TECHNICAL COMMENT

# Botulinum Toxin Injections as a Method for Chemically Denervating Skeletal Muscle to Test Functional Hypotheses: A Pilot Study in *Lepomis cyanellus*

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## ABSTRACT

In this study, we demonstrate that botulinum toxin can be used to chemically denervate muscles to test functional hypotheses. We injected research-grade type A botulinum toxin complex into pectoral fin abductors (abductor superficialis) of green sunfish (*Lepomis cyanellus*) to determine whether chemical denervation would eliminate the ability of a particular muscle to contribute to overall pectoral fin movements. Reduction of target muscle activity occurred within 8 d of the injection, and paralysis was confirmed using electromyography. No paralysis was seen in the adjacent muscles (abductor profundus) or in positive controls (saline injections). Paralysis occurred more slowly and at lower doses than previously documented for mammals. However, botulinum toxin complex (500 kDa) was used here, whereas previous studies have used purified toxin (150 kDa). Therefore, differences in physiological responses between fish and mammals cannot yet be distinguished from differences caused by the toxin type. However, we note that the toxin complex is less likely to diffuse across muscle fascia (because it is large), which should minimize paralytic effects on adjacent muscles. We suggest that botulinum toxin holds great promise as a chemical denervation agent in functional studies of animal locomotion and feeding behaviors.

## Introduction

Functional morphologists and comparative anatomists often seek to determine the function of an individual muscle, but this can be difficult in complex systems. For example, 14 muscles span the human femur-tibia (knee) joint (Saladin 2001). All of these muscles may serve to extend the lower leg; thus, identifying the specific action of an individual muscle can be difficult. A variety of techniques has been used to test hypotheses about muscle function, including measuring muscle activity using electromyography (EMG), measuring muscle length changes with sonomicrometry, measuring muscle force using force buckles attached to tendons or strain gauges mounted on bones, and disabling the muscle by various methods, including severing ligaments or tendons that transmit muscle force and physically denervating the muscle by transecting nerves. Although important information has been gained from studies using these methods, the invasive nature of some techniques may limit their effectiveness for testing functional hypotheses, while others will not work with very small animals.

Chemical denervation is a potentially useful tool for studying muscle function because injections are minimally invasive. Botulinum toxin type A (commonly sold as Botox [Allergan] or Dysport [Ipsen]) is a zinc endopeptidase produced by the bacteria *Clostridium botulinum* that has been used for decades to denervate individual muscles in mammalian systems (Simpson 1981; Schiavo et al. 1992; Brin 1997). In humans, Botox has been used therapeutically for a variety of muscular diseases and conditions, including hyperfunctional facial lines (Blitzer et al. 1997), spasmodic torticollis (Borodic et al. 1992), focal dystonia (Brin et al. 1986), cerebral palsy (Calderon Gonzalez et al. 1994; Chutorian et al. 1995; Denislic and Meh 1995), and muscle spasticity (Chutorian and Root 1994), and has recently been used as a cosmetic treatment to relax the muscles that cause facial wrinkles (Huilgol et al. 1999; Lehrer and Benedetto 2005). In addition to pharmacological work, botulinum toxin has also been used to paralyze muscles for developmental studies and nerve regeneration studies (Pytte and Suthers 2000; Landers et al. 2002). For these purposes, the neurotoxin is injected into the target muscle, where it diffuses through neuromuscular junctions into presynaptic motor neurons and prevents the release of acetylcholine from the presynaptic neuron (Gunderson 1980; Simpson 1981; DasGupta 1994; Brin 1997).

There are seven serotypes of botulinum toxin, each of which targets a different structure in the neuromuscular system (Borodic et al. 1992, 1994; Brin 1997; Aoki and Guyer 2001; Comella

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and Pullman 2004; Gracies 2004). Type A toxin, the most commonly used toxin for medical procedures, destroys the SNAP-25 protein, one of the proteins that enables neurotransmitter vesicles to fuse to the presynaptic cell membrane and release acetylcholine into the synaptic cleft (Schiavo et al. 1993). Muscle deactivation in mice and rabbits may appear immediately; however, it takes 3–4 d for complete paralysis to occur, and paralysis in human treatments may take up to 7 d (Pearce et al. 1995; Brin 1997). Motor neurons will eventually sprout new terminal butons, which will restore nerve function and muscle activity (Alderson et al. 1991). Recovery of muscle function in mice and rabbits typically takes 4–6 mo; recovery takes approximately 3 mo in humans (Habermann 1974; Girlanda et al. 1992; Borodic et al. 1994; Brin 1997) but can be more rapid with lower toxin doses (Pearce et al. 1995). A side effect of botulinum toxin paralysis is that muscles can show atrophy

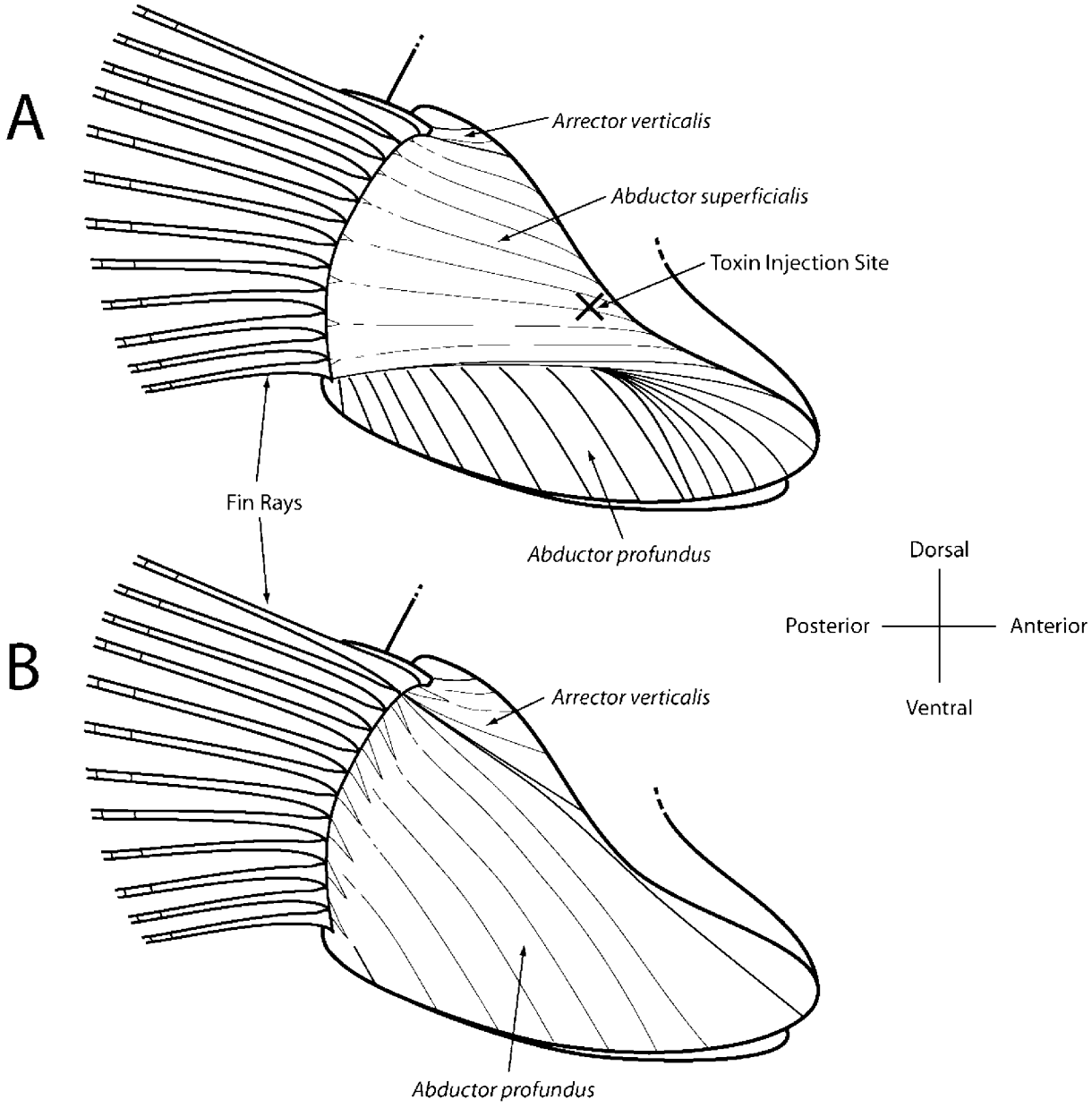



Figure 1. Line drawing (after Drucker and Jensen 1997) illustrating right-side pectoral fin abductor muscles in *Lepomis cyanellus*. *A*, Right lateral aspect of the pectoral fin abductor muscles after removal of the skin. *B*, Right lateral aspect with the abductor superficialis removed. $x$ = approximate location of injection of toxin into the right abductor superficialis.

after treatment and fiber types may be different after recovery (Ansved et al. 1997).

The effectiveness of botulinum toxin is determined by a number of variables. Artificial stimulation of an injected muscle has been shown to increase the effectiveness of the toxin in rabbits (Kim et al. 2003), presumably by increasing the amount of toxin taken up by the motor neurons. Because the spread of the neurotoxin is limited (but not entirely blocked) by muscle fascia (Shaari et al. 1991; George et al. 1992; Borodic et al. 1994; Eleopra et al. 1996), the injection dose, volume, location, and the number of injections must be controlled to limit the movement of the toxin into adjacent muscles (Lingua 1985; Biglan et al. 1988; Borodic et al. 1992, 1994; Aoki and Guyer 2001; Comella and Pullman 2004; Gracies 2004). A higher quantity of toxin and higher injected toxin solution volume will increase muscle denervation but at the risk of regional or systemic effects by diffusion into adjacent muscles and/or axonal transport of the toxin (Habermann 1974; Girlanda et al. 1992; Borodic et al. 1994).

In this experiment, we use the relatively simple suite of muscles that serve to abduct the pectoral fin in the green sunfish *Lepomis cyanellus* (Rafinesque 1819) as a model system in which to study botulinum toxin as a tool for chemical denervation. Here, we denervate a target pectoral fin muscle using research-grade type A botulinum toxin complex and confirm the denervation using EMG to monitor muscle activity during station-holding behavior. We demonstrate that botulinum toxin effectively eliminates electrical activity of the muscle and thus removes the ability of the muscle to produce active force during the fin beat cycle. This is a unique and valuable application of botulinum toxin as a tool for functional morphology.

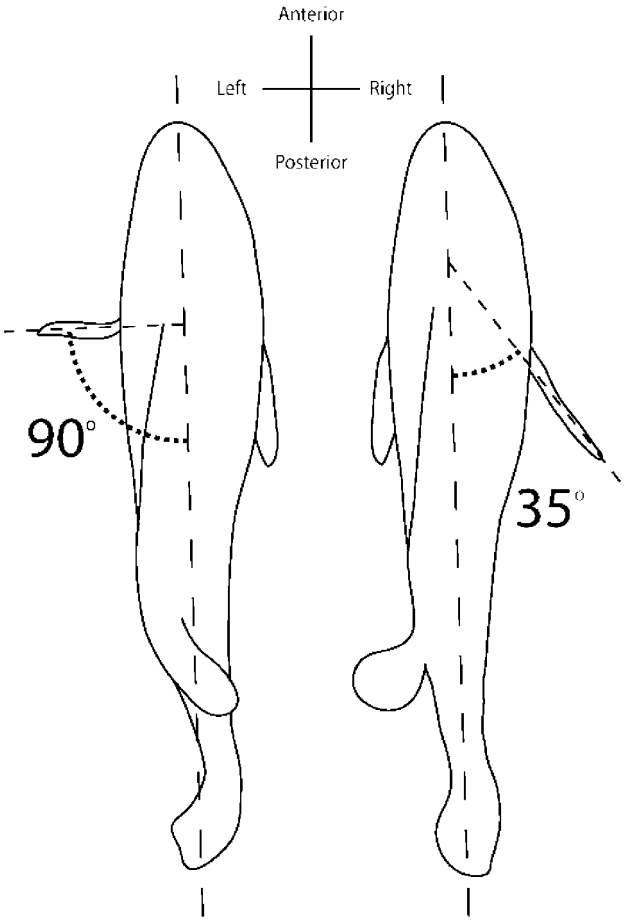



Figure 2. Line drawings illustrating a dorsal view of treated *Lepomis cyanellus* during station holding. On the side where botulinum toxin compound was injected (*right*), the pectoral fin extends approximately 35° from the midsaggital plane. On the side where saline solution (a positive control) was injected (*left*), the pectoral fin extends approximately 90° from the midsaggital plane.

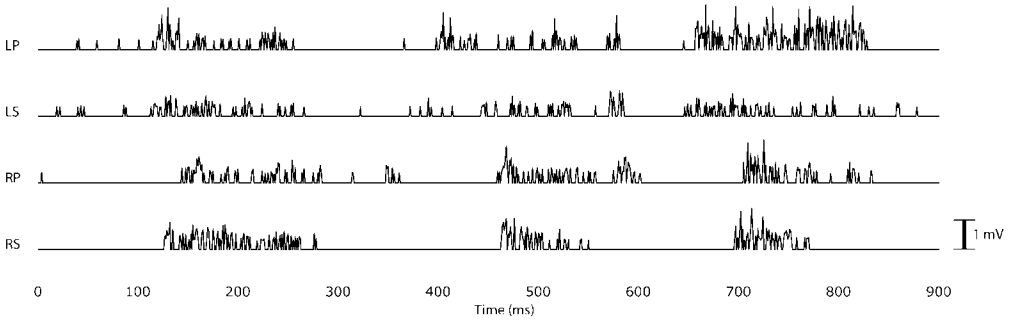
## Material and Methods

Four green sunfish *Lepomis cyanellus* were obtained from Fossil Creek, Arizona, by electrofishing. Fish ranged in standard length from 87 to 138 mm (40.1–90.0 g) and were housed in 38-L aquaria at 21° ± 2°C. All animal care and experimental procedures were conducted in accordance with Northern Arizona University Institutional Animal Care and Use Committee protocol 04-007.

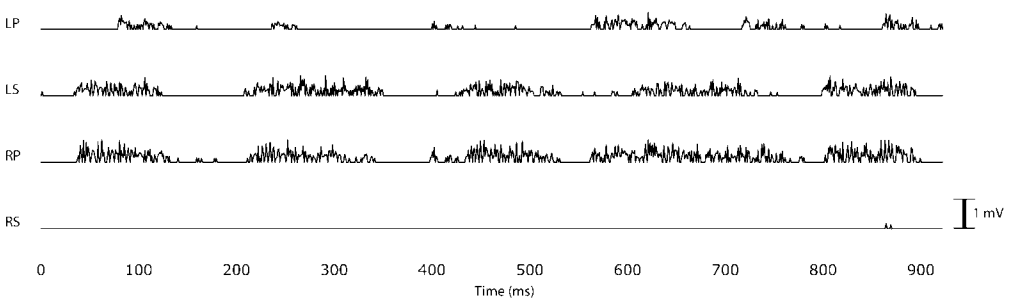
An initial set of observations was made on untreated fish to determine the baseline electrical activity of the paired muscles during station-holding behavior. For these experiments, bipolar hook electrodes were made from bifilar wire (stainless steel, 0.002 in; California Fine Wire Company, Grover Beach). Individual green sunfish were anesthetized using tricane methanosulfate (0.15 g/1 L water), and two or three scales were removed to facilitate the placement of each electrode. After external morphological landmarks (e.g., base of the pectoral fin rays) were used to determine the appropriate implantation location (Fig. 1), electrodes were inserted into the belly of each muscle using 27-ga, 0.5-in syringe needles. Electrodes were implanted into two muscles on each side of the fish: the abductor superficialis and the abductor profundus (Fig. 1). Both muscles serve to abduct and depress the pectoral fin during steady swimming and are active concomitantly during locomotion in many fish species (Westneat 1996; Drucker and Jensen 1997; Westneat and Walker 1997). Because of this simultaneous activity, distinct functions for each muscle cannot readily be determined from kinematic studies and EMG recordings.

After surgery, fish were returned to their home aquaria and allowed to recover for 1–2 h. During this time, electrode leads were attached to an A-M Systems model 1700 amplifier (low cutoff 100 Hz, high cutoff 1,000 Hz), which amplified EMG signals, and a MacLab 4e system captured and recorded EMG signals at 1,000 Hz. Simultaneous EMG recordings were made from the abductor superficialis and abductor profundus from the left and right sides of each fish. Observations of fish be-

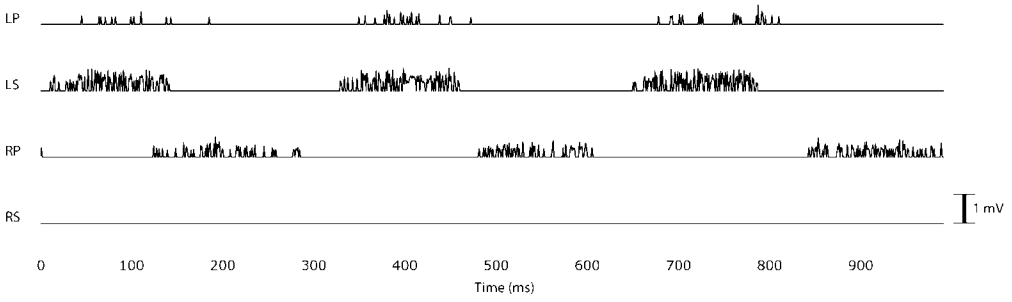
A: Fish A, Before Treatment



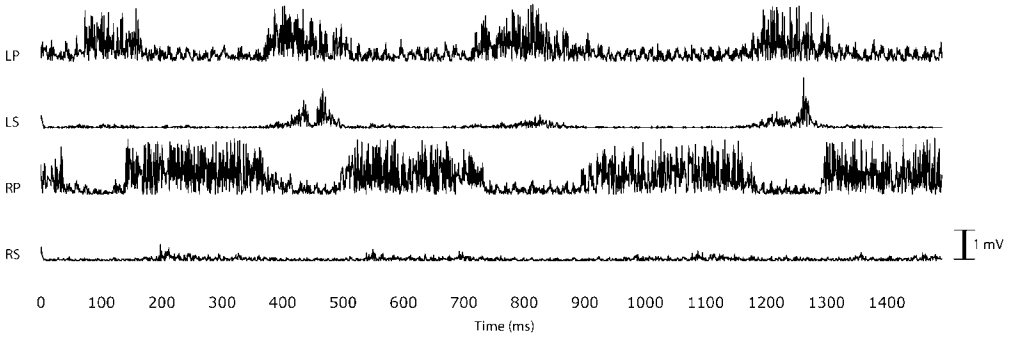

B: Fish C, After Treatment




C: Fish E, After Treatment




D: Fish D, After Treatment



havior were also made for two individuals with a JVC GR-DVL 9800 camera recording at 60 Hz to obtain dorsal views of fish station holding in the tank. After the experiment, EMG signals were filtered with a Butterworth filter (threshold 0.05 V, high cutoff 500 Hz, low cutoff 100 Hz) using a custom LabView program (Stephen Deban, University of South Florida, Tampa).

Subsequently, another set of experiments was conducted using type A botulinum toxin complex to chemically denervate a target muscle. Despite its frequent and long-standing use in human subjects, both the toxicity of solutions and the concentrations of botulinum toxin necessary to induce muscle paralysis are relatively unstandardized (Brin and Blitzer 1993; Brin et al. 1993; Pearce et al. 1995; Brin 1997). "Mouse units" (MUs; i.e., mouse LD50) is the accepted terminology for toxicity, but MUs differ for the same mass of a given toxin isolated by different companies. Therefore, different isolates of toxin yield different physiological results, even when the two major commercial type A toxins (Botox and Dysport) are compared (Pearce et al. 1995; Brin 1997). Furthermore, there are differences in the relative toxicity of a given isolate across species (Brin 1997) and even individuals (Pearce et al. 1995), which means that caution must be used when extrapolating dosages from one species to another (Brin 1997). For these reasons, when working with botulinum toxin, it is critical to report where the toxin is obtained, the injected amount, as well as injection (ng/$\mu$L) and muscular (ng/muscle mg) concentrations.

For this study, type A botulinum toxin complex was purchased from Metabiologics (Madison, WI). The complex is a less expensive, unpurified form of the type A toxin and has six associated proteins that serve to extend the shelf life of the toxin and increase its molecular weight to 500 kDa. The botulinum complex's toxicity is identified by http://www.metabiologics.com as 1 MU/0.25 ng of toxin. A study assessing the paralytic ability of Botox and Dysport in mice (Pearce et al. 1995) found that approximately 0.7 MU of Botox toxin would paralyze (i.e., reduce the compound action potential of the muscle to 10% of baseline or less) a muscle weighing 0.1 g. Because the efficacy of the toxin as a muscle paralytic on aquatic, poikilothermic vertebrates had never been established, we began our pilot experiments with a very low toxin concentration and progressively increased the dose to approximately 0.4 MU (0.1 ng) of toxin per 0.1 g muscle, at which point muscle paralysis was achieved within 10 d. After we established the appropriate dosage, we injected 0.3–0.4 MU of type A botulinum toxin complex (diluted in teleost saline to a final volume of 1.5 or 2 $\mu$L, depending on the size of the animal) into the right-side abductor superficialis (our target muscle) for three individuals (hereafter, treated fish). As a positive control, we injected 1.5 or 2 $\mu$L of teleost saline into the left-side abductor superficialis of all three treated fish. Because the abductor superficialis masses ranged from 0.06 to 0.12 g in our treated animals (determined by removing and weighing the muscles after the experiments), final injected concentrations ranged from 3.0 to 6.8 MU/g muscle.

Approximately 1 wk after injection of the toxin complex, we inserted electrodes and recorded EMG activity (using methods described above) in the four fin abductor muscles while the fish were holding station inside their tanks. To estimate the time course for recovery in this species, one fish was held for several weeks after the experiment until full pectoral fin movement reappeared. All other fish were killed immediately following EMG recordings to confirm electrode placement.

To determine the effects of botulinum toxin on muscle activity, we quantified a number of variables for all four muscles in two baseline (untreated) and three treated fish (injected with toxin and a positive control). Burst frequency of each muscle was determined by calculating the time period in which three sequential bursts occurred (beginning with the untreated left abductor superficialis) in an arbitrarily selected section of the EMG recording where fish were station holding and activity was anticipated for all four pectoral fin abductors. Onset of muscle activity was defined as electrical signal greater than two times the background electrical activity (noise) lasting for more than 10 ms, and offset was defined as the end of activity meeting these requirements. The relevant time interval was measured from onset of the first burst to offset of the last burst for each individual muscle; thus, three bursts divided by the time period for these bursts yielded burst frequency (Hz). Burst frequency was determined for each fish for three arbitrarily selected periods of muscle activity, which were separated from each other by distinct periods of inactivity (i.e., no electrical activity).

Frequency data were examined using a series of ANOVAs (JMP IN, ver. 5.1) to identify potential differences between muscles (the four pectoral fin abductors) and treatment effects. A one-way ANOVA examined the data for potential frequency differences among muscles in untreated fish. A second one-way ANOVA examined the data for potential differences among muscles in treated fish. For both ANOVAs, Tukey-Kramer HSD post hoc tests were used to determine which muscles were

Figure 3. Representative rectified electromyography signals of four pectoral fin muscles in *Lepomis cyanellus* during station holding. LP = left abductor profundus, LS = left abductor superficialis, RP = right abductor profundus, and RS = right abductor superficialis. *A*, Untreated sunfish (sunfish A) showing asynchronous electrical activity in the right- and left-side pectoral fin abductors. *B*, Treated sunfish (sunfish C) showing synchronous electrical activity in the right- and left-side pectoral fin abductors. *C*, Treated sunfish (sunfish E) showing asynchronous electrical activity in the right- and left-side pectoral fin abductors. *D*, Treated sunfish (sunfish D) showing asynchronous electrical activity in the right- and left-side pectoral fin abductors and low-level activity in the right abductor superficialis.

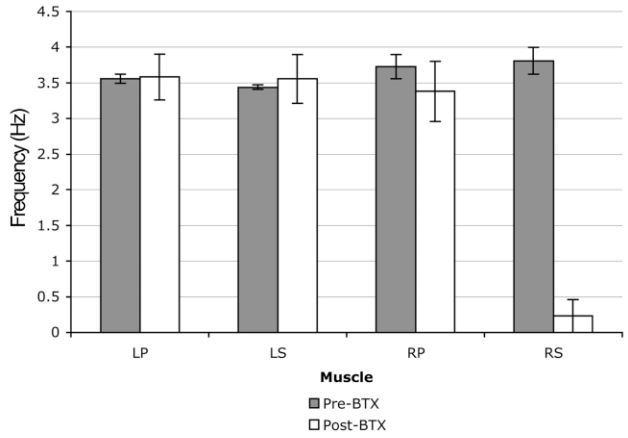



Figure 4. Mean burst frequencies (Hz) with standard error for each muscle in two untreated (*filled bars*) and three treated (*open bars*) fish; for each individual, three periods of muscle activity were examined. LP = left abductor profundus, LS = left abductor superficialis, RP = right abductor profundus, and RS = right abductor superficialis.

significantly different from one another in burst frequency. To examine the data for differences in muscle activity across treatment, four one-way nested ANOVAs (individual nested within treatment) were used to examine each of the four muscles for potential treatment effects. For all ANOVAs, a $P$ value less than 0.05 was considered significant.

## Results

During the recovery period, fish were observed daily to determine the time course of the toxin complex's effects. Between 3 and 5 d following injection, we noted a disruption of certain behaviors in treated fish. Although feeding and prey-tracking abilities remained largely unaffected, treated fish preferred to rest on the bottom of the tank and rarely held station midwater voluntarily. However, temporary station holding could be elicited by providing the fish with food or gently prodding them to leave the bottom.

Video recordings made of treated fish indicated that the pectoral fin on the side of the fish treated with the toxin did not move as far anteriorly during station holding as the pectoral fin on the untreated side. For example, one fish extended its untreated fin approximately 90° out from its body but extended its treated fin only approximately 35° (Fig. 2). During periods of time when the fish were station holding, we also observed apparent compensation for the lost range of motion with increased frequency and amplitude in movements of the dorsal, caudal, and anal fins.

Electromyographic recordings made of station-holding behavior in untreated individuals yielded patterns of muscle activity similar to those reported in studies of pectoral fin locomotion in other taxa (Westneat 1996; Drucker and Jensen 1997; Westneat and Walker 1997). For station holding, the left abductor superficialis and abductor profundus muscles are active simultaneously and show asynchronous periods of activity relative to the right-side muscles (Fig. 3*A*). Following toxin injection, the station-holding behavior is disrupted such that the asynchronous fin beat becomes inconsistent and the left and right muscles are active synchronously (Fig. 3*B*) as often as they are asynchronously (Fig. 3*C*). One fish treated with botulinum toxin complex did show some activity in the treated muscle (right abductor superficialis), but the activity appeared greatly reduced in magnitude relative to the paired untreated muscle in the same fish (left abductor superficialis) and the same muscle in untreated fish (Fig. 3*D*).

In the untreated fish that served to document baseline muscle activity, the mean frequency of burst activity did not differ significantly among all four muscles (3.6 Hz; $F = 0.0496$). In posttreatment individuals, the frequency of burst activity did not differ significantly for the two unmanipulated muscles (right and left abductor profundus) and the muscle that served as the positive control (left abductor superficialis; 3.5 Hz; $F = 0.0698$). However, the muscle that received a botulinum toxin injection (right abductor superficialis) showed significantly reduced activity (Fig. 4; 0.2 Hz; $F = 23.1010$). The four nested ANOVAs revealed a significant treatment effect for the right abductor superficialis but not for the other three muscles (Table 1; Fig. 4).

## Discussion

Behavioral observations of both fish station holding and burst frequency of pectoral fin muscles demonstrate that the abductor superficialis was chemically denervated during this experiment. After treatment with botulinum toxin complex, the fish showed a change in pectoral fin movements on the affected side during station-holding behavior, with little or no electrical activity in the targeted abductor superficialis. We conclude that the fish were no longer able to voluntarily stimulate this muscle with the motor neurons; thus, the muscle was unable to contract and produce force.

These data suggest that the abductor superficialis muscle can be chemically denervated with little effect on the adjacent abductor profundus. In fact, although we were not able to quantify this pattern because of the nature of our equipment (1,000-Hz capture rate is too slow to allow accurate measurements of rectified integrated area), there appears to be an increase in magnitude of abductor profundus activity on the denervated side (Fig. 3). We suggest that increased motor unit recruitment in the abductor profundus may compensate for the loss of the abductor superficialis. We also observed increased use of other fins during station-holding behavior, including the use of the dorsal and anal fins, presumably to compensate for the reduced effectiveness of the pectoral fin.

This study demonstrates that type A botulinum toxin com-

Table 1: Mean (Hz ± SE) of muscle activity and *F* statistics from four one-way nested ANOVAs

| | LP | LS | RP | RS |
|---|---|---|---|---|
| Pretreatment | 3.56 ± .06 | 3.44 ± .03 | 3.72 ± .17 | 3.81 ± .19 |
| Posttreatment | 3.58 ± .32 | 3.55 ± .34[a] | 3.38 ± .42 | .23 ± .23[b] |
| *F* statistic: | | | | |
| Treatment | .0006 | .0161 | .2363 | 33.9965 |
| Individual | .2054 | .2408 | .6253 | .2864 |

Note. Muscle activity shown in muscle bursts per second. ANOVAs were with individual nested within treatment. LP = left abductor profundus, LS = left abductor superficialis, RP = right abductor profundus, and RS = right abductor superficialis.

[a] Muscle injected with saline.

[b] Muscle injected with botulinum toxin.

plex can be used to selectively denervate a target muscle in a teleost fish, with little or no effect on the surrounding muscles. In addition, we note that denervation of the muscle caused a suite of behavioral changes in the animal that, under more controlled circumstances, could be used to elucidate muscle function. For example, treated fish showed asymmetry in fin abduction after the right-side abductor superficials was injected with botulinum toxin. As denervation of this muscle yielded decreased fin abduction, it appears this muscle may be responsible for the anteriormost excursion of the fin during swimming. Thus, we propose that this methodology may be successfully employed in future functional studies in teleost fishes and other vertebrates where traditional methods of determining muscle function are impractical.

There are some potential concerns with using botulinum toxin for chemical denervation. One potential problem that has been noted in previous studies is the ability of the toxin to diffuse out of the target muscle and into adjacent muscles (Shaari et al. 1991; George et al. 1992; Borodic et al. 1994; Eleopra et al. 1996), although developmental studies in finches and rats have effectively tested developmental hypotheses (Pytte and Suthers 2000; Landers et al. 2002). We propose that using research-grade botulinum toxin complex on nonhuman vertebrates, instead of the purified neurotoxin required for human use, may reduce or eliminate this problem. The botulinum toxin complex consists of six proteins plus the toxin protein (Brin 1997) and is approximately 500 kDa in size—whereas the purified neurotoxin is only 150 kDa. On the basis of cytoplasmic diffusion rates of other proteins (Arrio-Dupont et al. 2000), the passive diffusion rate of the toxin complex through muscle cytoplasm may be as much as three orders of magnitude less than that of the smaller, purified toxin. This implies that higher concentrations of the complex can be injected with little or no diffusion of the toxin complex out of the target muscle, which would allow complete paralysis of the target muscle without affecting adjacent muscles.

In addition to possible diffusion between muscles, there are other potential drawbacks to using botulinum toxin that have been observed in previous studies. The toxin can travel up motor neurons into the central nervous system, and thus the effects could potentially spread to other muscles or organ systems (Habermann 1974; Girlanda et al. 1992; Borodic et al. 1994), although we did not see evidence of this in our study. There is also some evidence that repeated injections create resistance to the toxin through antibodies (Greene et al. 1994; Jankovic and Schwartz 1995; Kessler and Benecke 1997), although limiting injections to one per experimental animal should avoid this problem. Finally, the positive correlation between frequency of muscle activity and completeness of paralysis (Hughes and Whaller 1962; Nathan et al. 1985; Kim et al. 2003) makes it likely that an infrequently used muscle will not be paralyzed as effectively as a continuously active muscle, although artificial stimulation of the targeted muscle may be employed to circumvent this potential problem (Kim et al. 2003).

We also noted two differences in the efficacy of the toxin in our study when compared with previous studies in mammalian systems. First, a considerably smaller amount of toxin was required for full paralysis in fish (0.4 MU) than in mammals (0.7 MU). Second, we observed a slower time to complete paralysis (7–9 d) than expected from mammalian studies (3–4 d). These discrepancies could be caused by any of a number of factors. (1) The toxin compound used here is larger in size than that used in previous studies. It is possible that keeping the toxin with its associated proteins improves the ability of the toxin to remain intact in the muscle, allowing effective paralysis to occur at lower doses. The associated proteins may also reduce the ability of the toxin to diffuse throughout the muscle, which may extend the time course required to paralyze the muscle. (2) Ectotherms have substantially lower metabolic rates than endotherms. A lower metabolic rate may contribute to a slower time to complete paralysis (because the rate of toxin uptake by the motor neuron will be lower), but it is not clear that a low metabolic rate would explain differences in sensitivity to the toxin. (3) Our experimental system was at a lower temperature (22°C) than mammalian studies (39°C); increased

temperature will increase diffusion and metabolic rates. As above, this effect would likely contribute to a slower time to complete paralysis in our system but not to the increased sensitivity to the toxin. (4) It is possible that fish use their fins less frequently than mammals used the locomotor muscles examined in previous studies. However, our fish muscles operated at about 3.5 Hz during station holding, whereas previous studies using artificial stimulation to enhance toxin uptake in rabbits stimulated muscles at only 0.1 Hz (Kim et al. 2003). In addition, we expect fish (which are continuously swimming) to use their locomotor muscles more often than mammals (which can lie on the ground or stand using postural muscles). We suggest that fish use the injected muscle more than injected mammals in any previous study and therefore consider this fourth possibility unlikely. Clearly, future studies comparing purified toxin and toxin complex in fishes will be necessary to distinguish potential phylogenetic physiological effects, temperature effects, and frequency-of-use effects from effects due to differences in the type of toxin compound injected.

We conclude that botulinum toxin is a tractable tool for examining muscle function in vertebrates. However, additional research is necessary to (1) clarify the ramifications of using botulinum toxin complex instead of the purified toxin and (2) determine the appropriate dosages to use for animals from different vertebrate groups because metabolism and/or muscle physiology may determine the susceptibility of a given group. In addition, botulinum toxin may not be useful in targeting very deep muscles because puncturing an overlying muscle with the needle delivering the toxin to an underlying muscle will likely render the incidentally punctured muscle more susceptible to the effects of the toxin (because the toxin will more readily diffuse across ruptured fascia; Shaari et al. 1991). However, for small muscles or muscle systems that cannot be studied using more traditional techniques, chemical denervation holds great promise for use in functional studies.

## Acknowledgments

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