific males, as seen in the results from the principal components analysis (Table 2.2). The first three axes of the PCA explain a cumulative total of 53.5% of the variation in the primary data. The first axis of the PCA explains 22.1% of the variation and contrasts sexual behaviors (i.e., body grip, jerk, mount) with agonistic behaviors (i.e., flee, high posture, offensive attack, tail slap). Note that the term agonistic refers to all behaviors associated with the contest or struggle between two lizards, whereas aggression refers to the initiation and attack phase of the agonistic encounter (King 1973). The second axis explains 16.7% of variation and contrasts aggressive behaviors (i.e., high posture, offensive attack) from defensive behaviors (i.e., defensive attack, flee). The third axis of the PCA explains 14.7% of variation and contrasts general social behaviors (i.e., tail wave, watch) often displayed by lizards of either sex during social interactions, from submissive behaviors (i.e., flee and tail slap).

During trials with a control male opponent, the first axis of the PCA indicates that males from all treatment groups responded with agonistic behaviors ($F_{2,28}$=2.86, P=0.0742; x-axis in Fig. 2.5 and Fig. 2.6A). During these same trials, the second axis of the PCA indicates that MCON and MTEST males displayed aggressive behaviors whereas MCAST displayed defensive behaviors ($F_{2,28}$=13.65, P<0.0001; y-axis in Fig.

2.5 and Fig. 2.6B). The third axis of the PCA did not differentiate among the male groups ($F_{2,28}$=0.08, P=0.92).

In trials involving two males, the treatments of two opposing males had a significant effect on the types of behaviors displayed. Males of all treatment groups responded agonistically toward MCON and MTEST opponents, whereas males tended to display sexual behavior toward MCAST opponents (Fig. 2.6A). Control (MCON) and T-replaced (MTEST) males were aggressive in trials with an MTEST opponent, while MCAST were consistently defensive ($F_{2,22}$=14.61, P<0.0001 Fig. 2.6B). Similarly, MCON and MTEST tended to behave more aggressively than MCAST toward MCAST opponents, but here the difference among groups was not significant ($F_{2,27}$=3.23, P=0.0551).

Females rarely displayed aggressive behavior, and T supplementation only slightly increased the likelihood that females would behave aggressively. Intact females (i.e., FCON) never displayed aggressive behaviors, such as high posture or attack, toward females or males. Only two of nine FTEST females displayed high posture but none of them attacked an intact male.

Discussion

Effect of androgens on courtship behavior

I hypothesized that sex differences in trait expression would be due to differences in circulating levels of T, and that the selective loss of courtship from the sexual repertoire would be due to a specific alteration in the endocrine control module rather than a general reduction in the availability of T. In support of these hypotheses, intact

males exhibit high and variable levels of circulating T (Fig. 2.2), which mediates the expression of most sexually dimorphic traits, and courtship behavior could not be induced by exogenous T. The effectiveness of experimental treatments was verified by measuring plasma T, which was reduced to undetectable levels by surgical castration and was restored via T implants to levels within the physiological range of intact males of this species. Furthermore, the observed changes in androgen-sensitive physiological (e.g., activity of precloacal pores) and morphological (e.g., size of the hemipenes) traits indicate the effectiveness of exogenous T implants. Therefore, the data presented here do not support the hypothesis that a change in circulating levels of T is associated with the evolutionary loss of courtship in this species.

In *C. elegans*, elevated levels of T were required for the expression of all other sexually dimorphic traits (i.e., head width, pore secretions, aggression), similar to results from *E. macularius* (Rhen and Crews 1999, 2000; Rhen et al. 2005). Therefore, the data suggest the androgenic control of the suite of sexually dimorphic traits is generally conserved in a species that express different combinations of traits within their individual suites.

Androgen control of copulatory behavior

In the present study, copulatory behavior in mature males of *C. elegans* was not significantly affected by manipulations of T for up to 10 weeks following treatments (Fig. 2.4). Male sexual behavior persists following castration in some mammals (25 weeks, Clemens et al. 1988; 7 weeks, Costantini et al. 2007). In lizards, however, previous studies have reported a significant decline in male copulatory behavior within a few weeks after castration (2 weeks *Anolis carolinensis* Crews et al. 1978, Neal and Wade

2007; 24 days *A. sagrei* Tokarz 1986; 3 weeks *Cnemidophorus inornatus* Lindzey and Crews 1986). In the present study, behavioral trials were initiated five weeks after experimental treatment. In our design, all experimental individuals were socially naïve to preclude the possibility that prior social experience might prolong the maintenance of sexual behavior after castration (Phelps et al. 1998*;* Costantini et al. 2007), although the effect of prior sexual experience did not significantly affect the frequency of copulatory behaviors in *E. macularius* following castration (Sakata et al. 2002a,b; Sakata and Crews 2004).

In a field study, some castrated males of the lizard *Sceloporus jarrovi* continued to express courtship and copulatory behavior toward females introduced to their home territory three weeks after surgery (Moore 1987). This observation suggests the persistence of copulatory behavior in castrated males of *C. elegans* may be associated with the males' familiarity with the home cage. Similar retention of sexual behavior in castrates has been observed in some laboratory rodents (Wee and Clemens 1989), but this type of home-cage effect had not previously been reported in lizard species studied in the laboratory (Crews et al. 1978; Lindzey and Crews 1986; Tokarz 1986; Sakata et al. 2002b, Ch. 4). In my subsequent study on the eublepharid lizard *Goniurosaurus lichtenfelderi* (Ch. 3), males from all treatment groups displayed similar levels of courtship and copulatory behaviors during trials in the home cage, but these behaviors were significantly reduced in castrated males during trials conducted in a neutral arena. Thus, the persistence of sexual behavior following castration of *C. elegans* may have been influenced by a home-cage effect. If trials in the present study had been conducted

in a neutral arena, sexual behavior may not have persisted for such a long period of time following castration.

In the eublepharid *E. macularius*, castration reduced courtship but not body grip behavior in males during trials conducted in a neutral arena (Rhen and Crews 1999). In a subsequent study, males of this species were observed to express copulatory behaviors (i.e., body grip and mount) in repeated trials over several weeks after castration (Sakata et al. 2002a). However, a more recent study indicated that castration significantly decreased the frequency of body grip and mounts during repeated trials (Sakata and Crews 2004). These inconsistencies in reports from *E. macularius* indicate the duration of persistence is variable, even within a species, and somewhat dependent on experimental circumstances. Collectively, the data from geckos suggests that male sexual behaviors can persist for a period of time, but likely declines at some point in the absence of elevated plasma T.

In females of *C. elegans*, exogenous T promoted the expression of male-typical copulatory behavior (Fig. 2.4). Supplementation with T significantly increased the likelihood that a female of *C. elegans* would body grip and mount an intact female, consistent with findings in other lizards (e.g., Adkins and Schlesinger 1979; Ch. 4). The observation that females, even as adults, retain the ability to express male-typical sexual behaviors suggests that sex-limited organization of neural circuits that underlie these behaviors does not occur during an early developmental period. Furthermore, the expression of mounting behavior by adult females of *C. elegans* suggests that these neural circuits are sensitive to androgens (e.g., have androgen receptors).

Androgen control of aggression

Agonistic interactions are typical during trials between two males of *C. elegans*, and elevated levels of T are necessary for the expression of aggressive behaviors (e.g., high posture and attack). This finding is consistent with reports from other species of lizards, and for vertebrates in general (Moore 1987; Rhen and Crews 2000; Weiss and Moore 2004; Kabelik et al. 2006; reviewed in Adkins-Regan 2005; Trainor et al. 2009). It is important to note that steroid hormones do not cause aggression per se, but modulate the motivation for expression of aggressive behaviors given the appropriate behavioral context (Nelson 2005; Kabelik et al. 2008). Although the plasma T in castrated males with T-replacement (i.e., MTEST) was somewhat lower than in control males at the end of the treatment period (Fig. 2.2), the level of aggressive behaviors were similar between these two groups (Figs. 2.5, 2.6). The display of aggressive behaviors (e.g., dewlap extension) in the lizard *Anolis sagrei* was not different from levels of intact males when castrates were treated with a moderate or high dose of T, suggesting a threshold level of plasma T is required to produce a maximal behavioral response (Tokarz et al. 2002). A threshold likely exists in males of *C. elegans*, whereby the T-implants used in the present study were sufficient to achieve such levels required to elicit behavioral responses given the proper context.

Aggressive behavior is expressed only by males of *C. elegans.* Long-term supplementation with T in adult females only induced expression of male-typical threat display (i.e., high posture) in two individuals and no females ever physically attacked a male. This result is in striking contrast to the dramatic increase in male-typical copulatory behaviors displayed by females supplemented with T. Although adult females

retain androgen-sensititive circuits for male-typical sexual behavior, these data suggest that the circuits for aggressive behaviors are not functional, or at least are not sensitive to T. The observed T-insensitivity of aggression in mature females suggests that the capacity to respond aggressively has to be organized early in development and that the neural circuitry responsible for aggressive behavior cannot be reorganized in adults following reproductive maturity.

Androgen control module: comparison between two eublepharid species

From an evolutionary perspective, the lineages that gave rise to *C. elegans* and *E. macularius* diverged from a common ancestor approximately 150 million years ago (Jonniaux and Kumazawa 2008). The early divergence of these genera may have led to genetic divergence, random genetic drift, differences in environmental conditions and selection pressures (Garland and Adolf 1994). These or other evolutionary factors may have had a role in shaping the observed differences in biological characteristics, specifically in breeding phenology and mode of sex determination. In terms of breeding phenology, *E. macularius* breeds seasonally, whereas *C. elegans* breeds opportunistically. In terms of mode of sex determination, *E. macularius* is characterized by temperature-dependent sex determination (Viets et al. 1993), whereas *C. elegans* has genetic sex determination (Pokorna et al. 2010). Despite these differences, males of *C. elegans* and *E. macularius* share androgenic control of most sexually dimorphic traits, whereby the size of the hemipenes, precloacal pore secretions, aggressive and sexual behaviors require activational effects of elevated levels of circulating T.

The main difference between *C. elegans* and *E. macularius* is the loss of tail vibration courtship behavior in the former. Tail vibration could not be induced by T-

treatment in males or females in the present study, indicating this behavior has become dissociated from the T control module. Males of *C. elegans* have high levels of circulating T. Insensitivity of tail vibration to circulating T suggests a selective change in components of the androgen control module that regulate the expression of this behavior (e.g., change in AR in the brain or motor neurons or tail muscle). I hypothesize that an evolutionary change in neural and/or tissue sensitivity to activational effects of T has enabled the dissociation of the tail vibration courtship behavior from the conserved suite of traits.

Alternatively, in eublepharid species that express courtship, organizational effects in the brain of males may be necessary for the subsequent activation of the behavior in adulthood. Finding that aggression cannot be induced in adult females of *C. elegans* suggests that organizational effects occur in the brain of this species. Thus, the potential exists for changes in organizational processes leading to evolutionary differences in brain and behavior, such as the loss of courtship. If tail vibration behavior is organized during development in the male brain, then T-supplementation should not induce this behavior in adult females. In *E. macularius*, tail vibration can be induced in females, suggesting sex-limited organization of tail vibration behavior does not occur in this species (Flores and Crews 1995). However, organizational processes in the brain can potentially be affected by differences in mode of sex determination. In species with temperature-dependent sex determination, the windows for the processes of sex specification, determination and differentiation overlap; whereas these three processes are fixed and sequential during the development in species with genetic sex determination (Valenzuela 2008). It is thought that this developmental difference allows the relatively earlier

expression of sexually dimorphic traits in species with genetic versus temperature-dependent sex determination (Valenzuela 2008). Future studies will be needed to determine whether organizational and activational effects are necessary for the expression of tail vibration courtship in species with genetic sex determination. If organizational effects are necessary, then the loss of tail vibration in *C. elegans* may be explained by changes during development rather than changes in T-sensitivity in adulthood.

Summary

To address intersexual differences in trait expression, I hypothesized that sex differences in trait expression would be due to differences in circulating levels of T and predicted that male-typical traits can be induced by T-supplementation in females of *C. elegans*. Females were highly sensitive to T treatment, which induced the expression of all male-typical morphological, physiological and behavioral traits except aggression. Therefore, sexual dimorphism in the expression of traits in this species is due in most cases to sex differences in the availability of T, but females appear to have evolved a selective loss of sensitivity to T in the neural substrate responsible for aggression.

To address interspecific differences in trait expression, I hypothesized that the selective loss of courtship from the sexual repertoire in *C. elegans* would be due to a specific alteration in the androgen control module rather than a general reduction in the availability of T. In support of this hypothesis, intact males had high and variable levels of plasma T, which was required for the secretory activity of precloacal pores and expression of aggressive behaviors. In comparing *C. elegans* to *E. macularius*, the androgenic control mechanism has been retained for the expression of sexually dimorphic traits that are present. Between species, differences in level of T do not explain presence

versus absence of courtship. Therefore, a specific alteration in some other component of the endocrine signaling system must underlie the selective loss of sensitivity to T of tail vibration courtship behavior in *C. elegans.* Future studies are needed to determine which components have the ability to enable the dissociation of one or more traits from a conserved suite.

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Tables

Table 2.1. Informative behaviors displayed by *C. elegans* during social interactions between two experimental males. These behaviors describe the experimental lizard responding to the opponent lizard. Counts or durations of these behaviors were entered into principal components analyses (see statistical methods).

Behavior	Description
Approach	Directed movement toward opponent animal
Body grip	Bite and grip the opponent animal, usually near the neck
Body roll	Bite and grip the opponent and twist causing both lizards to rapidly roll over
Chase	Rapidly pursues a moving opponent
Defensive Attack	Rapidly bites in response to attack initiated by the opponent animal
Flee	Rapid and frantic movement away from the opponent animal
High posture	Extends limbs to stand tall; threat display
Jerk	Movement of the head in short, rapid jerks, typically occurring after body grip
Mount	Straddles and attempts copulation
Offensive Attack	Initiates aggression with a rapid bite, but does not grip the opponent
Tail slap	Attack the opponent using rapid side-to-side movements of the tail
Tail wave	Tail lifted high above body with waving motion
Watch	Orientation of the head toward the opponent animal

Table 2.2. The principal components analysis (PCA) of behaviors displayed during trials between two experimental male lizards; the first three axes explain a combined total of 53.5% of the variation in the primary data. The first axis contrasts sexual behaviors (i.e., body grip, jerk, mount) and agonistic behaviors (i.e., flee, high posture, offensive attack, tail slap). The second axis contrasts aggressive behaviors (i.e., approach, high posture, offensive attack) from the defensive behaviors (i.e., defensive attack, flee). The third axis contrasts general social behaviors (i.e., tail wave, watch) from submissive behaviors (i.e., flee, tail slap).

Variable	First Axis		Second Axis		Third Axis	
	Coefficient	Significance	Coefficient	Significance	Coefficient	Significance
Approach (min)	-0.174	0.109	**0.632**	**<0.0001**	0.147	0.178
Body grip (min)	**0.786**	**<0.0001**	0.179	0.100	-0.176	0.104
Chase	0.287	0.007	0.264	0.014	0.095	0.382
Defensive attack	-0.180	0.097	**-0.429**	**<0.0001**	-0.177	0.103
Flee	**-0.379**	**0.0003**	**-0.549**	**<0.0001**	**-0.428**	**<0.0001**
High posture (min)	**-0.470**	**<0.0001**	**0.656**	**<0.0001**	-0.007	0.946
Jerk	**0.692**	**<0.0001**	0.220	0.042	-0.130	0.234
Mount	**0.804**	**<0.0001**	0.183	0.092	-0.128	0.242
Offensive attack	**-0.421**	**<0.0001**	**0.492**	**<0.0001**	-0.027	0.808
Tail slap	**-0.471**	**<0.0001**	**0.382**	**0.0003**	**-0.454**	**<0.0001**
Tail wave (min)	0.030	0.784	-0.218	0.044	**0.768**	**<0.0001**
Watch (min)	-0.044	0.687	-0.260	0.016	**0.812**	**<0.0001**

Figures

Figure 2.1. The sequence of male sexual behavior for *Coleonyx elegans* (Yucatan banded gecko) is a simplified version of the complex ancestral mating behavior repertoire, which includes precopulatory displays including tail vibration and scent marking, as observed in *Eublepharis macularius* (leopard gecko). Adapted from Golinski et al. 2011.

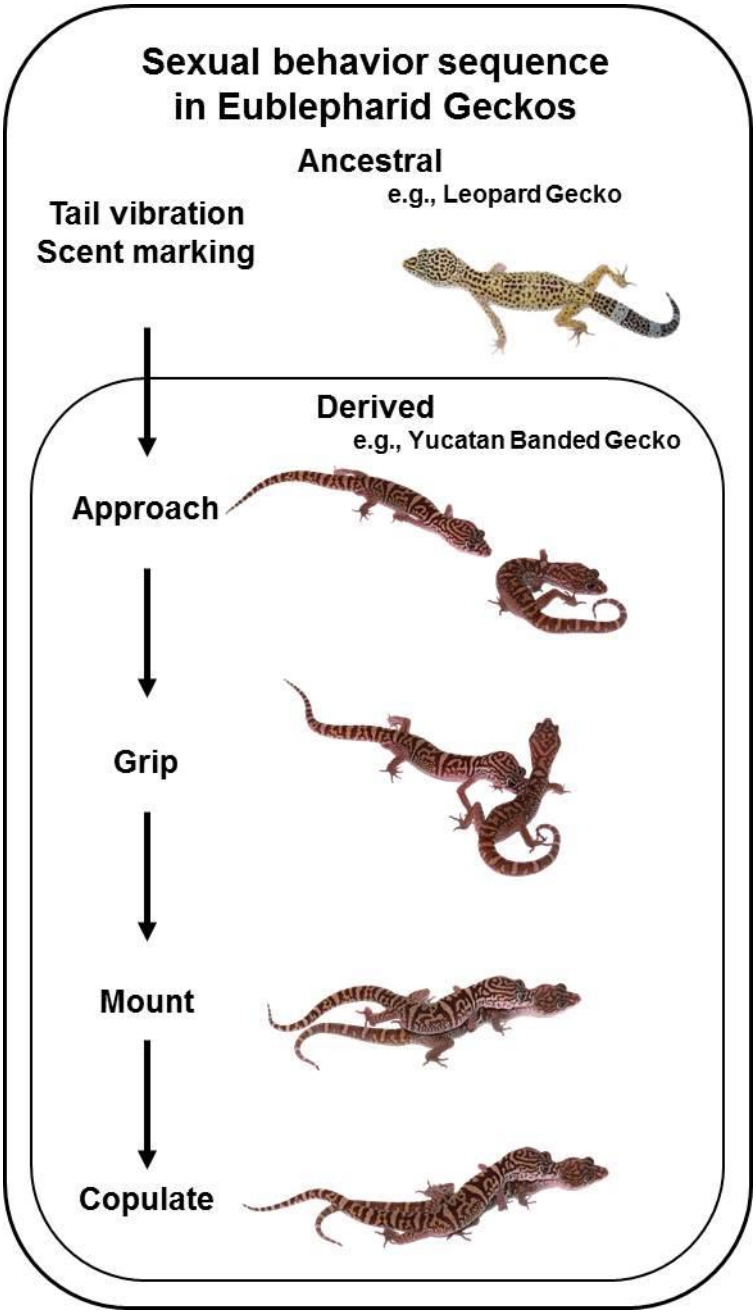


Figure 2.2. Plasma testosterone (T) in experimental males and females of *Coleonyx elegans*, measured 11 weeks after surgery. Lines indicate group means. Lowercase letters indicate post hoc statistical groupings (see statistical methods). Treatment groups are designated as: MCON = intact control males, MCAST = castrated males, MTEST = castrated males with T-implant, FCON = intact control females, FTEST = intact females with T-implant. Note the log10-scale on the y-axis. Modified from Golinski et al. 2011.

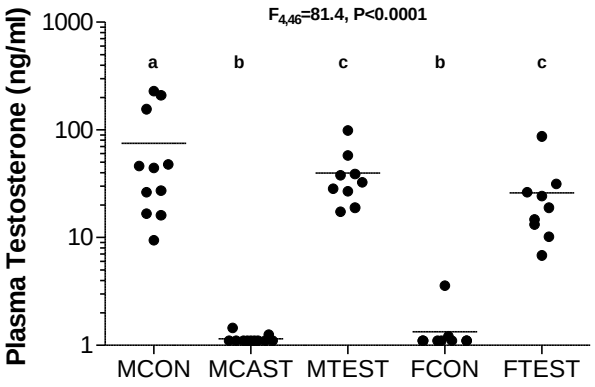


Figure 2.3. Number of active precloacal pores (A: median, interquartile and range), mass of the hemipenes (B: mean ± SEM), head width (C: mean ± SEM) and Snout-vent length (D: mean ± SEM) in males and females of all treatment groups. The number of precloacal pores and mass of hemipenes were reduced by castration (MCAST) and restored by T-replacement (MTEST) in males, and were increased by T-supplementation in females. Treatments had no significant effects on head width in males, but head width was increased in T-supplemented (FTEST) females relative to controls (FCON). Undetectable value indicated by "u.d.".

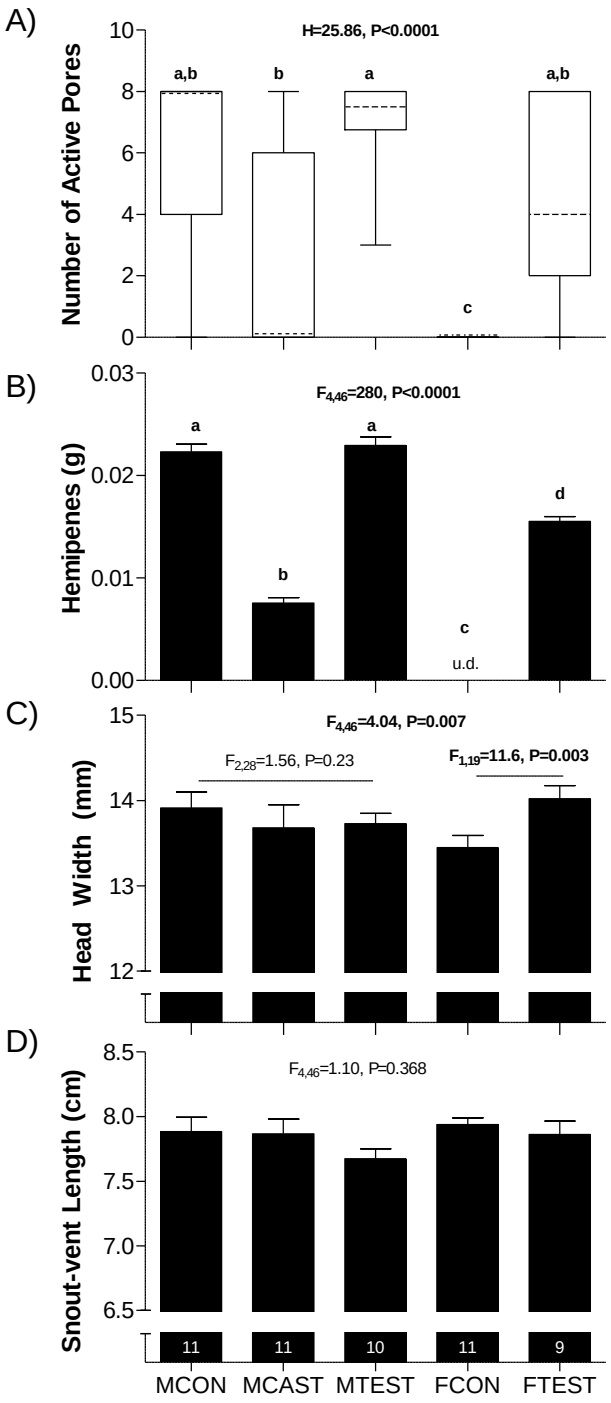

A)
H=25.86, P<0.0001
Number of Active Pores
10
8
6
4
2
0
a,b
b
a
c
a,b
B)
$F_{4,46}$=280, P<0.0001
Hemipenes (g)
0.03
0.02
0.01
0.00
a
b
a
c
u.d.
d
C)
$F_{4,46}$=4.04, P=0.007
$F_{2,28}$=1.56, P=0.23
$F_{1,19}$=11.6, P=0.003
Head Width (mm)
15
14
13
12
D)
$F_{4,46}$=1.10, P=0.368
Snout-vent Length (cm)
8.5
8.0
7.5
7.0
6.5
11
11
10
11
9
MCON
MCAST
MTEST
FCON
FTEST

Figure 2.4. The proportion of experimental lizards that displayed body grip behavior toward an intact female. Number of trials indicated within each bar. Experimental treatments had no significant effect on copulatory behaviors of males. In females, body grip was rare and never proceeded to mounting behavior in controls (FCON), and was increased by T-supplementation (FTEST).

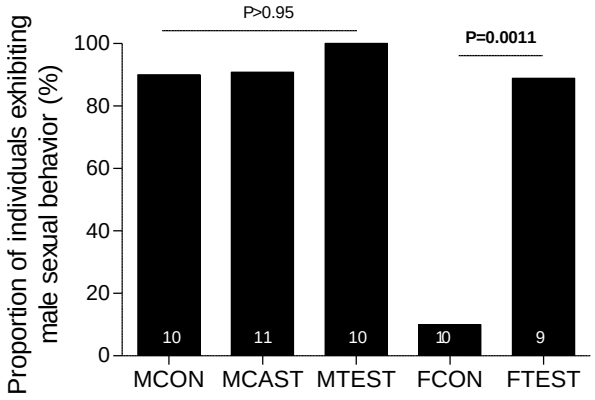


Figure 2.5. Behavioral responses of males from each treatment group when presented with an intact control male (see Table 2.2). The PCA's first axis (x-axis) contrasts agonistic behaviors indicated by negative values, from sexual behaviors indicated by positive values. The PCA's second axis (y-axis) contrasts defensive behaviors (i.e., defensive attack, flee) indicated by negative values, from aggressive behaviors (i.e., offensive attack, high posture, tail slap) indicated by positive values. Males from all treatment groups typically responded with agonistic behaviors toward intact males, rather than sexual behaviors. The clustering of castrated males (MCAST) in the lower left quadrant indicates they displayed low levels of agonistic behaviors and were subordinate during trials with intact control males, relative to the behavioral responses of intact (MCON) and T-replaced (MTEST) males.

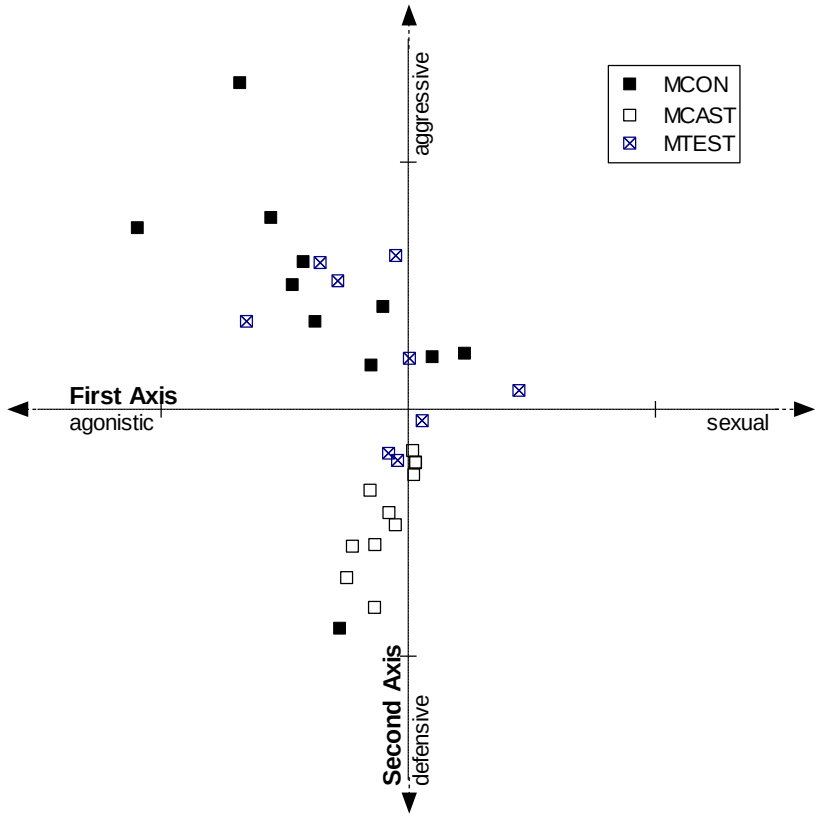
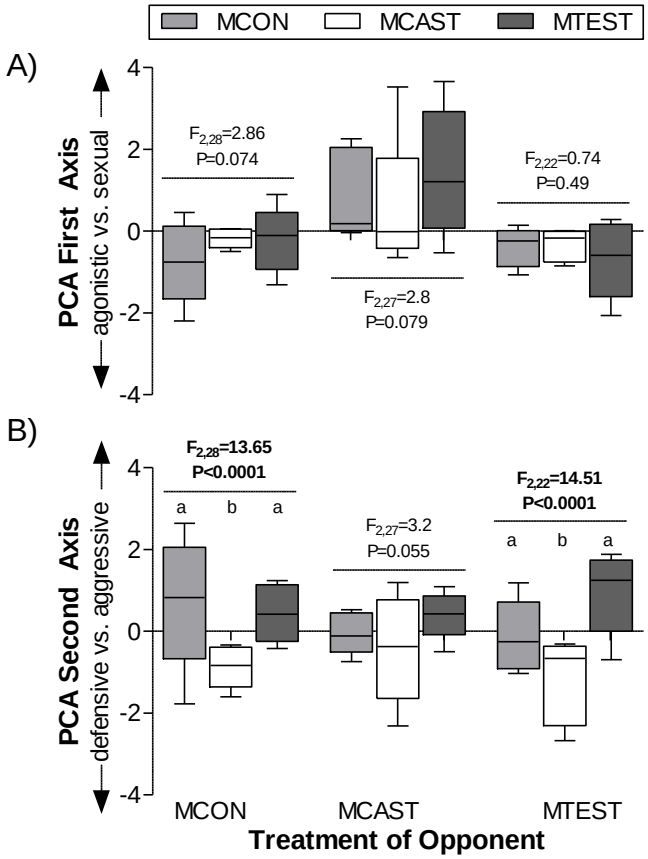


Figure 2.6. Behavioral responses of experimental males from each treatment group toward male opponents from each treatment group (see Table 2.2). Treatment of the experimental males is indicated by shading of the boxes and treatment of the opponents is listed on the x-axis. Box and whisker plots indicate median, interquartile (box) and range (whiskers). Lower case letters indicate post hoc statistical groupings (see statistical methods). A) The first axis of the PCA indicates that experimental males from all treatment groups responded with agonistic behaviors toward intact (MCON) and T-treated (MTEST) male opponents, whereas experimental males tended to display sexual behaviors toward castrated male (MCAST) opponents. B) The second axis of the PCA indicates that castrated males (MCAST) displayed defensive behaviors whereas intact (MCON) and T-treated (MTEST) males displayed aggressive behaviors during trials with intact (MCON) and T-treated (MTEST) male opponents. This treatment effect on the display of aggressive versus defensive behaviors is not as pronounced during trials with MCAST opponents.



CHAPTER 3

EFFECTS OF TESTOSTERONE ON THE EXPRESSION OF SEXUALLY DIMORPHIC TRAITS IN LICHTENFELDERI'S GECKO (EUBLEPHARIDAE: *GONIUROSAURUS LICHTENFELDERI*)

Abstract

Lizards from the family Eublepharidae (eyelid geckos) have a well-supported phylogeny and exhibit a suite of sexually dimorphic traits which are expressed in males. The full suite of traits, including male-larger head and body size, functional precloacal pores, aggressive behavior between males, and both the courtship and copulatory phases of sexual behavior, are hypothesized to be ancestral. However, one or more traits have been evolutionarily lost in some species, suggesting the proximate mechanism has changed to enable individual traits from the suite to evolve independently from each other. I studied *Goniurosaurus lichtenfelderi* to investigate the androgenic regulation of sexually dimorphic trait expression in a "type-species," representative of the ancestral condition in eublepharids. Most sexually dimorphic traits require T in the eublepharid *Eublepharis macularius*, which also expresses the full suite of sexually dimorphic traits, but differs from *G. lichtenfelderi* in mode for sex determination. I hypothesized that elevated levels of T are required for trait expression in adult males of *G. lichtenfelderi*, similar to the androgenic control reported in *E. macularius*. Experiments included 3 groups of males (intact control, surgically castrated, castrated with T replacement) and 2 groups of females (intact control, T supplemented). Testosterone stimulated activity of precloacal pores in males but not in females. For behavioral traits, T activated the

expression of aggressive, courtship and copulatory behaviors in males, and T-supplementation induced courtship but not aggression or mounting in females. Therefore, the suite of sexually dimorphic traits requires activational effects of T in males of *G. lichtenfelderi*, but females appear to have evolved a loss of sensitivity to T in pore activity and the neural substrate responsible for aggression and mounting. The sensitivity to T of sexually dimorphic trait expression is very similar between *G. lichtenfelderi* and *E. macularius*, suggesting that the androgen control module is not significantly affected by differences in mode of sex determination. Subsequent analysis of androgen receptors in the brain may provide insight as to why T-sensitivity differs between sexes and how behavioral traits could be dissociated from the conserved androgen control module in some species.

Introduction

Sexually dimorphic traits evolve due to selection for reproductive advantage (Andersson 1994). Sexually selected traits often exhibit patterns of phenotypic correlation involving multiple levels of biological organization (e.g., behavior, physiology, morphology), suggesting that their expression is coordinated by a common pleiotropic regulatory mechanism (Stearns 1991; Adkins-Regan 2005; Hau 2007; McGlothlin and Ketterson 2008). In reproductively active males of many species, for example, the expression of suites of sexually dimorphic traits suggest coordination by a common androgenic signaling mechanism (Badyaev 2002; Adkins-Regan 2005). Regulation by a shared proximate mechanism may constrain the independent evolution of individual traits within suites (i.e., evolutionary constraint hypothesis, Hau 2007;

McGlothlin and Ketterson 2008). However, the observation that phenotypic differences in sexually dimorphic trait expression, including the loss of individual traits from a suite, exist even among closely-related species, suggests that specific components of the androgen signaling system (i.e., control module) have the potential to evolve independently and give rise to differences in trait expression (i.e., evolutionary potential hypothesis, Hau 2007; McGlothlin and Ketterson 2008). My studies on pleiotropic regulation of correlated traits in gecko lizards address these alternative hypotheses.

Eublepharid geckos (Eublepharidae; eyelid geckos) provide an advantageous model system to investigate how alterations in a hormonal control module could allow loosening or severance of correlations between functionally related traits. Eublepharids have a well-supported phylogeny in which most species are characterized by an ancestral suite of sexually dimorphic traits expressed in males. The male-specific traits include larger head and body size, secretory precloacal pores, courtship and copulatory phases of sexual behavior, and aggressiveness toward conspecific males (Rhen and Crews 1999, 2000; Rhen et al. 2005; Kratochvíl and Frynta 2007). However, one or more of these traits are not expressed in some extant species (Kratochvíl and Frynta 2007; Ch. 2), presumably due to evolutionary loss. Therefore, comparative studies of eublepharids can address hypotheses on how and why the link between androgens and trait expression has been selectively lost.

The present study investigates the role of testosterone (T) in regulating the expression of sexually dimorphic traits in males of *Goniurosaurus lichtenfelderi*, an important species to include in a broader comparative study of eublepharids. In common with *Eublepharis macularius*, *G. lichtenfelderi* expresses the full suite of traits typical of

eublepharids. However, *E. macularius* is characterized by temperature-dependent sex determination (Viets et al. 1993), while *G. lichtenfelderi* is tentatively characterized by a genetic sex-determining mechanism (Pokorna et al. 2010). This difference could influence the development of sexually dimorphic traits in eublepharids because species that utilize genetic sex determination are thought to express genes for sexually dimorphic traits earlier in development than species that utilize environmental sex determination (Valenzuela 2008). Based on the pattern of sexually dimorphic traits and mechanisms for sex determination, *G. lichtenfelderi* is more typical of its family and presumably more representative of the ancestral condition. Furthermore, in contrast to *Coleonyx elegans* which breeds opportunistically and fails to express courtship, *G. lichtenfelderi* is a seasonal breeder and displays courtship in its behavioral repertoire. Finally, the expression of sexually dimorphic traits shows an unusually high degree of evolutionary lability in the genus *Goniurosaurus*, in which all traits are expressed in *G. lichtenfelderi* but none is expressed in the congeneric *G. splendens*. Thus, *G. lichtenfelderi* can be considered a good "type-species" to provide a baseline view of androgenic regulation of sexually dimorphic traits, and a good point of reference for comparison with species in which trait expression has been altered.

I examined whether the suite of sexually dimorphic traits in *G. lichtenfelderi* is sensitive to activational effects of androgens by conducting surgical manipulations to alter circulating levels of T in adult males and females. Based on previous findings in *C. elegans* and *E. macularius* (Rhen and Crews 1999, 2000; Rhen et al. 2005; Golinski et al. 2011; Ch. 2), I hypothesized that eublepharid species with differing modes of sex determination (i.e., genetic versus environmental) would have similar mechanisms for

regulating the expression of sexually dimorphic traits, and that elevated levels of T are required for trait expression in adult males of *G. lichtenfelderi*. I predicted that castration would reduce, and T-replacement would restore, precloacal pore secretions and the expression of aggressive and courtship behavior. I also predicted that expression of these traits would be induced by T-supplementation in females. At the termination of this study, I collected tissues from these lizards to investigate whether interspecific or intersexual differences in observed behavioral sensitivity to T are reflected by differences in androgen receptors in the brain (reported in Ch. 5).

Material and methods

Animals

Goniurosaurus lichtenfelderi (Lichtenfelderi's gecko) are small, nocturnal, ground-dwelling lizards that live on rocky granite banks of forest cascade streams in Southeast Asia (Orlov et al. 2008). Eggs from the captive breeding colony in the laboratory of Lukáš Kratochvíl at Charles University were collected within 24 h of oviposition. Eggs were placed in individual cups filled with moist vermiculite and incubated at 28°C. Upon hatching, animals were individually housed with coconut husk substrate, a water dish and a shelter. The animal facility was maintained with a 12L:12D photoperiod with lights off at 1800 h, ambient temperature set at 26°C and relative humidity of approximately 60%. Animals were fed crickets dusted with calcium powder (Roboran, Univit, Czech Republic) three times per week. Drinking water was supplemented with vitamins A, D_3 and E once every two weeks. *Goniurosaurus lichtenfelderi* reach sexual maturity at approximately one year of age (Holfert and Seufer

2005). Experiments were conducted in accordance with research protocols approved by the Czech Central Commission for Animal Protection (protocol #18847/2003-1020) and the Rutgers University Animal Care and Facilities Committee (protocol #01-019).

Surgical and hormone treatments

Reproductively mature individuals of *G. lichtenfelderi* were assigned to size-matched treatment groups. Males (n=25) were assigned to one of three groups: sham-operated intact control (MCON: n=8, mean SVL=80.8 ± 0.8 mm), surgically castrated (MCAST: n=8, 80.4 ± 0.8 mm), and castrated with T replacement (MTEST: n=9, 80.2 ± 0.9 mm). Females (n=15) were assigned to one of two groups: intact control (FCON: n=8, 82.8 ± 1.2 mm) and intact with T supplement (FTEST: n=7, 82.6 ± 1.2 mm). For surgery, animals were anaesthetized using a combination of an intramuscular injection of ketamine (Narkamon 5%, Spofa a.s., Prague, Czech Republic; 170 μg/g body mass) and cold anesthesia (i.e., placed on ice). The testes were exposed via a medial ventral incision. Bilateral castration (orchiectomy) was done on males from both MCON and MTEST groups by ligating each spermatic cord with surgical silk, then ablating each testis. For MCON and all females, "sham" surgeries were performed, in which ventral incisions were made to expose and manipulate the testes or ovaries while leaving the gonads completely intact. Empty implants (MCON, MCAST, FCON) or T implants (MTEST, FTEST) were then inserted into the coelomic cavity, and the incision was closed using Chromic Gut surgical suture (Ethicon INC, Somerville NJ, USA) and covered with Glubran ®2 surgical glue (GEM S.r.l., Viareggio, Italy). Tonic-release T implants were constructed as described in Cox et al. (2005) and Golinski et al. (2011). Animals were allowed 4 weeks to recover prior to behavior trials. Hormone treatments

were verified by assaying plasma T levels after completion of all experimental procedures (see below).

Behavioral trials

Behavioral trials were begun four weeks after surgical manipulations and were completed over the course of six weeks. Experimental males experienced five behavioral trials while experimental females experienced four. Consecutive trials for any given experimental animal were separated by a minimum of 6 days. The first set of behavior trials was designed to assess sexual behavior in response to the introduction of a lizard of the opposite sex. Here, I refer to the introduced lizard as the "stimulus lizard", or simply the "stimulus". In these trials, a separate pool of unmanipulated males and females was used to provide size-matched stimulus for behavior trials (n=10 stimulus females, mean SVL=79.4 mm; n=8 stimulus males, mean SVL=84.2 mm). Sequential trials involving the same stimulus animal were separated by a minimum of 48 hours. The second set of trials was designed to assess agonistic behavior in response to the introduction of an experimental animal of the same sex. In these trials, behavioral responses of members of all treatment groups were assessed in response to experimental lizards of all treatments of the same sex. All behavior trials and subsequent analyses were conducted without the observer having knowledge of experimental treatment.

Goniurosaurus lichtenfelderi is nocturnally active, thus all behavioral trials were conducted between 1830 and 2130 h under dim red light. To minimize disturbance, trials were not observed directly but were monitored in real time from a second room via closed circuit television. Each trial was video-recorded (Sony Handycam® DCR SR42) and subsequently analyzed for the occurrence and duration of observed behaviors using

JWatcher™ Video software (Dan Blumstein's Laboratory, UCLA and The Animal Behavior Laboratory, Macquaric University, Sydney).

Sexual behavior trials

Sexual behavior of experimental males (i.e., MCON, MCAST, and MTEST) was assessed during two trials with a stimulus female: first in the home cage of the male (glass aquarium, 30x30 cm) and second in a neutral arena (glass aquarium, 30x40 cm). The same pairings between males and stimulus females were used for both trials to reduce novelty associated with the second trial in the neutral arena. Similarly, sexual behavior of experimental females (i.e., FCON, FTEST) was assessed during two trials with a stimulus male following the same protocol described above.

For trials conducted in the experimental animal's home cage, the water dish and shelter were removed five minutes before the introduction of a stimulus animal of the opposite sex. Behavior was observed for 15 minutes. Trials were terminated early if mounting occurred to prevent intromission and reduce the potential for ovulation in the female, allowing them to remain receptive in subsequent trials (Rhen et al. 2000). During the second round of trials, the experimental animal and the stimulus animal were placed into a neutral arena on opposite sides of an opaque central divider. After five minutes, the divider was lifted via a remote pulley system. Behavior was observed for 15 minutes or until mounting position was achieved. The neutral arena was cleaned with 70% ethanol between subsequent trials. Digital recordings were later viewed and analyzed for the occurrence and duration behaviors displayed by experimental males and females toward stimulus animals. Informative behaviors are listed in Table 3.1.

Agonistic behavior trials

Same-sex trials were begun one week after the completion of sexual behavior trials (6 weeks post-treatment) to assess the behavior of each experimental animal in all treatment groups in response to opponents of all treatment groups of the same sex. Each experimental male experienced three trials with a male opponent, and each experimental female experienced two trials with a female opponent. The sequence of pairing between treatment groups was randomized. Trials were conducted in a neutral arena (glass aquarium, 60x40 cm) to eliminate all possibility of residency biases. At the start of a trial, two lizards were placed into the neutral arena on opposite sides of an opaque central divider. After five minutes, the divider was lifted via a remote pulley system, and behavior of each lizard was observed for a maximum of 15 minutes. Trials were terminated early when mounting occurred and when behavior appeared to be highly stressful (i.e., repeated fleeing by one individual) or potentially injurious. The neutral arena was cleaned with 70% ethanol between trials.

Digital recordings were later viewed and analyzed for the occurrence and duration of behaviors displayed by each of the experimental lizards. Informative behaviors are listed in Table 3.1. Note that "agonistic" behavior refers to all behaviors associated with the contest or struggle between two animals, including the threat (i.e., high posture), attack, flee, approach, and retreat (King 1973). The role of aggressor versus defender often alternates between two individuals in a dyad during agonistic interactions (King 1973). The term "aggression" refers to the initiation and attack phase of the agonistic encounter (King 1973).

Morphological measurements

Body mass (g), snout-vent length (SVL, mm), head width (mm), and width of the tail base (i.e., and indicator of hemipenes size) were measured in each animal prior to surgical manipulations and after completion of behavioral trials, 11 weeks after surgical treatments. At these two time points, digital images of the dorsal and ventral surfaces of each lizard were made with a desktop digital scanner. In this species, precloacal pores are present on the ventral surface anterior to the cloacal vent, and scanned images were used to quantify the number of active pores. The presence of waxy secretions was used as the indicator of pore activity.

Radioimmunoassay (RIA)

After completion of all behavior trials (11 weeks after surgeries), blood samples were collected in heparinized microhematocrit capillary tubes from trunk and neck wounds following rapid decapitation. Whole blood was held on ice for a maximum of 4 hours and then centrifuged at 1500 rpm for 10 min at 4°C. Plasma was stored in plastic microfuge tubes at -20°C until assayed for T. Radioimmunoassay (RIA) for plasma T followed the methods of Smith and John-Alder (1999) and Golinski et al. (2011). Samples were extracted twice in diethyl-ether, dried under a stream of ultra-filtered air, and reconstituted in phosphate-buffered saline with gelatin (PBSG, pH 7.4). Reconstituted samples were assayed with ^{3}H-T (Perkin Elmer Life and Analytical Sciences Inc., Waltham, MA, USA) and T antiserum developed in rabbits by A. L. Johnson (The University of Notre Dame, Indiana, USA). Recoveries averaged 87%.

Samples were processed in a single assay with a detection limit of 13.1 pg T. Typical intra-assay variation is 6.7% (Smith and John-Alder 1999).

Statistical Analysis

Statistical analyses were conducted on behavioral data from all subjects with successful experimental treatment, which was verified via plasma hormone concentrations at the end of the 11 week study. All statistical analyses were conducted using SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) or GraphPad Prism (version 5.00; GraphPad Software, San Diego CA USA). Plasma T levels and tail base width (i.e., proxy for size of hemipenes) were analyzed using a one-way ANOVA with Ryan-Einot-Gabriel-Welsch multiple range test post hoc. The number of active pores was analyzed using a nonparametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons post hoc test. Statistical significance was accepted at $\alpha = 0.05$ based on the H-statistic, which is the non-parametric analogue of an F-statistic.

Principal components analysis (PCA) was used to reduce the dimensionality and help identify informative behavioral traits. Two separate PCAs were conducted: 1) male behavior toward stimulus females during trials in the home cage and neutral arena, 2) behaviors displayed during trials between two experimental males. For PCA 1) that involved trials in two different venues (i.e., home cage and neutral arena), t-tests were used to determine whether the venue had a significant effect on the PCA scores. For PCA 2) involving trials between two experimental males, I performed a repeated subsampling randomization analysis to ensure that no skew in interpretation of the data was created by use of data from both male opponents in each dyad. I verified that the variance in the computed impact of each axis from the PCA was relatively unaffected

(i.e., no effect beyond that expected by limiting sample size) by any sub-designation for groups of trials. Therefore results reported are from the PCA of the full dataset (n=54), regardless of the behavioral role of the male opponents in each dyad. Only the first two or three axes from each PCA are reported because these axes explain more of the variation in the primary data relative to the later axes and the trait loading patterns of these axes were most amenable to interpretation. Effects of treatment on PCA axis scores were analyzed using one-way ANOVA with Ryan-Einot-Gabriel-Welsch multiple range post hoc test. For each of these analyses, the Dunn-Sidak method was used to control for experiment-wise error level at α=0.05.

For analysis of male-typical behaviors displayed by experimental females, a Fisher exact test was used to compare the proportion of trials in which such behaviors occurred.

Results

Plasma testosterone

Plasma T verified the efficacy of experimental manipulations (ANOVA: $F_{4,32}$=60.7, P<0.0001; Fig. 3.1). In males, intact controls had high and variable levels of T (MCON n=8; mean 50.79 ng/ml, range=5.40-135 ng/ml). Plasma T was significantly reduced by surgical castration (MCAST n=8; 0.46 ± 0.02 ng/ml) and restored to levels of controls by T-replacement in 6 of 9 MTEST (n=6 with successful manipulation; 17.7 ± 2.80 ng/ml). Three MTEST males lost their implants and had low T levels similar to MCAST; they were excluded from all subsequent analyses. In females, intact controls

had low levels of T (FCON n=8; 1.06 ± 0.59 ng/ml) and T-supplementation significantly elevated plasma T (FTEST n=7; 17.6 ± 4.25 ng/ml).

Morphological measurements

The number of precloacal pores actively producing secretions at the end of the 11-week experimental period were significantly greater in males than in females (Kruskal-Wallis: H=28.70, P<0.0001; Fig. 3.2A). In males, the number of active pores was reduced by castration and restored to levels of controls by replacement of T ($F_{2,19}$=15.72, P<0.0001). In females, T-supplementation had no effect on the activity of precloacal pores. The width of the tail base (i.e., proxy for hemipenes size) was significantly smaller in FCON and MCAST than MCON and was increased by T in both sexes ($F_{4,32}$=20.15, P<0.0001; Fig. 3.2B). Head widths were similar among all treatment groups ($F_{4,32}$=1.552, P=0.21; Fig. 3.2C). Snout-vent length also did not differ among treatment groups ($F_{4,32}$=2.194, P=0.09; Fig. 3.2D).

Sexual behavior

During behavioral trials with a female, a male typically approaches, displays tail vibration, bites near the neck of the female (i.e., body grip), and walks over the top of the female to align his body with hers. The male and female typically hold this position for several minutes, and then the male displays tail vibration one or more times before proceeding to mount for copulation.

Behavioral responses of males from each treatment group when presented with an intact female during trials conducted in the home cage and neutral arena were entered into a principal components analysis (PCA). The results of the PCA are shown in Table 3.2. The first axis of this PCA explains 28.1% of variation in the primary data and

contrasts sexual behaviors (i.e., body grip, jerk, mount, tail vibration, walk-over) from anti-social or submissive behaviors (i.e., flee and motionless). The second axis of the PCA explains 17.9% of variation and groups ambulatory behaviors (i.e., approach, chase, and explore). The third axis of the PCA explains 13% of variation and groups general social behaviors (i.e., tongue flick, tail wave) which are often displayed by lizards of either sex during social interactions. Treatment did not significantly affect scores on the first axis of the PCA during trials in the home cage ($F_{2,19}$=1.46, P=0.26), but scores tended to be reduced in castrates during trials in the arena ($F_{2,19}$=3.50, P=0.051; Fig. 3.3A). The test venue (i.e., home cage versus neutral arena) had a significant effect on males from all treatments, whereby the display of sexual behaviors was increased during trials in the home cage relative to the neutral arena (t-test: t=2.60, P=0.013). The scores from the second axis were not different among treatment groups (home: $F_{2,19}$=0.56, P=0.58; arena: $F_{2,19}$=1.64, P=0.22), but were increased during trials in the neutral arena versus the home cage (t=-3.17, P=0.004; Fig. 3.3B). The scores from the third axis were not different among treatment groups (home: $F_{2,19}$=0.64, P=0.54; arena: $F_{2,19}$=1.11, P=0.35), but were increased in the home cage versus the neutral arena (t=2.82, P=0.007).

During behavioral trials between two experimental females, the animals typically displayed general social behaviors (i.e., tongue flick, tail wave) as well as exploration. Only rarely, females were observed to display male-typical sexual behaviors (i.e., jerk, mount, tail vibrate) toward an intact female. Four out of 7 T-supplemented (FTEST) and 1 out of 8 intact (FCON) females displayed the male-typical sexual behaviors of tail vibration and body grip toward an intact female (Fig. 3.4). Only 1 out of 7 T-supplemented female proceeded to mount. The differences between treatment groups are

not statistically significant due to the low sample size and low incidence of occurrence (Fisher exact test: for body grip P=0.119, for tail vibration P=0.119, for mount P=0.467).

Aggressive Behavior

Results of the PCA on behaviors displayed during trials between two experimental males in the neutral arena are shown in Table 3.3. The first two axes explain a cumulative total of 53% of the variation in the primary data. The first axis of the PCA explains 27.9% of variation in the primary data and groups aggressive behaviors (i.e., attack, body roll, faceoff, high posture, and tail slap). The second axis of the PCA explains 25.7% of the variation and contrasts behaviors typical during sexual interactions (i.e., body grip, jerk, tail vibration and walk-over) but did not include mount, from the submissive behavior (i.e., flee).

During trials with an intact male opponent, the first axis of the PCA indicates that males from all treatment groups displayed aggressive behaviors (see x-axis of Fig. 3.5 and Fig. 3.6A) with equivalent intensity ($F_{2,17}$=0.93, P=0.41). During the same trials, the second axis indicates that MCAST males displayed more submissive behavior whereas MCON and MTEST displayed sexual-like behaviors ($F_{2,17}$=4.29, P=0.031; see y-axis of Fig. 3.5 and Fig. 3.6B). These results may appear contradictory: how can MCAST be both aggressive and submissive? When behaviors displayed by experimental males during trials with intact males are analyzed separately rather than in the PCA, MCAST males displayed significantly reduced levels of aggressive behaviors including high posture (MCON: mean=6.5±2.1 min; MCAST: mean=0.7±0.4 min; $F_{2,17}$=3.586, P=0.05) and offensive attacks (MCON: mean=3.8±0.9 attacks; MCAST: mean=0.7±0.7 attacks;

$F_{2,17}$=3.931, p=0.0395). The term offensive attack is used here because data in which the experimental male was rejecting a body grip by attacking the intact male opponent were excluded. This distinction was not made in the PCA. When data for all attacks was analyzed, the results were similar to those from the first axis of the PCA in that male treatment groups did not significantly differ ($F_{2,17}$=0.075, p=0.928). Thus, MCAST males displayed less aggressive behaviors than MCON and MTEST males, but they would retaliate with attacks when provoked by an aggressive opponent.

During trials with male opponents from the MCAST and MTEST treatment groups, male treatment groups did not significantly differ in scores from the first axis of the PCA (with MCAST: $F_{2,18}$=1.75, P=0.20; with MTEST: $F_{2,10}$=2.87, P=0.10) or the second axis of the PCA (with MCAST: $F_{2,18}$=3.44, P=0.054; with MTEST: $F_{2,10}$=0.89, P=0.44). Trials between two MTEST opponents appeared to have high scores on the first axis (i.e., high levels of aggressive behaviors) and low scores on the second axis (i.e., submissive behavior), however, the failure to attain significance is due to the small sample size of trials (n=2).

Females rarely displayed aggressive behaviors, and T-supplementation only slightly increased the likelihood that a female would behave aggressively toward an intact male. During trials conducted in a neutral arena, 2 out of 7 T-supplemented females (FTEST) versus 0 of 8 controls (FCON) attacked the intact male. During trials conducted in the female's home cage, 1 out of 7 FTEST and 1 out of 8 FCON females attacked the intact male. None of the experimental females expressed the aggressive behavior of high posture or body roll.

Discussion

The goal of this study was to investigate the androgenic control of sexually dimorphic traits in *G. lichtenfelderi*, a "type-species" or representative of the ancestral condition of eublepharid geckos; characterized by the full suite of sexually dimorphic traits, genetic sex determination, and a seasonal breeding pattern. Thus, the regulation of trait expression by T in this species provides a good baseline for the function of the androgen control module in members of this family. *Eublepharis macularius* is similar to *G. lichtenfelderi* in trait expression and breeding phenology, but differs in mechanism of sex determination. Based on previous findings from eublepharids (Rhen and Crews 1999, 2000; Rhen et al. 2005; Golinski et al. 2011; Ch. 2), I hypothesized that mode of sex determination (i.e., genetic versus environmental) does not have a significant effect on androgenic regulation of trait expression and that elevated levels of T are required in adult males of *G. lichtenfelderi*.

Androgen control of sexual behavior

As predicted based on previous findings from *E. macularius* (Rhen and Crews 1999; Sakata et al. 2002a), courtship and copulatory behaviors were reduced in castrated males of *G. lichtenfelderi* during trials conducted in a neutral arena and T-replacement restored the expression of these behaviors to levels observed in intact males. Therefore I conclude that activational effects of contemporaneously elevated levels of T increase the likelihood for the expression of tail vibration courtship behavior in adult males of *G. lichtenfelderi*. Male-typical sexual behaviors were also expressed by T-supplemented females of *G. lichtenfelderi*, consistent with reports from other lizards (e.g., Adkins and

Schlesinger 1979; Flores and Crews 1995; Golinski et al. 2011). Because the cellular and molecular signaling pathways that mediate androgen actions are similar in males and females (Foecking et al. 2008), the most parsimonious explanation for the expression of male-typical behaviors in females is via activational effects of T on existing neural substrates.

The interspecific comparison of the androgenic regulation of male sexual behaviors is very similar between *G. lichtenfelderi* and *E. macularius*, in terms of the activational effects of T in males and in females of these species. However, both species possess intersexual differences in sensitivity to T. Whereas T in males increased the likelihood for both courtship and copulatory behaviors, T in females increased the likelihood for tail vibration courtship behavior but not male-typical mounting (Flores and Crews 1995; Rhen and Crews 1999). The observed difference in behavioral responsiveness to T between sexes suggests females have evolved a selective loss of sensitivity to T in the neural substrate responsible for mounting behavior.

Overall, the results from the present study on *G. lichtenfelderi* are consistent with those in *E. macularius*, a species characterized by temperature-dependent sex determination, which together indicate that androgenic regulation of sexual behavior is not affected by mechanism of sex determination.

Effect of test venue on androgen control of sexual behavior

Courtship and copulatory behavior were expressed more frequently by males of all treatment groups during trials in home cages compared to the neutral arena, and the effect of T was only evident in the neutral arena. The absence of a treatment effect in

home cages indicates prolonged retention of sexual behavior following castration. Sexual behavior in male lizards is typically reduced within a few weeks following castration even during tests in the home cage (Crews et al. 1978; Lindzey and Crews 1986; Tokarz 1986; Sakata et al. 2002b; Ch. 4), but familiarity with the test environment may have been a factor in the retention of behaviors in castrated males of *S. jarrovi* studied in the field on their home territory (Moore 1987). During trials conducted in the male's home cage, retention of sexual behavior following castration has been reported in some laboratory rodents (Wee and Clemens 1989) and also in *C. elegans* (Golinski et al. 2011; Ch. 2).

The observed difference between behavioral results from trials in the home cage versus neutral arena could have been due to differences in general activity between the venues. In a study investigating general activity of *E. macularius* placed alone into a neutral arena, castrated males showed a reduction in general activity, and implantation with T restored levels of general activity to that of intact males (Sakata et al. 2002a). The authors of that study suggested that the inactivity of lizards in neutral arenas could be analogous to freezing behavior in rodents during open-field trials and a proxy for fear or anxiety of the animals (Dennenberg 1969; Sakata et al. 2002a). However, male treatment groups of *G. lichtenfelderi* did not differ in the display of ambulatory behaviors (i.e., a proxy for general activity), but these behaviors were significantly increased in the neutral arena relative to the home cage (Fig. 3.3B). These data suggest that the difference between trials in expression of sexual behavior was not associated with a venue effect on general activity.

Evidence from neuroendocrine studies in *Anolis carolinensis* suggests that T may alter activity of neurotransmitter systems to modulate motivation for social interaction and sexual behavior (Neal and Wade 2007; Wade 2011). Therefore, it is likely that the male's familiarity with the venue overwhelmed these effects of hormonal modulation of activity and motivation during trials in the home cage, masking the effects of castration on male sexual behavior in *G. lichtenfelderi*. *Goniurosaurus lichtenfelderi* is the only eublepharid that has been tested with a female in both the home cage and neutral arena, as previous studies in lizards only used one venue or the other for behavior trials conducted in the laboratory (Crews et al. 1978; Lindzey and Crews 1986; Flores and Crews 1995; Rhen and Crews 1999; Golinski et al. 2011). Based on the observed differences in behavioral responses of *G. lichtenfelderi* between the two venues, I predict that a treatment effect on male sexual behavior in *C. elegans* may have been observed if trials had been conducted in a neutral arena.

Androgen control of aggression

Physical aggression between two males of *G. lichtenfelderi* often appears very similar to sexual interactions with females, whereby males often body gripped and held their opponent for extended periods of time and displayed tail vibration. In contrast to true sexual interactions, however, high posture, faceoff and attacks were common during trials between two males, and mounting never occurred. Aggressive behaviors were more frequent and more likely to escalate and continue during trials between two males with elevated T. In contrast, during trials with a castrated male, after a brief aggressive interaction, the castrated male typically fled from the opponents with T. Therefore, castrated males displayed fewer aggressive behaviors and more submissive behaviors

relative to intact or T-treated males, indicating that elevated T levels increase the likelihood for the expression of typical aggressive behaviors. This result in *G. lichtenfelderi* is consistent with previous findings in other lizards (Moore 1988; Rhen and Crews 2000; Tokarz et al. 2002; Weiss and Moore 2004; Kabelik et al. 2006; Ch. 2) and consistent with reports among vertebrates (reviewed in Adkins-Regan 2005).

Summary of the androgen control of the male phenotype in eublepharid geckos

The goal of this experiment was to examine the androgenic control of sexually dimorphic traits in *G. lichtenfelderi*, a "type-species" or representative for the ancestral condition of eublepharid geckos. In males of this species, contemporaneously elevated levels of T are necessary for the expression of sexually dimorphic behaviors: aggressive behaviors toward males, courtship and copulatory behaviors toward females. Testosterone is also required for the expression of morphological and physiological traits in males, namely the enlargement of the hemipenes and the production of waxy secretions from precloacal pores. During this 11-week study, T treatment had no significant effect on body size or head width. In order to study the effect on these morphological traits, a study should be conducted earlier in ontogeny during the phase of rapid growth (e.g., Tousignant and Crews 1995). The T-sensitivity of sexually dimorphic traits in this "type-species," or model for the ancestral condition, can be used as a baseline and a good point of reference for comparison with species in which trait expression has been altered.

The comparison between *G. lichtenfelderi* and *E. macularius* indicates that the endocrine control module is largely the same in species that share the expression of the

full suite of sexually dimorphic traits and a seasonal breeding pattern but contrast in mechanisms of sex determination (i.e., genetic versus temperature-dependent). Therefore, these data suggest that the presence or absence of sex-determining genes does not have a significant effect on the regulation of sexually dimorphic traits by androgens.

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T**ables**

Table 3.1. Behaviors displayed by *G. lichtenfelderi* during social interactions of all types (i.e., male-male, male-female, female-female). These behaviors describe the experimental lizard responding to the introduced stimulus lizard. Counts or durations of these behaviors were entered into principal components analyses (see statistical methods).

Behavior	Description
Approach	Directed movement toward stimulus animal
Attack	Rapidly bites, but does not grip stimulus
Body grip	Bite and grip the stimulus animal, usually near the neck
Body roll	Bite and grip the stimulus animal and roll the body rapidly
Chase	Rapidly pursues a moving stimulus
Exploration	Movement without apparent regard for the other lizard
Faceoff	Stand parallel to the stimulus animals in a mutual lateral orientation
Flee	Rapid and frantic movement away from stimulus animal
High posture	Extends limbs to stand tall; threat display
Investigate	Press snout against the stimulus lizard; typically the first physical contact
Jerk	Movement of the head in short, rapid jerks, typically occurring after body grip
Motionless	Passive, no movement
Mount	Straddles and attempts copulation
Pinned	Immobilized by physical contact of stimulus animal
Retreat	Movement away from stimulus animal
Tail slap	Attack the stimulus using rapid side-to-side movements of the tail
Tail vibration	Rapid movements of the distal third of the tail, producing audible sound
Tail wave	Tail lifted high above body with waving motion
Tongue flick	Rapid and sequential protrusion and retraction of the tongue
Walk-over	Climbs over top of the stimulus animal

Table 3.2. Principal components analysis (PCA) of behavioral responses of experimental males from each treatment group when presented with a female during trials conducted in the home cage and neutral arena; the first three axes explain 59% of the variation in the primary data. The first axis contrasts sexual behaviors (i.e., body grip, jerk, mount, tail vibration, and walk-over) from anti-social behaviors (i.e., flee and motionless). The second axis groups the ambulatory behaviors of approach, chase and explore. The third axis groups general social behaviors of tail wave and tongue flick.

	First Axis		Second Axis		Third Axis	
Variable	Coefficient	Significance	Coefficient	Significance	Coefficient	Significance
Approach (min)	0.018	0.906	**0.870**	**<0.0001**	-0.160	0.301
Body grip (min)	**0.801**	**<0.0001**	0.003	0.983	-0.320	0.034
Chase (s)	-0.043	0.783	**0.789**	**<0.0001**	-0.129	0.403
Exploration (min)	-0.390	0.009	**0.551**	**0.0001**	0.303	0.045
Flee (s)	**-0.582**	**<0.0001**	-0.309	0.041	-0.156	0.313
Jerk	**0.601**	**<0.0001**	-0.318	0.035	0.460	0.002
Motionless (min)	**-0.792**	**<0.0001**	-0.083	0.594	-0.247	0.107
Mount	**0.809**	**<0.0001**	0.001	0.993	-0.301	0.047
Tail vibration (s)	**0.560**	**<0.0001**	-0.071	0.649	0.200	0.193
Tail wave (s)	-0.130	0.401	-0.156	0.311	**0.488**	**0.001**
Tongue flick	0.119	0.441	0.376	0.012	**0.792**	**<0.0001**
Walk-over (s)	**0.495**	**0.001**	0.301	0.047	-0.182	0.238

Table 3.3. Principal components analysis of behaviors displayed during trials between two experimental males; the first two axes explain a total of 53% of variation in the primary data. The first axis groups aggressive behaviors (i.e., attack, body roll, faceoff, high posture, and tail slap). The second axis separates dominant behaviors typical of sexual interactions (i.e., body grip, jerk, tail vibration and walk-over) from submissive behavior (i.e., flee).

	First Axis		Second Axis	
Variable	Coefficient	Significance	Coefficient	Significance
Attack	**0.558**	**<0.0001**	-0.370	0.006
Body grip (s)	-0.043	0.758	**0.815**	**<0.0001**
Body roll	**0.584**	**<0.0001**	-0.011	0.939
Faceoff	**0.895**	**<0.0001**	0.077	0.581
Flee	0.231	0.093	**-0.491**	**0.0002**
High posture (s)	**0.753**	**<0.0001**	0.135	0.330
Jerk	0.292	0.032	**0.787**	**<0.0001**
Tail slap	**0.712**	**<0.0001**	-0.118	0.395
Tail vibration (s)	0.211	0.125	**0.805**	**<0.0001**
Walk-over (s)	-0.268	0.050	**0.466**	**0.0004**

Figures

Figure 3.1. Plasma testosterone (T) in experimental males and females of *G. lichtenfelderi* measured 11 weeks after surgical manipulations. Lines indicate group means. Lower case letter indicate post hoc statistical groupings (see statistical methods). Treatment groups are designated as: MCON = intact control males, MCAST = castrated males, MTEST = castrated males with T-implant, FCON = intact control females, FTEST = intact female with T-implant. Note the $\log_{10}$-scale on the y-axis.

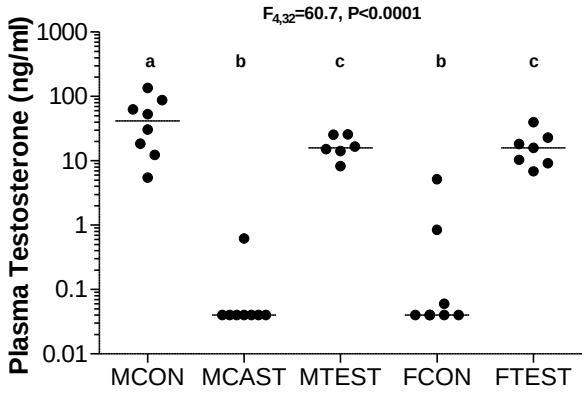


Figure 3.2. Number of active precloacal pores (A: median, interquartile and range), width of tail base (B: mean ±SEM), head width (C: mean ±SEM) and snout-vent length (D: mean ±SEM) in males and females of all treatment groups. The number of active precloacal pores were reduced by castration (MCAST) and restored by T-replacement (MTEST) in males, and were absent in both groups of females. The width of the tail base was reduced by castration (MCAST) and restored by T-replacement (MTEST) in males, and was increased by T-supplementation in females. Experimental treatments had no significant effects on head width. Sample sizes indicated within the bars are the same for all morphological measurements. Lower case letters indicate post hoc statistical groupings (see statistical methods); "u.d." indicates undetectable values.

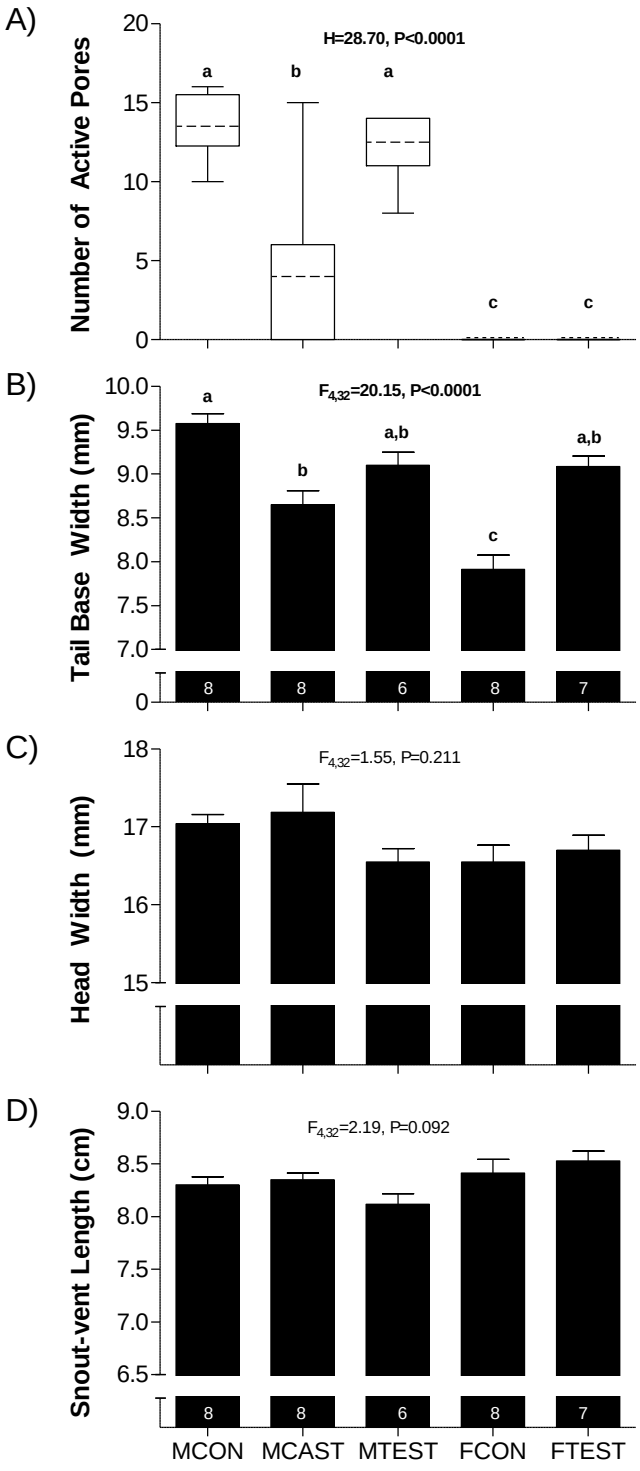

A)
H=28.70, P<0.0001
Number of Active Pores
20
15
10
5
0
a
b
a
c
c
B)
$F_{4,32}$=20.15, P<0.0001
Tail Base Width (mm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
0
a
b
a,b
c
a,b
8
8
6
8
7
C)
$F_{4,32}$=1.55, P=0.211
Head Width (mm)
18
17
16
15
D)
$F_{4,32}$=2.19, P=0.092
Snout-vent Length (cm)
9.0
8.5
8.0
7.5
7.0
6.5
8
8
6
8
7
MCON
MCAST
MTEST
FCON
FTEST

Figure 3.3. Behavioral responses of experimental males from each treatment group when presented with a female in the home cage and in a neutral arena (see Table 3.2). Test venue is indicated on the x-axis. Box and whisker plots indicate median, interquartile (box) and range (whiskers). A) The first axis of the PCA indicates that males from all treatment groups expressed sexual behaviors during trials conducted in the home cage, but this behavior was reduced in males from all treatment groups during trials conducted in the neutral arena and more so in castrates (MCAST). B) The second axis indicates treatment did not significantly affect the display of ambulatory behaviors (i.e., approach, chase and exploration), but these behaviors were increased during trials in the neutral arena relative to the home cage.

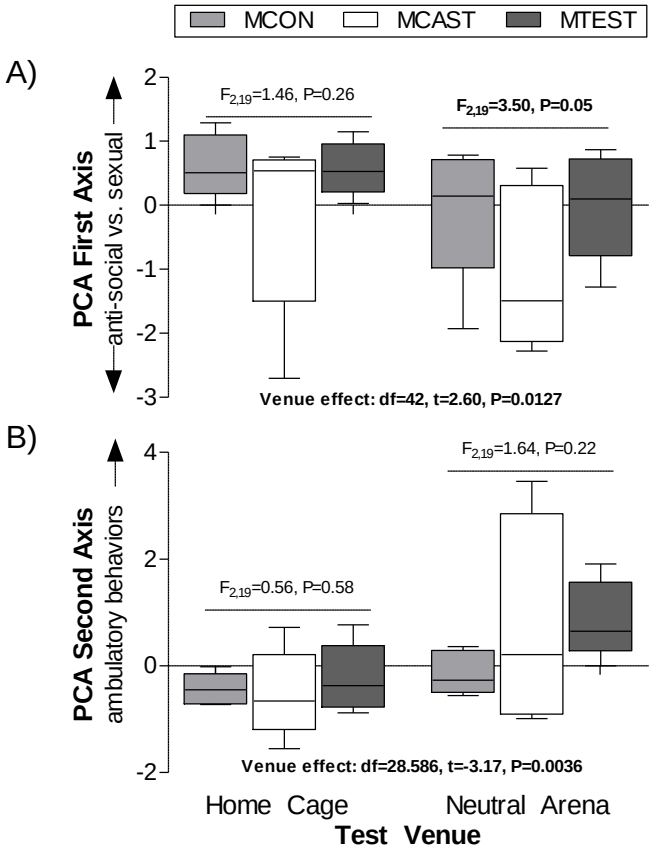


Figure 3.4. The percent of experimental females responding to the presence of an intact female with the male-typical sexual behaviors of tail vibration, body grip and mount during trials conducted in the neutral arena. T-supplementation induced male-typical sexual behaviors displayed by females (FTEST), but not significantly above levels observed in intact females (FCON). Sample sizes and Fisher exact test statistics shown.

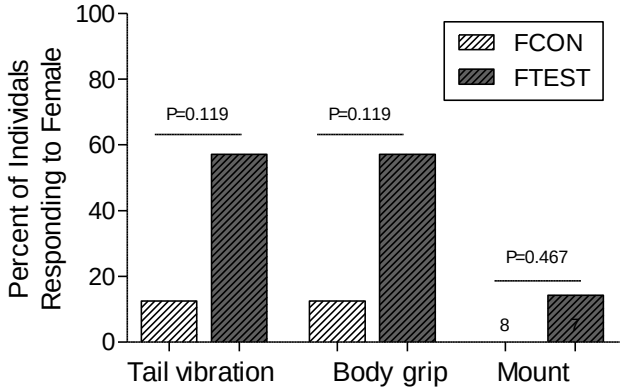


Figure 3.5. Behavioral response of experimental males from each treatment group when presented with an intact control male (see Table 3.3). The PCA's first axis (x-axis) is an index of aggression, whereby more positive values indicate a higher score for aggressive behaviors (i.e., attack, body roll, faceoff, high posture, and tail slap). The second axis (y-axis) contrasts dominant sexual-like behaviors (i.e., body grip, jerk, tail vibration, walk-over) indicated by positive values and submissive behavior (i.e., flee) indicated by negative values. The clustering of castrated males (MCAST) in the lower half of the figure indicates that they were consistently subordinate during trials with an intact control male.

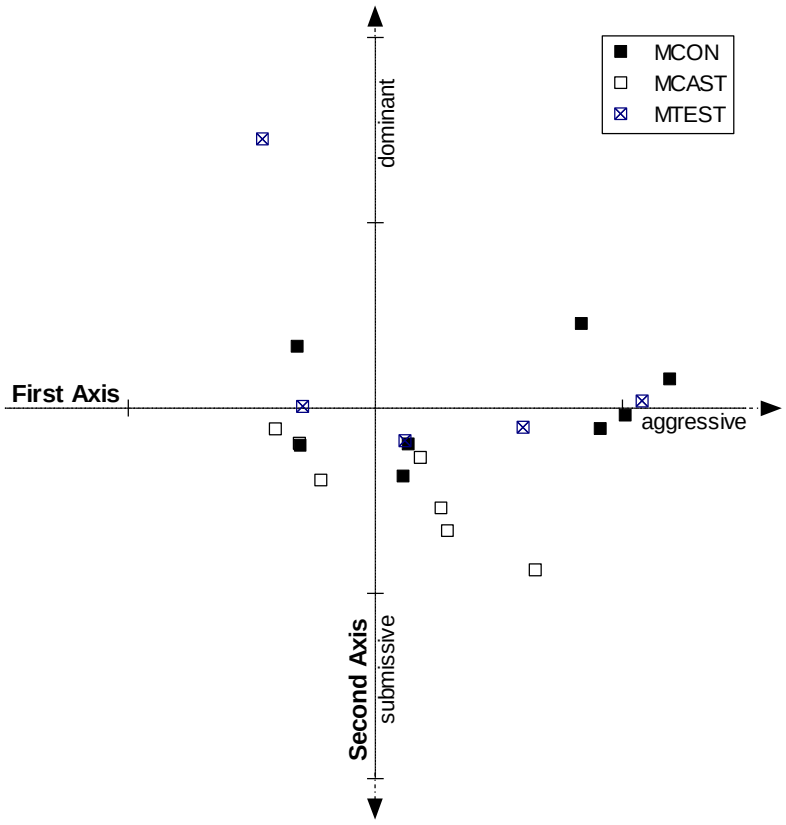
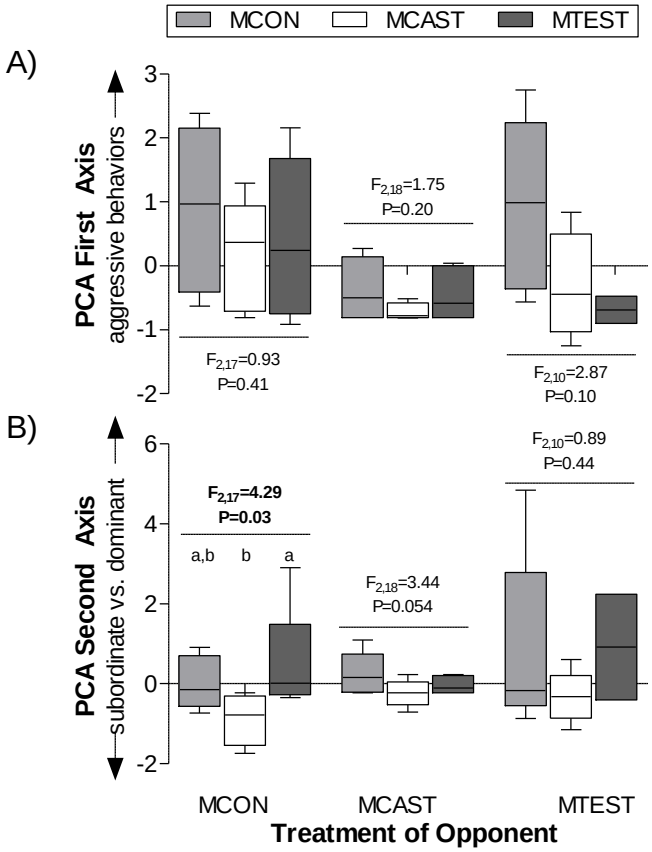


Figure 3.6. Behavioral responses of experimental males from each treatment group toward male opponents from each treatment group (see Table 3.3). Treatment of the experimental males is indicated by shading of the boxes and treatment of the male opponents is listed on the x-axis. Box and whisker plots indicate median, interquartile (box) and range (whiskers). Lower case letters indicate post hoc statistical groupings (see statistical methods). A) The first axis of the PCA indicates that experimental males from all treatment groups responded with aggressive behaviors toward intact (MCON) male opponents, whereas aggression was reduced in trials with opponents from the MCAST group. B) The second axis of the PCA indicates that intact (MCON) and T-treated (MTEST) males displayed dominant sexual-like behaviors whereas castrates (MCAST) displayed submissive behaviors during trials with intact (MCON) male opponents. This significant treatment effect is absent during trials with MCAST opponents. Note, for both axes of the PCA, the comparison among treatment groups of behavioral responses toward MTEST opponents are not clear due to the small sample size (n=2) of trials between two MTEST males.



CHAPTER 4

EFFECTS OF TESTOSTERONE ON THE EXPRESSION OF SEXUALLY DIMORPHIC TRAITS IN MADAGASCAR GROUND GECKOS (GEKKONIDAE: *PAROEDURA PICTA*)

Abstract

Androgens mediate the expression of suites of sexually dimorphic morphological, physiological, and behavioral traits in all classes of vertebrates including reptiles. Recent experimental studies on eublepharid geckos demonstrated that sexual dimorphism in the expression of behavioral and physiological traits is due in most cases to sex differences in the availability of T. However, intersexual and interspecific differences in the sensitivity of individual traits to T indicate that specific components of endocrine signaling system have the potential to evolve independently. In the present study, I examined hormonal control of sexually dimorphic traits in males and females of the Madagascar ground gecko (*Paroedura picta*). *Paroedura picta* (Gekkonidae) is characterized by male-larger dimorphism in body size. However, unlike most eublepharids, this species does not exhibit dimorphism in head size, and precloacal pores are absent in both sexes. Behaviorally, males of *P. picta* express aggression during interactions with males and copulatory behavior without courtship during interactions with females. I hypothesize that T is the primary mediator for the development and expression of sexual dimorphism in body size and behaviors in *P. picta*. This experiment included 3 groups of males (intact control, surgically castrated, castrated with T replacement) and 3 groups of females (intact control, T supplemented and ovariectomized females) and was conducted

over a 6-month period in the laboratory. Aggressive behavior and mounting were reduced by castration and restored by T in males, and were induced by T in females. Body size was not affected by treatments in males, but was increased by ovariectomy and T-supplementation in females. These data indicate that expression of sexually dimorphic behaviors is due to sex differences in availability of T, but gonadal androgens are not the primary mediator for the development of sexual dimorphism in body size in *P. picta*. Instead, these data implicate a role for ovarian function in the expression of sexual dimorphism in body size. The sensitivity of growth/body size to ovarian hormones in this species provides evidence that mechanisms other than changes in pleiotropic effects of T in males can enable individual traits within a suite of sexually dimorphic traits to evolve independently from the others.

Introduction

Pleiotropic effects of gonadal hormones and their metabolites mediate the expression sexually dimorphic morphological, physiological and behavioral traits in all classes of vertebrates, including reptiles (reviewed in Adkins-Regan 2005). The pleiotropic effects of a single hormone that regulate suites of functionally correlated traits may constrain the evolution of changes to individual traits within the suite (i.e., evolutionary constraint hypothesis, Hau 2007; McGlothlin and Ketterson 2008). Alternatively, selective changes in components of the endocrine signaling system may enable individual traits to evolve independently from one another (i.e., evolutionary potential hypothesis, Hau 2007; McGlothlin and Ketterson 2008). In gecko lizards from the family Eublepharidae, sexual dimorphism in the expression of traits is due in most

cases to sex differences in the availability of T. However, interspecific and intersexual differences in responsiveness to T have been reported for various traits. For example, in males of most species, courtship behavior requires activational effects of T, but this behavior cannot be induced by T in some species. For an intersexual example, T increases the likelihood for the expression of aggressive behavior in males but does not have this effect in females. These observations indicate that different species and sexes within species have evolved selective losses in sensitivity to T in the physiological or neural substrates that underlie the expression of sexually dimorphic trait, and provide support for the hypothesis that specific components of the endocrine signaling system have the potential to evolve independently (i.e., evolutionary potential hypothesis, Hau 2007; McGlothlin and Ketterson 2008; Ch. 2; Ch. 3). Is T the primary mediator of the development and expression of sexually dimorphic traits in gekkotan lizards that have evolved derived patterns of trait expression?

Paroedura picta is a gecko lizard from the family Gekkonidae (Gamble 2008; Jonniaux and Kumazawa 2008). Both families Gekkonidae and Eublepharidae are within the infraorder Gekkota, which is a relatively basal lineage of squamate reptiles. Gekkotans diverged from the sister group which gave rise to Serpentes, Anguimorpha and Iguania approximately 165-225 million years ago (Kluge 1987; Vitt et al. 2003; Vidal and Hedges 2005; Jonniaux and Kumazawa 2008; Wiens et al. 2012; but see Losos et al. 2012; Fig. 1.2). Therefore, *P. picta* shares a more recent common ancestor with the eublepharid geckos than with all other lineages of squamate reptiles previously studied such as iguanids and teiids.

Although geckos share evolutionary history, a number of factors (e.g., genetic drift, natural selection) since their divergence may have led to the observed differences in sexually dimorphic trait expression between members of these two families. Whereas most eublepharids exhibit a suite of traits, which includes male-larger head and body size, functional precloacal pores, aggressive behavior between males, and both the courtship and copulatory phases of sexual behavior (Kratochvíl and Frynta 2007), *P. picta* exhibits male-larger dimorphism in body size and aggressive behaviors in males (Starostová et al. 2010). However, this species lacks courtship behavior, precloacal pores, and dimorphism in head size (Brillet 1991, 1993; Jirků 2007). *Paroedura picta* also differs from eublepharids in the age at sexual maturation. Males and females of this species become sexually mature at an early age prior to attaining adult body size. Thus, *P. picta* represents a suitable model organism to investigate effects of T on the development and expression of sexual dimorphism in growth and body size as well as behaviors.

In my earlier studies on *Coleonyx elegans* and *Goniurosaurus lichtenfelderi*, I was unable to investigate effects of T on growth and body size because the animals were nearly fully grown at the age of sexual maturation when experiments were initiated (Ch. 2; Ch. 3). Nevertheless, evidence from several species of iguanid lizards strongly suggests that T regulates male growth and the development of both male- and female-larger sexual dimorphism in body size (Cox et al. 2005; Cox and John-Alder 2005; Cox et al. 2009). In the present study, I hypothesize that sex differences in trait expression are due to sex differences in the availability of T, which predicts that sexual dimorphism in growth and body size as well as the expression of aggressive and copulatory behaviors is

due to presence of T in males. Specifically I predict that castration will reduce growth rate and the occurrence of aggressive and sexual behavior in behavioral trials, and that T-replacement will reverse these effects. Further, I predict that T-supplementation will induce these traits in females.

By definition, sexual dimorphism is a function of trait expression in one sex relative to the other (Badyaev 2002; Ellengren and Parsch 2007). Thus, sex-limited and sex-biased expression of traits can be due to genetic, epigenetic, and regulatory factors operating in one or both sexes (Badyaev 2002; Ellengren and Parsch 2007). Thus, to better understand how gonadal hormones may influence the development of sexually dimorphic traits in both males and females, I included a group of ovariectomized females in the present experiment even while focusing primarily on the role of T.

Materials and Methods

Animals

Paroedura picta (Madagascar ground gecko) is a small, nocturnal, ground-dwelling lizard, found living at coastal shores to dry, thorn scrubs and forest leaf litter in Madagascar. This species is easily bred and maintained in the laboratory, grows rapidly and matures at an early age (~3 months). Among reptiles, this species is known for its extremely short intervals between clutches of one or two relatively large eggs, and it can breed continuously in suitable environmental conditions (Kubička and Kratochvíl 2009; Kubička et al. 2012; Starostová et al. 2012). Eggs from the captive breeding colony of Lukáš Kratochvíl at Charles University were collected from mated females that were individually housed in a climate-controlled chamber set at 27 °C and a 12L:12D light

cycle (lights off at 1800). Eggs were then placed in individual cups filled with moist vermiculite and incubated at 27 °C. Upon hatching, animals were individually housed in standardized plastic boxes (approximate size 20x20 cm) with a substrate of sand, a shelter, and a water dish. Geckos were fed crickets (*Gryllus assimilis*) dusted with vitamins (Roboran, Univit, Czech Republic) twice weekly to satiety. Water enriched by calcium (Vitacalcin, Zentiva, Czech Republic) was always provided but was replaced once every two weeks by water supplemented with vitamins A, D3 and E (Combinal A+D3 and Combinal E; IVAX Pharmaceuticals, Opava, Czech Republic). This experiment was conducted with the approval of the Ethical Committee of Charles University (permit number 34711/2010-30).

Surgical and hormone treatments

Paroedura picta reach sexual maturity at approximately 3 months of age. At this time it becomes possible to determine the sex of individuals according to external morphology (enlarged hemipenal sacs in males). Males (n=40) and females (n=30) of *P. picta* between the ages of 12 to 16 weeks were assigned to treatment groups that were balanced with respect to age, body mass and snout-vent length within each sex. Males were assigned to one of three treatment groups: sham-operated intact controls (MCON: n=13, SVL=64.5 ± 0.7 mm), surgically castrated (MCAST: n=13, 63.2 ± 1.0 mm), and castrated with T replacement (MTEST: n=14, 63.2 ± 1.0 mm). Females were assigned to one of three treatment groups: intact control (FCON: n=10, 63.5 ± 1.0 mm), intact with T supplement (FTEST: n=10, 62.7 ± 1.0 mm) and ovariectomized (FOVX: n=10, 62.9 ± 1.0 mm). For surgery, animals were anaesthetized using a combination of an intramuscular injection of ketamine (Narkamon 5%, Spofa a.s., Prague, Czech Republic; 130 μg/g body

mass) and cold anesthesia (i.e., placed on ice). The gonads were exposed via a medial ventral incision. Bilateral orchiectomy was done on MCAST, MTEST, and FOVX by ligating each spermatic cord or oviduct (respectively) with surgical silk, then ablating each testis or ovary with all visible follicles. For the intact control groups, "sham" surgeries were performed, in which ventral incisions were made to expose and manipulate the testes or ovaries while leaving the gonads completely intact. Empty implants (MCAST, FCON, FOVX) or T implants (MTEST, FTEST) were then inserted into the coelomic cavity, and the incision was closed using Prolene® surgical suture (Ethicon INC, Somerville NJ, USA) and covered with Glubran ®2 surgical glue (GEM S.r.l., Viareggio, Italy). Tonic-release T implants were constructed as described in Cox et al. (2005) and Golinski et al. (2011).

Blood samples were collected approximately 28 weeks after surgical manipulations to verify hormone treatments prior to the start of behavioral trials. Blood plasma was assayed at the Institute of Endocrinology (Prague, Czech Republic) using the method published by Hampl (1994). The method consists of extracting hormones from plasma with diethyl-ether followed by radioimmunoassay using rabbit polyclonal antiserum to testosterone-3-(carboxymethyloxime) bovine serum albumin conjugate, and homologous [^{125}I]tyrosine methyl ester derivative as a tracer. Intra-assay and inter-assay coefficients of variation for the analyses are typically 8.2% and 10.7%, respectively.

Morphological measurements

Body mass (g), snout-vent length (SVL, mm) and head width (mm) of all experimental geckos were measured monthly during the experimental period. Before surgery and prior to termination, the width of the hemipenes (nearest mm) was also

measured and digital images of the dorsal and ventral surfaces of each individual were scanned.

Behavioral trials

Behavioral trials were begun 29 weeks after surgical manipulations, and one week after blood samples had been collected. Experimental males experienced three behavior trials and experimental females experienced a total of two. Behavior of each experimental animal was first assessed during a trial with a stimulus of the opposite sex followed by a trial with a stimulus of the same sex. A separate pool of unmanipulated males and females was used to provide size-matched 'stimulus' animals for behavior trials (n=14 stimulus females, mean SVL=76mm, age=37 weeks; n=27 stimulus males, mean SVL=85mm, age=45 weeks). Consecutive trials for any given experimental animal were separated by a minimum of one week. Sequential trials involving the same stimulus animal were separated by a minimum of 48 hours. All behavior trials and subsequent analyses were conducted without the observer having knowledge of experimental treatment.

The first trials assessed behaviors of experimental males (i.e., MCON, MCAST, MTEST) when presented with a stimulus female in a neutral arena (glass aquaria, 30x40 cm). However, the stimulus females escaped from the test arena during 10 of these trials, and those trials were excluded from further analysis. All subsequent behavior trials were conducted in the home cage of the experimental animals (size: 20x20 cm), from which lizards were unable to escape. In these trials, a stimulus animal was introduced into the home cage of each experimental animal and behaviors were observed for 15 minutes.

Trials were terminated early when mounting occurred and when behaviors appeared to be highly stressful (i.e., repeated fleeing by one individual) or potentially injurious.

Paroedura picta is nocturnally active, thus all behavior trials were conducted between 1830 and 2130 h under dim red light. To minimize disturbance, trials were not observed directly but were monitored in real time from a second room via closed circuit television. Trials were video-recorded (Sony Handycam® DCR SR42) and subsequently analyzed using JWatcher™ Video software (Dan Blumstein's Laboratory, UCLA and The Animal Behavior Laboratory, Macquaric University, Sydney). All behaviors displayed by the species were programed into JWatcher's global definitions file. Digital recordings were later analyzed for the duration and occurrences of behaviors of interest (Table 4.1).

Statistical Analysis

Statistical analyses were conducted on behavioral data from all subjects with successful experimental treatment, which was verified via plasma T measured 28 weeks after surgical manipulations. Statistical analyses were conducted using SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) or GraphPad Prism (version 5.00; GraphPad Software, San Diego CA USA). Plasma T levels and snout-vent length (SVL) were analyzed using one-way ANOVA with the Ryan-Einot-Gabriel-Welsch multiple range test for post hoc analysis. Hemipenes size was analyzed using a non-parametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons post hoc test. Statistical significance was accepted at $\alpha = 0.05$ based on the H-statistic, which is the non-parametric analogue of an F-statistic.

Principal components analysis (PCA) was used to reduce the dimensionality and help identify informative behavioral traits displayed by experimental animals during the

behavioral trials conducted in the home cage. Three separate PCAs were conducted: 1) male behavior toward stimulus females, 2) female behavior toward stimulus female, and 3) male behavior toward stimulus males. Only the first two or three axes from each PCA are reported because these axes explain more of the variation in the primary data relative to the later axes and the trait loading patterns of these axes were most amenable to interpretation. Effects of treatment on PCA axis scores were analyzed using one-way ANOVA with Ryan-Einot-Gabriel-Welsch multiple range post hoc test. For each of these analyses, the Dunn-Sidak method was used to control for experiment-wise error level at α=0.05.

Results

Validation of manipulations with plasma T levels

Plasma T measured 28 weeks after manipulation validated the efficacy of the experimental procedures (ANOVA: $F_{5,61}$=41.4, P< 0.001; Fig. 4.1). In males, plasma T levels were high and variable in intact controls (MCON n=13; mean=31.58 ng/ml, range=3.30 - 144.22 ng/ml; note, T levels in one MCON was above the standard curve and was not included in Fig. 4.1). Plasma T was reduced by surgical castration (MCAST n=13; 0.202 ± 0.02 ng/ml) and restored to physiological levels by T-replacement in 11 out of 14 MTEST (25.54 ± 0.64 ng/ml). Plasma T was nearly undetectable in three MTEST males. I assumed that the implants in these males had been depleted and I excluded them from all analyses. In females, plasma T was very low in intact controls (FCON n=10; 0.397 ± 0.08 ng/ml) and ovariectomized females (FOVX n=10; 0.199 ± 0.03 ng/ml) and was elevated into physiological range of males by T-implantation

(FTEST n=10; 12.44 ± 3.33). Although treatments were successful in all females, some of these individuals were excluded from subsequent analysis of morphology and behavior because the blood sampling procedure negatively affected their health status.

Morphology

As a species, *P. picta* is characterized by male-larger sexual dimorphism in body size and in the present study, at the end of the 6-month experiment, snout-vent length (SVL) was significantly larger in males than in females ($F_{5,59}$=9.617, P<0.0001; Fig. 4.2A). Castration and T-replacement did not significantly affect SVL in males ($F_{2,34}$=1.430, P=0.25). In females, SVL was greater after 28 weeks of treatment in FOVX and FTEST than FCON ($F_{2,25}$=3.959, P=0.0321). Although FTEST and FOVX females grew more than FCON during the experiment, the final SVL of these females was significantly smaller than that of MCON ($F_{2,29}$=11.772, p<0.001).

Testosterone treatment significantly affected the mass of the hemipenes in both males and females (H=72.05, P<0.0001; Fig. 4.2B). In males, the mass of the hemipenes was reduced in MCAST relative to MCON and MTEST (H=24.7, P<0.0001). In females, the hemipenes were undetectable in FCON and FOVX, but were significantly larger in FTEST. Head width is not sexually dimorphic in *P. picta*; males and females follow more or less the same allometric growth of limb, head and tail dimensions relative to trunk (Jirků 2007). This species lacks precloacal pores, and long-term T-treatment did not change this trait in either sex.

Sexual behavior

During social trials between a male and a female of *P. picta*, both individuals display tongue flick, a behavior common in lizards that use chemical cues to investigate

their surrounding environment (see Houck 2009). One animal typically approaches to investigate the other animal. This species does not perform a sexual body grip behavior that involves the male biting the female, usually near the neck. Instead, a male typically moves his body over the top of the female (i.e., walk-over; see Table 4.1) until finding the correct alignment of their bodies (i.e., straddle) required to achieve mounting for copulation. The male is dominant to the female in the position of walk-over, which is analogous to body grip displayed by other lizards. Males of *P. picta* do not perform precopulatory courtship display behaviors prior to walk-over and mount (also see Brillet 1991, 1993). During sexual behavior trials, the test venue (i.e., neutral arena versus home cage) had no significant effect on behaviors expressed by experimental males including tongue flick (Wilcoxon signed rank test: $P>0.6$ for each treatment group), investigate and walk-over (Unpaired t-test: $P>0.2$ for all comparisons), and mount (Fisher exact test: $P>0.35$ for each treatment group).

Results from the principal components analysis (PCA) on behaviors displayed by experimental males and females toward intact females introduced into the home cage are shown in Table 4.2. The first three axes explain a total of 74.7% of variation in the primary data. The first axis of the PCA explains 38.8% of the variation and contrasts male-typical sexual behaviors (i.e., investigate, mount, straddle) from behavioral inactivity (i.e., total inactivity). The second axis explains 22.2% of the variation and groups general investigative behaviors (i.e., approach and tongue flick). The third axis explains 13.7% of the variation and groups investigate and tail wave. The first axis of the PCA shows that intact (MCON) and T-replaced (MTEST) males as well as T-supplemented females (FTEST) responded to the stimulus female with sexual behaviors

whereas castrated males (MCAST), intact (FCON) and ovariectomized females (FOVX) were inactive ($F_{5,60}$=38.36, P<0.0001; Fig. 4.4A). Treatment had no significant effect on the display of general investigative behaviors from the second axis of the PCA ($F_{5,60}$=0.84, P=0.527; Fig. 4.4B) or the third axis of the PCA ($F_{5,60}$=1.02, P=0.412).

Aggressive behavior

Results from the principal components analysis conducted on behaviors displayed by experimental males and females towards intact males are shown in Table 4.3. The first three axes of the PCA explain a combined total of 73.5% of the variation in the primary data. The first axis of the PCA explains 39.3% of the variation and contrasts all active behaviors (i.e., approach, attack, investigate, tail wave, and tongue flick) from inactivity. The second axis of the PCA explains 21.2% of the variation and contrasts general investigative behaviors (i.e., approach and tongue flick) from attack. The third axis of the PCA explains 13.0% of the variation and contrasts tail wave from investigate. All treatment groups had similar scores on the first axis of the PCA ($F_{5,58}$=0.89, P=0.495; Fig. 4.5A), the second axis of the PCA ($F_{5,58}$=1.21, P=0.318; Fig. 4.5B), and the third axis of the PCA ($F_{5,58}$=2.08, P=0.081).

Experimental animals in this study rarely displayed aggressive behavior, and T only slightly increased the likelihood that a male or female would attack an intact male introduced into their home cage. Only 3 out of 7 intact (MCON) and 4 of 7 T-replaced (MTEST) males, and 1 out of 7 T-supplemented (FTEST) female displayed attack, whereas castrated males (MCAST), intact (FCON) and ovariectomized (FOVX) females never displayed this behavior (H=11.52, P=0.0421). Note the low sample size (i.e., out

of 7 trials) indicates that only 7 experimental animals from those treatment groups first investigated the stimulus males, a prerequisite for subsequent attack to be observed.

Discussion

The goal of this study was to examine the role of gonadal hormones in regulating the expression and development of sexually dimorphic morphological and behavioral traits in *P. pica*. This species exhibits sexual dimorphism in the expression of copulatory and aggressive behaviors as well as body size. Based on previous findings in other lizards, I predicted that these traits are dependent on effects of T.

Androgen control of sexual behavior

Previous studies in gekkotan lizards from the family Eublepharidae revealed that copulatory behavior persists in males for weeks after castration (Lindzey and Crews 1986; Rhen and Crews 1999; Sakata et al. 2003; Golinski et al. 2011; Ch. 2; Ch. 3), a longer period than previously reported in other lizards (2 weeks *Anolis carolinensis* Crews et al. 1978, Neal and Wade 2007; 24 days *A. sagrei* Tokarz 1986; 3 weeks *Cnemidophorus inornatus* Lindzey and Crews 1986). The persistence of male sexual behavior for weeks after castration in eublepharid geckos was an unusual finding among lizards and among vertebrates in general (see Golinski et al. 2011). The observed persistence of behavior was speculated to be caused by unusually long-lasting effects of androgens on neural circuitry but also potential influences of a home-cage effect, which was documented in *G. lichtenfelderi* (Ch. 3). In *P. picta*, male copulatory behavior was significantly reduced six months after castration, indicating that the neural circuitry is not maintained in reproductive condition throughout this extended experimental period and

suggesting that T must be present for the expression of sexual behaviors. However, it is not clear if *P. pica* differs from *C. elegans* and *G. lichtenfelderi* in this regard. Comparisons of these three species must take into account significant differences in experimental methods, including the age of the animals and the duration of treatment.

Although all studies involved surgical manipulations conducted after the age of sexual maturation, this occurs at the age of 3 months in *P. picta* and approximately 1 year in the eublepharids. At the age of 3 months in *P. picta*, animals are not fully grown, and rapid growth continues until adult (i.e., asymptotic) body size is attained at approximately 9 months of age (see Starostová et al. 2013 in press). In contrast, eublepharids were nearly fully grown at the time of surgery. The period marked by rapid physical growth in *P. picta* may coincide with further brain development (e.g., organization of neural circuits by androgens, similar to puberty in mammals; see Schulz et al. 2009), and castration at three months of age may have been too early to observe potential long-lasting effects of androgens on sexual behavior.

The second issue to consider when comparing results among these studies is the difference in duration of experimental treatments. The six-month experimental period in *P. picta* is significantly longer than the studies conducted on *E. macularius*, *C. elegans*, and *G. lichtenfelderi* (Rhen and Crews 1999; Sakata et al. 2002; Sakata and Crews 2004; Golinski et al. 2011; Ch. 3), in which copulatory behavior persisted for unexpectedly long periods of time following castration. However, because of substantial differences in the duration of experiments, it is unclear whether the three species of eublepharids differ from *P. picta* with regard to long-term persistence of copulatory behavior. Collectively, the data from species of geckos indicate that male sexual behaviors can persist for a

period of time before gradually decaying in the absence of elevated plasma T. The relative persistence of copulatory behavior in these nocturnal geckos may be associated with their low body temperature and metabolism relative to diurnal lizards and to vertebrates in general (e.g., Feder et al. 1981; Al-Sadoon and Abdo 1989; Autumn et al. 1994, 1997).

No venue effect on expression of male sexual behavior

In *P. picta*, male behavior did not differ between trials conducted in the neutral arena versus the home cage, contrasting behavioral findings from *G. lichtenfelderi* (Ch. 3). The comparison between species indicates that venue affects behavioral responses in some lizards (e.g., *G. lichtenfelderi*) but not others (e.g., *P. picta*). These two experiments are the only studies on lizards where behavioral responses of the same animals were assessed in two different test venues, as laboratory studies always use either a home cage (e.g., Crews et al 1978; Adkins and Schlesinger 1979; Flores and Crews 1995; Tokarz 1986; Lindzey and Crews 1986; Neal and Wade 2007; Golinski et al. 2011; Ch. 2) or a neutral arena (e.g., Rhen and Crews 1999, 2000; Sakata et al. 2002a,b; Sakata and Crews 2004). The effects of venue, novel stimuli, and other laboratory conditions are well known in rodent models (e.g., see Boleij et al. 2012), but are not as well documented in non-mammalian systems. Future laboratory studies investigating behavior of lizards or other non-traditional model organisms should be aware that different species and even different phenotypes within a species (e.g., incubation temperature morphs: Rhen and Crews 1999; Trnik et al. 2011; Huang and Crews 2012) may yield varying behavioral responses depending on the experimental design.

Androgen control of aggressive behavior

Paroedura picta is not very aggressive, as attacks directed toward males introduced into their home cage were rare. Although behavior of this species has not been studied in nature, the low levels of aggression observed during behavioral trials in the laboratory suggest this species is not likely to be territorial. In non-territorial lizard species, low levels of aggression may be adaptive based on ecological factors such as habitat and resource availability (e.g., *Phrynosoma platyrhinos* Trollestrup 1981; *Gambelia wislizenii* Trollestrup 1983; *Lacerta vivipara* Bauwens et al. 1987). Regardless, the expression of aggressive behavior in this species required elevated levels of androgens, which is consistent with reports from other lizards and in vertebrates in general (Moore 1987, 1988; Rhen and Crews 2000; Civantos 2002; Weiss and Moore 2004; Kabelik et al. 2006; reviewed in Adkins-Regan 2005). Long-term treatment with T in females increased the likelihood for the expression of aggressive behavior, but only one of seven attacked an intact male. The low levels of responsiveness suggest that the neural substrates responsible for aggression are relatively insensitive to T in adult females, which is consistent with reports in eublepharid geckos (Flores and Crews 1995; Rhen and Crews 2000; Ch. 2; Ch. 3).

Effects of gonadal hormones on growth and body size

Paroedura picta is characterized by male-larger body size due to a longer period of rapid growth in males relative to females (Starostová et al. 2010). Experimental studies involving castration and T-replacement in several iguanian lizards suggest that gonadal androgens are the primary mediator leading to sex differences in adult body size (reviewed in Cox et al. 2009). More specifically, gonadal androgens stimulate growth in

species with male-larger dimorphism in body size (e.g., *Sceloporus jarrovi* Cox and John-Alder 2005; *Anolis sagrei* Cox et al. 2009) and inhibit growth in species with female-larger dimorphism in body size (e.g., *S. undulatus* Cox et al. 2005; *S. virgatus* Cox and John-Alder 2005). Therefore, I predicted that T would stimulate growth and the development of male-larger body size in *P. picta*. Contrary to predictions, at the end of the six month treatment period, male treatment groups did not significantly differ in asymptotic body size (Fig. 4.2A). This result indicates that the effects of gonadal androgens during the period of rapid growth are not the primary mediator for the development of male-larger body size in *P. picta* (see Starostová et al. 2013 in press), at least not under conditions of this laboratory study (see Cox et al. 2006).

Ovarian effects on female growth and body size

Ovariectomy of female lizards in previous studies suggest that removal of female gonads not only prevents reproduction but also has effects on growth and other aspects of physiology (Cox 2006; Cox and Calsbeek 2010; Cox et al. 2010). In females of *P. picta*, ovariectomized females and intact females supplemented with T attained larger body size than intact controls (Fig. 4.2), although they still fell short of the asymptotic size of males. Because gonadal androgens did not significantly affect growth and body size in males of *P. picta*, the effect of exogenous T in females is not likely due to androgenic effects on growth. The similarity in body size between the ovariectomized and the T-supplemented females suggests the effect of T-supplementation may be due to interference (i.e., negative feedback) with normal function of the ovaries. At termination, the ovaries in all T-supplemented females were small and with no enlarged vitellogenic follicles, which is indicative of negative feedback of high levels of exogenous steroids on

gonadal function. Together, these data suggests that normal ovarian function leads to a decrease in growth of females in *P. picta,* which supports the hypothesis that ovarian hormones in females play a more important role than gonadal androgens in males with regard to the development of sexual dimorphism. Evidence from studies in other lizards also support the hypothesis that ovarian hormones affect growth in females and thus the sexual dimorphism in body size of the species (reviewed in Starostová et al. 2013 in press). Notably, ovariectomy or T-supplementation had positive effects on female body size in male-larger species, as demonstrated here in *P. picta*, while the same treatments had negative effects on female body size in female-larger species (reviewed in Starostová et al. 2013 in press).

In the present study, ovariectomy of females led to increased body size relative to intact females, but all male groups were still significantly larger than all female groups. Thus, the effects of gonadal hormones alone do not explain the mechanism responsible for the development of sexual dimorphism in body size in *P. picta*. Potential alternative mechanisms could include processes during embryonic development or postembryonic ontogeny before the age at surgical manipulation in this study or the possibility that final body size may be linked to sexual differences in genotype (i.e., sex chromosomes; Blumberg et al. 2002; Kratochvíl et al. 2008).

Androgen control of sexually dimorphic traits in gekkotan lizards

When male-specific sexually dimorphic traits are present in a species, T is almost always the primary mediator for the expression of the trait. Among gecko lizards studied, T-sensitive traits include behaviors (i.e., courtship, copulatory mounting, and aggression), physiological activity of functional precloacal pores, and morphology (i.e., hemipenes,

head width). Although head width in adult males was not significantly altered by experimental treatments, T-supplementation in females of *C. elegans* induced the enlargement of head width (Ch. 2), suggesting the development of this dimorphic trait requires T. As for the other morphological, physiological and behavioral traits in males, activational effects of T were necessary for their expression if the traits were present in the species. The linkage of traits to androgenic control is a simple and effective mechanism to coordinate expression of traits at the appropriate time and conditions (Finch and Rose 1995; Adkins-Regan 2005; Hau 2007; McGlothlin and Ketterson 2008). In species with an associated reproductive pattern, these traits are accordingly expressed during the breeding season when T levels rise to coordinate the suite of traits necessary for reproductive success via intersexual and intrasexual competition (Andersson 1994; Woolley et al. 2004; Adkins-Regan 2005).

However, in some species, certain traits are absent (e.g., courtship in *C. elegans* and *P. picta*) and treatment with exogenous T cannot induce trait expression, suggesting that phenotypic differences have evolved via selective loss of sensitivity to T of such traits. Although gecko species share the androgenic control for the expression of sexually dimorphic traits, T-sensitive traits differ among species, which suggests the targets for activational effects of T differ in species-specific manner leading to the observed diversity in phenotypes. For example, T stimulates the expression of tail vibration courtship, aggression and pore secretions in males of *E. macularius* and *G. lichtenfelderi* (Ch. 3; Rhen and Crews 1999) whereas the same hormone stimulates expression of blue coloration of ventral patches and aggression in males of *S. undulatus* (Hews and Quinn 2003; Cox et al. 2005). Traits favored for reproductive success within individual species-

specific conditions (e.g., environment, mating system, etc.) are often linked to androgen control module. Once such suites of androgen-dependent traits have evolved, they are not constrained from further evolutionary changes, but rather the loss of trait expression can occur by targeted change in sensitivity to T of individual traits allowing plasticity in linkage among traits mediated by a common proximate mechanism (Hau 2007; McGlothlin and Ketterson 2008).

In gekkotan lizards, certain male-typical traits can consistently be induced by T in females, which support the idea that selection for trait expression in males led to the correlated evolution of the underlying components of the endocrine signaling system in females (Ketterson et al. 2006; Hau 2007). In each species studied, T-supplementation induced enlargement of the hemipenes and male-typical courtship behaviors (if present; Ch. 2; Ch. 3; Flores and Crews 1995; Rhen et al. 1999). However, T-supplementation in female geckos did not induce the expression of aggressive behaviors observed in males, which suggests females have evolved a loss of sensitivity to T in the neural substrate responsible for aggression. The responsiveness of various sexually dimorphic traits in females was not always the same among species. For example, T-supplementation in females of *C. elegans* and *P. picta* induced male-typical copulatory behavior to levels observed in intact males, whereas this effect was not observed in *E. macularius* and *G. lichtenfelderi*. Although these species generally require elevated levels of T to coordinate the expression of the male phenotype, females of these species differ in their level of sensitivity to T.

Unlike the majority of sexually dimorphic traits in geckos, body size was not affected by T in males, but rather the present study in *P. picta* implicates ovarian

hormones reduce growth in females. Because sexual dimorphism is defined as the relative difference in trait expression between males and females, this finding indicates that sexually dimorphic traits not only have the potential to evolve via selective changes in sensitivity to gonadal androgens in males, but also mediated by sensitivity in females to the effects of gonadal hormones. In this species, the potential that the mechanism responsible for the development of sexual dimorphism in body size switched from androgens to estrogens as the primary growth regulators is very interesting. However, future studies investigating the effects of estrogens on growth in gecko lizards would be needed to verify this possibility.

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Tables

Table 4.1. Behaviors displayed by *P. picta* during social interactions of all types (i.e., male-male, male-female, female-female). These behaviors describe the experimental lizard responding to the introduced stimulus lizard. Counts or durations of these behaviors were entered into principal components analyses (see statistical methods).

Behavior	Description
Approach	Movement toward stimulus animal
Attack	Rapidly bites, but does not grip stimulus
High posture	Extends limbs to stand tall; threat display
Inactivity	Passive, no movement
Investigate	Presses snout against the stimulus lizard; typically the first physical contact
Mount	Straddles and attempts copulation
Pinned	Immobilized by physical contact of stimulus animal
Retreat	Movement away from stimulus animal
Straddle	On top with forelimbs on either side of stimulus lizard's body
Tail wave	Tail lifted high above body
Tongue flick	Rapid and sequential protrusion and retraction of the tongue
Walk-over	Climbs over top of the stimulus animal

Table 4.2. Principal components analysis (PCA) of behavioral responses of experimental males and females from each treatment group when presented with a female; the first three axes of the explain 74.6% of the variation in the primary data. The first axis contrasts sexual behaviors (i.e., investigate, mount, straddle) from behavioral inactivity (i.e., tongue flick, total inactivity). The second axis groups general investigative behaviors (i.e., approach, tongue flick). The third axis loads investigate and tail wave.

Variable	First Axis		Second Axis		Third Axis	
	Correlation	Significance	Correlation	Significance	Correlation	Significance
Approach (min)	-0.244	0.049	**0.873**	**<0.0001**	-0.053	0.671
Investigate (min)	**0.432**	**0.0003**	0.360	0.003	**0.391**	**0.001**
Mount	**0.824**	**<0.0001**	0.056	0.654	-0.046	0.715
Straddle (min)	**0.821**	**<0.0001**	0.148	0.236	0.012	0.926
Tail wave	-0.333	0.006	-0.164	0.189	**0.877**	**<0.0001**
Tongue flick	**-0.546**	**<0.0001**	**0.729**	**<0.0001**	0.005	0.969
Total inactivity (min)	**-0.839**	**<0.0001**	-0.278	0.024	-0.168	0.177

Table 4.3. Principal components analysis of behavioral responses of males and females from each treatment group when presented with a male; the first three axes explain 73.5% of the variation in the primary data. The first axis contrasts all active behaviors (i.e., approach, attack, investigate, tail wave, tongue flick, walk-over) from inactivity (i.e., total inactivity). The second axis contrasts general investigative behaviors (i.e., approach and tongue flick) versus aggressive behavior (i.e., attack). The third axis contrasts investigate from tail wave.

Variable	First Axis		Second Axis		Third Axis	
	Coefficient	Significance	Coefficient	Significance	Coefficient	Significance
Approach (min)	**0.618**	<.0001	**0.556**	<.0001	-0.143	0.259
Attack	**0.468**	<.0001	**-0.524**	<.0001	0.017	0.896
Investigate (min)	**0.707**	<.0001	-0.250	0.047	**-0.551**	<.0001
Tail wave	**0.653**	<.0001	-0.180	0.155	**0.670**	<.0001
Tongue flick	**0.653**	<.0001	**0.752**	<.0001	0.093	0.465
Total inactivity (min)	**-0.802**	<.0001	0.162	0.201	0.009	0.945

Figures

Figure 4.1. Plasma testosterone (T) in experimental males and females of *P. picta*, measured 28 weeks after surgical manipulations. Lines indicate group mean. Lower case letters indicate post hoc statistical groupings (see statistical methods). Treatment groups are designated as follows: MCON = intact control males, MCAST = castrated males, MTEST = castrated males with T-implants, FCON = intact control females, FOVX = ovariectomized females, FTEST = intact females with T-implants. Note the $\log_{10}$-scale on the y-axis.

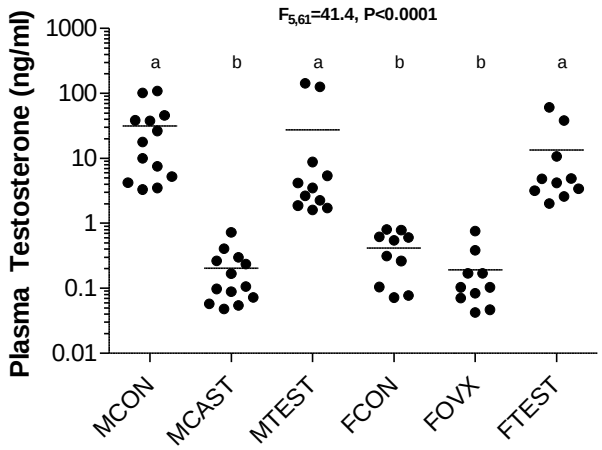


Figure 4.2. Snout-vent length (A: SVL; mean ±SEM) and mass of the hemipenes (B: median, interquartile and range) in males and females of all treatment groups. Experimental treatments had no significant effects on SVL in males. In females, SVL was lower than in males, and was lower in controls (FCON) than in ovariectomized (FOVX) and T-supplemented (FTEST) females. Mass of hemipenes was reduced by castration (MCAST) and restored by T-replacement (MTEST) in males, and was increased by T-supplementation in females. Lower case letters indicate post hoc statistical groupings (see statistical methods); "u.d." indicates undetectable values.

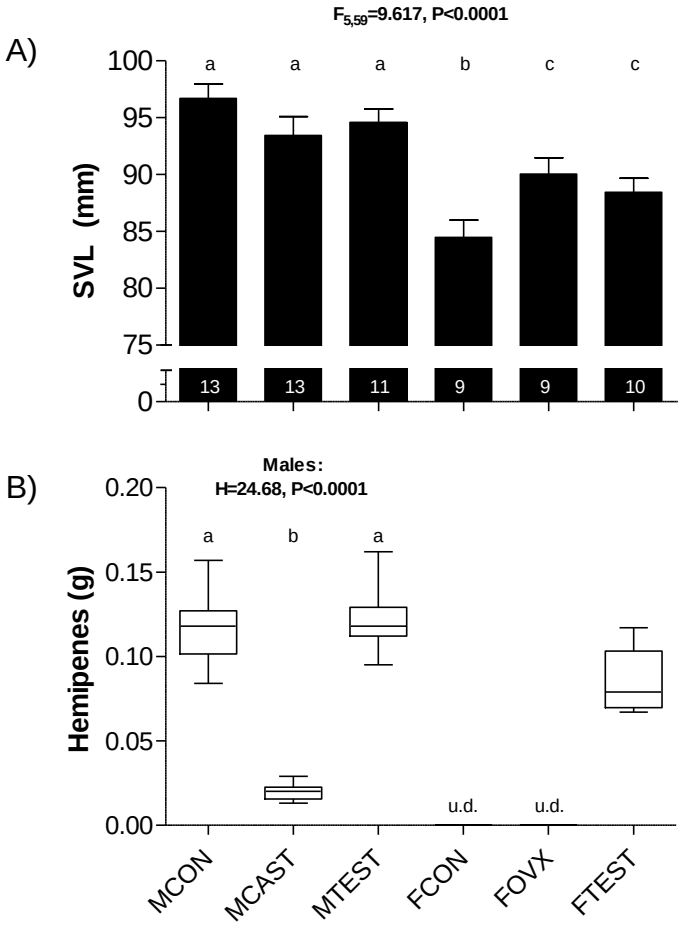


Figure 4.3. Behavioral responses of experimental males and females from each treatment group when presented with a female (see Table 4.2). Box and whisker plots indicate median, interquartile (box) and range (whiskers). Lower case letters indicate post hoc statistical groupings (see statistical methods). A) The first axis of the PCA indicates that intact (MCON) and T-treated (MTEST) males responded to the female with sexual behaviors whereas castrates (MCAST) were inactive. Supplementation with T induced male-typical sexual behavior in females (FTEST). B) The second axis of the PCA shows that treatment had no significant effect on the display of general investigative behaviors.

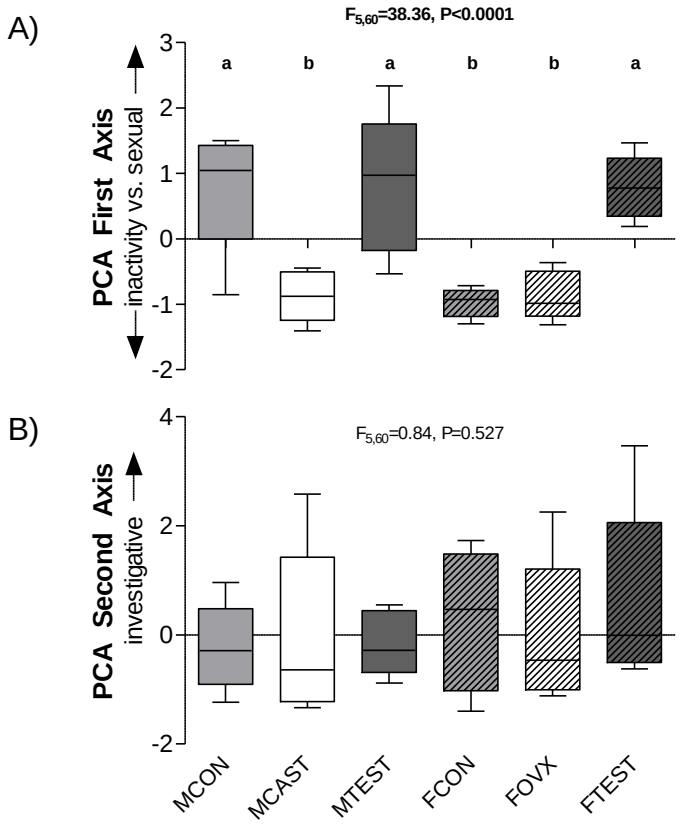
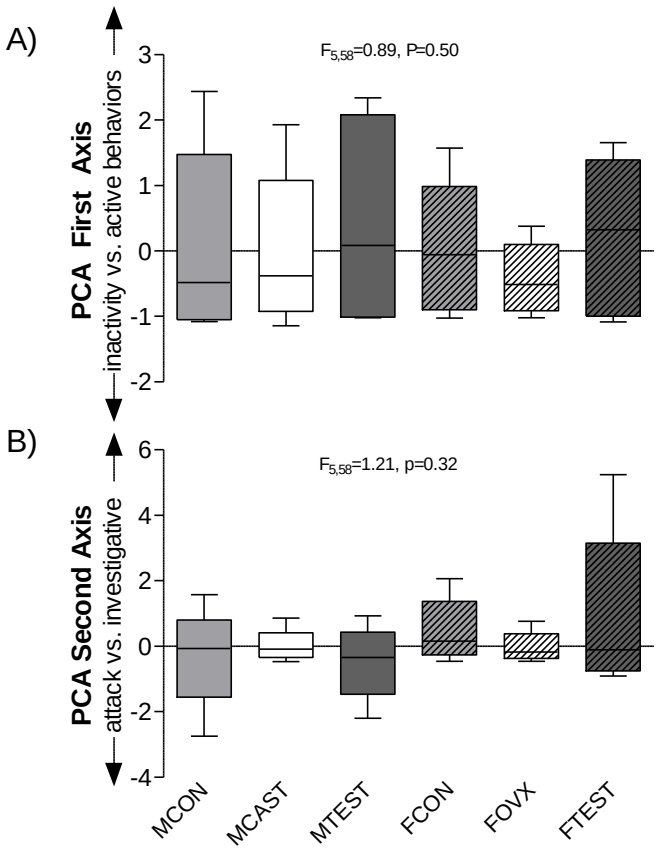


Figure 4.4. Behavioral responses of males and females from each treatment group toward intact males (see Table 4.3). A) The first axis of the PCA indicates treatment groups did not differ in terms of active behaviors versus inactivity. B) The second axis of the PCA indicates that treatment groups did not differ in display of general investigative behaviors (i.e., approach and tongue flick) versus attack.



CHAPTER 5

ANDROGEN RECEPTOR IMMUNOREACTIVITY IN THE BRAIN OF GECKO LIZARDS (*GONIUROSAURUS LICHTENFELDERI* AND *PAROEDURA PICTA*)

Abstract

In many vertebrates, androgens act on steroid-sensitive neural circuits to increase the likelihood of the expression of male-typical aggression, territoriality and courtship, all of which facilitate successful reproduction. Androgen receptor immunoreactive (AR-ir) neurons have been identified in brain regions associated with the control of reproductive behaviors in all vertebrate classes. However, taxonomic and species differences in brain AR-ir have been reported in groups with more specialized aspects of reproductive behavior (e.g., bird song). In gekkotan lizards, components of the reproductive behavioral repertoire differ markedly among species. For example, males of *Goniurosaurus lichtenfelderi* are highly aggressive and display precopulatory courtship behavior, whereas males of *Paroedura picta* show little aggression and no courtship. Furthermore, these species differ in behavioral sensitivity to T, suggesting differences in AR distribution or abundance in the brain. I conducted immunohistochemical assays for brain AR-ir in *G. lichtenfelderi* and *P. picta* to determine whether species-specific differences in sexual and aggressive behaviors can be explained by differences in the distribution or abundance of brain AR-ir. The percent of cells with AR-ir in the amygdala, preoptic area (POA), and ventromedial hypothalamus (VMH) did not differ between males and females in either species. In *G. lichtenfelderi*, castration increased the

nuclear AR-ir labeling in the POA and T-implantation restored levels to that of intact males. Between species, *P. picta* had increased abundance of nuclear AR-ir in the POA and VMH relative to *G. lichtenfelderi*. However, this dissimilarity in AR-ir does not likely explain the dramatic behavioral differences between *G. lichtenfelderi* and *P. picta*.

Introduction

In many vertebrates, gonadal androgens are necessary for the expression of male-typical morphology, physiology and behaviors, including aggression, territoriality and courtship, which together facilitate successful reproduction (reviewed in Adkins-Regan 2005). Most behavioral effects of androgens are mediated through steroid-sensitive neural circuits, and androgen receptors (AR) are consistently present in brain regions associated with control of reproductive behaviors across different vertebrate taxa (reviewed in Guerriero 2009). On a large scale, differences among vertebrate taxa in the patterns of sex steroid receptors in the brain appear to be associated with the evolution of taxa-specific behaviors (Gahr et al. 1993). For example, oscine passeriformes, commonly known as songbirds, have characteristic estrogen receptors in the caudal forebrain and androgen receptors in song control areas, which are absent in the brain of non-song birds (Gahr et al. 1993; reviewed in Gahr 2001). On a smaller scale, patterns of sex steroid receptors in the brain among closely-related species seem to be associated with the evolution of species-specific behaviors, although few comparative studies have been conducted within taxa. A comparative study of two galliforms (i.e., Japanese quail and chicken), in which the species differ in two components of their androgen-dependent courtship behavior, reported species-specific differences in the distribution of AR in the

caudal hindbrain (Shaw and Kennedy 2002). Thus, both macro- and micro-evolutionary patterns suggest that the evolution of specialized reproductive behavior (e.g., bird song; reviewed by Guerriero 2009) involves changes in patterns of steroid receptors in the brain. These observations raise the question of whether changes in AR are a common alteration in the endocrine signaling system associated with the evolutionary changes in androgen-mediated behaviors (i.e., evolutionary plasticity, Hau 2007; McGlothlin and Ketterson 2008).

Gekkotan lizards have several characteristics that make this group a suitable model to investigate whether species differences in reproductive behaviors are associated with differences in steroid hormone receptors in the brain. In contrast to most lizards, which are diurnal and reliant on visual communicative signals, most gekkotan lizards are nocturnal and use nasal olfaction for species and sex recognition. Nocturnality and the reliance on nasal olfaction rather than visual sensory input are associated with the evolution of courtship that differs from that of diurnal lizards (Nobel and Bradley 1933; Vitt et al. 2003). Males of some gekkotan species display courtship behaviors, which are conserved in closely related species (Kratochvíl and Frynta 2007). In other gekkotans, however, male courtship and aggression are absent from the behavioral repertoire (Brillet 1993; Ch. 2; Ch. 4). In addition to interspecific differences in aggressive and courtship behavior, gekkotan lizards differ in testosterone-sensitivity of various behavioral traits (Golinski et al. 2011; Ch. 3 and Ch. 4). Although conversion of androgens to estradiol may have a modulatory effect on copulatory behavior (Latham and Wade 2009), T and the androgenic metabolite DHT are the primary mediators of sexual and aggressive behaviors in male lizards (e.g., Rhen and Crews 1999, 2000; reviewed in Wade 2011).

Therefore, gekkotan lizards can be used as a model to investigate whether species-specific differences in the repertoire for male courtship and copulatory behavior are associated with differences in androgen-sensitivity in the brain, measured by the distribution and abundance of AR-immunoreactivity (AR-ir).

Prior to the present study of brain AR-ir, I experimentally manipulated testosterone (T) to investigate the androgen-sensitivity of male-typical behavior in *Goniurosaurus lichtenfelderi* and *Paroedura picta* (Ch. 3; Ch. 4). In *G. lichtenfelderi* (family Eublepharidae), males are highly aggressive toward conspecific males and display precopulatory courtship behavior toward females, which involves rapid vibrations of the distal portion of the tail (Kratochvíl and Frynta 2007). In males of this eublepharid species, courtship and copulatory behaviors are unexpectedly persistent after removal of the endogenous source of T (Ch. 3). In *P. picta* (family Gekkonidae) males display little aggression toward conspecific males and no courtship behaviors toward females. In males of this species, aggressive and copulatory behaviors require elevated levels of T, as indicated by the restoration of these behaviors by T-replacement after castration (Ch. 4). The observed differences in behavioral sensitivity to circulating T levels may reflect species differences in AR.

In both *G. lichtenfelderi* and *P. picta*, aggression, courtship and copulatory behaviors are sexually dimorphic and thus are not typically expressed by females. However, exogenous T masculinized reproductive behavior in females of both species; T-supplementation increased the likelihood that females displayed male-typical courtship or copulatory behaviors when presented with an intact female. Behavioral sensitivity to T

treatment in females suggests that females possess androgen receptors (AR) in the same brain regions as males.

In most lizards studied to date, androgens increase the likelihood of the expression of male reproductive behaviors, and androgen receptors (AR) are present in regions of the brain underlying male reproductive behaviors (reviewed in Woolley et al. 2004; Crews et al. 2009). The amygdala (AME, referred to as the external amygdala, see Crews et al. 2009, or ventromedial nucleus of the amygdala, see Rosen et al. 2002) functions in general arousal and interest in a stimulus, serving as a relay for sensory input from the olfactory bulb (Martinez-Garcia et al. 1991; Lanuza and Halpern 1997, 1998) to the ventromedial hypothalamus (VMH) (Bruce and Neary 1995; Lanuza et al. 1997). The VMH is critical for the expression of sexually receptive behaviors in females (e.g., Kendrick et al. 1995) and is also involved in the expression of aggression in males (Kabelik et al. 2008). The medial preoptic area (POA) and the anterior hypothalamus (AH) are necessary for the performance of male sexual behaviors, including mounting (reviewed in Godwin and Crews 1997; Adkins-Regan 2005; Hull and Dominguez 2007).

Androgen receptor-ir (AR-ir) cells and AR mRNA are present in the POA, VMH and AME (Rosen et al. 2002; Rhen and Crews 2001; Moga et al. 2000; Hews et al. 2012). The distribution of AR in the brain is generally similar between male and female lizards (Rosen et al. 2002; Rhen and Crews 2001; Moga et al. 2000; Guerriero 2009), consistent with reports on other vertebrates (e.g., fish, Kah et al. 1993; birds, Gahr 2001; reviewed in Adkins-Regan 2005). However, studies on lizards have generally reported qualitative descriptions of AR distribution rather than quantitative comparisons between sexes or experimental treatments (Rhen and Crews 2001; Moga et al. 2000; Guerriero 2009; but

see Rosen et al. 2002 and Hews et al. 2012). Androgen receptors have not previously been investigated in lizard species that differ in the expression of male courtship behavior or traits that have evolved to be expressed without contemporaneously elevated circulating levels of androgens.

In the present study, I performed immunohistochemical assays on brain sections from experimental males and females of the gecko species *G. lichtenfelderi* and *P. picta*. Based on the behavioral sensitivity to T in females of both gecko species and the conserved distribution of AR between sexes reported in other vertebrates (e.g., Belle and Lea 2001; Rhen and Crews 2001; Shaw and Kennedy 2002; Rosen et al. 2002), the most parsimonious prediction is that males and females do not differ in the distribution of AR in the brain. Alternatively, females may have fewer AR than males, as seen in some mammals (e.g., rats, Feng et al. 2010) and in the lizard *Sceloporus undulatus* (Hews et al. 2012). The most parsimonious predictions for *G. lichtenfelderi* and *P. picta* include that AR-ir distribution does not differ between species or sexes. In terms of regulation of AR-ir by hormone manipulations, in vertebrates, AR in the brains of both males and females is generally reduced following gonadectomy and restored to levels found in intact animals following androgen replacement (reviewed in Burnstein 2005; Ing 2005). Therefore, I predict that AR-ir in the brain of these lizard species should similarly be reduced by castration and restored by T-replacement.

Materials and Methods

Validation of IHC protocol

The immunohistochemistry (IHC) protocol used in this study followed methods previously used in *Anolis carolinensis* (green anole) in the laboratory of Dr. Juli Wade at Michigan State University (e.g., Rosen et al. 2002; Holmes and Wade 2005; Neal and Wade 2007). Preliminary IHC validation trials were conducted using samples of kidney tissue, which are known to contain androgen receptors (e.g., see Rosen et al. 2002). Initially, two types of primary anti-AR antibodies were tested. The PG-21 (Millipore, Temecula, CA) antibody binds the first 21 amino acids at the N-terminus of AR protein (see Prins et al. 1991 for details about preparation and characterization), whereas C-19 (Santa Cruz Biotechnology, Inc., Dallas, Texas) binds the last 19 amino acids at the C-terminus of the same protein (see Fig. 5.1). Both primary antibodies yielded a similar pattern of nuclear staining in kidney sections from three species of geckos (i.e., *G. lichtenfelderi*, *P. picta*, and *Aeluroscalabotes felinus*), as well as the positive control tissue from *A. carolinensis*. This first trial indicated successful binding of both anti-AR antibodies to proteins in the gecko kidney.

I selected the PG-21 antibody for use in the present study because it has been successfully used in diverse vertebrate species (see Fig. 5.1), including other lizards (e.g., Moga et al. 2000; Holmes and Wade 2005; Hews 2012). Various dilutions of PG-21 were tested for use in both kidney tissue (1-2µg/ml) and brain tissue (3.5-4.0µg/ml). A concentration of 4.0 µg/ml PG-21 was selected for all subsequent assays with brain tissue because this concentration yielded AR immunoreactivity (AR-ir) in sections from *A. carolinensis* (positive control tissue) consistent with previously published results using

the same antibody (at 2.0 µg/ml concentration, obtained from G. Prins; Rosen et al. 2002). Three types of negative controls were used to test the anti-AR specificity of PG-21 in gecko tissues. As a final step, negative controls were incubated as follows: 1) in the absence of PG-21 or secondary antibody, 2) with rabbit IgG in place of PG-21, and 3) after preadsorption of PG-21 with AR-21 peptide at 10-fold or 20-fold molar excess. None of these negative controls produced the labeling pattern that was observed when tissues were incubated with all reagents of the IHC protocol.

Protein isolation and Western immunoblot

Western immunoblot was done to validate the anti-AR specificity in lizard tissues (recommended by Burry 2011). Kidney and brain samples (20 mg) from gonadally intact males of three lizard species (*G. lichtenfelderi*, *P. picta*, and *A. carolinensis*) were homogenized in 200 ml lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 300 mM NaCl, 1 mM $MgCl_2$, pH 7.5), then centrifuged (12,000 g, 4 °C) for 15 min. The supernatant was immediately removed and stored at −80°C until use. Protein concentration was measured using the DC Protein Assay kit (Bio-Rad Laboratories) following standard protocol with standard curves generated using bovine serum albumin.

Twenty micrograms of protein from each sample were run on a 12% NuPAGE gel (Invitrogen) and transferred to nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk and 5% Normal Goat Serum (Jackson ImmunoResearch) in Tris-buffered saline with Tween-20 (TBST; 25 mM Tris (pH 7.5), 0.14 mM NaCl, 3 mM KCl, and 0.05% Tween-20) for 1 h and incubated overnight at 4 °C in a primary antibody solution containing the rabbit PG-21 anti-AR antibody (1:10,000). After washing with TBST, blots were incubated with HRP-conjugated goat anti-rabbit (1:20,000) for 1 h at

room temperature. Bound antibodies were detected by ECL Chemiluminescent Substrate (Novex®). Antibody specificity was confirmed for PG-21 by using Green Anole (*A. carolinensis*) tissues as positive controls, whereby preadsorption of the primary antibody with 20-fold excess AR-21 peptide eliminated two AR-specific bands at ~51 and ~38 kDa (see Fig. 5.2).

Experimental animals and hormone treatments

Brains were collected from experimental animals of *G. lichtenfelderi* and *P. picta*, which were previously described in Ch. 3 and Ch. 4. Briefly, eggs from the captive breeding colony at Charles University were incubated in individual cups filled with moist vermiculite. Upon hatching, animals were housed in standardized plastic boxes and raised in social isolation. Upon reaching sexual maturation at approximately 1 year in *G. lichtenfelderi* and 3 months in *P. picta*, animals were assigned to size-matched treatment groups. Males were assigned to one of three treatment groups: (1) intact control (MCON), (2) castrated (MCAST), and (3) castrated with T replacement (MTEST). In *G. lichtenfelderi*, females were assigned to one of two treatment groups: (1) intact control (FCON) and (2) intact with T supplement (FTEST). In *P. picta*, a third group included (3) ovariectomized females (FOVX). Hormonal manipulations in *G. lichtenfelderi* were conducted in June 2008, and animals were terminated 11 weeks later, following a series of social behavior trials (reported in Ch. 3). Manipulations in *P. picta* were conducted in October 2009, and animals were terminated approximately six months later, following a similar series of behavior trials as *G. lichtenfelderi* (reported in Ch. 4).

IHC brain analysis

After the completion of all behavioral trials, lizards were killed by rapid decapitation. Brains and kidneys were extracted, immediately frozen on dry ice, and stored at -80°C. Condition of the gonads (size, color, and vascularization), when present, was noted. Tissues were embedded in CRYO-OCT (TissueTek, Andwin Scientific) and sectioned on a cryostat at -16°C. Subsequent 20 µm tissue sections were thaw mounted onto four series of Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) so that the same brain regions from each individual could be processed in different assays. Slides were stored at -80°C with desiccant.

A single series of each brain was processed for AR IHC following a similar procedure as Rosen et al. 2002. Slides were warmed to room temperature and fixed for 10 min in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4), then incubated for 30 min in 0.5% H_2O_2, and blocked for 1 h in 4% normal donkey serum in PBS with 0.2% Triton X-100. To block endogenous biotin and avidin (which is common in frozen kidney, liver and brain tissue; see Wood and Warnke 1981; Bourne 2001), tissue sections were first incubated in 0.1mg/ml avidin (EMD Calbiochem, Billerica, MA) in 0.1M PBS, then in 0.1mg/ml biotin (Sigma, Louis, MO) in 0.1M PBS for 15 min with each blocking solution. PG-21 rabbit polyclonal antibody (4 µg/ml; Millipore, Temecula, CA) in 4% normal donkey serum in 0.1 M PBS with 0.2% Triton X-100 was applied to each slide placed in a humidified chamber and incubated for 30 min at room temperature followed by overnight (~20 h) at 4°C. AR were visualized with biotinylated donkey anti-rabbit secondary antibody (1 µg/ml for 2 h; Jackson Laboratories, West Grove, PA), Elite ABC peroxidase reagents (Vector Laboratories, Burlingame, CA), and

diaminobenzidine. Slides were rinsed with 0.1 M PBS between all steps. All washes and incubations were done on a shaker at room temperature unless otherwise indicated. Tissues were dehydrated and coverslipped with DPX mountant for microscopy.

To aid in anatomical localization, alternate brain sections of all animals were stained with thionin (a Nissl stain). These thionin-stained slides of gecko brain sections were compared with detailed brain maps of other reptiles (Greenberg 1982; Smeets et al. 1986; Coomber et al. 1997; Krohmer et al. 2011) to identify the preoptic area (POA), amygdala (AME) and ventromedial hypothalamus (VMH).

Stereological analysis of AR staining

The distribution of AR-immunoreactivity (AR-ir) was visualized using a light microscope. To estimate the densities of AR-ir within the POA, VMH and AME, images were taken at high magnification (40x optical on Nikon Eclipse E800 light microscope with 0.5x adapter on digital camera DXM 1200F). For each experimental animal, AR-ir labeling was counted within10 randomly selected counting frames (70.71µm x 70.71µm) within each brain region of interest. Labeled cells were categorized as type 1= uniform, pale color throughout cell body, type 2= pale cytoplasm with dark staining within one portion of the cell body (i.e., nuclear label), and type 3= dark color throughout cell body (see Fig. 5.3).

Neurons categorized as type 1 were widespread throughout the brain and largely resembled labeling of Nissl stain for morphology, suggesting these cells may represent non-specific background. However, in a random subsample of individuals, the number of type 1 cells per 5000 μm^2 was consistently less than the mean number of cell bodies with Nissl stain (paired t-test, one-tailed: t=6.913, P=0.0005); indicating that the type 1 label

from this IHC protocol did not stain all neuron cell bodies. Regardless, neurons labeled as type 1 (but not type 2 or 3) were observed in the nucleus rotundus, which has not previously been reported to contain AR and is used as a negative control region in the green anole (Rosen et al. 2002), suggesting the observed staining was non-specific background. No AR-ir labeling of any type was present in the medial nucleus sphericus, a region shown not to concentrate sex steroids in other reptiles (Halpern et al. 1982).

For each brain region of each experimental animal, labeled cells were counted manually within 10 counting frames (size ~70.7x70.7 μm =5000 μm^2; see Fig. 5.4) using ImageJ software. The counts from each brain region of each individual were then used to calculate mean number of labeled cells from each category as well as the mean number of total cells counted per 5000 μm^2. Subsequently, the percentage of cells categorized as type 1, 2, or 3 were calculated by taking the mean number of cells of a given category divided by the total number of labeled cells. Mean values or percentages were then grouped by treatment.

Statistics

For each species, ANOVA followed with the Ryan-Einot-Gabriel-Welsch multiple range post hoc analysis was used to compare treatment groups, and subsequently for treatment effects within each sex. Unpaired t-tests were used to determine if intact males (i.e., MCON) differed from intact females (i.e., FCON) in any variable. ANOVA was used to compare the main effects of sex and species, and the interaction on the counts of AR-ir of intact control animals from *G. lichtenfelderi* and *P. picta*. All statistical analyses were conducted in SAS (version 9.2; SAS Institute Inc., Cary, NC, USA).

Results

In the present study, all observed AR labeling was abolished in control experiments when the primary antibody was omitted or preabsorbed with immunogenic peptide, therefore indicating specificity of immunostaining for AR in *G. lichtenfelderi* and *P. picta*. Furthermore, the immunostaining protocol used in this study yielded patterns of AR-ir labeling in brain sections from *A. carolinensis* similar to those previously published (Rosen et al. 2002).

Goniurosaurus lichtenfelderi

Androgen receptor-ir was quantified in the AME, POA, and VMH; the location of these regions is illustrated in Figure 5.5. The total counts of cells (including types 1, 2 and 3; see Fig. 5.3) per 5000 μm^2 measured in the AME ($t=0.87$, $P=0.41$), POA ($t=-0.59$, $P=0.57$), or VMH ($t=0.27$, $P=0.79$; see Table 5.1) did not differ between MCON and FCON. No sex differences in the counts of nuclear (type 2) or cytoplasmic (type 3) labeling were observed for the any of these regions ($P>0.15$ for each comparison).

Total counts of cells (including types 1, 2 and 3; see Fig. 5.3) per 5000 μm^2 were similar among all treatment groups in the AME ($F_{4,24}=0.65$, $P=0.6310$), the POA ($F_{4,25}=0.90$, $P=0.4814$), and the VMH ($F_{4,22}=0.52$, $P=0.7199$; Table 5.1). The percent of cells labeled with nuclear AR-ir did not significantly differ among treatment groups in the AME ($F_{4,24}=1.15$, $P=0.3611$; Fig. 5.6A). Hormone manipulations had significant effect on the percent of cells labeled with nuclear AR-ir (i.e., type 2) within the POA ($F_{4,25}=3.69$, $P=0.0200$; Fig. 5.6B) and also the absolute counts of cells with nuclear AR-ir ($F_{4,25}=4.01$, $P=0.0143$; Table 1). Castrated males had a greater percent of cells labeled with nuclear AR-ir in the POA compared to MCON or MTEST males ($F_{2,12}=6.78$,

P=0.0096). The percent of cells labeled with nuclear AR-ir did not significantly differ among treatment groups in the VMH ($F_{4,22}$=0.64, P=0.6388; Fig. 5.6C).

Paroedura picta

Androgen receptor-ir was quantified in the AME, POA, and VMH; the location of these regions is illustrated in Figure 5.7. The total counts of cells (including types 1, 2 and 3; see Fig. 5.3) per 5000 μm^2 were similar between MCON and FCON in the AME (t=-0.42, P=0.68), POA (t=-1.8, P=0.10) and VMH (t=0.27, P=0.79; Table 2). No sex differences in the counts of nuclear (type 2) or cytoplasmic (type 3) labeling were observed for any of these regions (P>0.5 for each comparison).

Total counts of cells per 5000 μm^2 were similar among all treatment groups in the POA ($F_{5,32}$=0.55, P=0.7355), the VMH ($F_{5,31}$=0.76, P=0.5848), and the AME ($F_{5,32}$=2.42, P=0.0620). Males and females from all treatment groups did not significantly differ in the percent of cells with nuclear AR-ir within AME ($F_{5,32}$=1.58, P=0.198; Fig. 5.8A), POA ($F_{5,32}$=0.59, P=0.7094; Fig. 5.8B), or the VMH ($F_{5,31}$=0.72, P=0.6124; Fig. 5.6C).

Comparison of AR-ir abundance between species

Nuclear AR-ir was increased in *P. picta* relative to *G. lichtenfelderi* in both the POA ($F_{3,22}$=3.07, P=0.049) and the VMH ($F_{3,19}$=11.14, P=0.0002; Fig. 5.9). Cytoplasmic AR-ir was also increased in *P. picta* relative to *G. lichtenfelderi* in the POA ($F_{3,22}$=5.35, P=0.0064). However, it is important to note that immunohistochemistry on samples of *G. lichtenfelderi* and *P. picta* were processed in separate assays, which could have influenced the results.

Discussion

Sex differences in androgen receptors in the brain

No sex differences were observed in the quantity of total AR-ir or cells with nuclear AR-ir in the POA, AME and VMH of *G. lichtenfelderi* or *P. picta*. This finding supports the prediction based on the behavioral responsiveness to T observed in females and is consistent with the conserved distribution of AR between sexes reported in other vertebrates, including birds and reptiles (e.g., Belle and Lea 2001; Rhen and Crews 2001; Shaw and Kennedy 2002; Rosen et al. 2002). This study demonstrates that the brains of female geckos have AR in the same regions as males, and thus treatment with exogenous T acted on these receptors that are typically only stimulated in males to enable expression of courtship and copulatory behaviors (Ch. 3). Together, the behavioral responsiveness to T-treatment combined with AR data indicate that androgen sensitivity in the POA, AME, and VMH is the same in males and females, and suggest the sexes only differ in the circulating androgen levels to activate them and is consistent with behavioral results from studies in other lizards (Adkins and Schlesinger 1979; Flores and Crews 1995; Golinski et al. 2011).

Behavioral sensitivity to testosterone and androgen receptors in the brain

I hypothesized that the testosterone (T) control mechanism regulates suites of sexually dimorphic traits, and that species differences in the expression of male-typical traits may be associated with evolutionary changes in the endocrine regulatory pathway. The occurrence of separate evolutionary trajectories for various components of the endocrine regulatory pathway has been termed evolutionary plasticity (McGlothlin and Ketterson 2008), or evolutionary potential (Hau 2007). In gekkotan lizards, I previously

studied how T affects the expression of sexually dimorphic traits in species that differ in the sexually dimorphic phenotype of males. Males of *Goniurosaurus lichtenfelderi* express of suite of traits including male-larger head and body size, secretions from precloacal pores, aggression, and courtship behavior. Although head and body size were not affected during the 11-week experimental period, pore activity and behavioral traits required activational effects of elevated T. However, male courtship and copulatory behaviors persisted following castration during trials conducted in the home cage (Ch. 3). *Paroedura picta* is characterized by male-larger sexual dimorphism in body size. Furthermore, the behavior of males obviously differs from females during the copulatory phase of sexual behavior, and male-specific copulatory behavior does require elevated T. However, they exhibit little aggression and no precopulatory courtship, and T cannot induce courtship and has little discernible effect on aggressive behavior (Ch. 4). In the present study, I investigated AR-ir in the brain regions critical for male reproductive behaviors to investigate whether androgen sensitivity (measured by presence of AR-ir) differs in these two gekkotan species, which have evolved differences in expression of behavioral traits.

Based on previous reports and on behavioral differences between species, I expected to find that males of *G. lichtenfelderi* have greater abundance of AR-ir than males of *P. picta*. Males and females of *G. lichtenfelderi* and *P. picta* possess AR-ir in the AME, POA, and VMH (Tables 1 and 2; Fig. 9). Although intact males and females within each species do not significantly differ in abundance of AR-ir, the comparison between species indicates *P. picta* have greater abundance of nuclear AR-ir in the POA and VMH relative to *G. lichtenfelderi*. These results are the opposite of what was

predicted, and suggest that courtship behaviors and high levels of aggression in males of *G. lichtenfelderi* are not associated with an increase in AR-ir relative to males of *P. picta*, which display low levels of aggression and no courtship at all. The relative similarity in AR-ir between species in the AME suggests that androgen function in this region is conserved. Furthermore, these findings suggest that evolutionary changes in sexually dimorphic behaviors in gekkotan lizards are not associated with changes in AR abundance in these brain regions.

A recent study by Hews and colleagues found differences in the abundance of AR-ir in the POA and VMH between two species of lizards from the genus *Sceloporus*, which differ in levels of male aggressive behaviors (Hews et al. 2012). More specifically, in the sexually dichromatic and aggressive species, *S. undulatus*, males had elevated circulating T levels and elevated AR-ir in the POA and VMH relative to conspecific females, but sex difference in plasma T and AR-ir were absent in the sexually monochromatic and less aggressive species, *S. virgatus* (Hews et al. 2012). This study on *Sceloporus* lizards supports the hypothesis that species differences in sexually dimorphic behaviors are associated with changes in the underlying T signaling system (i.e., control module). Data from the present study on gekkotan lizards do not support this hypothesis. One significant difference between the present study and that by Hews and colleagues is the degree of phylogenetic relatedness of the experimental species. Here, comparisons were done between representatives of different gekkotan families; in Hews et al. (2012), very closely related sister-species were compared within a single genus. Future studies investigating the AR-ir in the brain of species from the genera *Goniurosaurus* (family Eublepharidae) and *Paroedura* (family Gekkonidae) are needed to resolve whether the

abundance of AR is widely conserved among gekkotan species, or if comparative studies within genera are required to resolve potential species differences.

Effect of T treatment on brain androgen receptors

Circulating androgens are known to regulate AR mRNA levels (i.e., autoregulation) in virtually all vertebrate models, whereby androgens downregulate AR mRNA in most tissues studied. However, upregulation of AR mRNA has also been reported (reviewed in Burnstein 2005; Ing 2005). In *P. picta*, experimental manipulations of circulating hormone levels did not significantly affect the abundance of AR-ir in the AME, POA, or VMH in females or males. The absence of any change in AR-ir in the brain following six months of gonadectomy stands in contrast to reports from many other vertebrates, in which AR are significantly reduced following castration (mammals, Lu et al. 1998; Greco et al. 1996; Handa et al. 1996; He et al. 2012; fish, Larsson et al. 2002). The finding in *P. picta* suggests the abundance of AR in the brain is not regulated by circulating levels of T. A similar result has been reported in some mammals. For example, in guinea pigs, males castrated for four days showed no difference in AR relative to intact controls (Choate and Resko 1992), and in hamsters, eight to 12 weeks of castration had no effect on AR relative to sham-operated males (Clancy et al. 1994).

In *G. lichtenfelderi*, males and females with elevated levels of circulating T had lower levels of nuclear AR-ir in the POA relative to treatment groups with low T (Fig. 6B), although this effect attained statistical significance only in males. The observed absence of a T-mediated regulatory effect on AR in the brain of female geckos suggests that androgens regulate receptors in a sex-specific manner, perhaps similar to studies

reporting sex-specific regulation of receptors by steroid levels in the *Cnemidophorus* lizards. In *Cnemidophorus inornatus*, estradiol increases the expression of estrogen receptors in the VMH of females but not males (reviewed in Young and Crews 1995). In males of that same species, castration significantly increases levels of AR mRNA in the POA, whereby T-treatment restored AR mRNA levels to that of intact males; this observed downregulation of AR mRNA by T is limited to males of this species (Godwin et al. 2000).

The regulation of nuclear AR-ir by circulating levels of T is region-specific in males of *G. lichtenfelderi*, only affecting receptors in the POA, which is a region critical for male reproductive behaviors. The increased AR in the POA of castrated males of *G. lichtenfelderi* may be functionally tied to the persistence of male courtship and copulatory behaviors following castration. Perhaps this finding is similar to the seasonal changes in AR mRNA in the brain of the territorial tropical bird *Hylophylax n. naevioides*. In that species, year-round territorial aggression is hypothesized to be maintained during the non-breeding season by an increase in AR mRNA levels in the nucleus taeniae, which is the avian homologue to the mammalian amygdala, providing increased neural sensitivity to the low levels of circulating androgens (Canoine et al. 2006). The observed upregulation of AR in the POA of castrated males of *G. lichtenfelderi* suggests that the seasonal decline in circulating T (i.e., during the non-breeding season) may allow the persistence of male-typical tail vibration displays, which are not only used in the context of courtship but also in inter-male aggressive interactions. However, the functional significance of the T-mediated regulation of AR (or lack thereof) in these gecko species is not understood. Additional studies on gekkotan lizards are needed to delineate the

functional and evolutionary significance of the abundance and distribution of AR vis-à-vis changes in circulating T.

Androgen receptors and evolution

Extensive studies have investigated the role of sex steroids in the organization and activation of sexually dimorphic morphology, physiology, behavior and brain function, and have revealed general principles that are taxonomically conserved but also strikingly diverse in vertebrates (Adkins-Regan 2005). Because endocrine regulation depends on interactions between multiple components of the signaling system – including circulating hormones, specific receptors and other determinants of cellular sensitivity – evolutionary changes in individual components of the system have the potential to alter the expression of separate traits within a suite or even to allow traits to become dissociated from one another (i.e., evolutionary potential; Hau 2007; McGlothlin and Ketterson 2008; Fig. 1). Alternatively, all of these components may be constrained to evolve as a self-contained endocrine control module (evolutionary constraint; Hau 2007; McGlothlin and Ketterson 2008). As a test of these broader hypotheses, I investigated the relationship between circulating androgens, the expression of aggressive and sexual behavior, and androgen receptors in the brains of two species of gekkotan lizards that differ in trait expression and behavioral sensitivity to T. The results here indicate that the sexes do not differ in AR abundance in the AME, POA, or VMH in either species, consistent with findings in many other vertebrates and suggesting the components of the T control module are conserved between sexes within species (consistent with the evolutionary constraint hypothesis). Furthermore, the comparison of two species that differ dramatically in the

display of sexually dimorphic behaviors fails to support the hypothesis that changes in AR in the brain underlie evolutionary changes in sexually dimorphic behaviors.

Taxon-specific differences in androgen receptors?

The infraorder Gekkota is a relatively basal lineage of squamate reptiles that diverged approximately 165-225 million years ago (Kluge 1987; Vitt et al. 2003; Vidal and Hedges 2005; Kumazawa 2007; Jonniaux and Kumazawa 2008) from the sister group which subsequently gave rise to Serpentes, Anguimorpha, and Iguania (Wiens et al. 2012; but see Losos et al. 2012). Most gekkotan lizards are nocturnal and use nasal olfaction for species and sex recognition, which may have led to the evolution of courtship behaviors that differ from many other lizards which are primarily diurnal (Nobel and Bradley 1933; Vitt et al. 2003). The nocturnal nature of geckos may have led to changes in the brain associated with taxon-specific courtship behaviors as well as the reliance on nasal olfaction rather than visual sensory input. More specifically, the amygdala serves as a relay center for sensory input from the olfactory bulb and the accessory olfactory bulb. The reliance on nasal olfaction in gekkotan lizards may have led to an increase in androgen-sensitivity in the amygdala. In the present study, both males and females of *G. lichtenfelderi* and *P. picta* contain AR-ir in the AME. A previous study on the gecko species *Eublepharis macularius* reported that labeling was especially strong for the expression of AR mRNA within the external nucleus of the amygdala (Rhen and Crews 2001). Comparing patterns of AR in the brain between nocturnal gekkotan species with diurnal lizards from more distant lineages provides a test of whether the androgen control module differs on a macroevolutionary scale (i.e., between lineages of squamate reptiles). Although the distribution of AR-ir in the brain is

generally conserved between the sexes of diverse taxa (Belle and Lea 2001; Rhen and Crews 2001; Shaw and Kennedy 2002; Rosen et al. 2002), in diurnal lizards studied to date, AR-ir is present in the AME of males but is absent in this region in females of *Sceloporus undulatus* (Moga et al. 2000) and *A. carolinensis* (Rosen et al. 2002; Neal and Wade 2007). The data currently available for diurnal lizards suggests a sex difference exists in the AME, whereas this difference is not observed in nocturnal geckos. Future studies on a wider diversity of lizards would be needed to test whether the observed differences in AR-ir in the AME between diurnal versus nocturnal lizards are characteristics of different lineages.

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Tables

Table 5.1. Androgen receptor immunoreactivity in the AME, POA, and VMH of *G. lichtenfelderi*. Counts for nuclear (type 2), cytoplasmic (type 3), and total number of cells per 5000 µm^2 are listed as group means ± SEM (n). Treatment groups are designated as: MCON = intact control males, MCAST = castrated males, MTEST = castrated males with T-implant, FCON = intact control females, FTEST = intact females with T-implant.

	Amygdala			Preoptic area			Ventromedial hypothalamus		
Treatment	nuclear	cyto-plasmic	total	nuclear	cyto-plasmic	total	nuclear	cyto-plasmic	total
MCON	0.48 ± 0.19 (4)	1.53 ± 0.77 (4)	13.1 ± 2.00 (4)	0.22 ± 0.08 (5)	1.32 ± 0.21 (5)	16.5 ± 1.34 (5)	0.33 ± 0.18 (4)	0.93 ± 0.11 (4)	18.0 ± 1.02 (4)
MCAST	0.80 ± 0.23 (6)	2.26 ± 0.36 (6)	13.8 ± 0.91 (6)	1.0 ± 0.25 (5)	2.02 ± 0.54 (5)	18.4 ± 1.41 (5)	0.65 ± 0.21 (4)	2.23 ± 0.52 (4)	20.0 ± 0.66 (4)
MTEST	0.80 ± 0.13 (5)	2.56 ± 0.61 (5)	13.3 ± 1.51 (5)	0.24 ± 0.05 (5)	1.20 ± 0.30 (5)	16.7 ± 0.93 (5)	0.24 ± 0.09 (5)	1.34 ± 0.47 (5)	19.5 ± 1.19 (5)
FCON	0.70 ± 0.13 (5)	2.50 ± 0.49 (5)	14.9 ± 1.04 (5)	0.67 ± 0.19 (6)	1.17 ± 0.24 (6)	18.3 ± 0.57 (6)	0.44 ± 0.12 (5)	1.78 ± 0.63 (5)	18.8 ± 1.54 (5)
FTEST	0.92 ± 0.29 (5)	1.62 ± 0.38 (5)	12.2 ± 0.92 (5)	0.24 ± 0.10 (5)	1.04 ± 0.26 (5)	18.8 ± 0.83 (5)	0.60 ± 0.30 (5)	1.42 ± 0.33 (5)	18.3 ± 0.44 (5)

Table 5.2. Androgen receptor immunoreactivity in the AME, POA, and VMH of *P. picta*. Counts for nuclear (type 2), cytoplasmic (type 3), and total number of cells per 5000 μm^2 are listed as group means ± SEM (n). Treatment groups are designated as: MCON = intact control males, MCAST = castrated males, MTEST = castrated males with T-implant, FCON = intact control females, FOVX = ovariectomized females, FTEST = intact females with T-implant.

	Amygdala			Preoptic area			Ventromedial hypothalamus		
Treatment	nuclear	cyto-plasmic	total	nuclear	cyto-plasmic	total	nuclear	cyto-plasmic	total
MCON	0.69 ± 0.21 (7)	2.76 ± 0.34 (7)	12.3 ± 0.65 (7)	1.33 ± 0.51 (7)	3.64 ± 0.67 (7)	16.7 ± 0.87 (7)	2.56 ± 0.55 (7)	2.10 ± 0.44 (7)	16.1 ± 0.38 (7)
MCAST	0.27 ± 0.15 (6)	2.98 ± 0.97 (6)	12.5 ± 1.02 (6)	0.74 ± 0.36 (5)	4.49 ± 1.37 (5)	19.5 ± 1.92 (5)	2.64 ± 0.60 (5)	1.14 ± 0.18 (5)	17.5 ± 0.80 (5)
MTEST	0.25 ± 0.05 (6)	3.47 ± 0.97 (6)	10.4 ± 0.96 (6)	1.06 ± 0.29 (5)	3.23 ± 0.52 (5)	16.6 ± 1.15 (5)	1.98 ± 0.37 (5)	1.76 ± 0.69 (5)	16.2 ± 1.43 (5)
FCON	0.82 ± 0.21 (9)	2.36 ± 0.64 (9)	13.6 ± 0.66 (9)	1.65 ± 0.32 (9)	3.99 ± 0.71 (9)	17.4 ± 1.19 (9)	2.79 ± 0.27 (7)	1.99 ± 0.53 (7)	16.4 ± 0.94 (7)
FOXV	0.57 ± 0.29 (3)	3.27 ± 0.48 (3)	14.9 ± 0.84 (3)	1.46 ± 0.76 (4)	6.05 ± 1.21 (4)	16.7 ± 2.35 (4)	2.04 ± 0.39 (5)	2.20 ± 0.69 (5)	18.0 ± 1.58 (5)
FTEST	0.10 ± 0.10 (2)	5.70 ± 0.80 (2)	13.8 ± 3.10 (2)	1.20 ± 0.86 (3)	4.13 ± 0.86 (3)	17.9 ± 0.66 (3)	3.93 ± 1.72 (3)	2.03 ± 0.77 (3)	18.3 ± 1.22 (3)

Figures

Figure 5.1. Alignment of the N-terminus and C-terminus regions of the androgen receptor (AR) amino acid sequence for several species of vertebrate. Gray letters in the rat sequence indicate that at least one species differs in the amino acid at this site. In all other sequences, dashed lines represent similarities and letters indicate differences in amino acid sequence compared to rat AR. Periods were used to maximize alignment between species. Boxes highlight the epitopes used to generate the anti-AR antibodies PG-21 (Millipore) and C-19 (Santa Cruz). Note, only a partial amino acid sequence from the C-terminus of AR from *Anolis* has been reported.

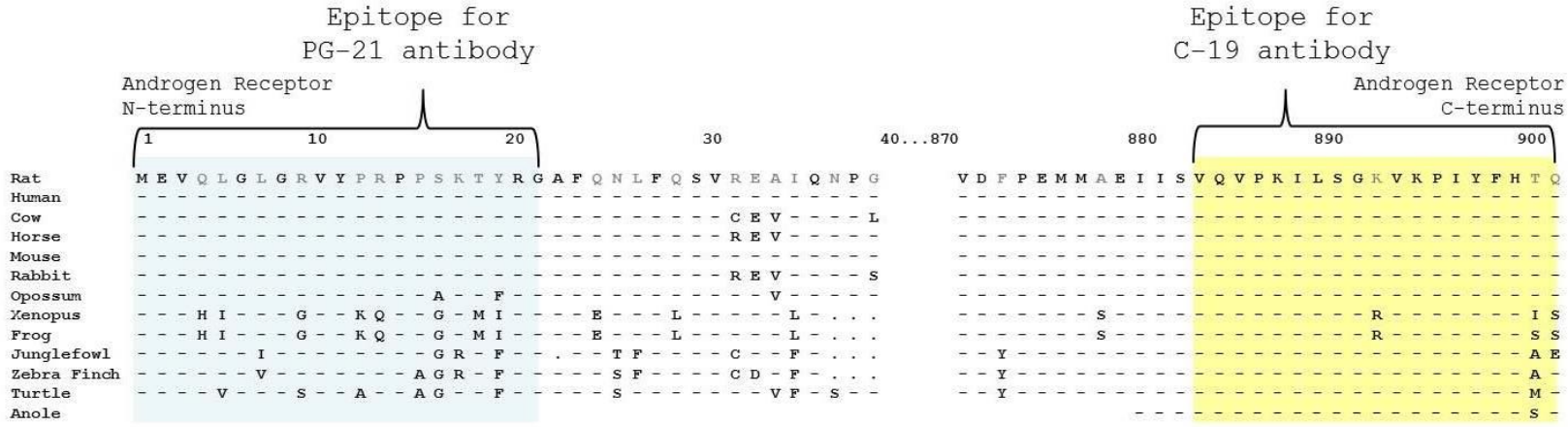

Epitope for
PG-21 antibody
Epitope for
C-19 antibody
Androgen Receptor
N-terminus
Androgen Receptor
C-terminus
1
10
20
30
40...870
880
890
900
Rat M E V Q L G L G R V Y P R P P S K T Y R G A F Q N L F Q S V R E A I Q N P G V D F P E M M A E I I S V Q V P K I L S G K V K P I Y F H T Q
Human
Cow C E V L
Horse R E V
Mouse
Rabbit R E V S
Opossum A F V
Xenopus H I G K Q G M I E L L S R I S
Frog H I G K Q G M I E L L S R S S
Junglefowl I G R F T F C F Y A E
Zebra Finch V A G R F S F C D F Y A
Turtle V S A A G F S V F S Y M
Anole S

Figure 5.2. Western blot using PG-21 anti-AR antibody without (left) and with (right) preadsorption with AR-21 peptide on protein extracted from brain and kidney tissues from three lizard species: *Anolis carolinensis* (AC), *Paroedura picta* (PP), *Goniurosaurus lichtenfelderi* (GL). Dashed lines indicate molecular weight markers at 97 (upper) and 39 (lower) kDa. Both blots were exposed together on the same film.

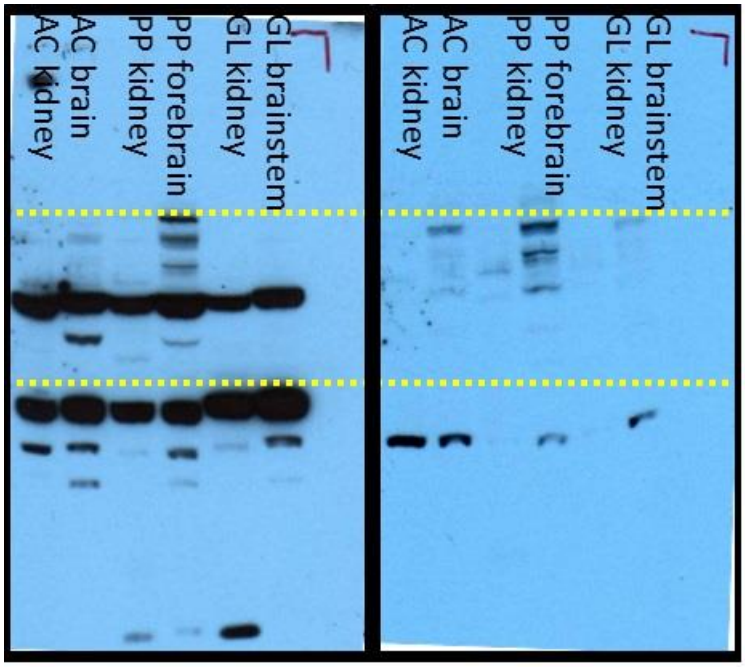


Figure 5.3. Digital image at 40x magnification of a brain section from *Anolis carolinensis* (positive control) processed in the immunohistochemistry validation trial using 4.0 µg/ml PG-21. Labeling of cells is categorized as 1= pale even color throughout cell body, 2= pale cytoplasm with dark staining within one portion of the cell body (i.e., nuclear label), 3= dark color throughout cell body.

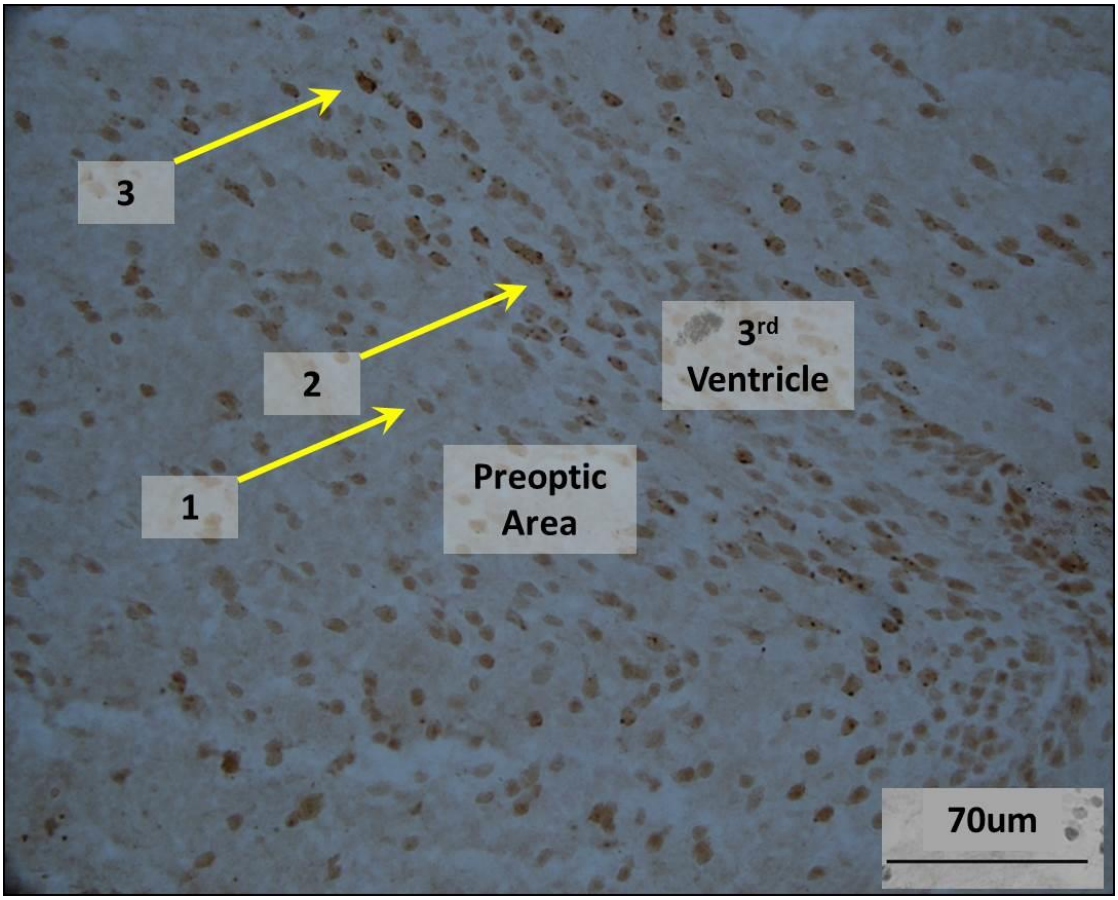


Figure 5.4. Steps for analysis conducted on brains of *G. lichtenfelderi* and *P. picta*.

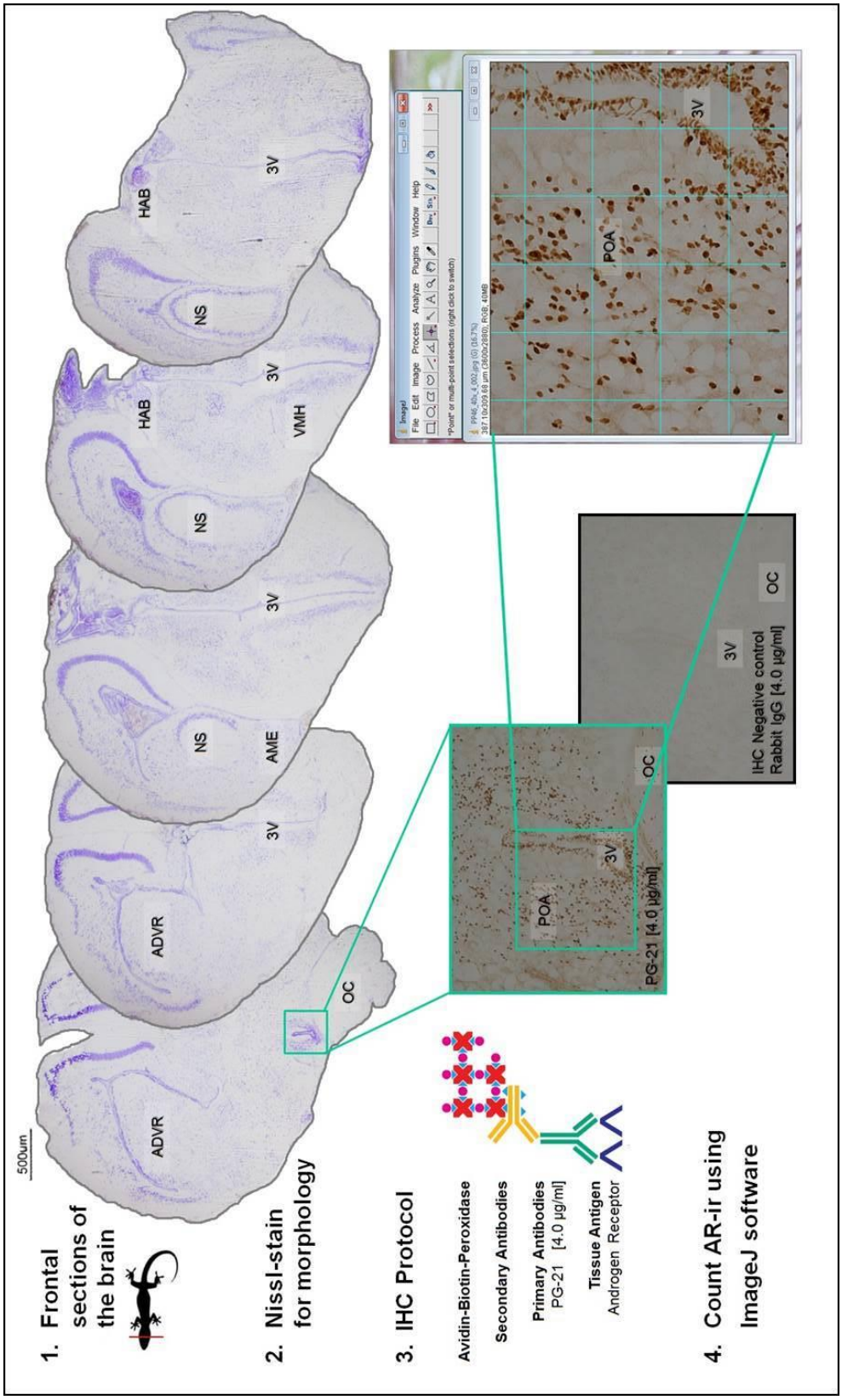


Figure 5.5. Brain morphology of *G. lichtenfelderi* traced from Nissl-stained sections. Regions designated by abbreviations: AC = anterior commissure; AH = anterior hypothalamus; AME = external nucleus of the amygdala; HAB = habenula; NS = nucleus sphericus; OPT = optic tract; POA = preoptic area; VMH = ventromedial hypothalamus; 3V = third ventricle.

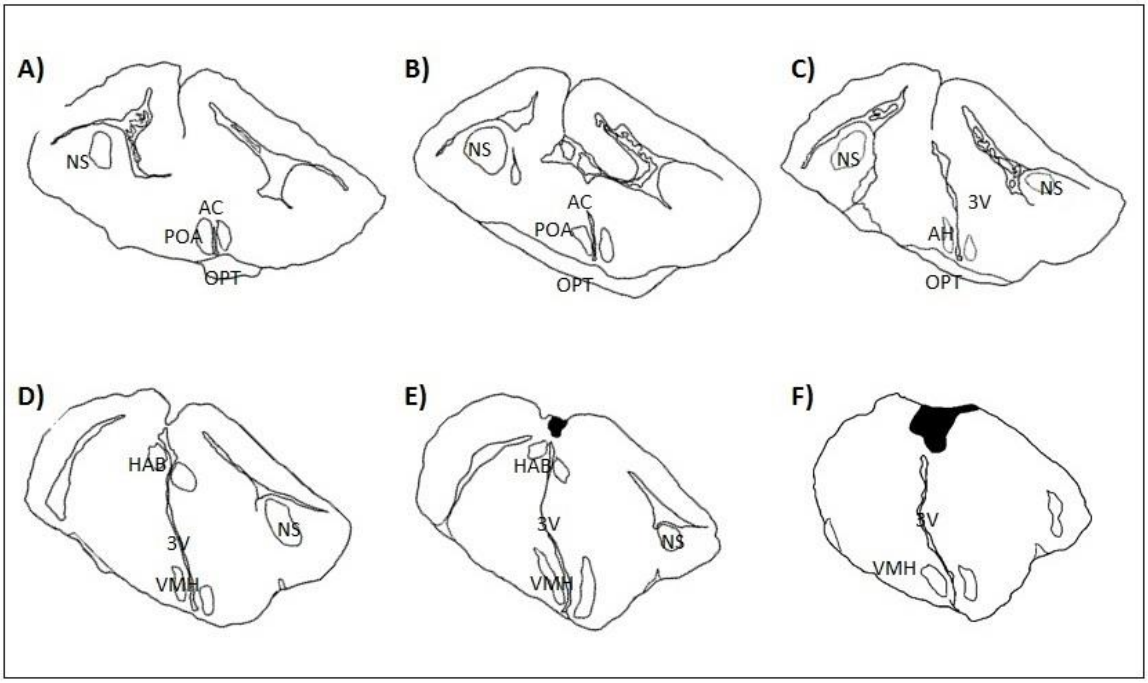


Figure 5.6. Percent of cells with nuclear androgen receptor immunoreactivity in the A) AME, B) POA, and C) VMH of *G. lichtenfelderi*.

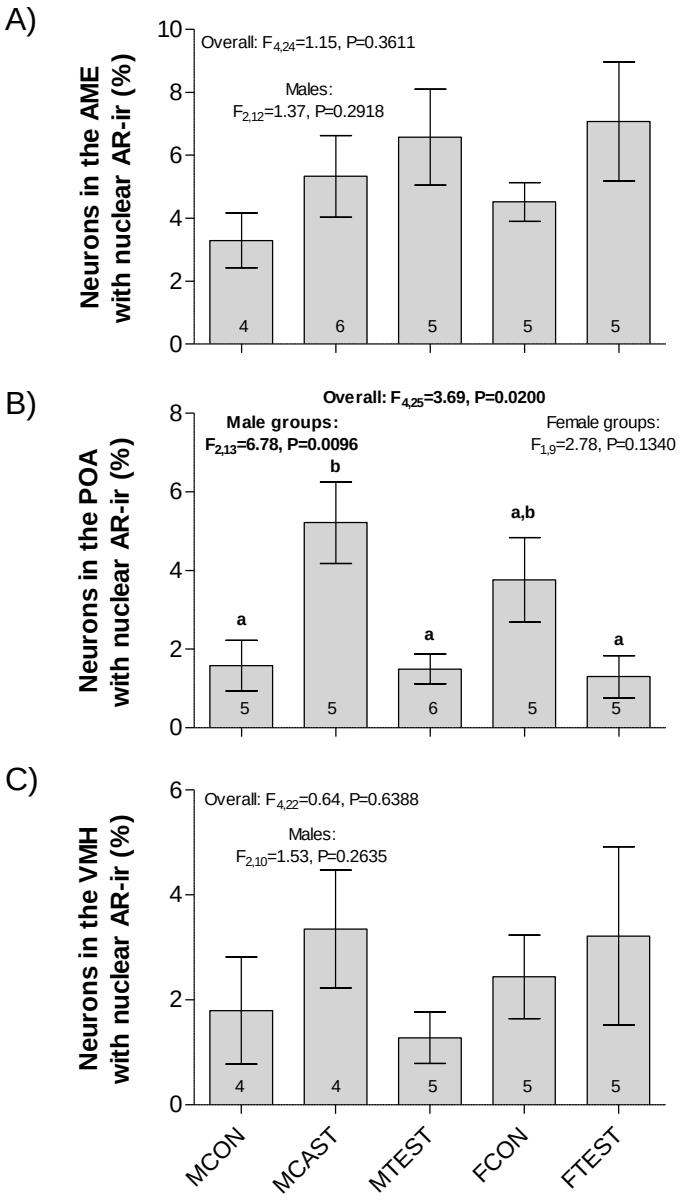


Figure 5.7. Brain morphology in *P. picta* traced from Nissl-stained sections. Brain regions designated by abbreviations: AC = anterior commissure; AH = anterior hypothalamus; AME = external nucleus of the amygdala; HAB = habenula; NS = nucleus sphericus; OPT = optic tract; POA = preoptic area; VMH = ventromedial hypothalamus; 3V = third ventricle.

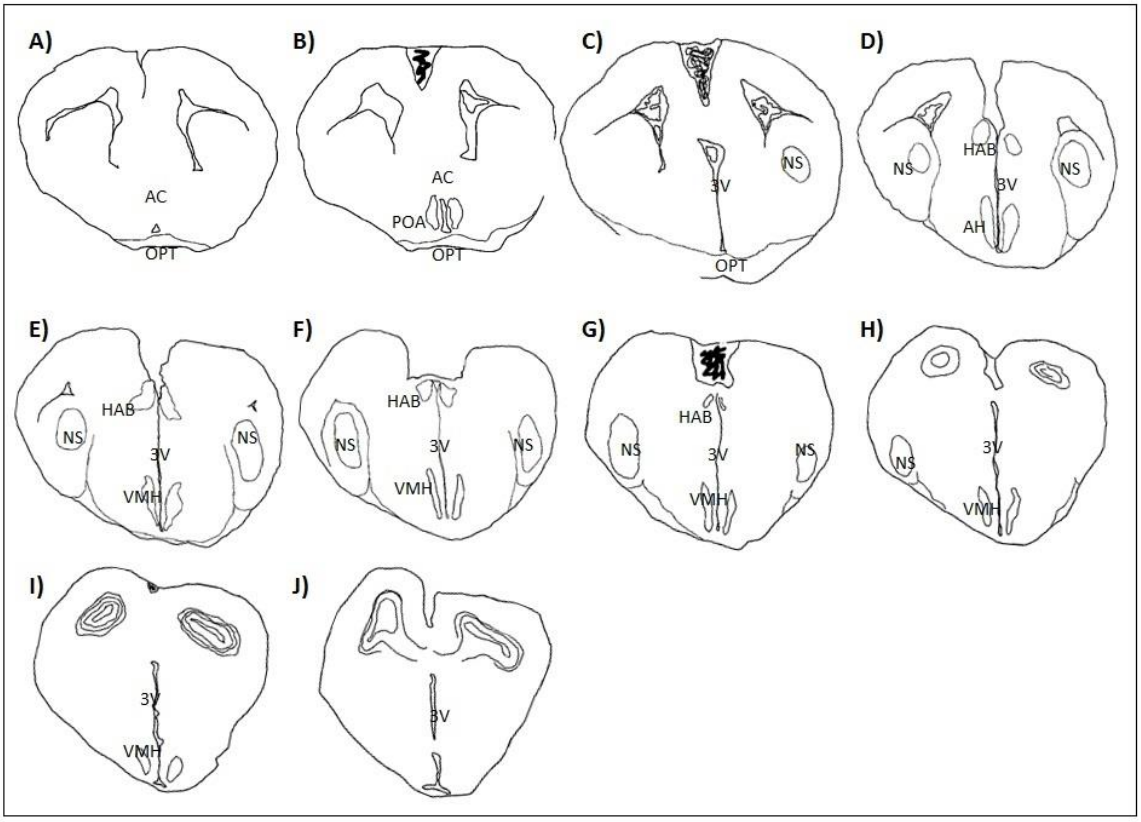


Figure 5.8. Percent of cells with nuclear androgen receptor immunoreactivity in A) AME, B) POA, and C) VMH of *P. picta*.

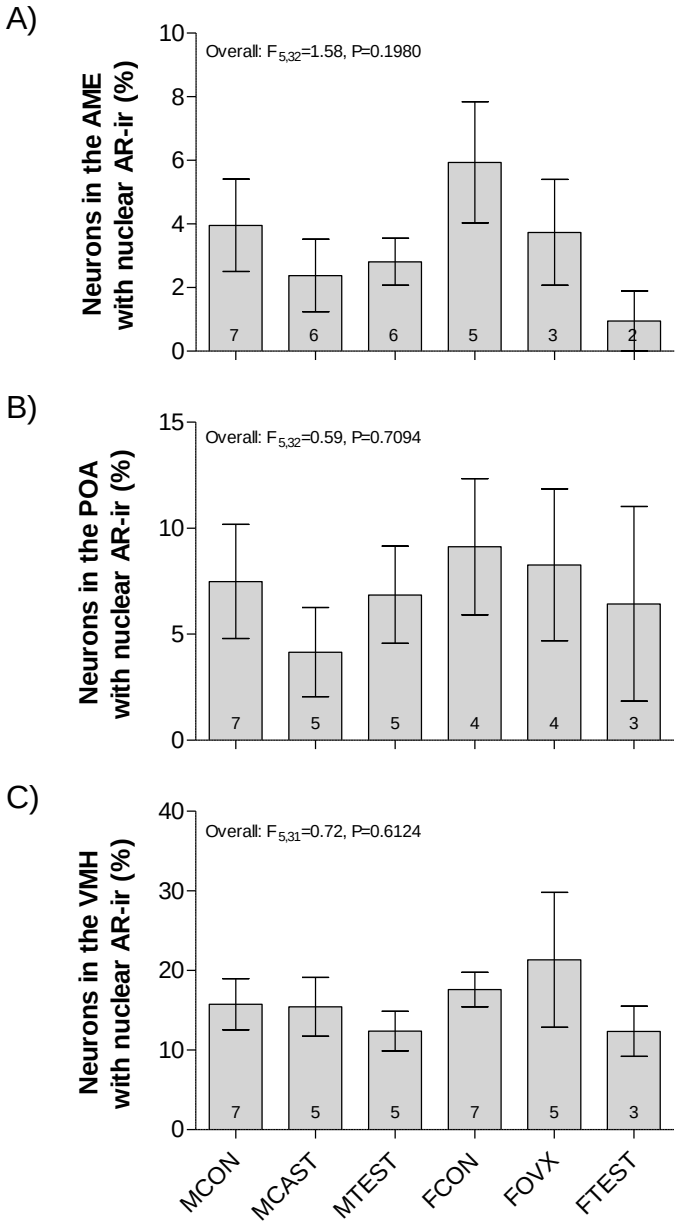
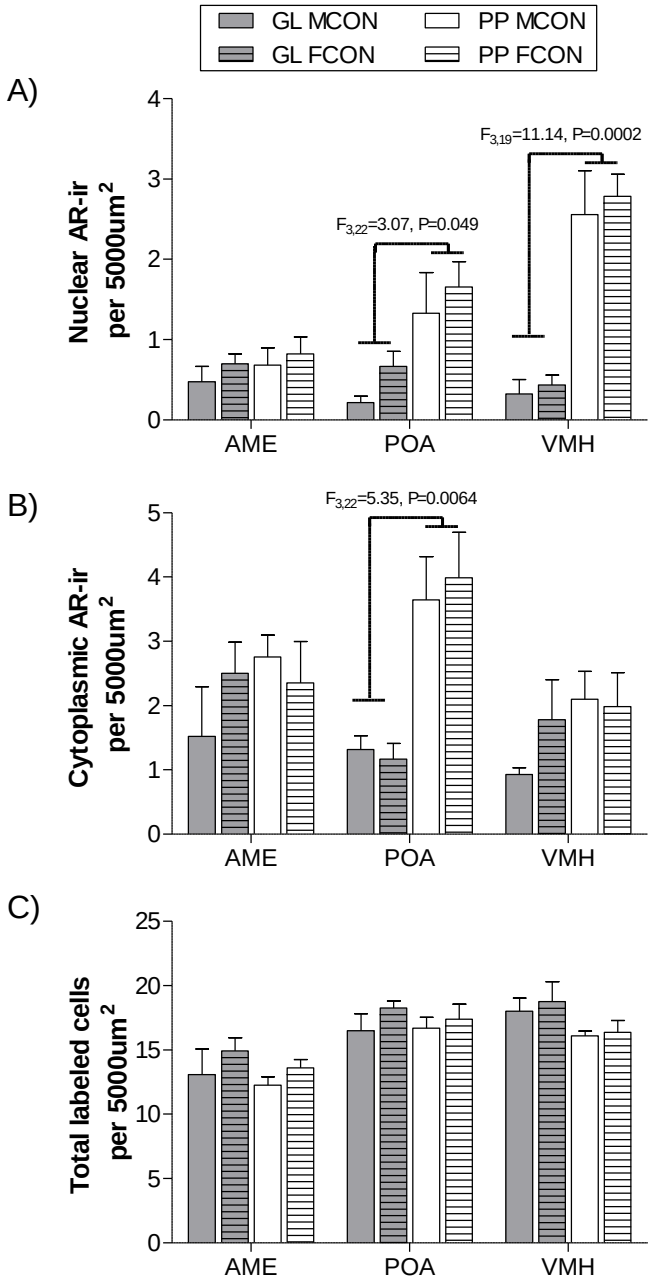


Figure 5.9. Comparison of AR-ir counts in the AME, POA, and VMH of intact males and females of *G. lichtenfelderi* (gray bars) and *P. picta* (white bars). Counts presented as group means ± SEM. For sample sizes, see Tables 5.1 and 5.2.



CHAPTER 6

SUMMARY AND CONCLUSIONS

Androgen control of sexually dimorphic traits in a comparative study of gekkotan lizards

Sexually dimorphic morphological, physiological and behavioral traits evolve due to selection for traits that confer reproductive advantage (Andersson 1994) and the expression of these correlated traits in male vertebrates is often regulated by the pleiotropic effects of gonadal androgens (Adkins-Regan 2005; Hau 2007; McGlothlin and Ketterson 2008). However, proximate mechanisms underlying evolutionary changes in suites of correlated sexually dimorphic traits are not well understood (Badyaev 2002; William and Carol 2009). The present dissertation helps to fill the gaps in our understanding of how traits controlled by one pleiotropic mechanism can follow different evolutionary trajectories and become dissociated. More specifically, I investigated whether changes in circulating testosterone (T) or androgen receptor (AR) abundance can explain sex-specific and species-specific differences in the expression of suites of sexually dimorphic traits in three species of gecko lizards.

Gekkotan lizards provide a suitable model system for testing hypotheses regarding proximate regulation of how suites of sexually dimorphic traits have evolved, as species in this group express several different patterns of these traits. By developing a comparative study based in a phylogenetic framework (Martins 2000; Adkins-Regan 2012), I was able test these hypotheses on whether differences in the androgen signaling system underlie evolutionary changes in sexually dimorphic phenotypes of three species

of gekkotan lizards. *Goniurosaurus lichtenfelderi* (family Eublepharidae) is characterized by male-larger body and head size, and by functional precloacal pores. Behaviorally, males of this species are highly aggressive and express both the courtship and copulatory phases of sexual behavior. *Goniurosaurus lichtenfelderi* not only express the full suite of sexually dimorphic traits for eublepharids, but this species is also characterized by genetic sex determination and a seasonal breeding pattern, which are common biological characteristics of most members of this family. Thus, *G. lichtenfelderi* can be considered a good "type-species", or model, of the ancestral condition to provide a baseline view of androgenic regulation of sexually dimorphic traits, and serve as a point of reference for comparison with species in which trait expression has been altered. *Coleonyx elegans* (family Eublepharidae) is similar to *G. lichtenfelderi* in most aspects, but has a derived pattern of sexual behavior in which the courtship phase has been lost. *Paroedura picta* (family Gekkonidae) is the least dimorphic of the three. It is characterized by male-larger body size, which is probably ancestral in all gekkotans, and by evolutionarily derived trait patterns including the absence of precloacal pores, a low level of intrasexual aggression and, similar to *C. elegans*, the absence courtship.

The presence versus absence of all or part of a suite of sexually dimorphic traits could be caused by evolutionary change in levels of circulating T, in which case males of species where traits have been evolutionarily lost would have low levels of T – too low to induce trait expression (i.e., evolutionary constraint hypothesis; Hau 2007; McGlothlin and Ketterson 2008). To test this hypothesis, I compared circulating T in intact males and females by investigating the effects of T on trait expression in *G. lichtenfelderi*,

which express multiple sexually dimorphic traits, with *P. picta*, which express few dimorphic traits. In *C. elegans*, a species that lost only one trait from the suite, this case cannot simply be explained by a change in the level of T, but more likely required a change in sensitivity to T in the neural substrates underlying courtship behavior (i.e., evolutionary potential hypothesis; Hau 2007; McGlothlin and Ketterson 2008). To understand the androgenic control of sexual dimorphism in trait expression and address these hypotheses, I investigated the effects of T on sexually dimorphic traits in adult male and females in three species of gekkotan lizards.

Aim 1: Effects of testosterone on sexually dimorphic trait expression

In males of *G. lichtenfelderi*, castration reduced and T restored secretory activity of the precloacal pores and the likelihood for the expression of aggression, courtship and copulatory behaviors during tests conducted in a neutral arena (Ch. 3; Table 6.1). T supplementation in females induced male-typical courtship behavior but not the other male-typical traits (i.e., pore activity, aggression and mounting behavior; Table 6.2). Therefore, the expression of sexually dimorphic physiology and behaviors require contemporaneously elevated levels of circulating androgens in males, but females appear to have evolved a selective loss of sensitivity to T in the precloacal pores and the neural substrate responsible for aggression and mounting behaviors. These results from *G. lichtenfelderi* are consistent with studies on *E. macularius* (Flores and Crews 1995; Rhen and Crews 1999, 2000; Rhen et al. 2005), another eublepharid species that shares the full suite of sexually dimorphic traits but is characterized by temperature-dependent sex determination (Viets et al. 1993). The comparison between *G. lichtenfelderi* and *E.*

macularius indicates the androgen control module has been conserved through evolutionary divergence, including the change in mechanism for sex determination.

In *C. elegans*, which evolutionarily lost courtship display but retained all other traits from the ancestral suite of sexually dimorphic traits, intact males had high and variable levels of plasma T. Thus the evidence does not suggest the selective loss of courtship in this species is associated with a reduction in levels of circulating hormone (Ch. 2). In males of *C. elegans*, elevated levels of androgens are required for the secretory activity of precloacal pores physiology and expression of aggressive behaviors (Table 6.1), indicating that the androgenic control mechanism has been largely retained for the expression of sexually dimorphic traits that are present. Interestingly, male copulatory behavior persisted for weeks following castration, suggesting this behavior is relatively insensitive to activational effects of T. Females of *C. elegans* were highly sensitive to T; supplementation with T induced the expression of all male-typical morphological, physiological and behavioral traits except aggression (Table 6.2). Therefore, sexual dimorphism in the expression of traits is due in most cases to sex differences in the availability of T, but females appear to have evolved a selective loss of sensitivity to T in the neural substrate responsible for aggression. Tail vibration courtship was not induced by T in either males or females of this species, indicating that the absence of courtship from the sexual repertoire of *C. elegans* must involve a selective loss of sensitivity to T.

The comparison of results between males of *C. elegans* and *G. lichtenfelderi* indicates that the androgen control module is generally conserved between species that differ in expression of individual traits within conserved suites of sexually dimorphic

traits. Whereas tail vibration courtship is sensitive to activational effects of T in both *G. lichtenfelderi* and *E. macularius*, the absence of this behavior and the inability to induce it in *C. elegans* indicates that a change in the ancestral mechanism occurred to selectively render this behavior insensitive to the activational effects of T. The loss of sensitivity to T of this trait may be due to a change in androgen receptors (AR) in the brain regions associated with sexual behavior such as the preoptic area (POA) or amygdala (AME), in the motor neurons, or in the musculature of tail.

In *P. picta*, from the family Gekkonidae, intact males had high and variable levels of plasma T. Thus, the evidence does not suggest a reduction in circulating levels of T is responsible for the absence of courtship behavior, precloacal pores and dimorphism in head width in this species (Ch. 4). In males, castration reduced and T-replacement restored the likelihood for the expression of copulatory and aggressive behaviors (Table 6.1). The data suggest that the androgenic control of aggressive and copulatory behaviors is widely conserved and present in species that exhibit few sexually dimorphic traits. In females of *P. picta*, T-supplementation induced male-typical copulatory behaviors but not aggression (Table 6.2). Like other gekkotan species, females appear to have evolved a selective loss of sensitivity to T in the neural substrate responsible for aggression. Similar to the findings in *C. elegans*, the inability to induce courtship or increase levels of aggression suggests *P. picta* may have relatively low sensitivity to T in brain regions critical for behavioral expression.

In contrast to the observed behavioral sensitivity in males of *P. picta*, T did not affect male growth and the development of male-larger body size, indicating that not all sexually dimorphic traits are mediated by androgenic regulation. In this species,

evidence from ovariectomized and T-supplemented females implicated a role for female ovarian function (e.g., hormones) in the development of sexual dimorphism in body size. The comparison to iguanid lizards, in which T regulates male growth and the development of sexual dimorphism in body size (Cox and John-Alder 2005; Cox et al 2005; Cox et al. 2009), suggests *P. picta* had a selective change in the components of the androgen control module leading to the loss of sensitivity to T of these traits. Furthermore, the experimental data from both males and females of *P. picta* suggests the mechanism may involve a switch from androgens to estrogens as growth regulators primarily responsible for the development of sexual dimorphism in body size. However, future studies investigating the effects of estrogens on growth in gecko lizards would be needed to verify this possibility.

Aim 2: Androgen receptors in the brain

Can sex- and species-differences in behavioral sensitivity to T be explained by differences in AR distribution or abundance in the POA, AME, or ventromedial hypothalamus (VMH) regions of the brain? To address this question, I conducted an immunohistochemical investigation of androgen receptor immunoreactivity (AR-ir) distribution in brains collected from individuals of *G. lichtenfelderi* and *P. picta*.

Within each species, males and females had similar abundance of AR-ir in the AME, POA, and VMH (Ch. 5), which indicates that sex-specific expression is largely dependent on the presence or absence of T to bind AR and modulate behavioral expression. However, T-supplementation in females of *G. lichtenfelderi* or *P. picta* did not induce the expression of aggressive behaviors to levels of males. And although this treatment activated mounting behavior in female of *P. picta*, it did not have the same

effect in *G. lichtenfelderi*. The sex differences in behavioral sensitivity to T cannot be explained by differences in AR in the adult brain, suggesting that sexes differ in the organization of the neural circuits prior to adulthood.

Nuclear AR-ir was similar in the AME between these two gekkotan species, but *P. picta* had increased nuclear AR-ir in the POA and the VMH. Males of both species express behavioral sensitivity to T and possess AR-ir in brain regions associated with regulations of sexual and aggressive behaviors. However, the species with low levels of aggression and no courtship possess more AR-ir than a species with high levels of aggression and courtship behavior. Therefore, the absence of behavioral traits in *P. picta* species cannot be explained by decreased levels of T or decreased abundance of AR-ir in these brain regions. The abundance of AR-ir in these brain regions fails to explain the differences in sexually dimorphic behaviors of gekkotan lizards. Although I did not analyze the brains of *C. elegans*, these data suggest AR-ir would be similar to the species studied here. These findings suggest that selection pressures for change in gecko behavior (e.g., gain or loss of courtship, change in level of aggression) did not act on sensitivity in the brain via changes in AR-ir, but perhaps changes in the mechanism located upstream or downstream of T-AR binding led to interspecific differences in behavioral trait expression.

Androgen control and implications for evolutionary change in trait expression

Together the data suggest that phenotypic diversity in suites of correlated traits among species can be explained by species-specific alterations in the androgen control module. This study provides support for the evolutionary potential hypothesis in that species exhibit differences in responsiveness to T for select traits (e.g., courtship) in an

otherwise conserved control module. The data on intersexual and interspecific comparisons of AR-ir in the brain indicate evolutionary change in this component of the androgen control module is not responsible for the sex-specific or species-specific differences in behavioral responsiveness to T. Therefore, the question remains unanswered: If the loss of tail vibration courtship in *C. elegans* evolved due to selective loss in sensitivity to T but the abundance of AR-ir in brain regions associated with mating behavior is not likely different between species with and without courtship behavior, how might the change in sensitivity occur? Below I have proposed alternative hypotheses to address this question.

Hypothesis 1. A change in androgen-sensitivity in brain pathways or subnuclei associated with control of courtship behavior has enabled the loss of this behavior in C. elegans.

In the present study, I was unable to resolve species-specific differences in T-sensitivity in the brain as measured by AR-ir in the AME, POA, and VMH. Recent reports suggest that courtship versus copulatory behaviors are regulated by different neural pathways or even subnuclei within the POA (see Been and Petrulis 2012). The field of neuroendocrinology is further advanced in mammals and birds than in squamate reptiles, and conducting a finer level of analysis (e.g., on pathways or subnuclei) is beyond the scope of my dissertation research. Future studies to delineate neural pathways in the lizard brain and how androgens act in these pathways are needed to uncover species-specific differences between geckos with or without courtship behavior.

Hypothesis 2. Species-specific difference in androgen sensitivity of motor neurons may be associated with the loss of courtship behaviors.

In many vertebrates, AR are present in the peripheral motor neurons connected to behavioral displays (e.g., frog, Kelly 1980; bird, Fuxjager et al. 2012) and species-specific differences in AR-ir in motor control regions have been reported (Shaw and Kennedy 2002). Eublepharids that exhibit tail vibration courtship behavior may possess AR in the motor neurons innervating the tail. *Coleonyx elegans* displays several controlled movements of the tail including 'tail wave' and 'tail slap', which indicates motor neurons underlie controlled movements of the tail in this species, but not tail vibration. Since T activates expression of tail vibration in *G. lichtenfelderi* (Ch. 3) and *E. macularius* (Rhen and Crews 1999), the specific loss of tail vibration in *C. elegans* could be due to the absence of androgen receptors in these motor neurons necessary for T-activated behavioral expression. Therefore, I suggest future studies investigate the presence of AR-ir in motor neurons of gecko species that either do or do not express tail vibration behavior. However, a study in the lizard *Anolis carolinensis* found relatively few cells labeled for AR-ir or AR mRNA in the main motor areas responsible for extension of the dewlap used for courtship displays (Rosen et al. 2002), suggesting the possibility that AR may not be present in the motor neurons of eublepharids.

Hypothesis 3. Behavioral insensitivity to T in C. elegans is mediated by effects from different components of the endocrine signaling system.

Differences in sensitivity to T for various behavioral traits may depend on whether individual traits require AR versus estrogen receptor (ER)-dependent responses. For example, during the breeding season in some bird species, exogenous T decreases parental care but does not increase aggression in males, and this difference in behavior sensitivity to T may relate to different mechanisms responsible for regulation of

aggression versus paternal behavior (Lynn et al. 2005; Lynn and Wingfield 2008; reviewed in Lynn 2008). The author suggested that T may enhance aggressive behavior by conversion to estradiol (E_2) and subsequent binding to ER, whereas parental care may be mediated by action of T binding AR (Lynn 2008). With differential target receptors for each behavior, alterations in the levels of the metabolizing enzyme aromatase would limit the availability of E_2 and thus E_2-ER dependent aggression (but not T-AR dependent parental care) would be insensitive to T (Lynn 2008).

However, this explanation for how an individual behavior can become insensitive to T in birds is not likely to explain the absence of T-sensitivity of courtship behavior in *C. elegans*. The scenario in birds pertains to seasonal changes in behavioral sensitivity of traits that are present in these species, whereas the trait in *C. elegans* is never present. Furthermore, a previous study in the *E. macularius* indicated that implants containing E_2, T, or dihydrotestosterone can restore tail vibration in castrated males (Rhen and Crews 1999). This suggests that behavioral responsiveness can be induced through AR- or ER-mediated pathways. If this is true for other eubelpharids, then changes in levels of metabolizing enzymes should not have a significant effect on the expression of tail vibration.

Hypothesis 4. An alternative to explaining the loss of tail vibration through potential changes in androgen-sensitivity of the behavior involves the potential changes in genetic mechanisms underlying sexually dimorphic behaviors.

Several types of genetic changes may be responsible for the absence of tail vibration in *C. elegans*. One possibility is that a change in the DNA sequence within the promoter region (i.e., upstream) of a gene can change the affinity of transcription factors

to bind and regulate (i.e., upregulate or downregulate) transcription of those genes (Williams and Carol 2009). Active steroid hormone-receptor complexes function as transcription factors and bind to specific sequences in the promoter regions of steroid-sensitive genes. An alteration in the DNA sequence of the binding site for the T-AR complex within the promoter region of a gene could lead to dissociation of individual genes from hormonal control and thus prevent hormone-mediated effects on transcription of those specific genes (i.e., loss of downstream effects of T-AR binding). Another possibility involves genetic change in the DNA sequence within the genes required for this behavior (e.g., gene mutation; loss of upstream effects of T-AR binding). For example, the *fruitless* gene in drosophila encodes a set of male-specific transcription factors present in the central nervous system, which form at least part of a male-specific neural network responsible for controlling the production of courtship (Baker et al. 2001; Manoli et al. 2005; Rideout et al. 2007). Mutations of the *fruitless* gene typically fail to express courtship. Although the gene or genes required for the expression of tail vibration behavior in geckos is unknown, a potential for evolutionary change in those genes or their regulatory sequences may underlie the observed insensitivity to T in *C. elegans*.

Loss of courtship behavior: speculation on ultimate causation

Regardless of how loss of tail vibration occurred (e.g., subnuclei or pathways in brain, motor neurons, genetic change), the present results suggest the behavior has been dissociated from the androgenic mechanism retained to regulate male aggression and other sexually dimorphic morphological and physiological traits important for intrasexual competition and sex recognition. A number of potential evolutionary mechanisms could

explain the specific loss of tail vibration from the conserved suite of traits, including natural selection, genetic drift, or antagonistic trade-off. Based on studies on the ultimate causation for phenotypic changes in sexually dimorphic traits in other organisms, I have proposed hypotheses for why *C. elegans* may have lost tail vibration from the ancestral suite of sexually dimorphic traits.

Hypothesis 1. Natural selection against tail vibration behavior in males of C. elegans led to the loss of this trait and female preference enabled the persistence of the derived male phenotype.

In other lizards, traits favored by sexual selection have been shown to differ across populations that vary in degree of predation, whereby male-limited traits such as bright coloration are reduced in areas with high predation risk (Endler 1983; Baird et al. 1997; McCoy et al. 2003). In these cases, sexual selection favoring expression of a "showy" trait is balanced by natural selection favoring a more muted phenotype. Studies in other vertebrates also demonstrated that sexual signals can differentiate across populations in response to local selection pressures (Verrell 1997; Wilczynski and Ryan 1997). The loss of tail vibration courtship in this species of *Coleonyx* may have been associated with natural selective pressures against (i.e., negative selection) this display behavior, perhaps due to increased risk of predation on individuals that performed the behavior.

When natural selection leads to changes in male phenotype, the coevolution of female preference to the derived male phenotypes has been demonstrated in several vertebrates. For example, in the fish *Gasterosteus aculeatus*, populations diverged in phenotypes adapted to different trophic habitats, which led to an associated change in

courtship behaviors, zigzag courtship swimming versus absence of this component of sexual behavior (Foster et al. 1998). Behavior studies demonstrated that females prefer male displays typical of their own populations (Foster et al. 1998). Similar scenarios where divergence between isolated populations led to alteration in male behaviors as well as female mate choice have also been reported in several amphibians (Kawamura and Sawada 1959; Verrell and Arnold 1989; Wilczynski and Ryan 1999) and birds (Schluter and Price 1993). These findings suggest that if natural selection pressures led to the loss of tail vibration in *C. elegans*, the phenotype could have been maintained by the coevolution of female preferences for the derived male behaviors.

Precopulatory courtship displays in lizards are not ubiquitous, as demonstrated by *P. picta* and other species that lack this behavior (e.g., *Phrynosoma platyrhinos* Trollestrup 1981; *Gambelia wislizenii* Trollestrup 1983; *Lacerta vivipara* Bauwens et al. 1987). However, precopulatory courtship appears to be necessary to induce female receptivity and willingness to mate in most eublepharid lizards (*G. lichtenfelderi*, Ch. 3; *E. macularius*, see Crews et al. 1998). This suggests that the evolutionary loss of male courtship in *C. elegans* would require a dramatic change in female requirements for the male display to induce physiological and behavioral receptivity. This change could have occurred if the ancestral females possessed some variation in this requirement, as suggested by a case study in field crickets (*Teleogryllus oceanicus* Bailey et al. 2008; Tinghitella and Zuk 2009). Whereas male courtship behavior in cricket species has generally reported to be required prior to copulation (Crankshaw1979; Adamo and Hoy 1994; Libersat et al. 1994), ancestral populations of the field cricket contain females that will mate with males that do not court (Bailey et al. 2008). This pre-existing condition

likely enabled the maintenance and spread of a genetic mutation rendering males unable to court in certain populations (Tinghitella 2008; Tinghitella and Zuk 2009). Similarly, if the ancestor of *C. elegans* had some variation in the strength of the requirement for courtship preceding the evolutionary loss of this trait in males, the subsequent relaxation of female mating preferences could have enabled the maintenance of the new male phenotype.

Hypothesis 2. Positive selection for a change in mating strategy may have led to the loss of male courtship in favor of an opportunist strategy involving forced copulations.

In most species of eublepharid lizards, males typically perform conspicuous, stereotyped courtship display involving tail vibration and scent-marking prior to physical contact with a potential female mate (see Fig. 2.1). Studies in other lizards have suggested that male courtship functions to relay cues advertising male quality (Martin and Lopez 2006; Martin et al. 2007) and to stimulate receptivity of the female (Crews 1975; Kelso and Martins 2008; Stapley 2008). In six eublepharids that exhibit tail vibration courtship, including *G. lichtenfelderi* (Ch. 3) and *E. macularius* (Sakata and Crews 2003), males court, body grip and then mount in a hierarchical pattern of behaviors for successful reproduction (Kratochvíl and Frynta 2007). In *E. macularius*, male courtship increases female receptivity, and only receptive females allow the male to body grip and mount for copulation (Crews et al. unpublished data, cited in Crews et al. 1998). Therefore, courtship display is an essential aspect for successful sexual interactions in that species. In a study involving anesthetized stimulus animals, males of *G. lichtenfelderi* were not able to copulate when attempting to mount an anesthetized

animal (personal observation); indicating that forced copulation is not possible in this species. Furthermore, this finding suggests that females have an active role during the mating process, providing a physiological or behavioral response to the male's courtship and body grip behaviors, in order to enable successful copulation. Therefore, male courtship behaviors in these two seasonally breeding eublepharids appears to be a requirement to stimulate female receptivity, as previously demonstrated in the seasonally breeding lizard *A. carolinensis* (Crews et al. 1975). In contrast, males of *C. elegans* were observed to body grip and mount both socially active and anesthetized animals (Golinski et al. unpublished data), and mounting was rapidly followed by intromission even when females were not receptive (see Ch. 2). But how could courtship be lost if it is vital for successful reproduction in most eublepharids? In a species that exhibits forced copulation (also see Faltusová 2005), female receptivity is not a prerequisite for reproduction. Accordingly, the fixed action pattern of sexual behavior in *C. elegans* does not include courtship, but rather begins with body grip followed by mounting for copulation.

Males of *C. elegans* exhibit a high level of forced copulation, which contrasts the mating behavior of other eublepharids. Forced or coercive copulations occur in diverse taxa, including birds (McKinney et al. 1983; Dunn et al. 1999), reptiles (Hews 1990; Rodda 1992; Olsson 1995; Olssen and Madsen 1995; Shine and Mason 2005), fish (Barbosa and Magurran 2006; Head and Brooks 2006; Griffiths et al. 2011), and insects (Dukas and Jongsma 2012; Arnqvist and Rowe 1995). Forced copulations can be detrimental to female reproductive success and lifespan in some species (McKinney et al. 1983; Dukas and Jongsma 2012) but has no such effect in others (e.g., Head and Brooks 2006). Forced copulation occurs in some lizards, typically involving a non-territorial

male that adopts this strategy because the females normally copulate with the territorial male in these mating systems (Hews 1990; Rodda 1992). In guppies, males display courtship when predation is low but switch to coercive mating when predation rates are high and this environment-dependent flexibility in mating decision enables both males and females to achieve a high reproductive success (reviewed in Barbosa and Magurran 2006). However, in all of these cases of forced or coercive mating in other organisms, males possess the ability to display courtship. Thus, the adaptiveness of courtship is dependent on ecological circumstances, even in species in which courtship is a regular component of the repertoire of sexual behavior.

Coleonyx elegans lives in forests of the Yucatan peninsula and is rarely observed during surveys of biodiversity (e.g., now classified as endangered by Mexican legislation, Luja et al. 2008). Therefore, nothing is known about the structure of their populations (e.g., local densities), mating systems (e.g., territoriality), or breeding phenology (e.g., may be seasonal or continuous as in the lab) in nature. If populations are characterized by a low density of animals, as suggested by the biodiversity surveys, sexual selection may have favored the opportunistic mating strategy involving forced copulation (i.e., if a male finds a female, copulation can occur even if she is not receptive). In terms of reproductive biology, females have the ability to store sperm in several species of eublepharid geckos (Holfert and Seufer 2005), and females of *C. elegans* also possess this ability when bred in the laboratory (L. Kubička, personal communication). Furthermore, our experiments demonstrated that females of this species developed enlarged vitellogenic follicles after behavior trials with males where copulatory interactions occurred but intromission was prevented, suggesting that male mounting

without courtship stimulates physiological responses in the female reproductive system (Golinski et al. unpublished data). Following copulation (forced or not), the female's follicles would develop and stored sperm would be used for fertilization. This description of reproductive physiology in *C. elegans* fits into the category of a mixed reproductive strategy, rather than associated or dissociated breeding patterns (Crews 1997). Together, the characteristics of female reproductive biology along with the little information regarding their ecology suggest the opportunistic mating strategy with forced copulations may have been advantageous for this species.

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Tables

Table 6.1. Responsiveness of sexually dimorphic traits to testosterone (T) in reproductively mature males: comparison among gecko lizards. Sexually dimorphic traits listed on the left. "yes" or "no" indicates whether the trait significantly differed between males with elevated T versus castrated males. "-" indicates the available data are insufficient based on the experimental design. "*" indicates the trait is absent from the species and cannot be induced by exogenous T. Data on *E. macularius* from Rhen and Crews 1999, 2000; Rhen et al. 2005.

Sexually Dimorphic Traits	*E. macularius*	*C. elegans*	*G. lichtenfelderi*	*P. picta*	Summary
Body Size	-	-	-	no	-
Head Width	-	no	no	no	**no**
Hemipenes	yes	yes	yes	yes	**yes**
Pore Secretions	yes	yes	yes	no*	**yes**
Aggression	yes	yes	yes	yes	**yes**
Mounting	yes	no	yes	yes	**yes**
Courtship	yes	no*	yes	no*	**yes**

Table 6.2. Responsiveness of sexually dimorphic traits to testosterone (T) in reproductively mature females; comparison among gecko lizards. Sexually dimorphic traits listed on the left. "yes" indicates male-typical trait was induced in females supplemeted with T. "no" indicates the trait did not significantly differ between intact control females versus females supplemented with T. "-" indicates the available data are insufficient based on the experimental design. "*" indicates the trait is absent from the species and cannot be induced by exogenous T. Data on *E. macularius* from Flores and Crews 1995; Rhen and Crews 1999, 2000.

Sexually Dimorphic Traits	*E. macularius*	*C. elegans*	*G. lichtenfelderi*	*P. picta*	Summary
Body Size	-	-	-	yes	-
Head Width	-	yes	no	no	**no/yes**
Hemipenes	yes	yes	yes	yes	**yes**
Pore Secretions	-	yes	no	no*	-
Aggression	no	no	no	no	**no**
Mounting	no	yes	no	yes	**no/yes**
Courtship	yes	no*	yes	no*	**yes**