Sexual Dimorphism in Neuromuscular Junction Size on a Muscle Used in Courtship by Green Anole Lizards

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ABSTRACT: The green anole lizard exhibits seasonal courtship behavior that is sexually dimorphic. This courtship consists of the extension of a bright red throat fan (dewlap) associated with head-bobbing display behavior. While males extend their dewlaps in aggressive encounters as well as in courtship, females use their considerably smaller dewlaps much less frequently and mainly in agonistic encounters. In parallel, a number of components of the neuromuscular system controlling dewlap extension are greater in males than in females during the breeding season, including dewlap motoneuron soma size and muscle fiber size and number. These features do not seem to change substantially in adulthood, despite a dramatic decline in dewlap use during the nonbreeding season. We explored the morphology of this neuromuscular system in more detail in the present experiment in males and females during both the breeding and nonbreeding seasons. Fiber and whole muscle length (approximately perpendicular to the fibers) were measured. Acetylcholinesterase histochemistry was used to visualize neuromuscular junctions (NMJs), and the surface area and density of NMJs were assessed for each animal. During the breeding season, NMJ size was larger in males than in females, but NMJ density along each fiber was equivalent between the sexes. In addition, whole muscle length and that of individual muscle fibers, was larger in males than in females. However, when corrected for body size, the sex difference in muscle fiber length disappeared. In the nonbreeding season, the sexual dimorphisms were maintained, suggesting that these features do not change substantially due to differences in circulating testosterone or a difference in use across seasons. Overall, these results are consistent with the idea that enhanced NMJ size is a relatively stable feature of the dewlap muscle in adulthood that either facilitates or is a consequence of using a larger muscle to extend a bigger dewlap in males compared to females.

INTRODUCTION

Parallels between nervous system structure and behavioral function have been examined in numerous vertebrate groups, including mammals, birds, frogs, and fish. These relationships have been extensively investigated in neuromuscular systems that mediate reproductive be-
example, sonic motoneurons and swimbladder muscle morphology are larger in courting males compared to noncourting males and females in the plainfin midshipman (Bass and Marchaterre, 1989a, 1989b). Similarly, motoneurons and larynx muscle fibers are larger and more numerous in male African clawed frogs compared to females (Kelley, 1988), and vocal motor nucleus volume is greater in male than female zebra finches, as is syrinx weight, and syrinx muscle fiber size (Wade and Buhlman, 2000).

The courtship behavior of the green anole lizard (Anolis carolinensis) has also proven useful for studying relationships between structure and function. In this species, courtship behavior is seasonal and sexually dimorphic. During the breeding season, males extend a red throat fan (dewlap) while giving headbobbing displays both in the context of courtship and territory defense (Greenberg and Noble, 1944; Crews, 1979, 1980). Females possess a very small dewlap compared to males (Jenssen et al., 2000), which is used much less often and primarily in aggressive encounters (Greenberg and Noble, 1944; Nunez et al., 1997). The dewlap is extended by the contraction of the bilaterally symmetrical ceratohyoid muscles that cause two small pieces of cartilage (the ceratohyals and first ceratobranchials) to act as a lever, pushing out the much larger second ceratobranchial cartilage to unfold the dewlap skin (Font and Rome, 1990; Fig. 1). Two pools of brainstem motoneurons innervate the ceratohyoid muscle via the ramus pharyngo-laryngeus (RPL nerve; Font, 1991; Wade, 1998).

The structures controlling the extension of the dewlap show male-biased sexual dimorphisms that parallel behavior during the breeding season (Wade, 1998; O’Bryant and Wade, 1999). Motoneuron size is consistently larger in males, but the most robust sex differences are in the periphery. The second ceratobranchials and the weight of the ceratohyoid muscle are larger in males than in females (Wade, 1998). Correspondingly, the cross-sectional area of individual muscle fibers, the number of fibers, and the cross-sectional area of the RPL nerve that projects to the muscle are also greater in males compared to females (O’Bryant and Wade, 1999).

The relationships between structure and function are not entirely parallel in the green anole, however. Dewlap extension by males is greatly reduced during the nonbreeding season (Jenssen et al., 1996) when testosterone (T) circulation is comparatively low (Lovern et al., 2001). However, dewlap motoneuron soma size and number and ceratohyoid weight are equivalent across seasons (O’Bryant and Wade, 1999; O’Bryant and Wade, unpublished). Removing endogenous T in adult males by gonadectomy during the breeding season also has no effect on neuromuscular morphology; motoneuron soma size, dewlap muscle fiber size, and RPL nerve cross-sectional area are equivalent in castrated adult males with or without T replacement (O’Bryant and Wade, 1999).

The idea that displays are sexually dimorphic and dependent on seasonal T in males, while gross morphology of the relevant motoneurons, nerve, and muscle remain relatively stable in adulthood led us to examine whether the structure of synaptic features might more closely parallel behavioral differences. The purposes of the present study, then, were to first investigate a new aspect of the 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 nonbreeding season.

METHODS

Animals

Adult green anoles were purchased from Fluker Farms (Louisiana), and were housed in group glass aquaria con-
sisting of one male and at least three 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 nonbreeding 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 nonbreeding 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 cerato-hyoid muscle and the second ceratobranchial, ceratohyal, and first 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 1 h. 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, nonbreeding 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 postsynaptic components of the NMJs. The whole muscle was incubated in the following medium at 37°C for 45 min, 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 min. Slides were counterstained with Harris hematoxylin, blue 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 ceratothyoid muscle of one individual from each sex and season (four total) in a specific inhibitor of acetylcholinesterase, 1.77 mM 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.

**Measurement and Analysis**

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; Fig. 1). These six measurements were averaged to obtain one value per individual. After incubation, the rostrocaudal length of the muscles (while still stretched between the ceratohyal and first ceratobranchial pieces of cartilage) was measured with calipers to get an estimate of the overall length of the muscle (Label 1; Fig. 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 two-tailed t-test (Statview, SAS Institute, Inc.). 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 three 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 nonbreeding seasons were generally ovoid overall, and fairly uniform in appearance (Fig. 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 \((t = 2.57, p = .022; \text{Fig. 3A})\), and this difference was due to the fact that they were longer as opposed to wider (major axis: \(t = 4.75, p = .003\); minor axis: \(t = .84, p = .413; \text{Table 1}\)). The density of NMJs along each fiber was equivalent between the sexes \((t = 1.79, p = .094)\). Both fiber length \((t = 4.47, p = .0005; \text{Fig. 4A})\) and overall muscle length \((t = 12.11, p < .0001)\) were significantly larger in males than in females. However, when corrected for body size (SVL, which is significantly greater in males than females, \(t = 6.39, p < .0001; \text{Fig. 4C}\)), only the length of the overall muscle \((t = 5.89, p < .0001; \text{Fig. 5A})\), and not individual muscle fiber length \((t = .92, p = .372; \text{Fig. 4E})\), was larger in males than in females.

Experiment 2

During the nonbreeding season, average NMJ area was larger in males than in females \((t = 2.17, p = .044; \text{Fig. 3B})\). As in Experiment 1, this overall difference in area was due to longer, as opposed to wider, junctions (major axis: \(t = 3.40, p = .003\); minor axis: \(t = .25, p = .804; \text{Table 1}\)). Also as in Experiment 1, muscle fiber length \((t = 18.60, p < .0001; \text{Fig. 4B})\), overall muscle length \((t = 11.30, p < .0001)\), and SVL \((t = 8.57, p < .0001; \text{Fig. 4D})\) were larger in males than in females. The length of the dewlap muscle divided by SVL was larger in males than in females \((t = 7.24, p < .0001; \text{Fig. 5B})\), but corrected individual muscle fiber length was equivalent between the sexes \((t = .72, p = .478; \text{Fig. 4F})\). 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 \((t = 1.60, p = .137)\). 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: 637.6–1084.4 μm²; males: 729.2–1643.2 μm²).

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

Figure 3  Neuromuscular junction size in Experiment 1 (A) and Experiment 2 (B) (mean ± standard error). *Significantly greater in males than females.
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 second 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). 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

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### Table 1 Average Length of Major and Minor Axes of Neuromuscular Juncions (in μm), and the Average Density of Neuromuscular Juncions (Number per mm)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Breeding Season</th>
<th>Nonbreeding Season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Major axis</td>
<td>40.07 (.99)</td>
<td>31.26 (1.56)</td>
</tr>
<tr>
<td>Minor axis</td>
<td>17.96 (.96)</td>
<td>16.79 (1.00)</td>
</tr>
<tr>
<td>NMJ density</td>
<td>12 (1)</td>
<td>15 (1)</td>
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Standard errors included in parentheses.

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**Figure 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. *Significantly greater in males than females.

**Figure 5** Overall length of the ceratohyoid muscle corrected for snout–vent length (SVL) in Experiment 1 (A) and Experiment 2 (B). *Significantly greater in males than females. Both values were measured in mm.
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 noncourting 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, in press), as well as a dramatic decrease in courtship behavior in the nonbreeding compared to the breeding season (Jenssen et al., 1996). Interestingly, the surface area of NMJs remains sexually dimorphic during the nonbreeding 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 nonbreeding 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 ($F = 11.21, p = .002$) one can pick up a small, but statistically significant, effect of season ($F = 4.62, p = .039$), with no sex by season interaction ($F = .18, p = .677$). Importantly, though, the magnitude of the seasonal change is nearly identical in males and females [compare Figs. 3A and 3B]. NMJ size is larger in both sexes during the nonbreeding 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, because 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 nonbreeding 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, or the morphology of other dewlap components, 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.

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REFERENCES


Lovern MB, Wade J. Maternal plasma and egg yolk testosterone concentrations during embryonic development in green anoles (Anolis carolinensis). Gen Comp Endocrinol, in press.


