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By

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ABSTRACT

DIMORPHISMS IN THE NEURAL AND MUSCULAR SYSTEM CONTROLLING MALE COURTSHIP BEHAVIOR IN THE LIZARD (*ANOLIS CAROLINENSIS*)

By

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Male courtship behavior in the green anole lizard includes the extension of a bright red throat fan, the dewlap. Males perform this androgen-dependent behavior during the breeding season to attract mates and defend territories. Females have smaller dewlaps that are used far less frequently (only in agonistic encounters). This set of five studies was designed to elucidate the relationship between structures involved in dewlap extension and the extent of the behavioral display. The 1st investigated whether sex differences in the soma size of caudal brainstem motoneurons critical for dewlap extension were stable between the breeding and non-breeding seasons and whether testosterone might influence this measure. The muscle and nerve regulating the display were also investigated. In addition to neuron soma size being larger in males, the cross-sectional areas of the nerve and muscle fibers as well as muscle fiber number were also larger in males, in parallel with behavioral differences. However, seasonal and experimental changes in androgen did not alter the morphology of the system. The 2nd study assessed dendritic length and arborization of dewlap motoneurons. They were not different between males and females, suggesting that input to the system is comparable between the sexes. However, output of the motoneurons may be increased in males due to larger neuromuscular junctions that were discovered in the 3rd study. Like soma size,

however, neuromuscular junctions were not smaller in the non-breeding season. The fact that dimorphisms in the neuromuscular structures are stable throughout the year and not altered by differences in adult testosterone suggests that they might be permanently organized during ontogeny. The development of some aspects of this system was thus examined in the 4th study. While the effects of gonadal steroids on development were beyond its scope, the goal of this study was to determine when the structures begin to differentiate, with the idea that hypotheses could then be generated about mechanisms. During the first 3 months after hatching, some dewlap structures began to differentiate well before overall body size. The timing in relation to a juvenile rise in testosterone suggests that the hormone may influence masculinization. Finally, the 5th study examined neuron soma size in the preoptic area and amygdala to determine if structure in these regions paralleled function. Both of these areas are critical to the display of masculine courtship behavior. Surprisingly, soma size was larger during the breeding season in both sexes, so a second experiment was done to address whether testosterone was the mechanism creating these differences. However, while testosterone administered in the non-breeding season increased behavior in both sexes, it did not alter soma size. Together, the studies indicate that numerous male-biased sex differences exist in dewlap neuromuscular structures, and that adult androgen and other seasonal differences do not alter their morphology. However, development of these structures is consistent with the idea that testosterone might act during ontogeny to organize sex differences, though future studies will have to test that idea empirically. Finally, it appears that the seasonal hormonal stimulation of male courtship behavior in the forebrain also does not require basic morphological changes.

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CHAPTER ONE:

GENERAL INTRODUCTION

Precisely how the brain is involved in the production of behavior is a question that is currently under investigation in many laboratories. One very simple way in which to address this fundamental question is to study the relationships between structures of the brain and the behaviors they are thought to mediate. These relationships have been studied by using courtship and copulatory behaviors for a number of reasons. For one, they are very conspicuous behaviors that are stereotyped and can be easily reproduced in the laboratory. Secondly, these sexual behaviors are usually sex-specific. It is therefore possible to identify any differences which might exist in brain regions known to be responsible for these behaviors between two types of individuals: one that naturally produces them and one that does not. Thirdly, many of these behaviors are dependent on the presence of circulating gonadal steroids such as androgens and estrogens (Meisel and Sachs, 1994). By manipulating the levels of these hormones, it is possible to study the role of these substances in eliciting these behaviors.

The relationships among sexual behavior, the structural elements that produce these behaviors, and their dependence on gonadal steroids have been studied in numerous vertebrate species from mammals to birds to lower vertebrates such as amphibians and fish. Perhaps the most extensively studied model of neuromuscular control of male sexual behavior has been conducted in a simple neuromuscular system controlling movement of the rat penis. Penile function in rodents requires contraction of the bulbocavernosus (BC) and levator ani (LA) muscles, which are innervated by

motoneurons in the lumbar spinal cord, contained in the spinal nucleus of the bulbocavernosus (SNB) (Breedlove and Arnold, 1980). Male rats have larger SNB motoneurons and cell nuclei than females (Breedlove and Arnold, 1981). Neuromuscular morphology can be modified later in life by changes in adult levels of androgen. For example, castration causes SNB somata, muscle fibers, and the size of neuromuscular junctions (NMJ) to shrink in adult males (Breedlove and Arnold, 1981; Balice-Gordon *et al.*, 1990). Alternatively, androgen at critical periods of development (see Breedlove and Arnold, 1983b) can affect BC/LA muscle morphology as well as SNB motoneurons number and size (Breedlove and Arnold, 1983a). Females do not have BC/LA muscles in adulthood, as they involute shortly after birth due to a lack of androgen (Cihak *et al.*, 1970). However, treating females perinatally with androgen can create similar morphological characteristics to those of males. More specifically, androgenized females develop BC/LA muscles that are comparable in size to that of males (Breedlove, 1984), and consequently develop masculinized SNB cells, both in terms of total number and overall size (Breedlove and Arnold, 1983a). Photoperiod studies in this model system reveal differences that mimic those seen after adult castration. For example, in the white-footed mouse (*Peromyscus leucopus*), SNB motoneuron soma and nucleus size, and dendritic arborization are reduced in non-breeding animals kept on short days, compared to individuals exposed to a long photoperiod (Forger and Breedlove, 1987). BC/LA muscle mass also decreases after exposure to short daylengths.

Similar studies have been done in non-mammalian vertebrates, including amphibians, fish, and avian species. Male courtship behaviors displayed by species of frogs, teleost fish, and songbirds are also activated in adulthood by androgens, and

changes in the cytoarchitecture of brain regions and muscles that regulate the behaviors are influenced by androgen (reviewed in Kelley, 1986; Bass, 1986; Arnold, 1992). For example, male South African frogs (*Xenopus laevis*) court females by producing a sex-specific vocalization that is under the control of motoneurons in the motor nucleus of cranial nerve IX-X and muscles of the larynx. Females do not produce this courtship call, and in parallel, the structures that are required for the call are diminished or absent in females. Specifically, somas of motoneurons that control muscle input for masculine courtship vocalization are larger in males than in females (Hannigan and Kelley, 1981; Kelley and Fenstemaker, 1983; Kelley *et al.*, 1988) and, like the SNB system, more larynx motoneurons exist in males than females (Kelley, 1988). Adult sex differences in motoneuron and motor axon number are created prior to metamorphosis due to higher circulating androgen in males (Robertson *et al.*, 1994; Kay *et al.*, 1999). Accompanying these neural differences, male *X. laevis* have larger larynges, which contain more and larger muscle fibers than those of females (Kelley, 1986). Males treated with the androgen receptor antagonist flutamide after metamorphosis have a lower laryngeal fiber number than control males, and an equal number of muscle fibers compared to flutamide- and control-treated females (Sassoon and Kelley, 1986). Treating females prior to metamorphosis with testosterone causes an increase in larynx weight, but never to the level of males and the females are not capable of producing a courtship call (Hannigan and Kelley, 1986).

The midshipman toadfish (*Porichthys notatus*) also provides a good model in which to study the relationships between structure and function in regards to male sexual behavior. In this species, two morphs of males (Type I and Type II) are capable of

fertilizing eggs from the female, but only the Type I male produces a courtship vocalization; Type II males fertilize eggs by sneaking (Brantley and Bass, 1994). Correspondingly, Type I males are larger than both Type II males and females, and they have higher levels of their biologically relevant androgen, 11-ketotestosterone (Brantley *et al.*, 1993). Courtship vocalizations in the Type I male are produced by a neuromuscular system consisting of caudal brainstem motoneurons innervating sonic muscles located on the walls of the swimbladder (reviewed in Bass, 1990). As you would expect based on the polymorphic behavior, Type I males have larger sonic motoneuron somas (Bass and Marchaterre, 1989a; Bass and Baker, 1990), more sonic motoneurons (Bass, 1986), and larger sonic muscles (Bass and Marchaterre, 1989b) than both Type II males and females. Specifically, sonic muscles contain larger and more muscle fibers in Type I males than Type II males or females. This larger neuromuscular system most likely allows Type I males to produce the vocalizations characteristic of courtship behavior in this species.

Some species of songbirds, including zebra finch and canary, have also sexually dimorphic courtship song. Male zebra finches sing to court females, whereas females never sing (Adkins-Regan and Ascenzi, 1987). Correspondingly, regions of the well-characterized forebrain song-control circuit are larger in volume in males than females (Nottebohm and Arnold, 1976). Androgen is thought to be involved in song production as castration leads to a reduction of singing behavior in males (Arnold, 1975), but fails to produce a concomitant decrease in the size of song-control nuclei (Arnold, 1980). On the other hand, females treated with androgen in adulthood still fail to produce song, and there is also no change in the size of song-control nuclei in these females (Arnold, 1980).

The volume of motor nucleus nXIIts which innervates the vocal organ (syrinx) is larger in male than female zebra finches, but not sexually dimorphic in soma size or number (Wade and Buhlman, 2000). Male songbirds also have a heavier syrinx than females (Wade and Arnold, 1996; Springer and Wade, 1997), which contains larger and more numerous muscle fibers in males than females (Wade and Buhlman, 2000). Adult treatment with testosterone fails to affect the volume of nXIIts, or the individual soma sizes of hypoglossal motoneurons in either sex (Wade and Buhlman, 2000). The androgen does, however, induce a modest increase in syrinx weight and muscle fiber size in adulthood (Wade and Buhlman, 2000).

Unlike zebra finches in which females never sing, female canaries sing, but much less often than males (Pesch and Güttinger, 1985). In addition to singing more, males have larger song control nuclei than females (Nottebohm and Arnold, 1976; DeVoogd *et al.*, 1991). However, treating adult females with exogenous androgen increases the size of one nucleus in particular (high vocal center, or HVC; Nottebohm, 1980). The volume of the hypoglossal nucleus which projects to the syrinx and the soma size of these motoneurons are also larger in male than female canaries (Nottebohm and Arnold, 1976).

While these relationships have been examined in other vertebrate groups, as outlined above, relatively little information is available on the neuromuscular regulation of sexually dimorphic reproductive behaviors in a reptile. The courtship display of green anole lizards (*Anolis carolinensis*) provides an ideal means to investigate these relationships. Male anoles court females and defend territories with a series of head-bobbing movements and the extension of a bright red throat fan called a dewlap (Greenberg and Noble, 1944; Crews, 1979, 1980). This behavior is highly sexually

dimorphic (Nunez *et al.*, 1997), occurs only in the breeding season (approximately April-July) and is androgen-dependent. During the non-breeding season, both males and females have a marked decrease in activity, and dewlap extension behavior is decreased in both sexes (Jenssen *et al.*, 1996). Masculine behaviors during the breeding season decrease within two weeks following castration, and androgen treatment will prevent or reverse this effect (Mason and Adkins, 1976; Adkins and Schlesinger, 1979; Winkler and Wade, 1998; Rosen and Wade, 2000). While females have only a very small dewlap that is not used in the context of courtship (Greenberg and Noble, 1944), they can be induced to dewlap more frequently during the breeding season with androgen treatment but still not as often as males (Winkler and Wade, 1998).

The mechanism by which this behavior is displayed is as follows. Dewlap extension is induced by the contraction of a bilaterally symmetrical muscle (M. ceratohyoideus, also called M. branchiohyoideus) in the throat, which causes a paired set of cartilage (2nd ceratobranchial cartilages, also called "retrobasal process"; Crews, 1980) that normally lays flat against the throat and chest to bow out (Bels, 1990; Font and Rome, 1990; Font, 1991), unfolding the dewlap skin (see diagram in Chapter 4). The ceratohyoid muscles are innervated by motoneurons located in (1) the vagal portion of nucleus ambiguus (AmbX) and (2) the region containing both the glossopharyngeal portion of nucleus ambiguus and the ventral motor nucleus of the facial nerve (AmbIX/VIImv) (Font, 1991; Wade, 1998). During the breeding season, these motoneurons are larger in males than in females (Wade, 1998). Peripheral structures are also sexually dimorphic, as M. ceratohyoideus is heavier, and the second ceratobranchial

cartilage is longer in males than in females (Wade, 1998) as is the size of the dewlap itself (Jenssen *et al.*, 2000).

The goal of this research was to elaborate on the structural differences in regions of the brain important for courtship behavior. I hoped to identify structural differences which might create or facilitate behavioral differences in dewlap extension behavior in this species either between the sexes or seasons. The behavioral dimorphisms might be produced at any or all of the following levels: increased soma size or soma number, increased input by way of more extensive dendritic arborization of dewlap motoneurons, or increased output from the system via an increase in neuromuscular junction morphology. Additionally, it was important to determine the development of these structures. The developmental time-course of neuromuscular structures involved in courtship behavior were unknown, as was the timing of their sexual differentiation. Therefore, it was necessary to know the critical period of structural development for these parameters in order to later identify the mechanism that promotes sex differentiation. Most often, sexual differentiation is caused by gonadal steroids, but could also be organized due to genomic factors (Wade, 1999).

Furthermore, the behavioral dimorphisms could be created by structural differences in a part of the brain that is involved in the motivation rather than the mechanisms of the behavior (see Crews and Silver, 1985). Two portions of the forebrain, the preoptic area (POA) and the amygdala, play important roles in the display of reproductive behaviors in many vertebrate species (Crews and Silver, 1985; Meisel and Sachs, 1994). Specifically, intracranial implant studies indicate that androgens act in the POA and amygdala to increase male sexual behavior in vertebrates ranging from reptiles

to mammals (Davidson, 1966; Rozendaal and Crews, 1989; Wood and Newman, 1995; Riters *et al.*, 1998). Additionally, parallels often exist between the morphology of these regions and the display of male sexual behavior, such that volume or neuron soma size are larger in males than in females. These sexual dimorphisms are in several cases maintained in adulthood by testosterone (T) or its metabolites (see Cooke *et al.*, 1998 for review). As the role of these regions in male sexual behavior in the anole has been described (see Chapter 6), I aimed to determine if parallels exist between the seasonal display of behaviors and the size and/or density of neurons in important regions in the forebrain.

CHAPTER TWO:

SEXUAL DIMORPHISMS IN THE NEUROMUSCULAR SYSTEM REGULATING COURTSHIP: EFFECTS OF SEASON AND ANDROGEN TREATMENT

RATIONALE

The sex and seasonal differences in male courtship display in the green anole might be facilitated by morphological differences in both neural and peripheral structures controlling this behavior. If so, then the size of dewlap motoneurons, the nerve that projects to the muscle, as well as characteristics of the dewlap muscle itself could be larger in males than in females, and larger in breeding males than non-breeding males.

These structural differences may be mediated by differences in testosterone (T) levels between males and females during the breeding season and between males across the two seasons. Androgens activate male sexual behavior in the anole when given systemically (Mason and Adkins, 1976; Adkins and Schlesinger, 1979; Winkler and Wade, 1998; Rosen and Wade, 2000). I predicted that structural changes would mirror behavioral ones, giving rise to larger dewlap motoneurons, nerve, and muscle fiber characteristics in testosterone-supplemented males compared to males that have relatively low circulating androgen.

Two experiments were conducted to assess in more detail the potential relationships between structure and function in this system. In Experiment 1, brain tissue was examined for the effects of sex, season, and androgen treatment on motoneuron number, soma size and nucleus size. In Experiment 2, peripheral tissues (muscle and

nerve) were examined during the breeding season only for effects of sex and androgen treatment.

METHODS

Animals

Experiments 1 & 2: Adult male and female green anoles were purchased either during the breeding season or in September, after it had ended (non-breeding season), from Buck's (LaPlace, LA), which supplies animals recently captured from the field. They were housed in glass aquaria with sticks and/or rocks for climbing and peat moss as substrate. Animals were exposed to ambient temperatures of 21-23°C and were allowed access to temperatures up to 38°C provided by heat lamps. Cages were sprayed with water daily to increase humidity. Photoperiod was maintained at 14:10 for the breeding season portion of the study (April through July) and 10:14 for the non-breeding season portion (September and October). All animals were fed with crickets or mealworms three times per week, and had water available *ad libitum*. Intact animals for this study were housed in 29-gallon tanks, each containing one male and 3-5 females per cage. Gonadectomized, implanted males were moved from 29-gallon aquaria to individual, visually isolated 10-gallon tanks immediately following surgery and hormone treatment (see below). Housing conditions were designed to minimize behavioral differences between the two groups of treated males within the breeding season. Morphological variables were assessed separately in untreated animals and in those that received hormone manipulations.

Treatments and Tissue Collection

Experiment 1: Four groups of animals were used during the breeding season: (1) intact males, (2) intact females, (3) males that had been gonadectomized and treated with a Silastic capsule containing testosterone propionate (Steraloids) (TP), or with (4) a blank capsule (BL) (both types: 7mm long x 1.65mm OD x 0.76mm ID). Implants were administered subcutaneously at the time of castration, two weeks prior to perfusion. All animals were given an overdose of Sodium Brevital (Eli Lilly and Co.) and perfused with 0.1M phosphate buffered saline (PBS) followed by 10% phosphate buffered formalin (PBF). The brains were then postfixed in PBF. The kidneys were also removed from each individual and stored in Bouin's fixative. The renal sex segment, which functions in a manner similar to the mammalian prostate, serves as a bioassay for circulating androgen levels in that epithelial cell height in these tubules increases in response to both testosterone and dihydrotestosterone (Cuellar *et al.*, 1972; Winkler and Wade, 1998). At the time of perfusion, intact animals taken during the breeding season had either developed testes or medium to large yolking follicles. The completeness of castration and the presence of a capsule were also confirmed in gonadectomized animals at the time of perfusion. The same treatment groups and all methods performed during the breeding season were repeated in the non-breeding season. However, in the intact animals, the gonads of all individuals were completely regressed when they were perfused during the non-breeding season.

Experiment 2: A separate set of animals (same four groups as above) was used to investigate the morphology of peripheral structures involved in dewlap extension during

the breeding season. The individuals were perfused with PBS and PBF as above, and the entire throat region underneath the dewlap skin, extending from just anterior to the hyoid apparatus to just anterior to the collarbone, was removed and stored in Bouin's fixative for three days. The forearm between the wrist and the elbow was also removed and placed in Bouin's fixative, and was used for measurement of control muscle tissue. Kidneys were not examined in Experiment 2, as the treatment groups were identical to those in Experiment 1.

Tissue Processing and Analysis

Experiment 1: Brains were embedded in a block of 10% gelatin and 30% sucrose, which was post-fixed in 20% sucrose in PBF overnight. The tissue was sectioned frozen at 20μm into PBS and mounted on gelatin-subbed slides. It was then stained with thionin, dehydrated, cleared in xylene, and coverslipped with Permount (Fisher). In the regions containing dewlap motoneurons (AmbIX/VIImv and AmbX, as defined in ten Donkelaar and Nieuwenhuys, 1979; Barbaras-Henry and Lohman, 1984; Wade, 1998), the number of neurons was counted, and the sizes of cell bodies and nuclei were measured using NIH Image software. The soma sizes of motoneurons in the intermediate portion of the oculomotor nucleus (IIIi) were measured as a control, since they are not involved in dewlap extension. In all cases, fifteen randomly selected neurons or cell nuclei on each side of the brain were used for measurements (30 neurons total). To be sure motoneurons were counted once, only large cells with characteristically angled morphology and an identifiable, in-focus nucleolus were quantified.

Kidneys were dehydrated in a series of alcohols, cleared in xylene, embedded in paraffin, and cut on a rotary microtome at 10μm. The tissue was then stained with hematoxylin and eosin, and coverslipped with Permount. In each individual, the height of epithelial cells (defined as the height from the basal membrane to the edge of the lumen) was measured in four locations in each of four tubules.

Experiment 2: The entire foreleg and the throat tissue were dehydrated in a series of alcohols and cleared in xylene before being embedded separately in paraffin. The tissue was then cut on a rotary microtome at 10μm, mounted on clean slides, and stained with a trichrome stain (Forger *et al.*, 1995).

Cross-sectional area of the nerve (ramus pharyngo-laryngeus IX+X; Willard, 1915) that runs along the ventral surface of M. ceratohyoideus, was measured in five sections randomly selected throughout the entire muscle. In addition, 30 fibers were randomly selected from either side of two muscles: M. ceratohyoideus and M. omohyoideus (both muscles are bilaterally symmetrical). M. omohyoideus was used as control because it is in a region of the throat similar to M. ceratohyoideus, but does not appear to be involved in dewlap extension (Font and Rome, 1990). The cross-sectional areas of the fibers in each muscle were measured using NIH Image software. The areas of 15 randomly selected fibers from the anterior radial muscle of the leg were measured using the same method. An estimate of fiber number and density in M. ceratohyoideus was determined as follows. Cross-sectional area of the entire left or right muscle was measured as above in 3 randomly selected sections at its largest point (the muscle narrows at the rostral and caudal ends). The total number of muscle fibers within those

areas was counted. Dividing the fiber count by total muscle area in each section provided an estimate of fiber density.

Statistical Analyses: Averages of each measurement for each individual were used in statistical analyses (*t*-tests and 2-way ANOVAs as indicated in Results; Statview: SAS Instruments). Measurements and counts of motoneurons appeared similar in the two halves of the brain. Differences were considered significant if $p \leq 0.05$. All measurements were taken in all individuals of each relevant group, unless they could not be assessed due to histological artifact. Tissues were examined from 5-8 animals in each group in Experiment 1. In Experiment 2, 6 animals per group contributed to each measure, except for radial muscle fiber size (4 males and 4 females).

RESULTS

Experiment 1: Effects of Season and Androgen Treatment on Dewlap Motoneurons

We addressed the following questions about motoneuron number, soma size, and nucleus size:

(a) Do sex differences exist during the breeding season (*t*-test; intact male vs. intact female)?

(b) Does the morphology change in either sex across the two seasons (2-way ANOVA; sex by season)?

(c) Are the structures regulated by testosterone in males during either season (2-way ANOVA; treatment by season)?

Motoneuron Soma Size. (a) As previously determined (Wade, 1998), the soma size of dewlap motoneurons was larger in males than females during the breeding season in both AmbIX/VIImv (1-tailed t=1.79, p=0.048) and AmbX (1-tailed t=2.30, p=0.019; Figure II-

1A,B). (b) In the analysis of sex by season, a main effect of sex existed in AmbIX/VIImv, such that soma size was larger in males than females (F=6.41, p=0.017; Figure II-1A). There was no main effect of season in AmbIX/VIImv (F=3.23, p=0.083), or sex by season interaction (F=0.02, p=0.896). In AmbX (Figure II-1B), no significant effects were detected when comparing intact males and females in the two seasons (all F<2.91, all p>0.099). (c) Testosterone treatment had no significant main effect on soma size in AmbIX/VIImv (Figure II-1C) (F=0.70, p=0.412) or AmbX (Figure II-1D) (F=3.22, p=0.087). No effect of season existed in AmbIX/VIImv (F=1.97, p=0.173), but a main effect of season did exist in BL- and TP-treated animals in AmbX (F=6.41, p=0.022), such that soma size was greater in the non-breeding season. There was no significant interaction between treatment and season on the soma size of either group of motoneurons (both F<1.20, both p>0.305).

In the control nucleus, IIIi, (a) there was no effect of sex during the breeding season (2-tailed t=1.37, p=0.193). However, (b) when the number of male and females was increased by adding non-breeding individuals in the sex by season analysis, males did have significantly larger soma sizes in this nucleus than females (F=3.28, p=0.029). There was no main effect of season (F=3.29, p=0.076), nor was there a significant interaction between sex and season (F=0.30, p=0.822). (c) No significant effects were detected in the comparison of males treated with either TP or BL in the two seasons (all F<1.05, all p>0.318) (data on IIIi not shown).

Motoneuron Nucleus Size. (a) No effect of sex on the size of nuclei in motoneurons was detected during the breeding season in either AmbIX/VIImv (2-tailed t=0.203, p=0.842;

Figure II-2A) or AmbX (2-tailed t=1.22, p=0.243; Figure II-2B). (b) We detected no main effect of sex or season, or sex by season interaction on dewlap motoneuron nucleus size in either AmbIX/VIImv or AmbX (all F<1.70, all p>0.202). (c) A main effect of testosterone treatment existed in AmbIX/VIImv (F=4.49, p=0.045; Figure II-2C), but not AmbX (F=0.229, p=0.637; Figure II-2D). In AmbIX/VIImv, nuclei were larger in TP- than BL-treated males. A main effect of season existed in this analysis in AmbX (F=4.17, p=0.053; Figure II-2D), but not AmbIX/VIImv (F=1.27, p=0.272; Figure II-2C), such that the nuclei were larger in the non-breeding season. There was no treatment by season interaction in either region (both F<0.27, both p>0.609; Figures II-2C,D). Nucleus size was not assessed in the control (IIIi) region.

Renal Sex Segment. (a) There was a significant effect of sex during the breeding season in renal sex segment cell height (2-tailed t=6.74, p<0.001), such that it was larger in intact males than females (Figure II-3). (b) Significant main effects of sex and season and a significant interaction existed (all F>37.02, all p<0.001). That is, cell height was larger in intact males than females and larger in the breeding season than in the non-breeding season, but the seasonal effect only occurred in males, since renal sex segment size was equivalently small in breeding and non-breeding females. The sex difference between intact animals in the breeding season disappeared in the non-breeding season (2-tailed t=0.069, p=0.946) as the gonads regressed. (c) In the analysis of androgen treatment in the two seasons, a significant effect of treatment existed (F=68.87, p<0.001), such that RSS cell height was larger in TP-treated than BL-treated males. A significant effect of season existed in that analysis as well (F=12.0, p=0.002), such that cell height

was larger in the breeding season. There was no treatment by season interaction (F=0.24, p=0.626) (Figure II-3).

Motoneuron Number. (a) During the breeding season, there was no effect of sex on motoneuron number in AmbIX/VII (2-tailed t=1.43, p=0.176) or AmbX (2-tailed t=0.74, p=0.476). (b) No statistically significant effects were detected when analyzing sex by season in AmbIX/VII or AmbX (all F<2.24, all p>0.145). (c) Among males, no significant differences in either brain area were detected due to treatment or season in the analysis of TP vs. BL treated individuals (all F<1.14, all p>0.298) (data on motoneuron number not shown).

Experiment 2: Sexual Dimorphisms in Peripheral Dewlap Structures

Effects of (a) sex (2-tailed t-test; male vs. female) and (b) testosterone treatment (2-tailed t-test; TP vs. BL) on nerve and muscle fiber size were assessed during the breeding season. However, because we considered it extremely unlikely that a substantial number of muscle fibers would be generated in adulthood, muscle fiber number and density were determined only in intact animals.

Ramus Pharyngo-laryngeus IX+X Size. (a) Cross-sectional area of the nerve was larger in males than females (2-tailed t=4.17, p=0.002). (b) TP treatment had no effect compared to BL treatment of castrated males (2-tailed t=0.22, p=0.828; Figure II-4).

Muscle Fiber Size. (a) Overall, M. ceratohyoideus appears substantially larger in males than females (Figure II-5). Specifically, cross-sectional area of dewlap muscle fibers was on average approximately twice as large in intact males compared to intact females in the breeding season (2-tailed t=5.27, p<0.001; Figure II-6). (b) There was no significant difference between BL- and TP-treated males on muscle fiber size (2-tailed t=0.30, p=0.766). A much smaller sex difference existed in M. omohyoideus, which was measured as a control (2-tailed t=2.73, p=0.021). In contrast to M. ceratohyoideus, in which the average fiber size in females was 46% of that in males, in M. omohyoideus average fiber size in females was 90% of that observed in males. No sex difference in fiber size existed in the anterior radial muscle of the leg (2-tailed t=0.60, p=0.572).

Muscle Fiber Number and Density. (a) There was an effect of sex on the number of dewlap muscle fibers such that males had more fibers than females (2-tailed t=3.64, p<0.005; Figure II-7A). Moreover, a significant difference in the density of these muscle fibers existed between intact males and females (2-tailed t=8.99, p<0.001; Figure II-7B), such that the M. ceratohyoideus of females was more densely packed with fibers than that of males.

DISCUSSION

In this study, I documented a variety of male-biased sex differences during the breeding season in green anole lizards. These dimorphisms include motoneuron soma size, cross-sectional nerve area, muscle fiber size, and muscle fiber number, and they parallel the behavioral dimorphism observed during the breeding season. That is, only

males normally extend the dewlap in courtship (Crews, 1979; 1980), and even with equivalent exogenous androgen treatment, males display this behavior significantly more than females (Winkler and Wade, 1998). This relationship between structure and function is not complete, however. Courtship behavior in males occurs seasonally (Greenberg and Noble, 1944) and is regulated by the presence of androgens (Mason and Adkins, 1976; Adkins and Schlesinger, 1979; Crews, 1980; Winkler and Wade, 1998), yet the morphology of dewlap structures is not reduced in the non-breeding season and does not appear to respond to androgen treatment in adulthood.

These results are consistent with the idea that motoneuron soma size, and especially muscle fiber size and number may be generally important in establishing functional differences between the sexes in the production of behavior. However, unlike some other systems, dewlap motoneuron number is not different between male and female anoles. M. ceratohyoideus is much larger overall in males than in females and contains more fibers in males. Thus, the similarity in neuron number suggests that motor unit is different in the two sexes, with each motoneuron innervating approximately twice as many muscle fibers in males as in females. It is presently unclear why some species differences exist, but even among rodents, perineal motoneuron number does not always parallel sex differences in muscle morphology; neuron number is equivalent in male and female guinea pigs (Freeman and Breedlove, 1995). While it is unlikely to explain the difference between rats and guinea pigs in the relationship between perineal muscles and motoneurons, in general varying degrees of function may influence the degree of sexual dimorphism. For example, although it is not used in a sexual context, female anoles do sometimes extend their rudimentary dewlap during aggressive encounters in a manner

similar to males (Greenberg and Noble, 1944). This situation does not completely parallel the SNB system, in which female rats do not use a penis, or the courtship systems of frogs and sonic fish, in which the vocalizations of females are very different from those of courting males (Kelley, 1986; Bass and Baker, 1990). In this respect, anoles may be more similar to Japanese quail, in which the motoneurons innervating the muscle of a gland used by males during copulation do not appear to be sexually dimorphic. As in the anole, female Japanese quail do use the muscle, during excretion and oviposition (Seiwert and Adkins-Regan, 1998).

Another possibility is that the level of control needed for a certain behavior might impose certain structural requirements. For example, dewlap extension is a simple behavior compared to courtship vocalizations or penile flips. The display must be timed, but the extension and retraction are gradual (each taking approximately 0.1-0.4 seconds), and the structure remains unfolded for 2-9 seconds (Bels, 1990; Font and Rome, 1990). Therefore, it is possible that males and females both have the minimum number of motoneurons required and motor unit size is not very meaningful. That is, males require more and larger muscle fibers because the hyoid apparatus that extends the dewlap skin is much larger than in females, but males do not require more motoneurons because the muscle contraction is not intricately controlled. One other possibility that cannot be ignored reflects the fact that motoneurons were counted in Nissl-stained material in this study, rather than in retrogradely labeled cells. Therefore, the counts may have included cells that projected to muscles other than M. ceratohyoideus. Finally, it may be that nerve size is more important than motoneuron number in facilitating the behavioral display. Because motoneuron number is similar in male and female anoles, the larger

cross-sectional area of the nerve in males compared to females suggests that axon size is larger in males. This increased cross-sectional area is likely to facilitate conduction along the axon.

Unlike other systems, behavioral changes in adult male anoles induced seasonally by testosterone are not accompanied by substantial alterations in motoneuron or muscle morphology. However, in some analyses soma size and nucleus size of dewlap motoneurons were slightly larger in the non-breeding season compared to the breeding season. Similar effects were seen in the SNB of sexually inactive rats compared to those that displayed copulatory behavior (Breedlove, 1997). It is important to note, though, that the average soma and nucleus sizes of intact male anoles in and out of the breeding season are similar, especially in AmbX (see Figures II-1B and II-2B). Therefore, statistical differences that include other groups of animals are unlikely to reflect naturally occurring seasonal changes. It is difficult to speculate about why the structure remains relatively consistent in anoles. However, one possibility reflects the fact that although all of the systems discussed involve sexual behaviors, they are different in two fundamental ways: (1) the SNB system regulates copulation, not courtship; and (2) among the courtship systems, while all controlled by caudal brainstem motoneurons (Nottebohm *et al.*, 1976; Kelley, 1980; Bass, 1985), the anole emits a visual signal in contrast to the auditory signals of the other species.

In these other systems, androgen receptors are expressed in either or both the muscles and motoneurons and, hence, those structures are potential direct sites of hormone action (Arnold *et al.*, 1976; Tremblay *et al.*, 1977; Lieberburg and Nottebohm, 1979; Breedlove and Arnold, 1980; Kelley, 1980; Gahr and Wild, 1997). It appears as

though AmbX contains some AR+ cells (detected using *in situ* hybridization and immunohistochemistry; Rosen *et al.*, in press), but this area is not as intensely labeled as portions of the POA and the VMN of the amygdala. No AR+ cells have been identified in the other dewlap motor nucleus, AmbIX/VIImv, and studies conducted in our lab have not been able to consistently detect AR in the dewlap muscle (unpublished observation).

It is important to note that, while the motoneuron somata, nuclei, nerve, and muscle fibers did not increase in size in the anoles with androgen treatment, the males used in these experiments were responsive to testosterone in both the breeding and non-breeding seasons, at least in peripheral tissues. That is, at both times of year TP-treated males had larger renal sex segments than BL-treated males and intact females. In fact, height of the renal sex segment tubules suggests that males treated with exogenous TP had levels of circulating androgens that were somewhat higher than intact males (Figure II-3). Further, similar regimes of hormone manipulation have produced significant effects on behavior (Mason and Adkins, 1976; Adkins and Schlesinger, 1979; Winkler and Wade, 1998; Rosen and Wade, 2000).

In sum, the present data contribute to the overall understanding of mechanisms through which the structure of both the brain and periphery can produce sexually dimorphic behaviors in adulthood. Similar to systems in other vertebrate groups, sexual dimorphisms exist in structures required for the display of masculine sexual behavior. However, while androgens initiate courtship behavior in males during the breeding season, they do not appear to exert their action via a change in the size of the various neural or peripheral characteristics measured in this study.

Rather than acting directly on structures such as the ceratohyoid muscle, androgens may in contrast alter input to the system, for example by increasing dendritic arborization of motoneurons. Alternatively, androgens might facilitate the motivation of courtship behaviors (see Crews and Silver, 1985). Such a change in motivation might involve a brain region other than those where the dewlap motoneurons are located, such as the preoptic area. This region is known in numerous vertebrate groups, including lizards, to be a location of androgen action in the facilitation of male sexual behavior (Morgentaler and Crews, 1978; Rozendaal and Crews, 1989; Meisel and Sachs, 1994). These behaviors in males have also been correlated with a male-biased sexual dimorphism in volume of regions in the preoptic area in species including rats, Japanese quail, and whiptail lizards (Gorski *et al.*, 1978; Viglietti-Panzica *et al.*, 1986; Wade and Crews, 1992; Wade *et al.*, 1993). The ventromedial nucleus of the amygdala (VMN) has also been implicated in male courtship behavior, as lesions to this region abolish courtship, but not aggressive, dewlap extensions (Greenberg *et al.*, 1984). AR has been localized in these two forebrain regions (Rosen *et al.*, in press), implicating these regions as potential mediators of androgen-dependent courtship behavior. While understanding the direct effects of androgen is not a primary goal of mine, subsequent chapters in this dissertation address the possibilities above by investigating at least sex (and sometimes seasonal) differences at each of these levels. It is also possible that androgens act primarily in ontogeny to permanently organize the dewlap system. The fifth chapter addresses when some of these dewlap structures develop, and discusses some possible mechanisms that might be involved in creating sexual dimorphisms during ontogeny.

Part II

APPENDIX

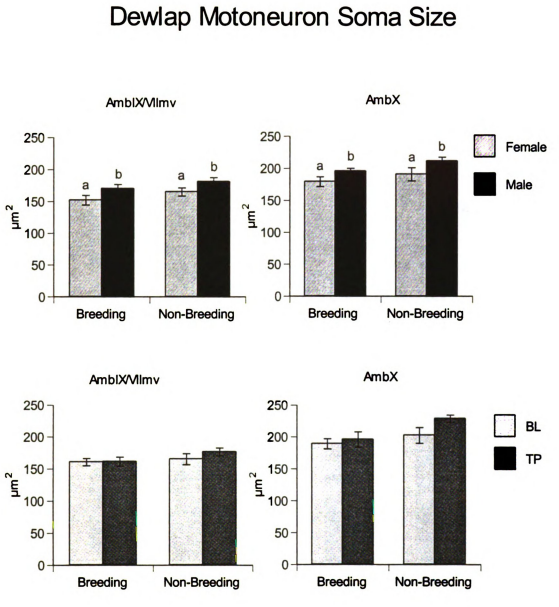

Figure II-1: Effects of sex and androgen treatment on dewlap motoneuron soma size. Amb IX/VIImv is shown in (A) the breeding and non-breeding seasons in intact animals and (C) both seasons in castrated males treated with either a blank (BL) capsule or a testosterone propionate (TP) capsule. AmbX is shown in (B) the breeding and non-breeding seasons in intact animals and (D) in castrated males treated with either a blank (BL) capsule or a testosterone propionate (TP) capsule. Letters **a** and **b** refer to statistically significant effect of sex detected by *t*-test ($p<0.05$).

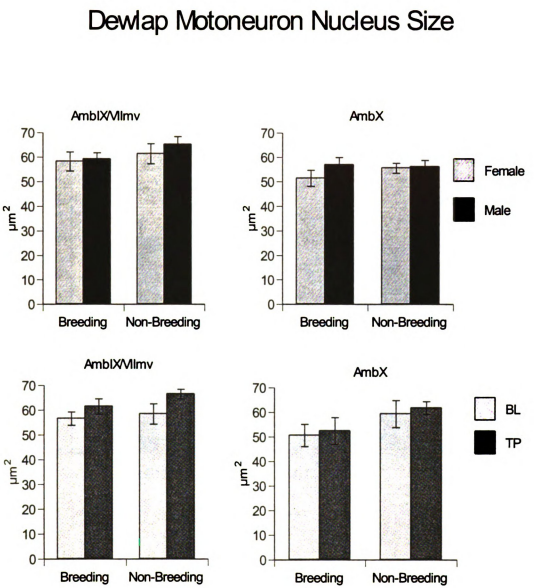

Figure II-2: Effects of sex and androgen treatment on dewlap motoneuron nucleus size. A: AmbIX/VIImv during both the breeding and non-breeding season in intact animals. B: AmbX during both seasons in intact males and females. C: AmbIX/VIImv in castrated animals with either a blank (BL) or testosterone propionate (TP) capsule during both seasons. D: AmbX in castrated animals with BL or TP capsule during both the breeding season and non-breeding seasons.

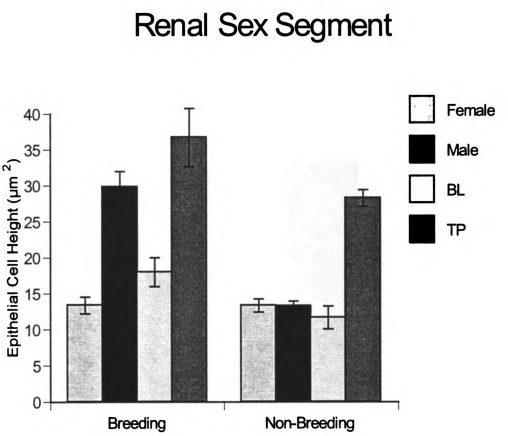

Figure II-3. Renal sex segment size during the breeding season and non-breeding seasons. The height of epithelial cells was measured as a bioassay of circulating androgen concentrations. All 4 groups of individuals [intact females, intact males, castrated males treated with a blank Silastic capsule (BL) and castrated males treated with a testosterone propionate capsule (TP)] are depicted together to provide an idea of relative androgen exposure across all conditions.

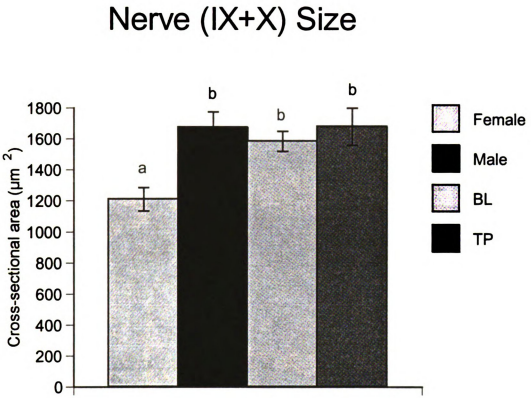

Figure II-4: Cross-sectional area of ramus pharyngo-laryngeus IX+X was larger in males than females. There was no effect of testosterone (TP) compared to blank (BL) treatment in gonadectomized males.

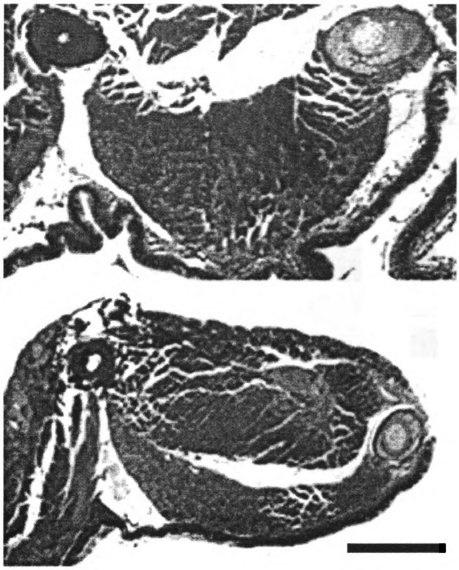

Figure II-5: Photograph of a cross-section through approximately half of the throat structure in the green anole. Top=male, bottom=female. Arrows point to the "smile-shaped" ceratohyoid muscle, which stretches between the two circular pieces of cartilage (ceratohyal and first ceratobranchial; Font and Rome, 1990; Bels, 1990). GG=M. genioglossus (Font and Rome, 1990). Scale bar=300μm.

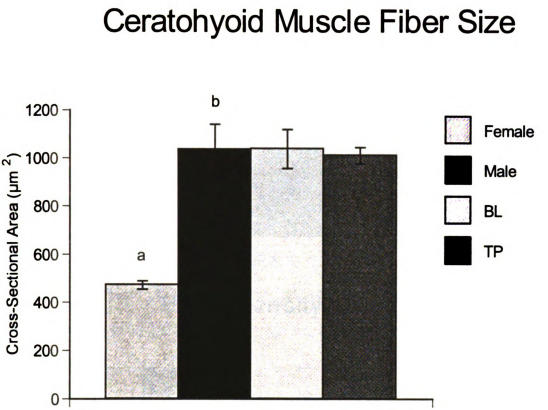

Figure II-6: Fiber size in M. ceratohyoideus. Measurements were taken in the breeding season in intact females, intact males, and castrated males given either a blank (BL) or testosterone-filled (TP) Silastic capsule. Letters **a** and **b** denote effect of sex detected by *t*-test ($p<0.05$).

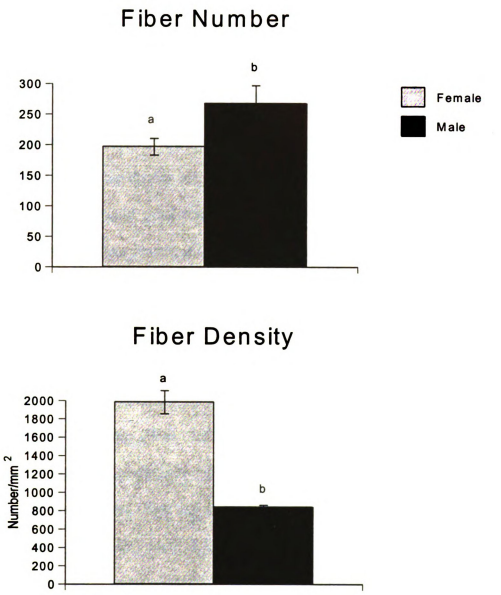

Figure II-7: Fiber number (A) and density (B) in M. ceratohyoideus. Letters **a** and **b** denote statistical significance detected by *t*-test ($p<0.05$).

CHAPTER THREE:

ARBORIZATION OF DEWLAP MOTONEURONS IN THE GREEN ANOLE LIZARD (*ANOLIS CAROLINENSIS*) IS NOT SEXUALLY DIMORPHIC

RATIONALE

Enhanced input to the dewlap neuromuscular system might provide a mechanism for the increased courtship display behavior in males during the breeding season in green anoles. If so, one would predict more elaborate dendritic arborization of dewlap motoneurons in male than female anoles. Sex differences in dendritic morphology of neurons are thought to contribute to the display of masculine courtship behaviors in some other vertebrate species. For example, a sex difference exists in the dendritic length and number of branch segments, but not the number of primary processes, of laryngeal motoneurons in *X. laevis* involved in the production of male-specific courtship vocalizations (Kelley *et al.*, 1988). In the vocalizing fish *Porichthys notatus*, the sonic motoneurons of courting males have a larger dendritic diameter than those of both non-courting males and females (Bass and Baker, 1990). Features of dendritic arborization, including total length (DeVoogd and Nottebohm, 1981), and numbers of primary processes and branch segments (Gurney, 1981), are also significantly greater in forebrain neurons of the nucleus robustus archistriatalis of male compared to female songbirds, both in zebra finches (Gurney, 1981) in which only males sing and in canaries (DeVoogd and Nottebohm, 1981) in which males sing a song much more complex than females.

While the important structural components of the dewlap display have been identified, details of the morphology of the motoneurons were unknown. Therefore, in

addition to assessing potential sex differences, the present study was conducted to characterize the arborization of neurons in the two critical brainstem regions.

METHODS

Animals and Tissue Collection: Intact males (n=7) and females (n=6) were measured for snout-vent length, weighed, and then perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Reproductive activity was confirmed in all animals by examination of gonads at the time of perfusion. The snout-vent length for males ranged from 55-65 mm, and from 47-55 mm in females; body weight ranged from 3.2-5.3 grams in males and 2.1-3.7 grams in females. Brains were removed and stored in 4% paraformaldehyde for 2 weeks, at which time they were exposed to the following regimen: three days in potassium dichromate/formalin at 4°C, followed by one day in silver nitrate in the dark at room temperature. The cycle was repeated two more times using potassium dichromate without formalin for the first step. Coronal sections were cut at 100μm on a Vibratome into cold PBS, stabilized (adapted from Geisert and Updyke, 1977), dehydrated, mounted on clean slides and coverslipped with Permount.

Nissl-stained dewlap motoneurons in alternate sections of the same regions have distinct features (Wade, 1998). Somata of motoneurons in AmbX are relatively full, having a round or occasionally more pointed (square) shape, whereas somata in AmbIX/VIImv tend to be more elongated, a feature emphasized by the fact that 2 processes often emerge from opposite ends of the cell body and extend in dorsomedial and ventrolateral directions. Motoneurons in this study were thus chosen based on their

previously identified anatomical location, size, morphology, and orientation (Wade, 1998; O'Bryant and Wade, 1999).

Analysis: Not all Golgi-impregnated cells could be analyzed, primarily due to ambiguities resulting from overlapping processes. However, each cell in AmbX and AmbIX/VIImv for which the entire extent could be traced and clearly seen was drawn with a camera lucida (2-7 cells per animal in AmbX; 2-6 per animal in AmbIX/VIImv), without knowledge of the sex of each individual. The number of primary processes and branch points was counted from the drawings. The total length of the processes was obtained by dividing the tracings into segments that were small enough so that they formed straight lines and by measuring these segments using a metric ruler. These measurements were then summed and adjusted for magnification. Data for each measure was averaged across the cells examined within each individual. The number of primary processes and branch points was analyzed by Mann-Whitney U test, since the data from these parameters was not continuous. Dendritic length was assessed by unpaired *t*-tests using Statview (SAS Instruments).

RESULTS

Dewlap motoneurons in both brain regions are relatively simple (Figure III-1). The cells in both AmbX and AmbIX/VIImv commonly had 2-4 primary processes in both males and females (one cell in AmbX had 5), and they tended to be relatively straight. The branching pattern in both regions was also similar in the two sexes, with the number of branch points generally ranging from 1-4 (one cell in each brain region had 6, and one

cell in AmbIX/VIImv had 8). Although the processes in both regions were on average slightly longer in males than in females, the difference was not statistically significant (AmbX: t=1.41, p=0.185; AmbIX/VIImv: t=0.90, p=0.388). There was also no sex difference in the number of primary processes (p=0.391) or the number of branch points (p=0.830) in AmbX. Similarly in AmbIX/VIImv, there was no effect of sex on the number of primary processes (p=0.475) or the number of branch points (p=0.668). Data are summarized in Table III-1.

DISCUSSION

Arborization of dewlap motoneurons in green anoles seems to be less complex than motoneurons regulating courtship in some other model systems. For example, Golgi-labeled laryngeal motoneurons of males and females in the frog *Xenopus laevis* contain roughly the same number of primary processes as those in anoles, but branching is more extensive than that of dewlap motoneurons. Additionally, total dendritic length of laryngeal motoneurons, particularly in male frogs, is greater than in dewlap motoneurons (Kelley *et al.*, 1988).

The arborization of motoneurons that control dewlap extension are sexually monomorphic in adulthood. This result suggests that the magnitude of input to these dewlap motoneurons is equivalent in male and female green anoles. The possibility does exist that, because the neurons were not retrogradely labeled in this study, some of the neurons measured were not dewlap motoneurons. It is also possible that with additional measures of more subtle morphological characteristics, we could have detected a sex difference in arborization. However, given the degree of similarity between males and

females on the measures taken, it seems unlikely that potentially undetected dimorphisms would have a large influence on the behavioral display.

In contrast to green anoles, sex differences in dendritic morphology of neurons are thought to contribute to the display of masculine courtship behaviors in some other vertebrate species. As stated in the Rationale for this study, sex differences exist in dendritic arborization of neurons involved in producing male sexual behavior in species of frogs (Kelley *et al.*, 1988), teleost fish (Bass and Baker, 1990), and some types of songbirds (DeVoogd and Nottebohm, 1981; Gurney, 1981). In these systems, the neurons themselves might be more complex than the simple structure of these dewlap motoneurons. Also, there might exist a more elaborate relay system that is involved in the production of these courtship or sexual behaviors, so that amassing input from many different areas is critical for the stereotyped production of these behaviors.

Unlike some of the other species in which females do not produce behaviors similar to males, female anoles do extend their small dewlaps. The display is less frequent than in males and normally occurs only during aggressive encounters, not in a sexual context (Greenberg and Noble, 1944; Winkler and Wade, 1998). The fact that a similar pattern of extension occurs in the two sexes, though, suggests that the neuromuscular machinery must be present in females as well as males, which use the dewlap in both courtship and aggression. It is possible that the quality or source(s) of input to the dewlap system are different in males and females. It also seems likely that dimorphisms in the periphery, and possibly at neuromuscular junctions as in the *Xenopus* larynx for example (Tobias *et al.*, 1995), are important in mediating the robust sex difference in the dewlap display.

Part III

APPENDIX

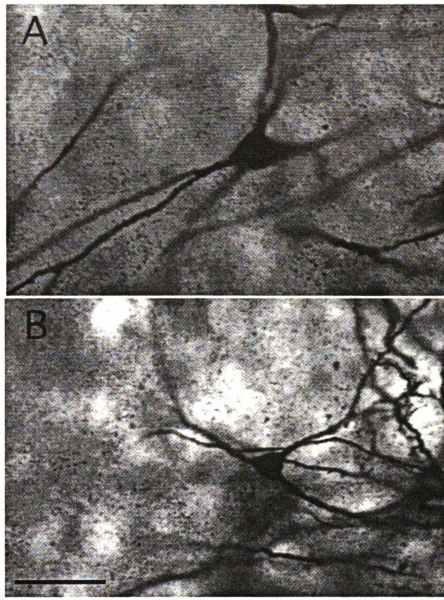

Figure III-1: Golgi-stained dewlap motoneurons from a breeding female: (A) AmbX and (B) AmbIX/VIImv. Scale bar = 50μm.

Table III-1. Dendritic measurements in AmbX and AmbIX/VIImv motoneurons (means±SE).

	AmbX			AmbIX/VIImv		
	Total Length (μm)	Primary Processes	Branch Points	Total Length (μm)	Primary Processes	Branch Points
Male	259 (35)	2.93 (0.25)	2.19 (0.38)	229 (48)	2.67 (0.16)	2.71 (0.57)
Female	195 (28)	2.6 (0.13)	2.02 (0.39)	177 (29)	2.53 (0.22)	2.22 (0.21)

CHAPTER FOUR:

SEXUAL DIMORPHISM IN NEUROMUSCULAR JUNCTION SIZE ON A MUSCLE USED IN COURTSHIP BY GREEN ANOLE LIZARDS

RATIONALE

The most robust sex differences identified thus far in the anole are found in the periphery, in the dewlap muscle and supporting cartilages. Again, the main piece of cartilage (2nd ceratobranchial process; Wade, 1998), the dewlap surface area (Jenssen *et al.*, 2000), as well as the weight of the muscle itself (Wade, 1998) is larger in males than in females. Correspondingly, the area of individual dewlap muscle fibers, the number of fibers, and the cross-sectional area of the nerve that projects to the dewlap muscle are also larger in males than in females (O'Bryant and Wade, 1999; Chapter 2). The nerve innervating these fibers is also larger in males than females, but the neuromuscular junction (NMJ) had yet to be assessed in these animals. To provide a more complete description of the relationship between structure and function, it was necessary to evaluate this structure, also known as the endplate region. It is the last unit of control before a behavior is produced and it is the "connection" between the neural and muscular components of the system.

The purposes of the present study, then, were to first investigate a new aspect of this system by describing the morphology of the dewlap neuromuscular junctions (NMJs) on the ceratohyoid muscles, and to determine whether sex differences exist at this level during the breeding season. Based on the results obtained, a second experiment was

designed to determine whether the existing sexual dimorphisms were also present in the non-breeding season.

METHODS

Animals: Adult green anoles were purchased from Fluker Farms (Louisiana) and were housed in group glass aquaria consisting of one male and at least 3 females, with sticks and rocks for climbing and peat moss as substrate. In addition to fluorescent room lights, each aquarium had full-spectrum bulbs and a heat lamp. During the breeding season, animals were housed on a 14L:10D cycle, with an ambient nightly temperature of 18°C and a daily temperature that ranged in each cage from 28-38°C depending on the distance from the heat lamp. During the non-breeding season, the light cycle was 10L:14D, with a nightly temperature of 15°C and a daily temperature ranging from 23-30°C. Relative humidity of 70% was maintained throughout the year. Animals were fed crickets and/or mealworms three times per week and had access to water *ad libitum*. Cages were also sprayed with water daily.

Tissue Collection and Incubation: Gonadally intact males and females from the breeding season (June and July; n=8 each) and non-breeding season (November; n=10 each) were euthanized by an overdose of Sodium Brevital (Eli Lilly and Co.) and snout-vent length (SVL) was recorded to the nearest mm. The ceratohyoid muscle and the 2nd ceratobranchial, ceratohyal, and 1st ceratobranchial cartilages were removed as a unit. The excess muscles not involved in dewlap extension were discarded, and the tissue was

stored in 4% paraformaldehyde/5% glutaraldehyde in phosphate buffered saline (PBS) for one hour. The muscle was then stored in PBS at 4°C for 5-10 days. Reproductive status was confirmed by inspection of the gonads under a dissecting microscope. In Experiment 1, breeding males had large vascularized testes, and breeding females had at least one yolking follicle or egg present. In Experiment 2, non-breeding animals of both sexes had completely regressed gonads.

Acetylcholinesterase histochemistry (adapted from El Badawi and Schenk, 1967 and Hirsch *et al.*, 1998) was used to visualize the post-synaptic components of the NMJs. The whole muscle was incubated in the following medium at 37°C for 45 minutes, and then rinsed with distilled water: 0.05% acetylthiocholine iodide, 0.82% sodium acetate, 0.6% acetic acid, 2.94% sodium citrate, 0.75% cupric sulfate, 0.137% iso-OMPA, and 0.165% potassium ferricyanide. Individual fibers were gently teased apart with forceps, placed onto gelatin-coated slides, and dried at room temperature for no longer than 30 minutes. Slides were counterstained with Harris hematoxylin, blued with 1% lithium carbonate, and then dehydrated, cleared, and coverslipped with permount. For comparison, the flexor tibialis externus posterior muscle of the hindlimb was removed in Experiment 2 and treated as above.

An enzyme control was run by incubating the ceratohyoid muscle of one individual from each sex and season (4 total) in a specific inhibitor of acetylcholinesterase, 1.77 m*M* 1,5-*bis*(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide. The inhibitor was combined with the incubation medium and the procedure continued as above. In all cases, staining of junctions was completely eliminated in tissue incubated with the inhibitor.

Measurements and Analyses: Prior to acetylcholinesterase staining, the lengths of individual muscle fibers were measured with calipers on each side of the bilaterally symmetrical muscles in three randomly chosen places that were approximately equidistant from each other (Labels 2A,B,C; Figure IV-1). These six measurements were averaged to obtain one value per individual. After incubation, the rostrocaudal length of the muscle (while still stretched between the ceratohyal and 1st ceratobranchial pieces of cartilage) was measured with calipers to get an estimate of the overall length of the muscle (Label 1; Figure IV-1). These two values were then averaged for each individual.

Separately within each experiment (season), an observer blind to sex collected the following information. NMJ areas were estimated by tracing their outlines using NIH Image software. Twenty NMJs per side were assessed, and an average of the 40 values was used in statistical analyses. The lengths of major and minor axes (the larger, major axis of the oval shape ran parallel to the fiber) were calculated simultaneously by the software program. NMJ interval (density) was also quantified in the breeding season. First, a straight length of fiber that contained at least three NMJs was measured from the beginning of one junction to the end of the last using NIH Image. Then the number of NMJs per length of fiber was counted and this number divided by the measured fiber length. Ten of these ratios were obtained for each side of the throat and the 20 values averaged together per individual for use in statistical analyses. Within each of the two experiments, each NMJ variable and the SVL were analyzed between the sexes by 2-tailed *t*-test (Statview, SAS Instruments). Also, fiber length and muscle length were divided by SVL in each individual, and analyzed in the same manner. In 12 of the 36

cases (both studies), a complete set of NMJ area measurements was impossible to obtain due to histological artifact. In 11 of these individuals, all 20 measurements were taken from the muscle on one side of the throat and an additional 4-19 measurements could be obtained from 3 of them on the other side of the throat. In one other case, 13 measurements were taken from only one side. Finally, in one individual, NMJ density was assessed on only one side.

As a control, NMJ area was measured in one flexor tibialis externus posterior muscle from animals in Experiment 2. In this muscle, the maximum possible number of distinct NMJs was assessed (mean = 6.6; range: 2-11).

RESULTS

NMJs (motor endplates) in both the breeding and non-breeding seasons were generally ovoid overall, and fairly uniform in appearance (Figure IV-2). Within these shapes, the acetylcholinesterase histochemistry revealed intense labeling of fine, rounded forms. In females, NMJs tended to be clustered near the middle of the muscle fibers (roughly equidistant between the cartilage pieces), whereas in males they were often located throughout the length of the muscle surface. Occasionally junctions were more elongated, with some branching present at the end. NMJ area and interval were assessed only for junctions that were on the top surface of the fiber (to be sure the entire extent was easily visible), were roughly elliptical and had clear, definable outlines.

Experiment 1: During the breeding season, NMJ area was on average larger in males than in females (t=2.57, p=0.022; Figure IV-3A), and this difference was due to the

fact that they were longer as opposed to wider (major axis: t=4.75, p=0.0003; minor axis: t=0.84, p=0.413; Table IV-1). The density of NMJs along each fiber was equivalent between the sexes (t=1.79, p=0.094). Both fiber length (t=4.47, p=0.0005; Figure IV-4A) and overall muscle length (t=12.11, p<0.0001) were significantly larger in males than in females. However, when corrected for body size (SVL, which is significantly greater in males than females, t=6.39, p<0.0001; Figure IV-4C), only the length of the overall muscle (t=5.89, p<0.0001; Figure IV-5A), and not individual muscle fiber length (t=0.92, p=0.372; Figure IV-4E), was larger in males than in females.

Experiment 2: During the non-breeding season, average NMJ area was larger in males than in females (t=2.17, p=0.044; Figure IV-3B). As in Experiment 1, this overall difference in area was due to longer, as opposed to wider, junctions (major axis: t=3.40, p=0.003; minor axis: t=0.25, p=0.804; Table IV-1). Also as in Experiment 1, muscle fiber length (t=18.60, p<0.0001; Figure IV-4B), overall muscle length (t=11.30, p<0.0001), and SVL (t=8.57, p<0.0001; Figure IV-4D) were larger in males than in females. The length of the dewlap muscle divided by SVL was larger in males than in females (t=7.24, p<0.0001; Figure IV-5B), but corrected individual muscle fiber length was equivalent between the sexes (t=0.72, p=0.478; Figure IV-4F). As NMJ density was not significantly different between males and females in Experiment 1, it was not assessed in Experiment 2.

Unlike the dewlap muscle, average NMJ area was not sexually dimorphic in the flexor tibialis externus posterior muscle (t=1.60, p=0.137). Unfortunately, due to difficulties encountered during processing, this muscle could only be analyzed in four

females and six males. However, sizes in the two groups overlapped substantially (females: 637.6-1084.4μm^2; males: 729.2-1643.2μm^2).

DISCUSSION

During the breeding season, several sexual dimorphisms were detected in the ceratohyoid muscle involved in dewlap extension. The size (surface area) of the NMJs was greater in males than in females, which was mainly due to increased length of each junction along the fiber. The rostrocaudal length of the muscle was also sexually dimorphic (whether or not it was corrected for body size), which reflects an increased number of fibers with larger cross-sectional area in males compared to females (O'Bryant and Wade, 1999). Similarly, while the corrected length of individual fibers did not differ between males and females, the uncorrected fiber length was significantly greater in males than females. The density of NMJs per fiber, however, was comparable between the sexes. These results suggest that the general organization of NMJs of males and females is similar, but that males have more NMJs, as the fibers are longer (present study) and more numerous (O'Bryant and Wade, 1999). These results are consistent with those from the developing frog pectoralis muscle (Grinnell and Harada, 1996), and suggest that NMJs may be added as the fiber grows longer.

The identification of robust sex differences in the dewlap musculature is not surprising, as we previously reported several sex differences in gross morphology of the muscle during the breeding season. In addition to fiber number and cross-sectional area (O'Bryant and Wade, 1999), the overall weight of the muscle and length of the 2nd ceratobranchials are larger in males than females (Wade, 1998). The cross-sectional area

of the RPL nerve, as well as the soma sizes of the brainstem motoneurons that innervate the dewlap musculature are also greater in males than females (Wade, 1998; O'Bryant and Wade, 1999).

In conjunction with these sexually dimorphic features of the neuromuscular system, the increased NMJ size and overall number in males might be related to the need for males to extend their larger dewlaps more often than females. For example, having larger NMJs might indicate greater acetylcholine release from the terminal (Herrera and Grinnell, 1985), or a delay in the onset of fatigue (Deschenes *et al.*, 1993; Hirsch *et al.*, 1998). More neurotransmitter release produces a stronger contraction in certain muscle fiber types (Gleeson *et al.*, 1980), however the composition of fiber types in the dewlap muscle of *A. carolinensis* has yet to be determined. The equivalent density of NMJs and length of individual muscle fibers relative to body size probably reflect the fact that females do extend their small dewlaps in a generally similar, although less frequent, pattern to males (Greenberg and Noble, 1944; Nunez *et al.*, 1997).

Similar to green anoles, structural differences exist (as assessed by electron microscopy) in neuromuscular junctions in the courtship vocalization system of midshipman fish. Specifically, presynaptic bouton area, terminal perimeter length, and the ratio between contact width and perimeter are all significantly larger in courting males than non-courting males and females (Fluet and Bass, 1990). However sex differences do not exist in all cases. For example, the length of presynaptic active zones and the density of channels within them are not sexually dimorphic at the larynx of the African clawed frog (Tobias *et al.*, 1995). Additionally, NMJ size (measured by

acetylcholinesterase histochemistry) is not sexually dimorphic in a sonic muscle used for courtship in the oyster toadfish (Hirsch *et al.*, 1998).

In green anoles, there is a large decrease in adult male and female T levels (Lovern *et al.*, 2001; Lovern and Wade, 2001), as well as a dramatic decrease in courtship behavior in the non-breeding compared to the breeding season (Jenssen *et al.*, 1996). Interestingly, the surface area of NMJs remains sexually dimorphic during the non-breeding season, suggesting that this neuromuscular construction is relatively stable in both sexes across seasons and does not fluctuate substantially in response to use or differences in circulating testosterone levels. Similarly, while dewlap displays are dependent on T in males (Mason and Adkins, 1976; Adkins and Schlesinger, 1979; Rosen and Wade, 2000), the sizes of the RPL nerve, motoneurons and muscle fibers that control dewlap extension are not affected by T treatment during the breeding season (O'Bryant and Wade, 1999). Thus, it appears that the hormone activates behavioral responsiveness in adults by a mechanism that does not involve substantial structural modulation of the neuromuscular machinery. However, it is possible that a subtle change in NMJ area due to season does occur. Because the tissue was processed and measured separately in the breeding and non-breeding seasons we felt it most appropriate to analyze the data from the two experiments individually. However, if a two-way ANOVA is used, in addition to the main effect of sex (F=11.21, p=0.002) one can pick up a small, but statistically significant, effect of season (F=4.62, p=0.039), with no sex by season interaction (F=0.18, p=0.677). Importantly, though, the magnitude of the seasonal change is nearly identical in males and females (compare Figures IV-3A and IV-3B). NMJ size is larger in both sexes during the *non-breeding* season. These points suggest

that an increase in T during the breeding season does not induce growth of NMJ size that is related to enhanced behavioral function in this species.

In contrast, increased muscle activity can cause hypertrophy of rat NMJs (Deschenes *et al.*, 1993, 1994), and androgens do influence morphology as well as behavior in other systems. For example, the sizes of SNB motoneurons and the BC/LA musculature decline in response to a decrease in androgen produced by short photoperiods (white-footed mouse: Forger and Breedlove, 1987; hamster: Hegstrom and Breedlove, 1999). Correspondingly, the sizes of NMJs on BC/LA muscles shrink with a photoperiod-induced reduction in testicular function in the male Siberian hamster (Hegstrom and Breedlove, 1999). Reducing T by castration in adult male rats causes a decrease in SNB muscle fiber size, as well as NMJ size, which is reversed by T replacement (Balice-Gordon *et al.*, 1990). The system appears different in anoles, since the male-biased dimorphism in muscle fiber size is apparently not altered by differences in circulating T (O'Bryant and Wade, 1999), and NMJ size remains sexually dimorphic in the non-breeding season.

As changes in adult levels of T do not enlarge neuromuscular morphology of the dewlap system, perhaps early hormonal influences have an effect on the development of sexual dimorphisms in these structures. It is possible that an increase in T in juvenile males (Lovern *et al.*, 2001) is important for permanently establishing the sexual dimorphisms documented here and in previous studies (Wade, 1998; O'Bryant and Wade, 1999). It is currently unknown, however, when NMJ morphology differentiates between male and female green anoles. Nonetheless, it appears that NMJ size is a relatively stable property of the adult dewlap neuromuscular system that either enables or

is a consequence of using a more robust muscle to extend a larger dewlap more often in males compared to females.

Part IV

APPENDIX

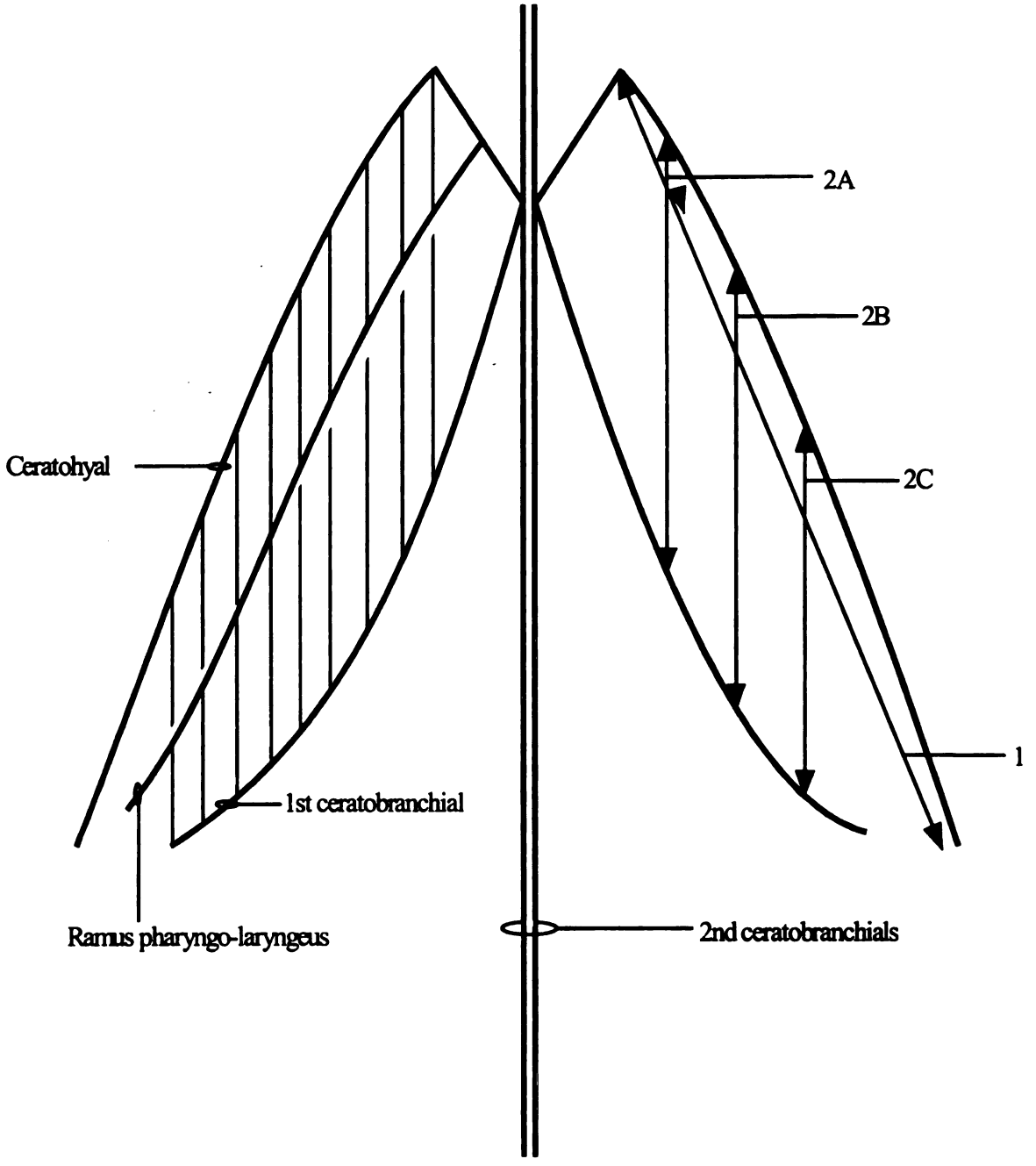

Figure IV-1. Schematic representation of bilaterally symmetrical dewlap cartilage and muscle organization in *A. carolinensis*. Muscle fibers run between the ceratohyal and 1st ceratobranchial pieces of cartilage. 1=measurement of rostrocaudal length of ceratohyoid muscle; 2a,b,c=individual muscle fiber measurements, averaged for analysis.

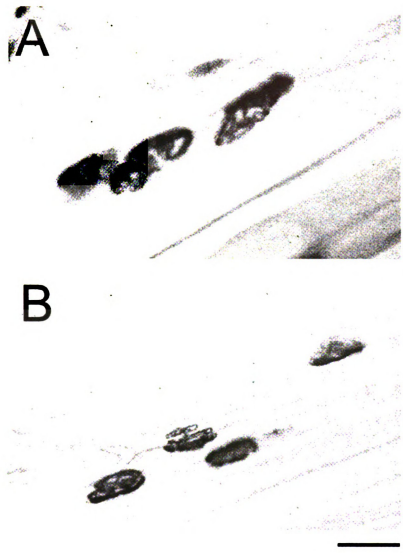

Figure IV-2. Representative neuromuscular junctions from Experiment 1: A=male, B=female. These samples were run through the incubation procedure without the hematoxylin and lithium carbonate, because while bluing and counter-staining enhanced visualization of muscle fibers (which facilitated measurement), the background made photography of NMJs difficult. Scale bar=50μm.

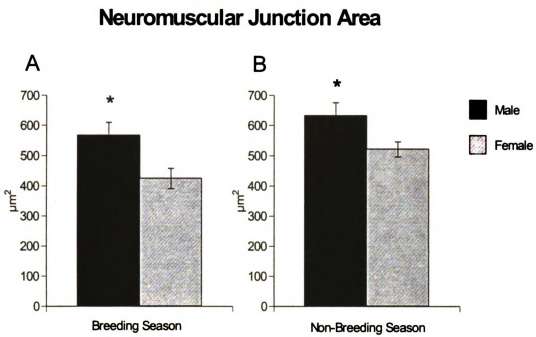

Figure IV-3. Neuromuscular junction size in Experiment 1 (A) and Experiment 2 (B) (mean ± standard error). Asterisk denotes significantly greater in males than females detected by *t*-test ($p<0.05$).

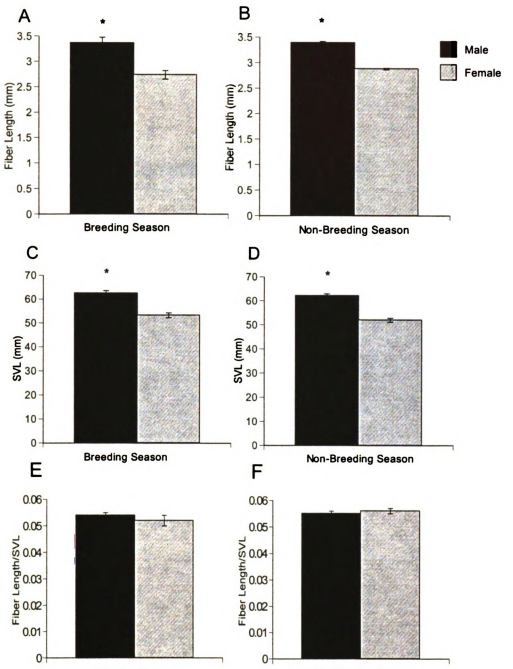

Figure IV-4. Ceratohyoid muscle fiber length and body size information (mean±SE) in Experiments 1 (A,C,E) and 2 (B,D,F). Fiber length is depicted in the top two panels, snout-vent length (SVL) in the middle, and the values obtained when fiber size for each individual was divided by the animal's length (both initially measured in mm) are shown at the bottom. Asterisk denotes significantly greater in males than females detected in each season separately by *t*-test ($p<0.05$).

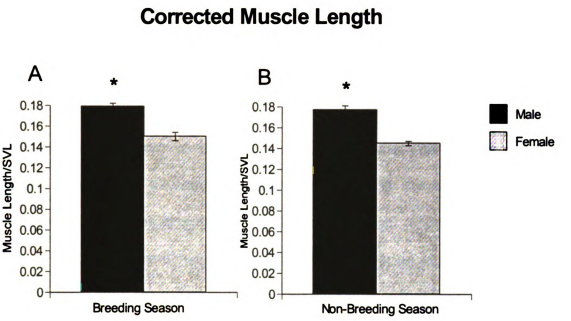

Figure IV-5. Overall length of the ceratohyoid muscle corrected for snout-vent length (SVL) in Experiment 1 (A) and Experiment 2 (B). Asterisk denotes significantly greater in males than females detected separately for each season by *t*-test ($p<0.05$). Both values were measured in mm.

CHAPTER FIVE:

DEVELOPMENT OF A SEXUALLY DIMORPHIC NEUROMUSCULAR SYSTEM INVOLVED IN GREEN ANOLE COURTSHIP BEHAVIOR

RATIONALE

In adulthood, neuromuscular structures involved in dewlap extension are sexually dimorphic, yet those investigated in Chapter 2 do not change morphologically in response to changing levels of circulating androgen. It was therefore possible that the size of these structures is organized at some point during development and remains fixed throughout adulthood. Sex differences in adult behavior are often organized during a critical pre- or perinatal period, and in many cases the organization is due to gonadal steroids permanently altering the structure of steroid-sensitive regions of the brain that mediate these behaviors (see Breedlove *et al.*, 1999). In order to begin to determine when the major morphological sex differences in this neuromuscular system arise, I examined the length of the 2nd ceratobranchial cartilages (which unfold the dewlap), muscle fiber size, nerve cross-sectional area, and motoneuron soma size, as well as measures of body size (snout-vent length and body weight) at post-hatching days 1, 30, 60, 75, and 90.

METHODS

Animals: Adult green anoles were purchased from Fluker Farms (Louisiana), and were housed upon arrival in the lab in 29-gal group aquaria consisting of one male and at least three females. Adult lizards were fed crickets or mealworms and had access to water daily. Aquaria were equipped with full-spectrum bulbs (Vitalite) and an incandescent

heat lamp directly above, and contained sticks and rocks to bask on, a water bottle, and a peat moss substrate. Additionally, each cage had a plastic nestbox filled with extra moist peat moss in which females laid their eggs. Females lay one egg every 7-14 days during the spring and summer breeding season (Smith *et al.*, 1973; Andrews, 1985). Nestboxes were checked daily, and eggs were placed individually in a plastic cup containing vermiculite and dH_2O (1:1, mass). The cups were then covered with a doubled plastic bag, secured with a rubber band, and incubated at a daily temperature of 28-29.5°C. Eggs hatched after 34±0.9 days. Hatchlings were toe-clipped for identification and housed in a group cage under the same environmental conditions as adults. They were fed pinhead crickets daily, and were moved to different cages as they grew larger to ensure that smaller hatchlings had sufficient access to food.

Adults and young were kept on three different environmental regimens which roughly approximated what they would have experienced in the field throughout the year. Overhead fluorescent lights were maintained on a 14L:10D cycle during the spring and summer breeding season, 12L:12D for a two-week intermediate period between the breeding and non-breeding seasons, and 10L:14D during the fall and winter non-breeding season. In all cases, full-spectrum bulbs (Vitalite) turned on one hour after and off one hour before the overhead room lights. The heat lamps came on two hours after and turned off two hours before the overhead lights. Daily temperature was set at 28°C during the breeding season, 26°C during the intermediate period, and 24°C during the non-breeding season. Animals could raise their body temperatures up to approximately 10°C higher than the set point by basking, depending on their distance from the heat lamp. Night temperatures were set at 18°C during the breeding season, 17°C during the intermediate

period, and 15°C during the non-breeding season. Relative humidity was maintained at 70% throughout the year.

Tissue Collection: Male and female hatchlings were sacrificed at the following ages: d1 (day of hatching, n=16), d30 (n=16), d60 (n=16), d75 (n=21), and d90 (n=12). The vast majority (84%) of these animals hatched during the breeding season. With the exception of d1 animals, which all were killed during the breeding season, roughly half of the individuals in the other age groups were killed following the breeding season. The "season" in which tissue was collected had no effect on any measure that differed between the sexes or among ages (all $F<2.98$, $p>0.089$).

Lizards were deeply anesthetized with Isoflourane and kept on ice while sex was determined by examination of post-anal scales. Males have two large scales which are located just caudal to the cloaca that are not present in females. Animals were then measured for snout-vent length (SVL) and body weight (BWT). Length of the 2nd ceratobranchial cartilage was measured with calipers after exposing it from underneath the throat skin. The head was then removed and the entire lower jaw separated from the rest of the head. The upper portion of the head containing the brain was placed in 10% phosphate buffered formalin, and the lower jaw was placed in Bouin's fixative. Tissues were fixed for three to five days. The brain was then removed from the skull and, along with the lower jaw, was soaked in 70% ethanol overnight. The tissues were then dehydrated, cleared in xylene, and separately embedded in paraffin.

Tissue was sectioned at 10μm, mounted on clean slides with gelatin water and dried overnight on a slide warmer. The lower jaw containing the ceratohyoid muscle and nerve

was stained with trichrome (see Forger *et al.*, 1995), and the brain was stained with thionin. Slides were then dehydrated in a series of alcohols, cleared in xylene, and cover-slipped with Permount.

Measurements and Analyses: All measurements were made on a PC with NIH Image software and an Olympus BX-60 light microscope, using techniques similar to O'Bryant and Wade (1999). As there are no obvious differences in size between structures on the left and right, measurements were randomly taken from the two sides of the animal except in the few cases in which histology was only good on one side. The cross-sectional areas of thirty randomly selected dewlap motoneurons in AmbIX/VIImv and AmbX were measured and averaged per individual (separately for the two brain regions). Cross-sectional area of the nerve was measured in five randomly selected sections that were roughly evenly distributed as it ran along the surface of the ceratohyoid muscle. Additionally, fifteen randomly selected fibers were measured from the ceratohyoid muscle.

A two-way (sex by age) factorial ANOVA (Statview, SAS Instruments) was used to analyze each variable. When significant effects of age were detected, Tukey-Kramer tests were used to detect which ages differed significantly from each other. When a main effect of sex was detected, unpaired two-tailed *t*-tests were used to compare between the sexes at particular developmental stages. Due to histological artifact, not all measurements could be obtained from every animal. Sample sizes used in statistical analyses are reported in each Figure and in Table 1.

RESULTS

There was no difficulty locating either pool of motoneurons, the ceratohyoid muscle fibers, or the nerve at any age, as they appeared remarkably similar to those found in adults (for description see Wade, 1998; O'Bryant and Wade, 1999; O'Bryant and Wade, 2000). Interestingly though, on the day of hatching, the ceratohyoid muscle was in contact with the 1st ceratobranchials, but had not yet extended completely to the ceratohyal cartilages. By the time animals had reached d30, however, the muscle was in contact with both, as it is in adults.

Body Size: SVL increased as a function of age (F=97.06, p<0.001; Figure V-1A); all developmental stages were significantly different from each other (all p<0.05). However, no difference existed in SVL between males and females (F=0.84, p=0.363), and no significant interaction between age and sex was detected (F=1.12, p=0.352). BWT also increased significantly as animals matured (F=73.91, p<0.001; Figure V-1B), such that all ages were different from each other (p<0.05) except days 1 and 30, and days 75 and 90. Male and female body weights were equivalent overall (F=0.07, p=0.786), and no significant interaction between age and sex was detected (F=1.26, p=0.292).

Cartilage Length: The 2nd ceratobranchial cartilages grew as animals matured (F=78.71, p<0.001; Figure V-2A); they were different between all ages (p<0.05) except d75 and d90. The cartilages were longer in males compared to females (F=27.46, p<0.001) and grew more in males than in females (age by sex interaction: F=7.18, p<0.001). The sex

difference was statistically significant at d60 (t=3.63, p=0.003) and d90 (t=5.17, p<0.001), and probably also at d75 (t=2.03, p=0.057).

Muscle Fiber Size: Ceratohyoid fibers increased in size over time (F=45.19, p<0.001; Figure V-3). They were statistically different (p<0.05) between all pairs of ages except d1 and d30, d30 and d60, and d75 and d90. Fibers were larger overall in males than in females (F=6.50, p=0.014), and the interaction between age and sex was marginally significant (F=2.49, p=0.057). At later ages the fibers seemed to grow more in males (Figure V-2B), such that they became significantly larger in males than females at d75 (t=2.27, p=0.037) and remained dimorphic at d90 (t=3.49, p=0.006; Figure V-3).

Nerve Size: The cross-sectional area of the ramus pharyngo-laryngeus IX+X also increased with age (F=36.25, p<0.001; Figure V-2C). However, unlike cartilage length and muscle fiber size, the nerve was comparable in size between males and females (F=0.95, p=0.333). The interaction between sex and age approached statistical significance, as the increase in nerve size over time was somewhat steeper in males compared to females (F=2.36, p=0.062).

Motoneuron Soma Size: The Nissl-defined soma sizes of neurons in AmbX and AmbIX/VIImv did not change in the first three months after hatching (both F<1.59, p>0.193), and did not differ between males and females (F<0.38, p>0.539). No

significant interaction existed in either brain region ($F<1.07$, $p>0.374$). Data on motoneuron soma size are summarized in Table V-1.

DISCUSSION

This study documents the developmental time-course of neuromuscular structures involved in the production of dewlap extension, as well as general growth parameters, in the first three months after hatching in the green anole lizard. The 2nd ceratobranchial cartilages that support the dewlap differentiated in the second month of life, followed by ceratohyoid muscle fibers, which became sexually dimorphic between days 60 and 75. The nerve that innervates these fibers, and the motoneurons that supply the axons for this nerve, however, had not yet sexually differentiated by the end of the three-month period examined. The results on the peripheral components are striking given the fact that two measures of body size (SVL and BWT) have not begun to diverge between the sexes. The data suggest that these structures differentiate independent of general growth patterns, and thus potentially via different mechanisms. The sequence of sexual differentiation is consistent with the idea that moving the larger cartilage of males requires the contraction of enhanced muscle fibers, and that the motoneurons differentiate still later, possibly due to tropic support from the muscle (see below).

The development of dewlap structures has not been reported before, but the present results on general growth patterns are generally consistent with those from several anole populations (Michaud, 1990). Females collected in five sites, from Tennessee in the north to southern Florida, produced young that hatched at approximately the same weight and length as the present animals. While the growth rates differed

among animals from the various populations, the average daily increases in BWT and SVL in our animals were within the ranges reported for both males and females. However, in the previous study, males gained weight faster than females by six weeks after hatching, and the pattern continued at least through 24 weeks of age. We collected data only out to 13 weeks, so it seems likely that males in the present study (or perhaps both sexes) developed somewhat more slowly. The reasons for this difference are not obvious, as if anything our animals had access to slightly warmer temperatures and more food.

While it would have been ideal to sample animals from hatching until adulthood, it is virtually impossible to obtain reasonable sample sizes, particularly at later ages. In the field, green anoles do not breed until the season after they hatch, so if natural conditions were mimicked, animals would need to survive for roughly 6 to 9 months, depending on when they hatched (Crews, 1980). While adults can thrive for years under laboratory conditions, and we have had some animals in the lab reach sexual maturity, the laboratory diet and/or environment is apparently not optimal for juveniles to live that long (Michaud, 1990). Additionally, given the variability of the age at which animals begin to reproduce and the fact that even after reproductive maturity they continue to grow (both sexes increase in SVL during the breeding season, but males gain while females lose weight, presumably due to the energetic demands of egg productions; Michaud, 1990), interpreting data from later ages would be difficult.

When comparing the information obtained at d90 to what we know about adults, it appears that while sexual differentiation of peripheral components of the dewlap system has already begun, in almost all cases the structures will grow substantially as the

animals increase in size. There is a large amount of variability in the sizes of adults, but females will more than double in BWT and increase another 30% in SVL between d90 and adulthood, and males will increase their weight 5-fold and their length by roughly 40% (based on routine measurements taken over a number of years from animals collected for our lab colony). Based on measurements from O'Bryant and Wade (1999), the 2nd ceratobranchial cartilage will increase in length roughly 40% more in females and 65% in males, and the nerve will grow in cross-sectional area roughly another 30% in females and 40% in males. Interestingly, the ceratohyoid muscle has reached its mature size in females by d90, but will grow approximately 25% more in males. Unlike the other measurements which were taken from tissue processed comparably, motoneuron soma sizes were assessed in adulthood from brains that were sectioned frozen (O'Bryant and Wade, 1999), while those in the present study were dehydrated and paraffin embedded, which induces more shrinkage. Thus, the sizes cannot be directly compared. The fact that the cartilage will proportionally increase in size far more than the muscle fibers will suggests that, while the timing is consistent with the sexual dimorphism in cartilage driving the early development of muscle fibers, growth in the two systems between 3 months of age and adulthood are not completely parallel.

Behavioral differentiation appears to follow morphological differentiation. Juvenile anoles can display at hatching, and the behavior increases in both sexes as the animals mature (Lovern and Jenssen, 2001). Males and females do not differ in the rate of the displays until after they have reached 36mm in length (after d90 in our study), after which they maintain a low level of activity into adulthood (Lovern and Jenssen, 2001).

It is difficult to draw parallels between the present study and other neuromuscular systems that have been more extensively studied. Developmental studies in those systems have focused primarily on the relationship between differentiating motoneuron and muscle fiber number. However, motoneuron number is equivalent between the sexes in adult anoles. Thus, while adult males have more fibers (O'Bryant and Wade, 1999), it seems unlikely that survival of cells in one structure is completely responsible for development of the other. Still the principles of other systems can guide future studies to address mechanisms of sexual differentiation of the size of structures, which was the focus of the present experiment. For example, motoneurons and muscles in the SNB system of rodents are initially equivalent between embryonic males and females, but become substantially enhanced in males compared to females around the time of birth (Cihak *et al.*, 1970; Nordeen *et al.*, 1985). The bulbocavernosus and levator ani muscles degenerate in females due to lack of androgen, and it is believed that motoneurons subsequently die in females due to lack of trophic support (Forger *et al.*, 1992). Similarly in *Xenopus laevis*, androgen is thought to maintain motoneurons that project to the muscles of the larynx and stimulate axon growth in male tadpoles and to facilitate the rapid addition of muscle fibers in males after metamorphosis (Sassoon and Kelley, 1986; Robertson *et al.*, 1994; Kay *et al.*, 1999). However, while neuronal differentiation seems to precede muscle differentiation, the innervation pattern does not drive muscle development. Instead, trophic support from the increasing larynx muscle fibers may act to sustain laryngeal axon numbers above their adult level in early post-metamorphic development (Roberston *et al.*, 1994). Similarly, while gonadal steroids may not be responsible for sexual differentiation of the zebra finch neuromuscular system (Wade,

Buhlman, and Swender, unpublished observations), input from the syrinx is important for the development of masculine soma size and volume of the brainstem motor nucleus (nXIIts) (Lohmann and Gahr, 2000).

Because muscle fiber size differentiates before motoneuron size in anoles, it is possible that a retrograde signal influences neuronal development. In addition, the timing of sexual differentiation of dewlap structures is consistent with the idea that testosterone could influence the masculinization. Testosterone is higher in males compared to females beginning at 26-30mm SVL (Lovern *et al.*, 2001). In the present study, that size was reached between the ages of d30 and d60, just before sexual differentiation of most peripheral components of the dewlap system. Therefore, the rise of testosterone in juvenile males prior to d60 might contribute to the differentiation of cartilage size, and perhaps muscle fiber size as well. As the distribution of steroid receptors during development has not been investigated, we do not yet know at which structures the androgen could act. In adulthood, dewlap motoneurons appear to express some, but minimal, androgen receptor mRNA and protein (Rosen *et al.*, in press), and it is unknown whether the muscle or cartilage of adults contains androgen receptor. However, the fact that changes in adult testosterone do not affect the morphology of the muscles or motoneurons (O'Bryant and Wade, 1999) is consistent with the idea that little or no androgen accumulates in these tissues in mature animals. It will be important to conduct hormone manipulation studies in order to determine if and when gonadal steroids play a role in sexual differentiation, and to selectively alter the development of individual structures to uncover the relationship in developmental processes among them.

Part V

APPENDIX

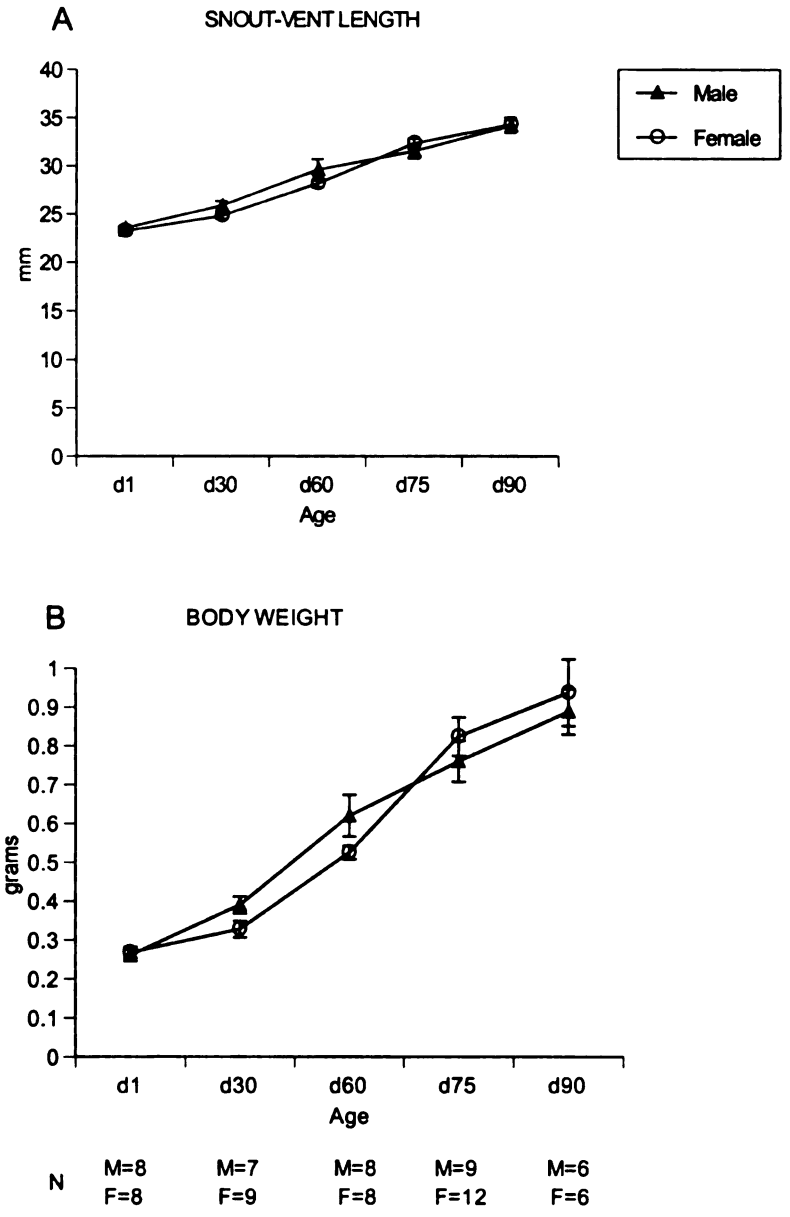

Figure V-1: Two measures of general body growth during development. A: Snout-vent length (SVL) increases over time, but is not different between males and females. B: Body weight (BWT) also grows as juveniles mature, but does not differ between the sexes. Sample sizes for males (M) and females (F) on both measures included at the bottom of the figure.

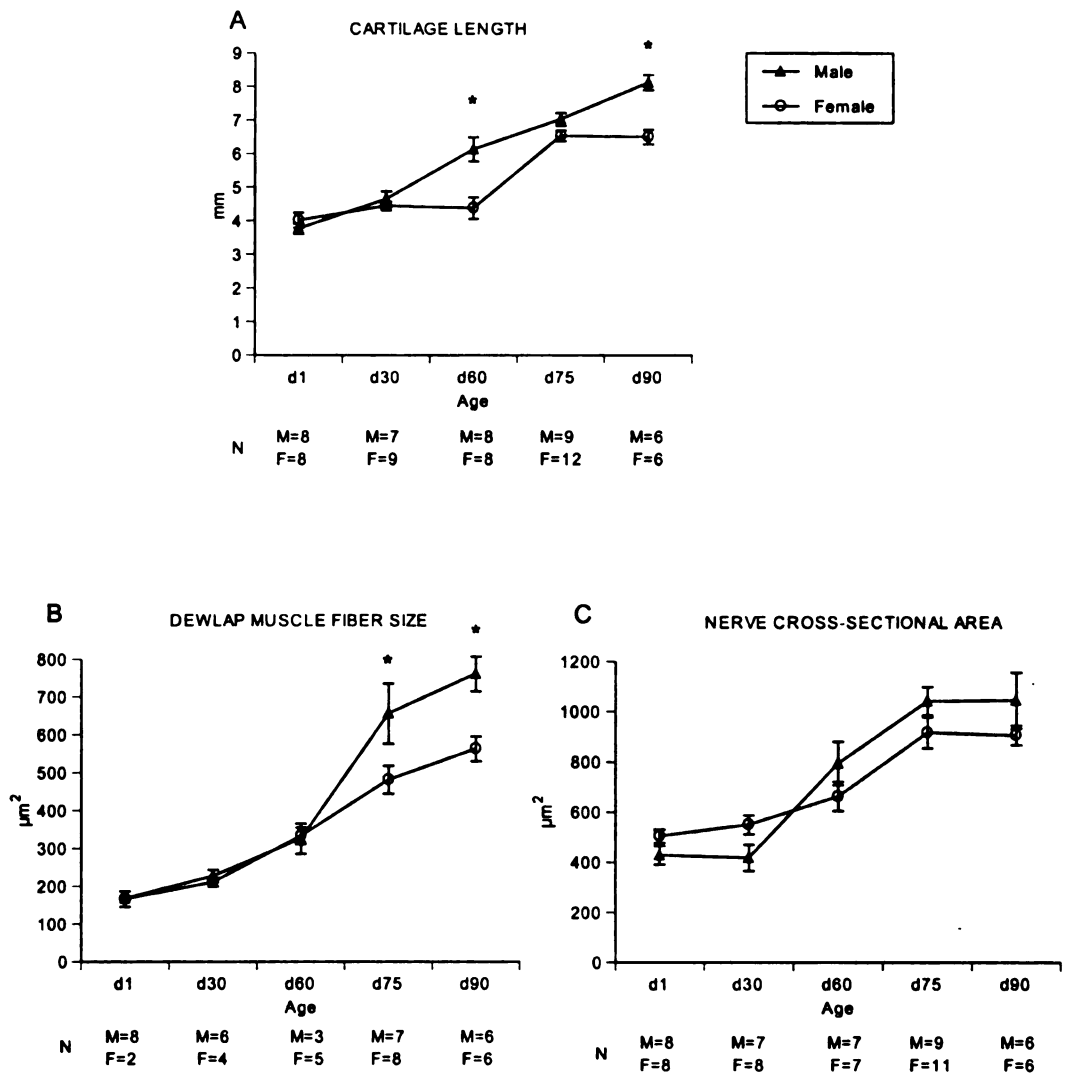

Figure V-2: The growth of dewlap structures during development. A: Length of the 2nd ceratobranchial cartilages; B: Size of ceratohyoid muscle fibers; C: Size of the ramus pharyngo-laryngeus IX+X. All measures increased over time, and significant effects of sex existed in the cartilage (A) and nerve (C). Asterisk denotes significantly different between males and females at a particular age detected by *t*-tests ($p<0.05$). Sample sizes in males (M) and females (F) are indicated at the bottom of each panel.

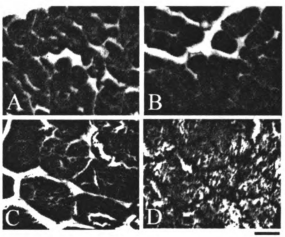

Figure V-3. Cross-section of muscle fibers in male and female hatchlings. Muscle fiber size is not sexually dimorphic on d1 (male: A, and female: B), but has sexually differentiated by d90, becoming larger in males (C) compared to females (D). Scale bar=10μm.

Table V-1. Mean soma size of dewlap motoneurons (μm^2) during post-hatching development. Standard errors in parentheses, N in brackets.

	AmbX		AmbIX/VIImv	
	Male	Female	Male	Female
d1	122.81 (5.7) [8]	139.51 (3.7) [3]	135.61 (7.6) [8]	138.57 (18.4) [3]
d30	115.52 (4.7) [6]	115.24 (9.9) [6]	120.45 (6.1) [6]	119.83 (10.6) [6]
d60	145.84 (3.4) [3]	125.10 (5.2) [6]	131.91 (20.6) [3]	139.04 (5.6) [6]
d75	124.54 (12.9) [7]	138.27 (8.7) [8]	124.95 (11.1) [7]	145.34 (10.5) [8]
d90	115.28 (9.0) [6]	124.95 (7.4) [6]	121.12 (13.7) [6]	110.64 (9.6) [6]

CHAPTER SIX:

SEASONAL AND SEXUAL DIMORPHISMS IN THE GREEN ANOLE FOREBRAIN

RATIONALE

I have identified several possible neuromuscular mechanisms for the increased dewlap display in males compared to females, but no differences that parallel seasonal changes in behavior. It is possible that forebrain areas involved in the courtship behavior may also play a role. As in other vertebrate species, lesion experiments demonstrate the critical role of the limbic regions in the production of male sexual behavior in the green anole. For example, ablating the POA disrupts sexual behavior (Wheeler and Crews, 1978). Lesions of the ventromedial nucleus of the amygdala (AMY; also called posterior dorsal ventricular ridge in Greenberg, 1982) diminish courtship, but not aggressive, dewlap extensions in males (Greenberg *et al.*, 1984). These regions contain androgen receptors (Morrell *et al.*, 1979; Rosen *et al.*, in press), and intracranial implants of androgens into the POA stimulate sexual behavior in males (Morgentaler and Crews, 1978), suggesting that T may locally influence the seasonal changes in behavior. The following experiments were therefore designed to determine whether structural differences accompany seasonal changes in behavior (see Chapter 1), and to test if these potential differences are hormonally regulated.

METHODS

EXPERIMENT 1:

Animals: Housing Conditions and Treatments: Recently captured adult male and female green anoles were purchased from Fluker Farms (La Place, LA). Animals were collected during the season in which their tissues were taken and were maintained under temperature, lighting and humidity conditions that approximated those they would have experienced in the field. All animals were housed in 29-gallon aquaria that each contained one male and at least three females. Each cage also contained a water bottle, sticks for climbing, rocks for basking, and peat moss as substrate. During the breeding season, intact males and females were maintained on a 14L:10D light cycle with room fluorescent lights, and full-spectrum bulbs and heat lamps over each cage that allowed animals access to basking temperatures up to 38°C. Average daily temperatures ranged from 28-38°C (depending on distance from heat lamp) and the nighttime temperature was 18°C. Intact males and females that had been collected during the non-breeding season were maintained on a 10L:14D light cycle, with average daily temperatures ranging from 23-30°C, and nightly temperature of 15°C. Throughout the year, humidity was maintained at 70%, and cages were also sprayed with water daily.

Tissue Preparation: All animals (n=10 of each sex during the breeding season; n=8 of each sex during the non-breeding season) were given an overdose of Sodium Brevital (Eli Lilly, Co.) and measured for snout-vent length (SVL) and body weight (BWT). Animals were then perfused transcardially with phosphate buffered saline followed by 10% phosphate buffered formalin. Reproductive condition was confirmed at the time of

perfusion. Breeding males had large testes with hypertrophied vasa deferentia, while breeding females had at least one yolking follicle. Non-breeding animals all had regressed gonads and ducts. Brains were removed and stored in phosphate buffered formalin overnight, then dehydrated in a series of alcohols, cleared in xylene, and embedded in paraffin. Tissue was then sectioned at 20μm, mounted onto clean slides, stained with thionin, and coverslipped with permount. Brains from both seasons were treated in an identical manner at every stage.

Measurements: The POA and AMY were defined with the aid of a green anole stereotaxic atlas (Greenberg, 1982; Figure VI-1). The POA was then further divided into dorsal and ventral portions, as it was clear from a cursory examination that the cells in these two areas were morphologically distinct. A difference in measured soma size between the two regions in both sexes was later confirmed by repeated measures ANOVA (F=13.39, p=0.001), such that cells from the dorsal region were larger than cells located in the ventral portion of the nucleus.

Soma size and density were assessed in this experiment by an observer blind to sex and season in all brain regions as follows: a box sized 107μm by 80μm was captured on a PC computer screen using NIH Image software and an Olympus BX-60 light microscope, and all cells with a clear, definable nucleolus were counted within this box. This procedure was repeated in 3 randomly selected sections throughout each region on both sides of the brain, and the 6 values were averaged. The cross-sectional area of 25 cells randomly selected from within these captured images was measured on each side of the brain, and an average of the 50 soma sizes was used in statistical analyses. Soma size

and density were also assessed in the same manner in nucleus rotundus, which does not contain androgen receptor message or protein in the green anole (Rosen *et al.*, in press). This region has also been used as a control nucleus in songbird studies (*e.g.*, Nottebohm and Arnold, 1976; Riters *et al.*, 2000). Additionally, to obtain an estimate of relative brain size in the region of interest, the height of the 3rd ventricle was measured at two levels using NIH Image, chosen because all of the soma size measurements (in the POA, AMY, and nucleus rotundus) were located in between them. First, the length of the 3rd ventricle between the optic chiasm and the anterior commissure was measured. Then, at the level of the ventromedial hypothalamus, the distance from the ventral edge of the brain (one end of the 3rd ventricle) to the dorsal edge of the habenula (other end of the 3rd ventricle) was assessed. Three measurements at each of the two locations were taken per animal, and the six values were averaged.

Statistical Analyses: For SVL, BWT, soma size, 3rd ventricle height and neuron density, the effects of sex and season were analyzed by 2-way ANOVA (Statview, SAS Instruments). Due to histological artifact, soma size data could not be obtained from all animals on all measures. Final sample sizes are indicated in the figures. Third ventricle height also could not be obtained from one animal. Based on the results (see below), simple regression analyses were run between SVL and each soma size measure, as well as between 3rd ventricle height and the soma size values to determine whether relationships existed between body or brain size and neuron some size (*e.g.*, whether some correction based on these variables was appropriate).

EXPERIMENT 2:

Housing Conditions and Treatments: Intact males and females obtained from the field during the non-breeding season (n=16 of each sex, n=8 of each treatment group) were first acclimated to the lab colony in group cages (as in Experiment 1) for 2 weeks, and then randomly selected from these cages and housed individually in 10-gallon aquaria that contained a small water dish, rocks for basking, and peat moss. Stimulus females were housed together without a male in a 29-gallon aquarium also containing a water bottle, sticks, rocks, and peat moss. Temperature and lighting conditions were as reported for the non-breeding season in Experiment 1. All experimental animals were deeply anesthetized with Isoflourane and gonadectomized while on ice. A small incision was made through both the skin and muscle wall on the side of the animal, just rostral to the hindlimb. The gonad was extracted through that hole, tied off with silk, and removed by a cauterizer. The incision was sutured and the procedure was repeated on the other side of the body. At the time of gonadectomy, each animal received a subcutaneous Silastic capsule filled with T-propionate (TP; Steraloids) or a blank (BL). Capsules were 7mm long and had 1mm at each end filled with sealant (1.65mm OD x 0.76mm ID). This implant size was chosen because it reliably stimulates masculine sexual behaviors under these housing conditions during the breeding season (Rosen and Wade, 2000). Stimulus females were gonadectomized as above, and then received subcutaneous injections in the neck of 4μg estradiol benzoate suspended in 20μl steroid suspending vehicle (for recipe see Wade *et al.*, 1997). These injections were administered daily starting one day prior to the first day of testing. This treatment has been used reliably in our laboratory to induce

female receptivity during the breeding season (Winkler and Wade, 1998; Rosen and Wade, 2000).

Behavioral Assay: In order to assess whether the brain was sensitive to gonadal hormones during the non-breeding season, animals were subjected to behavioral tests. Tests began two weeks after implantation, and ran for 10 minutes on three consecutive days between the hours of 10:00 AM and noon. The frequencies of the following behaviors were recorded: bouts of headbobbing accompanied by dewlap extension; mount attempts; and whether copulation occurred. The presence of a crest along the animal's dorsal surface and a dark spot behind the eye were also noted, as they are typical components of masculine aggressive displays (Greenberg, 1977; Jenssen *et al.*, 2000). The observer was seated behind a cardboard blind with a hole cut for viewing, and had no knowledge of the treatment of each individual. Behavior tests occurred in the test animal's home cage with a stimulus female that was introduced at the start of the testing period. Each stimulus female was used once per day and only once with each experimental animal.

Tissue Preparation: Immediately following the last behavioral test, SVL and BWT measurements were taken, animals were anesthetized and perfused as in Experiment 1. Castrated animals treated with T had hypertrophied vasa deferentia, while those treated with a blank capsule had diminished accessory sex structures. At the time of euthanasia, all capsules were present, with T remaining in those packed with the hormone, and no testicular remnants existed in any of the animals. Brains were removed and stored in

phosphate buffered formalin overnight and then embedded in paraffin, sectioned, and stained as in Experiment 1.

Measurements and Analyses: An observer blind to sex and treatment conducted the measurements for soma size in each of the four brain areas and 3rd ventricle height as in Experiment 1. Neuron density was not assessed for this portion of the study, as no main effects were detected in Experiment 1. Each behavior was averaged across the three tests for each animal. Data on body size, 3rd ventrical height, neuron soma size, and behavior were analyzed by 2-way ANOVA (Statview, SAS Instruments). In addition, SVL, BWT, and the two 3rd ventricle measurements were compared between Experiments 1 and 2 by unpaired t-test (Statview) to determine whether brain size and SVL were consistent across the two studies.

One animal died during the treatment period and was not available for behavior tests or other analyses, and the brains of a few animals had to be excluded due to problems with histology. Final sample sizes are included in the figures, and as for Experiment 1 degrees of freedom are included with statistical results.

RESULTS

EXPERIMENT 1:

Neuron Soma Size: Neuron somata were larger during the breeding season than the non-breeding season in both the dorsal (F=7.41, p=0.011) and ventral POA (F=7.32, p=0.011), as well as in the AMY (F=10.53, p=0.003; Figures VI-2 and VI-3). Soma size

was equivalent between the sexes in the ventral POA and AMY, although a trend for larger cells in males existed in the dorsal POA (F=3.96, p=0.055). No interactions between sex and season were detected in these limbic regions. In nucleus rotundus, no significant main effect of season, sex, or interaction was detected (Figure VI-2).

Neuron Density: Neuron density was not different between the sexes or seasons in any of the three forebrain regions assessed (Table VI-1). However, a significant interaction between sex and season was detected in the ventral POA (F=9.03, p=0.005). Neuron density appeared to increase in the non-breeding season in males, but decrease in females. As no overall significant main effect of season or sex existed in neuron density, this measure was not assessed in the control, nucleus rotundus.

General Measures: Males were larger than females, as assessed by both SVL (F=196.66, p<0.0001) and BWT (F=238.07, p<0.0001), although the estimate of 3rd ventrical height was equivalent between the sexes (Table VI-2). Animals were longer (SVL; F=11.60, p=0.02), as were their 3rd ventricles (F=9.20, p=0.005) in the breeding season than the non-breeding season. However, equivalent BWT indicates that general health was comparable across seasons.

The slightly smaller SVL between the seasons does not explain the soma size differences. Only one out of the 16 possible correlations between SVL and soma size in the 4 regions within each group was significant (non-breeding females: R^2=0.53, p=.041), and it was in the nucleus rotundus which did not show a seasonal difference in soma size (all others, R^2<0.39, p>0.13). A potential change in brain size is also not

responsible for the seasonal difference in POA and AMY soma size. In only one case was soma size in a limbic region positively correlated with 3rd ventricle height (AMY of non-breeding females; R^2=0.72, p=0.015). Soma size in nucleus rotundus in non-breeding males was also significantly correlated with this measure (R^2=0.70, p=0.018), and the association was marginal in non-breeding females (R^2=0.47, p=0.062). In all other cases a relationship between soma size and the estimate of 3rd ventricle height clearly did not exist (R^2<0.17, p>0.25).

EXPERIMENT 2:

Behavioral Assay: As described above, the green anole dewlap is used consistently in two contexts. During the breeding season in both the field and the lab, aggression and courtship are distinct behavioral classes that are easily distinguishable (Mason and Adkins, 1976; Jenssen *et al.*, 1995a; Winkler and Wade, 1998). Aggressive behavior in males is characterized by head-bobbing followed by a partial dewlap extension, and includes sagittal expansion of the body, an extended throat, and the formation of a crest along the animal's back and a black spot behind the eye (Greenberg, 1977). Courtship also involves a series of headbobs followed by a dewlap extension, but sagittal expansion, engorgement of the throat, and crest and eyespot formation do not accompany it. Instead, males raise themselves up on their forelimbs and extend the dewlap in a very rounded fashion, while turning the head sideways to allow for maximal viewing of the throat fan (Greenberg and Noble, 1944; Greenberg, 1977). In the lab, these two

behaviors are generally context-dependent; when with a female, a male performs a courtship display, with a male he performs an aggressive display.

While during the breeding season two discernable types of behavior exist in which the dewlap is used, displays during the non-breeding season in the present study were not entirely classifiable. All males exhibited a type of "mixed" behavior that had components of both aggression and courtship. For example, males would typically have an extended throat and sagittally expanded body, but without an eyespot or fully raised crest. In addition, males would often approach females, but would not engage in any physical contact. We therefore included all behavioral bouts with a dewlap extension in our analysis, because we could not confidently separate them into aggressive and courtship contexts.

Across treatments, males displayed more head-bobbing bouts accompanied by dewlap extension than did females overall (F=4.77, p=0.038; Figure VI-4), but TP treatment increased display behavior in both sexes (F=6.73, p=0.015; Figure VI-4). No significant sex by treatment interaction was detected, and no incidence of copulation (even mounting) occurred in any behavioral test.

Neuron Soma Size: Males had larger cells than females in the dorsal POA (F=40.48, p<0.0001), ventral POA (F=5.10, p=0.033), and AMY (F=4.31, p=0.049; Figure VI-5). No significant interactions were detected. In the control area, nucleus rotundus, no effect of treatment or interaction between sex and treatment existed (Figure VI-5). As there was no main effect of season or sex in Experiment 1, neuron density was not assessed for this portion of the study.

General Measures: As in Experiment 1, and common in this species (Jenssen *et al.*, 1995b), males were larger in size compared to females (Table VI-2). SVL was significantly greater in males (F=58.80, p<0.0001), as was BWT (F=87.29, p<0.0001; Table VI-2) In contrast, the length of the 3rd ventricle was equivalent both between males and females, and between the treatment groups (Table VI-2). There was also no difference in body size (by either measure) between animals treated with T or with a blank capsule. No significant interaction was detected on any measure. SVL and BWT were statistically equivalent between Experiments 1 and 2, as was brain size, determined by an estimation of the height of the 3rd ventricle.

DISCUSSION

In Experiment 1, we documented seasonal differences in soma size in three regions of the forebrain: the dorsal and ventral portions of the POA; and the AMY. Like neuron soma size, SVL and a rough estimate of brain size (3rd ventricle height in the general area of interest) were larger in the breeding than the non-breeding season. It is possible that the brains were smaller because the animals were smaller in the non-breeding season, or large areas of the brain may shrink in the non-breeding season due to factors such as a decrease in energy consumption or metabolism. It is highly unlikely that the bodies of the lizards shrink as a whole in the non-breeding season; the decrease in SVL is simply an unfortunate occurrence in these adult animals that were collected for us in the two seasons. In both the breeding and non-breeding seasons, we randomly selected animals for these experiments from those that were received from the field. It is clear,

however, that the changes in POA and AMY soma size are not due to differences in body or brain size. Importantly, SVL is not related to neuron soma size in the dorsal or ventral POA, or in the AMY. The variables are not correlated, and even with a large sex difference in SVL, soma size in Experiment 1 is statistically equivalent in males and females in all three limbic regions. Similarly, with one exception, 3rd ventricle height is not positively correlated with soma size in any of these areas. Finally, neurons in the control nucleus rotundus did not change seasonally, which suggests that even if several regions of the brain are modified, a change in soma size is not ubiquitous. The results from this experiment indicate that neuron soma size in regions of the forebrain involved in male sex behavior are plastic in adulthood (they are not simply due to differences in body or brain size), and that these structural changes mirror behavioral changes that occur across seasons.

In Experiment 2, we documented the ability of T treatment to facilitate masculine behavior in both males and females during the non-breeding season, although even with equivalent T-replacement males displayed more than females. While the behavior was somewhat modified in the present study, these results are consistent with data obtained on courtship in the breeding season (Winkler and Wade, 1998). However, T did not affect the size of neurons in any of the regions assessed. Surprisingly, we did find that neuron soma size in all three limbic regions was larger in males than in females, regardless of treatment. These data suggest a dissociation exists between the endocrine regulation of a behavior and the plasticity of relevant forebrain regions. Specifically, these experiments suggest that the seasonal changes and sex differences in forebrain regions involved in male sexual behavior are not mediated by seasonal fluctuations in T levels.

Seasonal Differences. The data from Experiment 1 are consistent with avian studies, which describe the seasonal change of volume and in some cases soma size of forebrain regions. For example, the volumes of song control nuclei increase during the breeding season when singing behavior is increased and regress in the non-breeding season when singing behavior is diminished (canary: Nottebohm, 1981; white-crowned sparrow: Smith *et al.*, 1995). Similarly, nucleus preopticus medianus volume and soma size are greater in Japanese quail exposed to long photoperiods that stimulate breeding compared to short daylengths in which gonads regress (Thompson and Adkins-Regan, 1994). In European starlings, preoptic median nucleus volume is larger in males during the summer breeding season than the post-breeding season (Riters *et al.*, 2000). The volume of the anterior hypothalamus/POA in whiptail lizards is also smaller in males that are reproductively inactive compared to breeding males (Wade and Crews, 1991). In these cases, the primary mechanism for the seasonal changes in neuron volume or cell size appears to be the natural fluctuations in T that occur across the breeding and non-breeding seasons (Wade and Crews, 1991; Wade *et al.*, 1993; Thompson and Adkins-Regan, 1994; Riters *et al.*, 2000). Similarly, removing endogenous T causes both volume and soma size of the dorsal portion of the medial amygdala to decrease with castration (Cooke *et al.*, 1999).

In anoles, T decreases during the non-breeding season in both sexes (Lovern, *et al.*, 2001; Lovern and Wade, 2001). Males also have higher levels of circulating T than do females, in both the breeding and non-breeding seasons (Lovern *et al.*, 2001). These data are consistent with the fact that T stimulates masculine sex behavior in both males

and females, and while it also facilitates female receptivity (via aromatization to estradiol), T is most critical for male reproduction (Winkler and Wade, 1998; Rosen and Wade, 2000). However, since T in the non-breeding season did not influence neuron soma size, it appears that the steroid affects behavior by a mechanism that does not involve a change in neuron soma size, as detected by a Nissl stain. The uncoupling of this measure from behavioral displays is similar to what occurs in the brainstem. Dewlap motoneuron soma size is not affected by T treatment in either the breeding or non-breeding season in green anoles, nor does this feature change seasonally (O'Bryant and Wade, 1999). It is currently unknown if T affects morphology in the forebrain regions during the breeding season. However, it may be that T increases cellular function that enhances either the motivation for or mechanisms of masculine behavior without a concomitant change in factors detected by a Nissl stain, such as the extent of endoplasmic reticulum.

This type of dissociation occurs in diverse species. For example, in the whiptail lizard (*Cnemidophorus inornatus*), T stimulates male-typical sexual behavior in both adult males and females, but only increases the volume of the sexually dimorphic anterior hypothalamus/POA in males (Wade *et al.*, 1993). Similarly, male and female bush shrikes (*Laniarius funebris*) have similar song repertoire size and structure, but males support a larger network of song control nuclei than do females (Gahr *et al.*, 1998). Finally, treating neonatal ferrets with T produces an increase in volume and soma size in a sexually dimorphic portion of the POA in males but not females, despite an increase in male-typical sexual behavior in both sexes (Cherry *et al.*, 1991).

Regardless of what may happen in the breeding season in anoles, it is not the case that animals become completely unresponsive to T in the non-breeding season. Both the brain and portions of the periphery are sensitive to T during the non-breeding season. For example, while no animal copulated, a neural response to T is indicated by the increase in display behaviors in the present study. We have also documented that the renal sex segment of the kidney (which performs functions similar to the mammalian prostate) responds to exogenous T during the non-breeding season with an increase in epithelial cell height (O'Bryant and Wade, 1999). It is of course possible that, while the renal sex segment was responsive to T, not all peripheral components necessary for copulation were adequately stimulated. Similarly, it is possible that the lack of copulatory behavior was due to decreased attractiveness or receptivity of stimulus females. Some evidence suggests that females are not as responsive to steroid hormones in the non-breeding as the breeding season (Wu *et al.*, 1985), but male anoles in our lab display to unreceptive females. So while firm conclusions cannot be drawn from the data on copulation, results on dewlap extension provide a clear indication that the animals were responsive to T.

If T does not induce the seasonal change in soma size, what might? Other gonadal steroids are of course possible, but melatonin also can modify cell morphology in at least one other species. It decreases the volume of the high vocal center of European starlings, regardless of circulating T levels (Bentley *et al.*, 1999). In the green anole, melatonin binds heavily in nucleus rotundus, but very lightly in the POA and is not detectable in the AMY (Wiechmann and Wirsig-Wiechmann, 1994). This pattern suggests that it is unlikely that melatonin is involved in the specific seasonal changes in soma size observed in this study (which occurred in the POA and AMY, but not nucleus

rotundus). It is not necessarily surprising that melatonin would not be a primary mechanism in anoles, since the hormone's secretion is regulated by photoperiod. Unlike the highly photoperiodic species in which melatonin directly influences neural morphology (see above), additional factors such as temperature play an important role in stimulating green anole reproduction. Males exposed to long daylengths and high daytime temperatures experience testicular recrudescence, but animals maintained in cold temperatures or treated with high temperatures only at night are impervious to the effects fluctuating daylengths (Licht, 1967). Females also depend on both temperature and photoperiod to maintain their ovarian cyclicity, but factors such as moisture and social interactions are also important (Licht, 1973).

Sex Differences: It is interesting that, while there was a suggestion of a sex difference in soma size in only one of the brain areas assessed in Experiment 1, sexual dimorphisms were present in soma size in each of the limbic regions measured in Experiment 2. These data are consistent with those in diverse model systems. For example, neuron soma size in the anterior hypothalamus/POA is larger in males compared to females in the whiptail lizard (Wade and Crews, 1992). Similarly, volume of these regions is often larger or contain larger components in males compared to females (rat: Gorski *et al.*, 1978; guinea pig, rat, hamster, mouse: Bleier *et al.*, 1982; Japanese quail: Viglietti-Panzica *et al.*, 1986; whiptail lizard: Crews *et al.*, 1990; rat: Hines *et al.*, 1992).

The question, of course, is why the same sexual dimorphisms were not present in the breeding season. Clearly, if neuron soma size is important for stimulating masculine behaviors, a sex difference would have been detected when animals display the highest

levels of behavior. There were no obvious differences between the animals in the two experiments; they were equivalent in body and brain size, at least by our rough estimate. Also, in both experiments, cells were on average larger in the two regions of the POA and the AMY in males than females. The magnitude of the sexual dimorphism was in all cases greater in Experiment 2, but it seems unlikely that the difference between the two experiments is biologically relevant (mean male-female difference across the 3 brain regions=6μm^2 for Experiment 2 and 4μm^2 for Experiment 1). While we can only speculate as to what may have contributed to this difference in main effects between the experiments, the studies differed in two ways. First, the animals in Experiment 1 were gonadally intact, but in Experiment 2 the animals had all been gonadectomized and given equivalent levels of T or an empty capsule. While T is probably not the cause, perhaps other gonadal secretions produced a change in Experiment 1 that prevented detection of a sexual dimorphism. Second, animals in Experiment 1 were housed in group cages whereas the animals in Experiment 2 were isolated (even visual communication was prevented by black dividers between cages). While other factors certainly contributed to the analysis, in the two regions of the POA the variability (standard errors) was generally increased in Experiment 1 compared to Experiment 2. It is possible that fluctuations in gonadal steroids or levels of social interaction enhanced the variability in the first study. Other studies have also shown that social interactions can affect brain structure (Tramontin *et al*, 1999; Cooke *et al.*, 2000).

Conclusion: The present experiments suggest that male sexual behavior and a seasonal change in soma size either have different sensitivities to T, or that the relationship

between these two factors is uncoupled. Because both the brain and the periphery are sensitive to the effects of androgens at a time when the natural circulating T is low, if T does induce the seasonal difference in neuron soma size as it does the difference in behavior, then the threshold must be substantially higher. This idea, combined with the fact that significant sex differences in neuron soma size are only detected in the non-breeding season, suggests that structural differences in the green anole POA and AMY do not necessarily accompany behavioral dimorphisms.

Part VI

APPENDIX

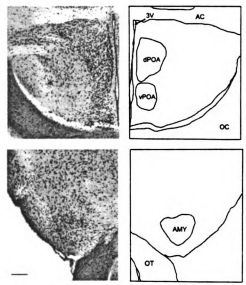

Figure VI-1: Photomicrographs and identification of the brain regions assessed. Top=dorsal (d) and ventral (v) POA, AC=anterior commisure, OC=optic chiasm, 3V=third ventricle. Botttom=AMY, OT=optic tract. Scale bar=100μm.

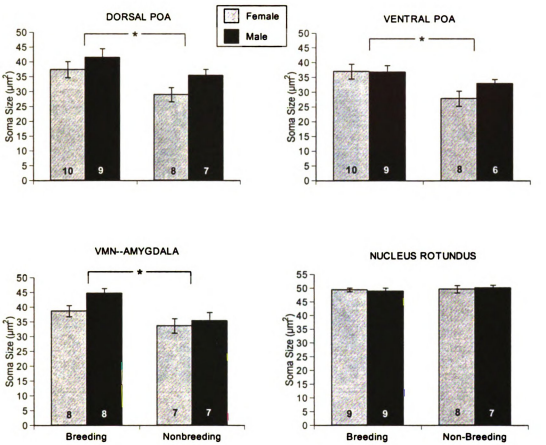

Figure VI-2: Soma size of forebrain regions in Experiment 1 (mean±SE). Final sample sizes are included for each group. Asterisk denotes statistical difference between breeding and non-breeding seasons regardless of sex detected by 2-way ANOVA ($p<0.05$).

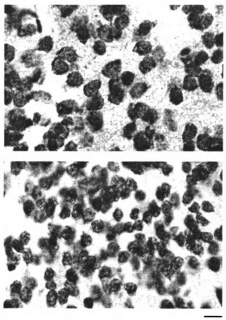

Figure VI-3: Photomicrographs of representing the seasonal difference in AMY soma size, shown in females. Top=breeding season, bottom=non-breeding season. Scale bar=10μm.

Table VI-1. Average neuron density (number per 107x80μm^2) of forebrain regions in Experiment 1. Standard errors included in parentheses.

SEASON	BREEDING SEASON		NON-BREEDING SEASON	
SEX	MALE	FEMALE	MALE	FEMALE
Dorsal POA	74.20 (3.54)	78.76 (2.58)	79.08 (3.12)	73.06 (2.49)
Ventral POA	69.20 (3.39)	74.50 (1.99)	76.43 (1.86)	66.86 (1.51)
VMN/amygdala	77.39 (2.75)	82.60 (3.72)	77.83 (3.25)	79.67 (2.33)

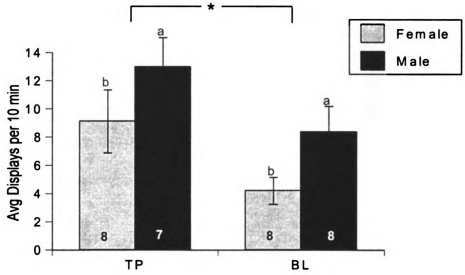

Figure VI-4: Effect of TP treatment on the rate of displays (mean±SE). Final sample sizes are included for each group. Asterisk denotes statistical difference between treatment groups and the different letters represent the main effect of sex ($p<0.05$).

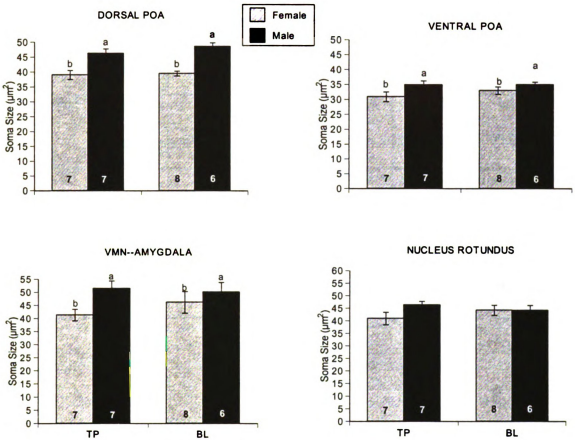

Figure VI-5: Soma size of forebrain regions in Experiment 2 (mean±SE). Final sample sizes are included for each group. Letters **a** and **b** indicate significant main effects of sex (male> female; $p<0.05$). No effect of hormone treatment existed.

Table VI-2. Average body weight (BWT), snout-vent length (SVL), and 3rd ventricle (3V) height in Experiments 1 and 2. Standard errors included in parentheses. BS=Breeding Season, NBS=Non-Breeding Season, TP=Testosterone Propionate, BL=Blank.

		BWT (g)		SVL (mm)		3V height (mm)	
		Male	Female	Male	Female	Male	Female
1	BS	4.62 (0.10)	2.22 (0.08)	62.60 (0.67)	49.10 (0.55)	1.33 (0.03)	1.20 (0.05)
	NBS	4.43 (0.23)	2.34 (0.17)	58.38 (1.00)	47.38 (1.30)	1.14 (0.02)	1.11 (0.06)
2	TP	4.89 (0.31)	2.75 (0.18)	61.57 (1.41)	51.71 (1.34)	1.19 (0.07)	1.19 (0.04)
	BL	4.81 (0.23)	2.81 (0.16)	61.13 (0.99)	53.25 (0.90)	1.18 (0.05)	1.23 (0.05)

CHAPTER SEVEN:

GENERAL SUMMARY AND CONCLUSIONS

In this dissertation, I have described a number of studies conducted for the purpose of identifying relationships between the structures regulating male courtship behavior in the green anole lizard and the degree to which the behavior is displayed. Briefly, males and females both extend the dewlap, but the display differs remarkably in two ways. First, the males have a much larger dewlap than females and they use it more frequently than do females. Second, males extend the dewlap in two behavioral contexts, for courtship and for aggression, while females use it only during agonistic encounters. In the first study, I replicated a previous male-biased sexual dimorphism in the soma size of brainstem motoneurons. However, unlike the SNB in rat, and the vocal motor nucleus in both frogs and fish (see Chapter 2), male and female anoles do not differ in the number of motoneurons involved in dewlap extension, which might simply reflect the fact that females use the dewlap as well, and must require the same neuromuscular machinery to produce this behavior, even if it is produced infrequently. The size of the nerve is also greater in males compared to females. Additionally, the size of each muscle fiber, as well as the number of fibers, is greater in males. The fact that the nerve as well as the number of muscle fibers is larger in males, but motoneuron number is equivalent with females, suggests that the size of the motor unit differs between the sexes. These results also suggest that the larger nerve of males contains more axons to innervate the increased number of muscle fibers, and the axons may be larger compared to females.

However, as none of these dimorphisms is related to differences in adult levels of testosterone, which is critical for the behavioral display, the relationship between structure and function isn't completely parallel. Perhaps the role of testosterone is not to increase the function of a structure by changing its size, but by merely activating a region already important for the display or by increasing cell excitability that does not involve a structural change. As it appeared unlikely that these dimorphisms were solely responsible for the robust sexual and seasonal dimorphisms in dewlap extension behavior, I next investigated possible structural differences in the level of input to (via motoneuron dendritic morphology) and output (via neuromuscular junctions) from this simple neuromuscular system.

The dendritic morphology of dewlap motoneurons suggested that input to the neuromuscular system at this level was equivalent in the sexes. While this result was not initially expected, it is logical as females do use this system to extend the dewlap in the same manner the males do (though markedly less frequently). Therefore they may require a similar level of input for appropriate stimulation. It seemed reasonable that enhanced display in males might be due to a difference in the output of the motoneurons. To address this possibility, I next examined NMJ morphology via acetylcholinesterase histochemistry. As shown in Chapter 4, breeding males have larger NMJs than females, which might reflect an increased release of acetylcholine at the synapse, allowing males to contract the dewlap muscle fibers more powerfully than females, or to produce a more sustained or more frequent display. However, these enhanced NMJs in males did not decrease in size during the non-breeding season in parallel with behavior, suggesting that

seasonal regulation of behavior might be mediated from elsewhere in the brain, such as the forebrain (see below).

To summarize the results from the first three experiments, it is clear that many of the neuromuscular components of this system are sexually dimorphic in adulthood. While these differences are consistent with the fact that males produce more behavioral displays than do females, they show a lack of congruency with the hormonal regulation of the behavior. In all cases, decreases in testosterone (either seasonal or experimentally induced) did not reduce the size of these structures. Clearly, fluctuations in this hormone do not modulate the basic morphology of these neuromuscular components. These results are different from those found in other systems, such as the rat SNB, in which testosterone can alter the morphology of neuromuscular structures in adulthood (see Chapters 2-4 for a more complete comparison to other species). It is, however, possible that testosterone acts on this system to stimulate behavior in a manner that does not alter structure size (see below).

If these sex differences (when detected, see Chapter 3) are not modified by adult testosterone, then perhaps these differences might have been permanently organized during development and therefore remain stable through adulthood. Based on the results of Chapter 5, it is clear that, while general body measures are not yet as sexually dimorphic by three months of life as they are in adulthood, structures involved in dewlap extension do differentiate in post hatching development. Thus, the development of these features is specific, and not part of the general growth of the animal, suggesting that different mechanisms mediate the development of each. Also, the pattern of differentiation implies that the development of some structures might depend on some

type of support or influence from others. Specifically, the cartilage might stimulate initial muscle development, and motoneuron differentiation could follow from that. As described in Chapter 5, the timing of these events is right for testosterone to induce this masculinization, but it will need to be determined whether that is the case and if so in which structures it may act.

Up until this point, I had identified a number of structural dimorphisms that might play a role in creating behavioral differences between males and females. However, it was still unclear, if the absence of testosterone could reduce behavioral displays, why structural components involved in the production of this behavior were not affected. Again, perhaps testosterone was inducing behavior in these regions in a manner that did not involve structural changes. It also seemed likely that it might be acting elsewhere in the brain to influence behavior. Previous work on anoles had documented the role of the preoptic area (POA) and the amygdala (AMY) as essential for male sexual behavior in adults, and that testosterone implanted in these regions was more effective in stimulating male sexual behavior than other steroids. We had established in our lab, via immunohistochemistry and *in situ* hybridization, that testosterone could bind in these areas. Therefore, I turned to the POA and AMY of the forebrain, to investigate whether structural differences might exist in males between the breeding and non-breeding seasons. I determined that cells in both of these regions were larger in the breeding season compared to the non-breeding season, regardless of sex. It therefore seemed possible that testosterone acting in these forebrain regions was the mechanism by which animals in the breeding season performed more behavioral displays. However, when animals were treated exogenously with testosterone during the non-breeding season,

soma sizes in the forebrain regions of interest were not reversed (i.e. increased), although behavior was stimulated. This result suggests that, as in Chapter 2, a dissociation exists between the hormonal control of dewlap extension behavior and the size of its anatomical substrates.

The experiments I have conducted in this project demonstrate that sexual dimorphisms exist at many levels of the dewlap neuromuscular system important for the production of a socially relevant behavior. I have also shown that at least some of these sex differences are initiated during juvenile development, along a trajectory that differs remarkably from that of general growth parameters. In addition to the sex differences observed in the neuromuscular system, I have identified seasonal dimorphisms in forebrain regions that parallel behavior.

Some sexual dimorphisms identified in these studies are consistent with those found in other systems. For example, neuromuscular structures that control male sexual behavior are sexually dimorphic in a wide variety of species (see Chapters 1 and 2). Perhaps this relationship is one that has been conserved over time as a means for producing a stable, increased behavior in one sex compared to the other. However, some of the mechanisms behind these differences, particularly those in the hindbrain and periphery, appear to be different to what is found in other systems. As described in the Introduction, adult testosterone plays a role in maintaining the size of many masculinized features of these neuromuscular systems in parallel with facilitating behavioral displays. However, it is clear that while behavior is certainly enhanced by testosterone in anoles, motoneuron soma size, muscle fiber size, and NMJ size, as well as the size of neurons in regions of the forebrain, do not show a concomitant increase in size with increased

androgen in adulthood. While the sex differences in these features are evident, these dimorphisms does not appear to depend on the presence of adult T to maintain them. If so, this mechanism would parallel the androgenic regulation of masculine features in a number of vertebrate models. However, the experiments necessary to determine whether androgen is important in organizing anole dewlap structures are beyond the scope of this dissertation.

Additionally, motoneuron number, a feature that is commonly different between males and females in sexually dimorphic neuromuscular systems, is not different between male and female anoles. In the SNB system for example, males and females have equal numbers of motoneurons before birth. However, females soon lose their perineal muscles due to a lack of androgen, and consequently more neurons die in females than in males around the time of birth because they aren't receiving support from the peripheral muscles. Although muscle fiber number in adult anoles is larger in males than females, the fact that males and females have equal numbers of dewlap motoneurons suggests that muscle fiber number isn't likely to be involved in regulating adult motoneuron number. This result suggests strongly that the relationship between motoneuron and muscle, and perhaps the mechanisms that regulate motor unit size, are different in the anole than in other more extensively studied systems.

Similarly, in many other vertebrate systems, a male performs a suite of behaviors in courtship or copulation that a female never produces. As discussed in the Introduction, male frogs, fish, and birds produce courtship vocalizations that females do not produce. Similarly, male mammals use a copulatory apparatus that females do not have. However, female anoles do extend the dewlap in certain agonistic situations, though remarkably

less often than do males. Perhaps it is not surprising that some features of this system would be held in common between males and females, as the basic neuromuscular machinery and connections to other regions of the brain would need to be in place in order for the behavior to be produced.

It is important to remember, though, that consistent species differences in the relationship between structures of the brain and the extent of the behaviors they mediate exist at many levels, suggesting that one model that examines the relationships between structure and function is not sufficient to arrive at a complete picture (see previous chapters for more complete comparisons). However, despite these differences, general principles can be obtained by performing careful and informed comparisons. For example, in most cases, a more developed musculature is required to produce a enhanced behavioral display. Oftentimes these muscles are controlled by more and/or larger motoneurons. These behaviors are often present at times that coincide with high levels of circulating testosterone, though the enhanced morphology of these structures need not necessarily depend on the presence of adult androgen. Early or juvenile increases in androgen often initiate masculinization, and results obtained from Chapter 5 suggest that testosterone might play a similar role in the anole. The fact that females can extend the dewlap more when treated with testosterone but do not perform the display as much as males, is consistent with the idea of an organizational effect of early testosterone on the system.

It is likely, then that these structural differences facilitate or permit increased display behavior in males, either via the ability to extend a larger structure, to perform a more frequent display, or both. Once permanently established, high testosterone acts to

seasonally induce the behavior in males, via a change in neuronal excitability that does not involve a structural change on the level examined in these studies. A biochemical change could involve modulations of neurotransmitter systems such as vasotocin/vasopressin (for review see Goodson and Bass, 2001). Arginine vasotocin induces male calling behavior in some fish (Goodson and Bass, 2000) and frogs (Boyd, 1994; Marler *et al.*, 1995), and may also interact with androgen to affect reproductive behavior in amphibian models (Penna *et al.*, 1992; Moore *et al.*, 1994; Burmeister *et al.*, 2001).

The study of structure/function relationships can allow us to draw conclusions about how changes in the brain might be related to changes in behavior, as an increase in structure size might imply increased function. For example, larger soma size might imply larger protein synthesis, larger dendritic arborization and/or larger axon diameter, which would produce faster conduction velocity. Large muscle fibers are more capable of providing a strong, sustained contraction than smaller fibers. Larger neuromuscular junctions might be related to increased release of acetylcholine at the synapse, which might also allow for a greater contraction and therefore an enhanced behavioral display. However, one fact that is important to consider when attempting to draw these parallels between structure and function, is how it relates to the behavioral model.

It is in this facet of behavioral neuroscience in which I believe comparative studies offer great value. By studying models in which animals produce different displays in different situations, or behavioral models in which the sexually dimorphic behavior is expressed in varying degrees in both sexes, one can begin to understand how the brain fashions neural systems to respond to different environmental or social needs.

For example, in the dewlap system, both males and females extend the dewlap. This fact, I believe, is largely responsible for the lack of parallels between structure and function in motoneuron number and the regulation of structure size by season or androgen exposure.

In conclusion, the neural and muscular systems regulating male courtship and sexual behavior in the green anole lizard represent a unique model that might provide an alternate view to what is commonly believed to be driving structure/function relationships of sexual behavior in vertebrates. These studies represent a first characterization of the structural features of this model system, and detail the effects of sex, and in some cases season, on their morphology. The work presented here therefore provides the groundwork for future studies to reveal similarities and differences across vertebrate groups in the principles and/or mechanisms of sex differences.

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