

Comparison of External Oblique Muscle Fiber Widths in Male and
Female Common Green Frogs, *Rana clamitans*

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Abstract: Contraction of the external and internal oblique muscles, in combination with the laryngeal musculature, produces vocalization in frogs. Male and female green frogs, *Rana clamitans*, display sexual dimorphisms with respect to vocalization. Rarely if ever do female green frogs produce calling sounds. The physiological basis of this dimorphism has been studied. This investigation focuses on differences in external oblique muscle fiber widths in male and female green frogs. It is concluded that external oblique fiber widths statistically differ in male and female green frogs. In addition, it is concluded that posterior and anterior external oblique muscle fiber widths in females statistically differ from centrally located muscle fiber widths.

Introduction

The purpose of this investigation is to quantitatively compare the external oblique muscle fibers of male and female common green frogs, *Rana clamitans*. The external and internal oblique muscles are used in both amphibian respiration as well as vocalization.

The physiological features of amphibian vocalization have been the focus of extensive investigations (Wetzel, et al. 1985, Schmidt 1972, de Jongh and Gans 1969, Marth and Gans 1972, Marsh and Taigen 1987, Muller et al. 1969, Rubinstein et al. 1983). There are two major muscle groups that produce and control amphibian calling. These are the laryngeal muscles and the external and internal obliques (Marsh and Taigen 1987). While the laryngeal muscles have been well investigated (Wetzel et al. 1985), the oblique muscles remain less studied on a quantitative level.

Vocalization in *Rana clamitans* is closely linked to respiration. Two types of "respiration" are generally recognized in frogs, pulmonary and buccal. However, only pulmonary respiration involves lung ventilation and flow of air through the glottis, which is of interest for this investigation. At the beginning of expiration in a relaxed frog, the glottis and nares open. After a slight pause, air is passively forced from the lungs due to their natural elasticity. After the inspiratory phase, a buccal or another

pulmonary cycle may then follow, or there may be a period of no movement (Schmidt 1966).

The respiratory cycle of an excited frog, such as after rough handling, will manifest several physiological differences from that of a relaxed frog. Respiratory movements become forceful and uninterrupted. Of significance for this investigation is the marked increase in the intensity of respiration. This can be observed externally as increasingly large constrictions in the abdominal muscles on the sides of the frog during expiration. If the anterior part of the abdominal musculature is exposed for visual observation and the animal is immobilized, sharp constrictions of the anterior end of the body wall can often be seen during expiration (Schmidt 1966).

During vocalization, the inspiratory phase appears to be identical to that of excited respiration, although the movements are somewhat greater in amplitude. Release calling seems to differ from breathing, therefore, only by an extreme elaboration of the expiratory phase. Of importance are the strong and vigorous contractions of the abdominal muscles. The result is a reduced body cavity volume coupled with an increased intrapulmonary pressure. Opening of the glottis then permits air to flow past the vocal cords, thereby causing them to vibrate and produce sound (Schmidt 1972).

Thus, production of a release call requires three mechanisms: a primary sound source (vocal cords), a pump to provide sufficient air pressure to set the vocal cords into vibration (internal and external oblique muscles), and a valve to regulate air flow (glottis).

Because of sexual dimorphisms, male and female green frogs do not exhibit identical calling characterizations. In fact, males give a robust and conspicuous advertisement call while females rarely do so. One of the underlying physiological reasons for this contrast is that the oblique and laryngeal muscles of the calling male are much larger than those present in females of the same body weight. (Emerson and Boyd 1999). Also, the oblique

muscles are composed of different fiber types (Marsh and Taigen 1987). The physiological result is increased contraction velocities and higher resistance to fatigue of the muscles in calling males (Marsh and Taigen 1987). A comparison of the fiber widths has the potential to reveal whether fiber widths play a role in the dimorphic characteristics.

Previous researchers have discussed the sexually dimorphic characteristics of auran vocalization. On its most basic and qualitative level, "most differences in temporal and spectral characteristics between female mating calls and the calls of conspecific males relate directly to sexual dimorphisms in laryngeal and oblique muscle morphology" (Emerson and Boyd, 1999). Males have oblique and laryngeal muscles that are more than twice the size of those of females of the same body weight (McClelland, et al, 1997). Comparisons of the chemical composition of male and female oblique muscles have also been performed (Marsh and Taigen, 1987). No attempts, however, have been made to quantify this sexual dimorphism in terms of muscle fiber width. This is important not only as a complement to the existing literature but also as an opportunity to better understand the physiological and developmental causes of the dimorphism.

Investigation Design

A. Site Description

The University of Notre Dame Environmental Research Center (UNDERC) encompasses approximately 7500 acres in Vilas County (Wisconsin) and Gogebic County (Michigan). The center of UNDERC is at 46'13' North by 89'32'. The altitude of the area ranges between 1640 ft (500 m) and 1700 ft (520 m). There were no restrictions on the selection of an appropriate site for the collection of specimens. Male and female *Rana clamitans* were collected on the property wherever they were found. Sites well populated with green frogs included Jude's Bog and Magic Bog.

B. Collection of Animals

All NIH guidelines for care and use of animals were strictly abided. A total of 15 females and 9 males were captured. The animals were identified by toe clippings. They were segregated by sex and placed in ten-gallon aquariums filled with tap water to a depth of approximately 0.75 inches in the wet laboratory. No more than four animals were placed in each tank. The tanks were cleaned and the water was replaced approximately every three or four days. The animals were fed insects collected in a light trap on the UNDERC property throughout the investigation.

C. Methods of Investigation

A NeuroTrace™ BDA-10,000 Neuronal Tracer Kit (Molecular Probes Product #N-7167) was used. All surgeries were performed under a benzocaine anesthesia. Each animal was immersed in a solution of 0.002% solution of benzocaine for approximately 20 minutes or until a toe pinch produced little reaction. The external oblique muscles were exposed through a 2-3 cm surgical incision on the dorsal side of the animal slightly distal to the vertebrae. A BDA-Phosphate buffer solution (pH= 7.3), a neural tracer, was prepared according to the manufacturer's protocol by dissolving 3 mg of BDA in 30 μ L of 0.1 M PBS. The solution was refrigerated and used within one week. Approximately 2 μ L of this solution was injected into a one cm^2 area of the external oblique muscle using a gas tight syringe. Two or three injections were made totally 2 μ L of solution. The side of the frog injected as well as the location on the oblique muscle was varied randomly for each frog. These designations were documented. The injections were confined to the area of the oblique muscles slightly distal (within approximately 1 cm) to the cartilaginous tissue on either side of the vertebrae. The animals were then sutured and returned to their tanks. In order to allow the dextran to travel, one half of the animals were kept alive for 7 days after

injection. One half of the animals were kept alive for 13 days. The animals were then sacrificed and a section of the oblique muscles and vertebrae surrounding the point of injection were removed from each animal and placed in fixative solution (4% paraformaldehyde in 0.1 M PBS).

In the laboratory at the University of Notre Dame, the muscle tissues were removed from the vertebrae and were embedded and then sectioned (cryostat, 50 μm sections). (Matz 1995). A working solution of avidin-HRP was prepared according to the manufacturer's printed protocol by diluting a 1 mg/mL stock solution of avidin-HRP to a working concentration of 2.0 $\mu\text{g}/\text{mL}$ with 0.1 M PBS, pH 7.3, containing 0.3% Triton X-100. The sections were incubated at room temperature in the avidin-HRP solution overnight.

A 5% DAB stock solution was prepared by dissolving 250 mg of DAB into 5 mL of distilled water. The DAB solution was filtered through a 0.2 μm syringe. The solution was divided into 0.5 mL aliquots and placed in the freezer. To produce a working solution of DAB, one 0.5 mL aliquot of 5 % DAB stock solution was diluted in 50 mL distilled water to a final working concentration of 0.05% DAB. Immediately before using the DAB working solution, 100 μL hydrogen peroxide was added to produce a concentration of 0.006% H_2O_2 . The sections were soaked in this solution for 30 minutes. The slides were then rinsed three times with PBS, dehydrated in alcohol, cleared in Hemo-DE, and mounted.

The finished slides were inspected under the microscope noting the external oblique musculature, which was defined as the thicker of the two muscle sheets present in each section (Wingerd 1988). A micrometer was used to make ten independent fiber width measurements for each animal. The measurements were distributed randomly across the entire length of the muscle tissue section. These measurements were recorded, means were calculated, and statistical analysis was performed.

Results

Table 1: Average External Oblique Muscle Fiber Width of Male and Female Common Green Frogs

	Male Mean Fiber Width (μm)	Female Mean Fiber Width (μm)
	801.5	355.5
	886.0	166.0
	879.0	191.5
	714.5	196.5
	1044.5	290.5
	834.0	295.5
	727.9	167.4
	927.5	309.5
Mean Fiber Width by Sex	851.9	246.6
Standard Deviation	439.6	127.4

Because of time constraints as well as the nature of the comparison, there was no benefit in analyzing more female tissues than male tissues even though more females were captured. As a result, eight (8) male and eight (8) female green frogs were analyzed. The average fiber width for the eight males was 851.9 μm with a standard error of 439.6 μm . The average fiber width for the eight females was 246.6 μm with a standard error of 127.4 μm . Using the t-test for statistical analysis, it can be stated with a confidence level of 99.9% that the differences observed in male and female external oblique muscle fiber widths is significant and not simply the result of random variation.

Table 2: Average External Oblique Muscle Fiber Width of Female by Location

	Female Mean Fiber Width Anterior (μm)	Female Mean Fiber Width Posterior (μm)	Female Mean Fiber Width Central (μm)
	191.5	166.0	355.5
	167.4	196.5	290.5
			295.5
			309.5
Mean Female Fiber Width by Location	179.5	181.3	312.8
Standard Deviation	52.3	47.5	106.0

Table 3: Average External Oblique Muscle Fiber Width of Male by Location

	Male Mean Fiber Width Anterior (um)	Male Mean Fiber Width Posterior (um)	Male Mean Fiber Width Central (um)
	886.0	879.0	801.5
	1044.5	727.9	714.5
			834.0
			927.5
Mean Male Fiber Width by Location	965.3	803.5	819.4
Standard Deviation	214.0	285.6	256.7

Among the eight males, two tissue samples each were taken from the anterior and posterior regions of the oblique musculature and four tissue samples were taken from the central region. The same was done for the females. These data are shown in Tables 2 and 3. The anterior region was defined as the area of the musculature extending posteriorly approximately 1.5 cm from the oblique muscle's incision point at the scapula. The posterior region was defined as the area of the musculature extending anteriorly approximately 1.5 cm from the incision point of the rectus anterior femoris at the vertebrae. The central region was defined as the area of the muscle lying between the anterior and posterior regions.

Using the t-test, there appears to be no variation in male fiber widths based on location within the musculature. Among the female samples, however, both the anterior and posterior samples show significant deviation from the samples taken from the central region of the tissue (90% confidence level).

No differences were observed based on survival time.

Discussion

The data presented in Table 1 is consistent with previous investigations of the sexual dimorphism of frog vocalization (Marsh and Taigen 1987, Emerson and Boyd 1999, McClelland et al. 1997). While previous investigations either qualitatively compared muscle size or quantitatively

compared muscle mass or chemical composition, this investigation goes further in that it not only quantifies the dimorphism but also provides some insight into the physiological causes of it. While it did not consider differences between the total number of fibers in male and female external oblique muscles, it does conclude that external oblique fiber width in the common green frog is sexually dimorphic.

This investigation also reveals a statistical difference in female fiber width based on location in the external oblique musculature. No statistically significant difference was evident in males. No such dimorphism has been reported in the literature. However, a more specific investigation of these differences with a larger sample size is necessary.

This investigation overlaps with several previous studies. There are two main differences, however. First, no relative masses were recorded in this investigation. While this may limit the usefulness of the data, it by no means disqualifies it. Secondly, this investigation focuses exclusively on the muscle fiber widths for comparison rather than a variety of characteristics. The similarities and differences shall be explored more closely.

Previous investigators have focused on the chemical activities and hormonal influences of specific muscles as the basis for studying sexual dimorphisms. (Rubinstein et al. 1983, Muller et al. 1969). They have suggested that there are significant differences in the numbers and types of muscle fibers between genders and between controls and castrated males. (Rubinstein et al. 1983). While the present investigation did not consider total fiber number, it may be consistent with the conclusion that muscle fiber types, differentiated by width, differ among males and females. The androgen-sensitivity of the forelimb muscles has also been studied (Muller et al. 1969). These studies suggest that sexual dimorphism results, at least in part, from hormonal control. Because sexual dimorphism was seen in the

external oblique muscles of the *Rana clamitans*, it is likely that there is a hormonal basis to these differences as well. A more focused investigation is needed to establish this correlation.

Also, the oblique muscles of *Rana virgatipes* have been compared to those of both the *Hyla versicolor* and *Hyla crucifer* (Heatwole 1990). The *R. virgatipes* has a lower call rate (1-4 calls/min) than the *Hylas* (15-100 calls/min). It was concluded that the sexual dimorphisms in aerobic capacity and the relative size of the trunk muscles may reflect the calling effort characteristic (defined as seconds of calling per hour) of a species. The *R. virgatipes* displayed a smaller relative trunk mass and a slower citrate synthase activity than the *Hylas*. Because the *Rana clamitans* is also a species with a lower call rate than the *Hylas*, it could be suggested that its dimorphisms are also less extreme. With interest to this investigation, it could be suggested that the differences in muscle fiber widths of males and females would be less pronounced than the same measurement in the *Hylas*. Unfortunately, only absolute measurements were taken. An additional investigation would be necessary to establish this.

There is one assumption that was made during this investigation that should be mentioned. Because the external oblique muscle is the primary muscle used in respiration and vocalization with the internal oblique muscles acting in a secondary capacity (Wingerd 1988), I assumed that the thicker of the two muscle layers was in fact the external oblique musculature. As a result, when making measurements of fiber widths, I measured the thicker of the two layers in each section.

Also of importance is the fact that the original purpose of this investigation was to trace neural pathway of the nerves innervating the oblique muscles. However, due to unknown factors, the neural tracer was not taken up by the neurons. As a result, it was impossible to study the

neuronal control. However, the muscle tissue sections were stained in such a manner as to allow the fiber widths to be measured.

During the survival period, which was either 7 or 13 days, two frogs died, one of which was female and one of which was male. The cause of death was likely infection of the surgical openings because the stitches used to close the openings on the backs of both animals became unattached. This opened the wound to the air and water.

Large error values were observed in this investigation. They can be attributed to two major sources. First, poor section techniques resulted in significant distortion and breakage of the muscle tissue during sectioning. The presence of cartilage and other non-muscular tissues in the samples caused difficulty when sectioning the samples. Second, the small sample size combined with variations in animal size and tissue sample location likely caused systematic errors. The small sample size was a result of adverse environmental conditions at UNDERC during the summer of 2000. Body weights were not recorded because the original purpose of the investigation did not take into account body size and weight. The variation in tissue sample location was also intended to provide more insightful data concerning neuronal control

Conclusions

It may be reported with 99.9% confidence that the external oblique muscle fiber widths of female and male common green frogs, *Rana clamitans*, differ. Male fiber widths are larger than female fiber widths.

It may be reported with 90% confidence that the female external oblique muscle fiber widths in the anterior and posterior regions of the musculature differ from the fiber widths in the central region of the external muscle. Samples taken from the anterior and posterior regions showed smaller widths. No such differences were evident in the male musculature.

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