

Effects of Restraint and Immobilization on Electro sensory Behaviors of Weakly Electric Fish

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Abstract

Weakly electric fishes have been an important model system in behavioral neuroscience for more than 40 years. These fishes use a specialized electric organ to produce an electric field that is typically below 1 volt/cm and serves in many behaviors including social communication and prey detection. Electrical behaviors are easy to study because inexpensive and widely available tools enable continuous monitoring of the electric field of individual or groups of interacting fish. Weakly electric fish have been routinely used in tightly controlled neurophysiological experiments in which the animal is immobilized using neuromuscular blockers (e.g., curare). Although experiments that involve immobilization are generally discouraged because it eliminates movement-based behavioral signs of pain and distress, many observable electro sensory behaviors in fish persist when the animal is immobilized. Weakly electric fish thus offer a unique opportunity to assess the effects of immobilization on behaviors including those that may reflect pain and distress. We investigated the effects of both immobilization and restraint on a variety of electro sensory behaviors in four species of weakly electric fishes and observed minor effects that were not consistent between the species tested or between particular behaviors. In general, we observed small increases and decreases in response magnitude to particular electro sensory stimuli. Stressful events such as asphyxiation and handling, however, resulted in significant changes in the fishes' electro sensory behaviors. Signs of pain and distress include marked reductions in responses to electro sensory stimuli, inconsistent responses, and reductions in or complete cessation of the autogenous electric field.

Key Words: curare; distress; electric fish; electric organ discharge (EOD); electro communication; immobilization; jamming avoidance response (JAR); pain; restraint; stress

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Introduction

Weakly electric fish emit an electric organ discharge (EOD¹) using a specialized electric organ located in the tail. EOD properties such as fundamental frequency are highly regulated by central nervous system (CNS) circuits (Heiligenberg 1991). Electroreceptors in the animal's skin detect perturbations of the EOD caused by nearby objects, prey, and the electric fields of conspecifics (Nelson and MacIver 1999; Turner et al. 1999; Zakon et al. 2002). For many species of electric fish, simple sine wave electric signals at species-appropriate frequencies and amplitudes can elicit behavioral responses from conspecifics (Heiligenberg 1991).

Most species of weakly electric fishes actively modulate their EODs depending on the behavioral context. For example, individual fish may produce stereotyped changes in the EOD during agonistic encounters and courtship (Zakon et al. 2002), to avoid jamming by conspecifics (Heiligenberg 1991), and in reaction to novel stimuli in the environment (Post and von der Emde 1999). These behaviors can persist in animals that have been pharmacologically immobilized (Heiligenberg 1991); because the pharmacological agents do not cross the blood brain barrier (Friedemann 1942) they do not directly affect brain processes.

The persistence of complex behaviors in awake, behaving but immobilized fish has been a principal reason for their extensive use as a model system for studying the neural mechanisms that control behavior (Carlson and Kawasaki 2006; Chacron 2006; Chacron and Bastian 2008; Chacron et al. 2003, 2005; Heiligenberg 1991; Heiligenberg et al. 1996; Juranek and Metzner 1998; Krahe et al. 2008; Oestreich and Zakon 2005; Ramcharitar et al. 2006; Sawtell et al. 2005, 2006; Zakon et al. 2002). In fact, the neural circuit used for the control of one such electrical behavior, the jamming avoidance response (JAR¹), has been described at a functional level that has not been achieved for any other model system (Fortune 2006; Heiligenberg 1991).

This progress, however, has relied on neurophysiological experiments conducted in awake, behaving animals that were immobilized using neuromuscular blockers without general

¹ Abbreviations used in this article: Δf , difference between a fish's EOD frequency and the stimulation frequency; EOD, electric organ discharge; JAR, jamming avoidance response

anesthesia. In most animals, behavioral signs of pain and distress are manifest in changes in behaviors that involve movement (Hargreaves et al. 1988; NRC 2008, 2009), but with immobilized animals it is impossible to use such indicators. The use of neuromuscular blockers is therefore generally not recommended. Further compelling evidence against the use of immobilizing agents without general anesthesia comes from mistakes in drug delivery during surgeries in humans (Sebel et al. 2004); patients who experience “anesthesia awareness” report intense distress during these surgeries, which may have dramatic long-term negative consequences on the psychology of the patient (Osterman et al. 2001).

It is well known that fish can display classical signs of stress such as heart rate elevation² or altered behavior (Mazeaud et al. 1977), and a growing body of evidence suggests that fish have all the necessary receptors and brain structures for experiencing distress and pain (Chandross et al. 2004; Sneddon 2009, Volpato 2009, in this issue). The complex electrical behaviors of weakly electric fish may therefore be a mechanism for monitoring possible pain and distress in these animals during experiments in which the animal is immobilized without general anesthesia. Although hundreds of studies have relied on such experimental preparations, there have been no systematic investigations of possible behavioral signs of pain and distress nor any quantitative comparisons of suites of electric behaviors in freely moving, restrained (confined), and immobilized animals.

We performed quantitative comparisons of electrical behaviors on four species of weakly electric fish under three different behavioral conditions: (1) unrestricted swimming in the experimental tank; (2) restraint (confinement) of the animal in a small enclosure in the experimental tank; or (3) immobilization of the animal by injection of a paralytic agent that blocks neuromuscular junctions.

These experiments are of general interest due to the difficulty of assessing behavioral state, pain levels, and distress in immobilized animals. They are also important because the use of general anesthetics in behavioral neuroscience experiments is problematic as anesthetic and analgesic drugs almost always have profound effects on the functional properties of the neural circuits under study. The activity of neurons in awake but restrained or immobilized animals without general anesthesia is certainly more typical of normal functioning.

Four Categories of Weakly Electric Fishes

There are four functional categories of freshwater weakly electric fishes. First, there are two phylogenetically independent clades of weakly electric fishes: the Gymnotiformes in South America and the Mormyriiformes of Africa. Although these clades evolved within two distantly related groups, the ostariophysans and osteoglossomorphs, respectively (Alvez-Gomes 2001), they share a remarkable number of convergent traits

related to the generation and sensing of weak electric fields, including neural and somatic specializations, electrosensory behaviors, and functional adaptations.

Second, species in both clades produce “pulse-type” and “wave-type” electric signals. Pulse-type electric signals are short, typically less than 2 milliseconds (ms) in duration, with longer (typically > 10 ms) intervals between pulses. The waveforms of the pulses exhibit enormous diversity among species, from monophasic up to at least six phases (Hopkins 1988). In some species the pulse rate is nearly constant, whereas others exhibit extraordinary variation in pulse rate over time. In wave-type electric signals, the pulses and interpulse intervals have roughly the same durations, ranging from as long as 50 ms to as little as 0.4 ms. As a result, the EOD signal of these fish appears quasisinusoidal and is best described by its fundamental frequency, which can range from 20 to 2500 Hz.

Thus the four categories of weakly electric fish are pulse- and wave-type gymnotiform and mormyriiform fishes. Examples of pulse-type gymnotiform genera are *Brachyhypopomus*, *Gymnotus*, and *Steatogenys*. Wave-type gymnotiform genera include *Apteronotus*, *Eigenmannia*, *Sternopygus*, *Sternarchorhynchus*, and *Sternarchella*. Examples of pulse-type mormyriiform genera are *Gnathonemus*, *Brienomyrus*, *Genyomys*, *Heteromormyrus*, and *Mormyrus*. The sole mormyriiform wave-type genus is *Gymnarchus*.

Experimental Methods

The animal care and use committees at both McGill University and the Johns Hopkins University approved all procedures for animal husbandry and electrosensory experiments described here. These experiments follow the guidelines for the use of animals in research established by the US National Research Council (NRC 1996), the Society for Neuroscience (www.sfn.org), and the Canadian Council on Animal Care (CCAC 2005).

Animal Husbandry

We used three gymnotiform wave-type species—*Apteronotus albifrons*, *A. leptorhynchus*, and *Eigenmannia virescens*—and one gymnotiform pulse-type species, *Brachyhypopomus brevirostris*.³ All fish were obtained from commercial ornamental fish dealers and acclimated in the laboratory for at least 2 weeks before experimentation.

These fishes are wild caught, since there is no commercial breeding at present, so their health on arrival at the laboratory facility can vary dramatically. The initial health and parasite load of the fish are critically dependent on the environmental conditions at the point of capture. Furthermore,

² In a preliminary measurement, however, we found that heart rate was not affected by immobilization in one *Apteronotus leptorhynchus*.

³ We attempted to use *Gymnarchus niloticus* in this study, but the individuals available to us did not, for unknown reasons, respond to any electrosensory stimuli and so were not included.

transportation can induce extreme stress in the animals; proper transportation involves placing them in plastic bags with a small amount of water (typically a liter or less) and a large volume of pure oxygen (typically 2 or more liters). Finally, maintaining temperatures above 24°C is critical for the health of the animals. In light of all these variables, some shipments of fish experience 100% mortality within 2 weeks of arrival from a commercial vendor. To our knowledge, there has not been a systematic attempt to assess the best treatment for newly arrived weakly electric fish. Traditional treatments such as formaldehyde are not well tolerated by these scaleless fishes. Compounds such as malachite green or methylene blue can be used to treat outbreaks of parasitic infections such as *Ichthyophthirius multifiliis* but only at doses that are lower than those recommended for scaly fish. The most common recommendation we have received from veterinarians and professional aquarists is to dip newly arrived fish in seawater (saltwater) for seconds to minutes before transferring them to the laboratory tanks.

Considerations for laboratory housing of gymnotiform fish are species dependent. We typically maintain a minimum of 1 gallon per fish in laboratory tanks. *Eigenmannia* and *Brachyhypopomus* are highly social fishes (Tan et al. 2005) and can be kept in large tanks with large numbers of fishes, whereas *A. leptorhynchus* and *A. albifrons* can be more aggressive toward conspecifics and so are kept in groups of 8 or 9 with tank dividers; communal housing of larger numbers of individuals of *Apteronotus* species is possible, however (Oestreich and Zakon 2005). *Steatogenys* and *Rhamphichthys* are also social and can be housed in large numbers in tanks. These animals typically rest on the substrate during light periods and are active only during darkness. Because these two genera and *Sternopygus* are more prone to jumping out of tanks than other groups of gymnotiform fishes, tank covers are advisable.

All of these gymnotiform species can be housed in multispecies tanks with no apparent negative effects on animal health as diseases often affect just one species in a communal tank (unpublished observations, ESF). This arrangement can reduce the overall space necessary to house the fish.

The same is not true, however, for *Gymnotus* and *Gymnarchus*, which can be aggressive toward both conspecifics and heterospecific weakly electric fishes. These animals should not be housed in multispecies tanks but instead either in small numbers in large tanks or in tanks with dividers. Whether these fish are compatible with nonelectrogenic fishes has not been determined in laboratory settings.

Weakly electric fishes readily eat frozen bloodworms, frozen or live brine shrimp, frozen mysis shrimp, or live blackworms, but they will not eat fish flakes. Feeding can be as often as daily or as infrequent as every 3 days. As with most aquatic species, it is imperative not to overfeed the fish as leftover food can rapidly foul the water and cause death. Live blackworms are advantageous as they can survive in the tank until they are eaten. It is advisable to provide frozen foods to the animals directly before or during dark periods

when the fish are active and will feed, to reduce the possibility that these foods will foul the water.

The pH of the water should be maintained between 6.5 and 7.5, the temperature 24–30°C, and conductivity between 25 and 2000 μS . In electric fish habitats in eastern Ecuador, the water is slightly acidic (between 6.5 and 7.0) and has very low conductivity (<50 μS). In general, most laboratories maintain conductivities on the order of hundreds of μS and pH that is slightly basic (between 7.0 and 7.5). As these animals most commonly live near the equator, day:night cycles of 12:12 are appropriate, although constant darkness does not have any deleterious effects on fish survival in the laboratory.

Experimental Setup

Recording the electric fields of fish does not require complicated hardware. A simple and inexpensive audio amplifier (e.g., Radio Shack model 277-1008) is effective for amplifying the signals. The probe can be constructed using two insulated wires with 1 cm of wire exposed at the end. These two ends can be attached to a stick, 4 to 15 cm apart, which can then be placed in the water within a few centimeters of the fish. The wires from the probe should connect to the two leads of a mono plug that fits into the input jack of the amplifier. The signal can be recorded using a variety of devices, including portable MP3 encoders and normal computer soundcards. Visualization of the signals can be achieved using any number of free or commercial software packages for sound display and analysis.

Our experimental setup was similar to that of in vivo neurophysiological experiments (Bastian et al. 2002; Chacron 2006; Chacron and Bastian 2008; Chacron et al. 2003, 2005; Fortune and Rose 2003; Krahe et al. 2008; Mehaffey et al. 2008; Ramcharitar et al. 2005, 2006; Rose and Fortune 1999). Briefly, a pump circulated water from the animal's home tank between an experimental tank and a heated reservoir, maintaining a constant temperature of 27–29°C for the water in the experimental tank. It is very important to maintain this temperature range as changes in the water temperature induce changes in the fishes' EOD frequency. A PVC tube in the tank provided a refuge for the animal. A pair of electrodes (E1–E2) at the front and back of the tank measured the EOD of the fish, and another pair (G1–G2) on each side delivered electrical stimuli to the animal (Figure 1).

For myogenic species, including those used in this study, curare and other neuromuscular blockers reduce the EOD amplitude 100- to 3000-fold. The residual EOD and neural command signal can then be monitored after immobilization using a pair of electrodes placed adjacent to the tail (Fortune and Rose 2003; Ramcharitar et al. 2005, 2006; Rose and Fortune 1999).

The amplitude of the EOD (for *Apteronotus*) or the residual amplitude (for all other genera of gymnotiform fishes) provides continuous information about the health of the immobilized animal. Stressful conditions like anoxia lead to

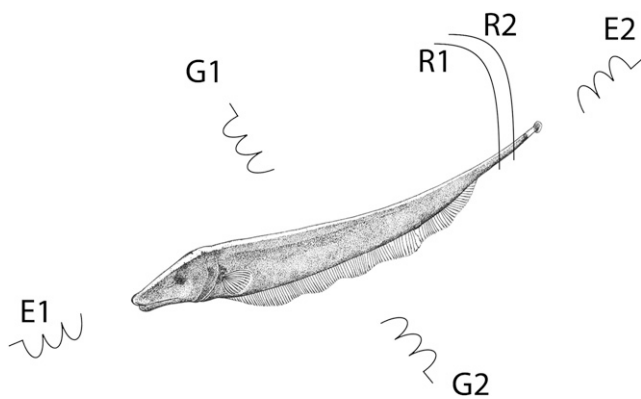


Figure 1 Schematic of the stimulus delivery system. The animal is placed in an experimental tank with electrodes located along the sides of the tank. Electrodes E1-E2 are used to monitor the animal's electric organ discharge (EOD) and electrodes G1-G2 deliver the stimuli. A hideaway (such as a PVC tube, not shown) can be placed at the center of the tank for freely moving conditions. For immobilized myogenic species, we used electrodes R1-R2 to monitor the residual EOD.

rapid (within seconds) decreases in amplitude and other more complex changes in electrosensory behavior, described below, although the amplitude can recover to its original value within perhaps 1 or 2 minutes after removal of the source of stress. Importantly, with proper respiration, the amplitude of the EOD or residual EOD can remain stable for over 7 hours (Bastian 1981; Chacron 2006).

Stimulation

Voltage changes across electroreceptors in the skin mediate the fish's electrosensory perception (Heiligenberg 1991). These electroreceptors are typically tuned to the fish's own EOD waveform (Zakon 1986) and detect amplitude and phase modulations of the animal's own EOD caused by any stimulus that alters the nearby electric field (i.e., any object with a conductivity different from that of the water). Experimenters can present signals with well-characterized parameters to elicit rapid and reliable electrosensory behaviors from these fishes (Heiligenberg 1991).

In the laboratory setting, we typically use sophisticated hardware for the generation of electrosensory stimuli. However, any battery-powered signal source is effective and used in field studies. For instance, an MP3 player can deliver signals to the fish via a probe similar to that described above; and inexpensive battery-powered sine wave generators, available as kits, are ideal for the study of the JAR and chirping (more on this below) in wave-type weakly electric fishes.

We used sinusoidal stimuli to elicit JARs and chirps in wave-type species. We produced sinusoids (delivered through electrodes G1-G2; Figure 1) at various frequencies near the fish's own EOD. The amplitudes of electrosensory stimuli for all species were between 0.1 and 2 mV/cm (amplitudes are adjusted to match the field strength of the subject). For *A. albifrons* and *A. leptorhynchus* ($n = 8$ for each), we presented

sinusoidal stimuli with frequencies 2 Hz, 4 Hz, 6 Hz, 8 Hz, and 10 Hz lower than the animal's EOD frequency. These stimuli elicit increases in the fish's EOD frequency as part of its JAR, and chirping in male *A. leptorhynchus* (Zakon et al. 2002). For *E. virescens* ($n = 7$; an eighth fish was stressed due to excessive handling and was not included in this group; data from this fish are discussed separately, under Experiment 4 below, and shown in a subsequent figure), we presented sinusoidal stimuli with frequencies that were 2.5 Hz, 5 Hz, 10 Hz, and 20 Hz lower and higher than the animal's EOD frequency. These stimuli elicited either increases or decreases, respectively, in the EOD frequency. Finally, we used a mechanical stimulus to elicit novelty responses in *B. brevirostrus* ($n = 8$) by dropping a hacky sack near the tank from a constant height of 30 cm.

We recorded a baseline measurement of the EOD for at least 10 seconds (typically 40 seconds) before stimulation. The stimulus was presented for at least 10 seconds, but usually 60 seconds, to permit sufficient time for the EOD frequency to reach an asymptote. We then recorded the poststimulation EOD for at least 10 seconds and more commonly 40 seconds, which is sufficient time for the EOD to return to baseline. Each fish in each of the three experimental conditions—freely moving, restrained, and immobilized—experienced each stimulus condition 2 to 6 times (but some trials were rejected because of technical problems that prevented reliable measurements of the response).

Data Analysis

We monitored the instantaneous EOD frequency at all times. In healthy wave-type weakly electric fishes, it showed little variance—typically less than 1 Hz—in the absence of stimulation.

We quantified the JAR by computing the frequency excursion during this behavior and by fitting exponentials to changes in the rise and decay phases of EOD frequency (see Figure 2). For male *A. leptorhynchus*, the JAR was frequently accompanied by brief, highly stereotyped increases in EOD frequency called chirps (Zakon et al. 2002). We computed the average chirp rate during the stimulus. *Eigenmannia* also produced chirps, but very infrequently (a total of 17 in the entire experiment); they occurred during all phases of the experiment and did not appear to be in response to any of the stimuli.

Some pulse-type species such as *B. brevirostrus* routinely display transient increases in EOD frequency when encountering novel stimuli. We quantified these novelty responses by computing the change in the electric pulse rate (Hz) and fitting an exponential to the baseline return after the response.

Behavioral Conditions

We tested each animal under three conditions: freely moving, restrained (confined), and immobilized. For the first of these, we transported the animal from its home tank to the experimental tank and allowed it to acclimate for at least

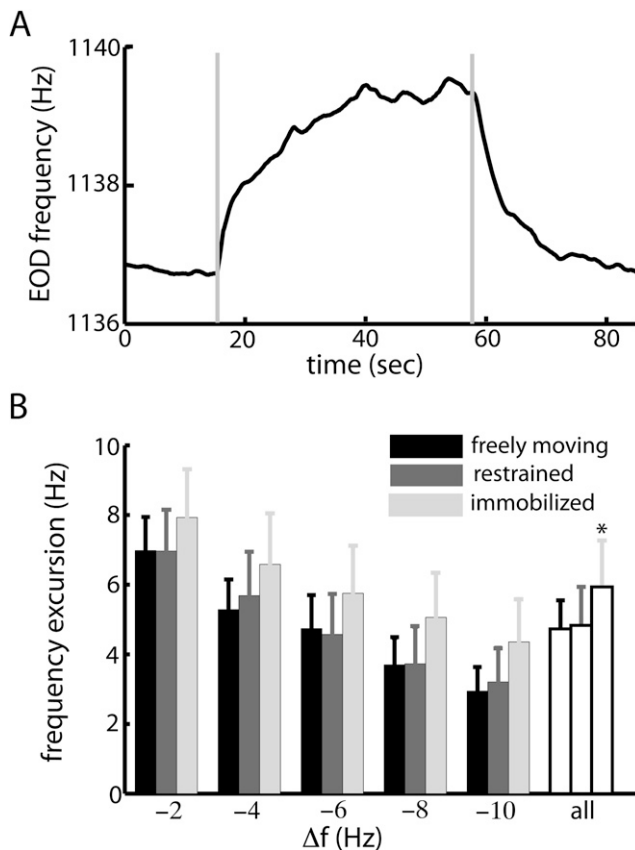


Figure 2 Effects of restraint and immobilization on the jamming avoidance response (JAR) in *Apteronotus albifrons*. (A) Sample recording showing the animal's EOD frequency as a function of time. The gray bars indicate the onset and offset of the sinusoidal stimulus frequency 10 Hz below the animal's EOD frequency. (B) Population-averaged ($n = 8$) frequency excursion as a function of Δf for all three conditions. * indicates a statistically significant difference (paired t-test, $p < 0.05$) between immobilized and freely moving conditions. EOD, electric organ discharge; Δf , difference between the fish's EOD frequency and the stimulation frequency.

20 minutes. After this we delivered either electrical or mechanical stimuli as described above and recorded the animal's EOD through electrodes E1-E2. For the restrained condition, we placed the animal in a "chirp chamber," a PVC tube covered with nylon mesh. For the final test condition, we used curare-like drugs (tubocurarine, pancuronium bromide, or gallamine triethiodide, 2 to 200 $\mu\text{g/g}$ fish, depending on the species and drug) to immobilize the animal; in general, we found that *Apteronotus* tolerated larger doses than *Eigenmannia* or *Brachyhypopomus*. After the cessation of gill movements, which typically occurred 30 seconds to 5 minutes after injection, we rapidly placed the mouth of the animal on a small pipe with a constant flow of tank water (~ 10 ml/min) (Bastian et al. 2002; Chacron 2006; Post and von der Emde 1999; Krahe et al. 2008) and repeated the stimulation protocol. For *Apteronotus*, which have neurogenic EODs, we used electrodes E1-E2 to measure the EOD. For species that have myogenic EODs, such as *E. virescens* and *B. brevirostris*, the curare-like drugs attenuated the EOD

amplitudes 100- to 1000-fold, so we used electrodes R1-R2 to measure the residual EOD (Figure 1).

The Four Experiments

Experiment 1: The Jamming Avoidance Response

Wave-type weakly electric fish maintain a nearly constant discharge frequency, the range of which depends on the species—for instance, *Eigenmannia* produce frequencies between 200 and 700 Hz. When two fish come in close proximity (i.e., a meter or less) their electric fields mix, producing amplitude and phase modulations that are detected by both fish. If the EOD frequencies are close (i.e., typically with a difference of less than 10 Hz), the interference patterns impair the electrosensory perception of both fish (differences in EOD frequencies of 20 Hz or more do not have this effect; Heiligenberg 1991). The JAR can be reliably elicited in experiments by delivering a sinusoidal electric field in the experimental tank that is within a few Hz of the animal's discharge frequency.

E. virescens, *A. leptorhynchus*, and *A. albifrons* solve the problem of impaired perception (i.e., "jamming") by changing their EOD frequencies. In *Eigenmannia*, the most intensively studied species for the JAR, the animal with the higher frequency increases it and the other fish lowers its frequency, and thus the two fish increase the difference between their EOD frequencies. *Apteronotus* have a unidirectional JAR—the fish with the higher initial EOD frequency will raise its frequency while that of the other fish remains unchanged; the frequency usually returns to its baseline value after the brief (i.e., 30 sec) presentation of the jamming stimuli.

Apteronotus albifrons

Sinusoidal stimuli that were a few Hz below the animal's EOD frequency elicited an increase in the frequency (Figure 2A) that returned to the baseline value after termination of the stimulus. We quantified this behavior by computing the change in frequency elicited by a variety of sinusoidal electrosensory stimuli. The difference between the fish's EOD frequency and the stimulation frequency is known as the Δf .¹ Smaller Δf s gave rise to higher-frequency JARs, as other studies have reported (Heiligenberg 1991).

We found that the JARs in this species were qualitatively similar under the three conditions. But when we pooled the data across all stimulus conditions we found a slight but statistically significant ($p = 0.001$, pairwise t-test, $n = 40$) increase in the amplitude of the JAR when the fish were immobilized (Figure 2B). We also measured the rise and decay time constants of the JAR and found that restraint caused a significant drop in both relative to unconstrained fish (rise: $p = 0.064$, decay: $p = 0.016$, pairwise t-tests, $n = 40$; Figure 3). Immobilization by curare, however, caused a statistically

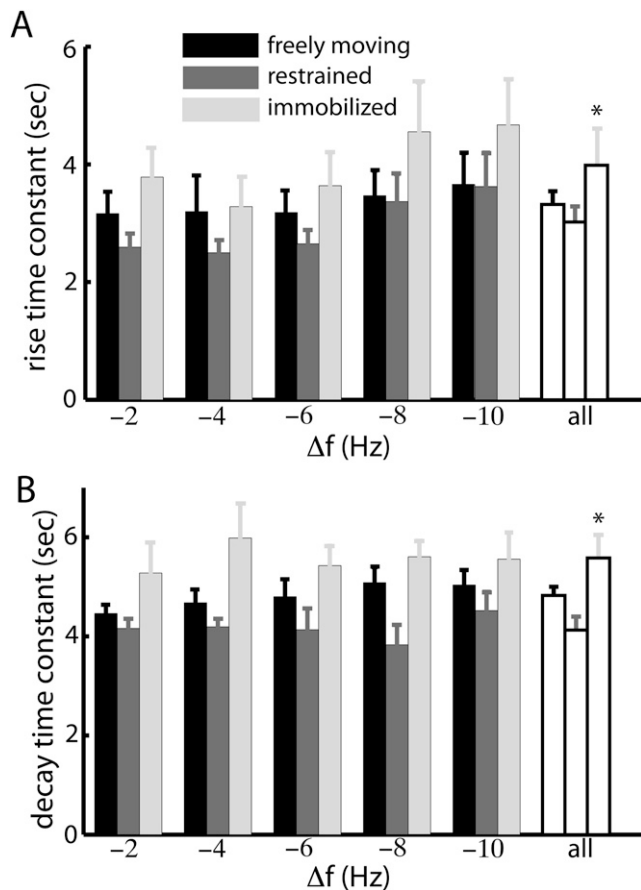


Figure 3 Effects of restraint and immobilization on rise and decay time constants of the jamming avoidance response (JAR) in *Apterionotus albifrons*. (A) Population-averaged ($n = 8$) rise time constants as a function of Δf for all three conditions. (B) Population-averaged ($n = 8$) decay time constants as a function of Δf for all three conditions. Δf , difference between the fish's electric organ discharge (EOD) frequency and the stimulation frequency.

significant increase in both time constants relative to unconstrained fish (rise: $p = 0.056$, decay: $p = 0.007$, pairwise t-tests, $n = 40$; Figure 3). As a consequence of the opposite effects of restraint and immobilization on these quantities, the rise time constant was significantly different under restraint and immobilized conditions when we pooled the data across these two stimulus conditions (rise: $p = 0.0009$, decay: $p = 0.0001$, pairwise t-tests, $n = 40$). The decay time constant was significantly different under restraint and immobilized conditions for $\Delta f = -4$, -6 , and -8 Hz as well ($p = 0.047$, 0.035 , 0.021 , respectively; pairwise t-tests, $n = 8$).

Apterionotus leptorhynchus

As with *A. albifrons*, we found with *A. leptorhynchus* that sinusoidal stimuli with frequencies a few Hz below the animal's EOD frequency elicited an increase in EOD frequency (i.e., a JAR) (Figure 4A). However, these stimuli also elicited brief (~40 ms) increases in EOD frequency called chirps. Restraint caused an increase in the frequency excursion that

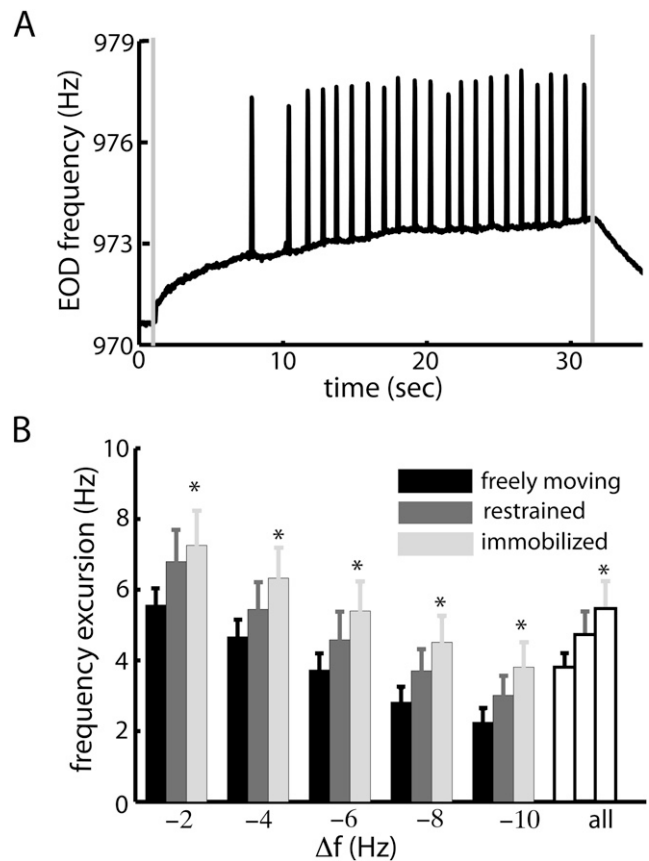


Figure 4 Effects of restraint and immobilization on the jamming avoidance response (JAR) in *Apterionotus leptorhynchus*. (A) Sample recording showing the animal's EOD frequency as a function of time. The gray bars indicate the onset and offset of the sinusoidal stimulus whose frequency was set 10 Hz below the animal's EOD frequency. The stimulus also elicited chirps (brief increases in the EOD frequency, shown as vertical bars). (B) Population-averaged ($n = 8$) frequency excursion as a function of Δf for all three conditions. EOD, electric organ discharge; Δf , difference between the fish's EOD frequency and the stimulation frequency; * indicates a statistically significant difference (paired t-test, $p < 0.05$) between immobilized and freely moving conditions.

was significant only when the data were pooled across all Δf s ($p = 0.004$, pairwise t-test, $n = 40$; Figure 4B). Immobilization caused an increase in the frequency excursion of JARs, which were significant for all values of Δf tested ($p < 0.04$, pairwise t-tests, $n = 8$). The differences in frequency excursion between restrained and immobilized conditions were significant only when the data were pooled across Δf s ($p = 0.001$, pairwise t-test, $n = 40$).

Restraint caused small decreases and increases overall in the rise and decay time constants, respectively (Figure 5). These were not statistically significant except for $\Delta f = -10$ Hz, where restraint caused a significant decrease in the rise time constants ($p = 0.045$, pairwise t-test, $n = 40$). Immobilization caused an increase in the rise and decay time constant that was statistically significant only when data from all Δf s were pooled (rise: $p = 0.0002$, decay: $p = 0.0001$, pairwise t-tests, $n = 40$).

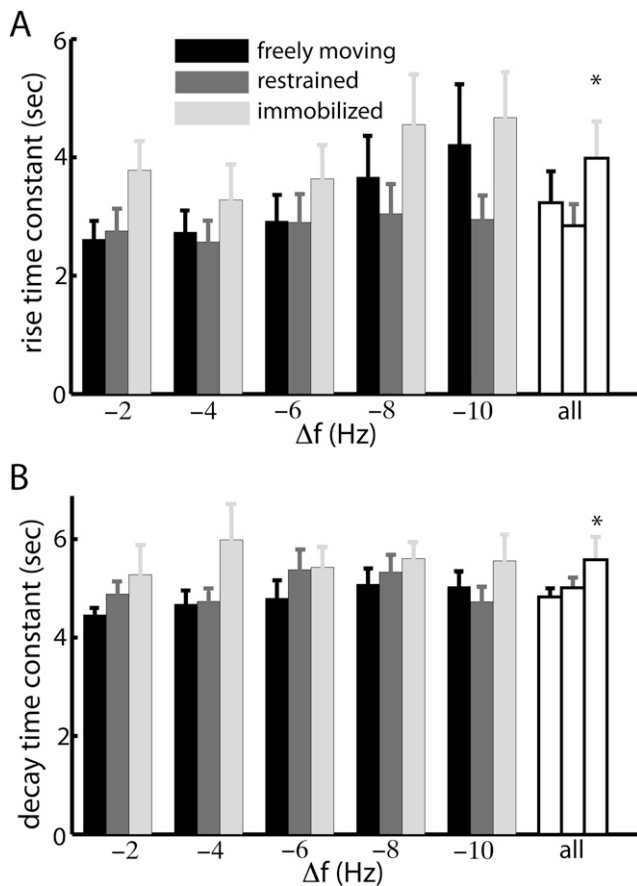


Figure 5 Effects of restraint and immobilization on rise and decay time constants of the jamming avoidance response (JAR) in *Apteronotus leptorhynchus*. (A) Population-averaged ($n = 8$) rise time constants as a function of Δf for all three conditions. (B) Population-averaged ($n = 8$) decay time constants as a function of Δf for all three conditions. Δf , difference between the fish's electric organ discharge (EOD) frequency and the stimulation frequency; * indicates a statistically significant difference (paired t-test, $p < 0.05$) between immobilized and freely moving conditions.

Eigenmannia virescens

As this species has a myogenic electric organ, immobilization by curare eliminates the EOD, so a different paradigm is necessary for stimulating the immobilized fish. One method is to replace the fish's own EOD with a sinusoidal electric field, called the S1, delivered by two electrodes positioned at the mouth and tail of the animal (Heiligenberg 1991). We monitored the residual EOD, composed of the command signal from the nervous system and a reduced-amplitude EOD, through a pair of electrodes (R1-R2) located near the animal's tail. This residual signal in the immobilized fish has the same frequency as the animal's original EOD.

It is possible to have the S1 stimulus freely running and thus independent of the EOD residual produced by the fish; we refer to this condition as "open-loop." In contrast, it is possible to measure the instantaneous frequency from the EOD residual and make the S1 frequency follow its changes; we refer to this condition as "closed-loop." The latter is more

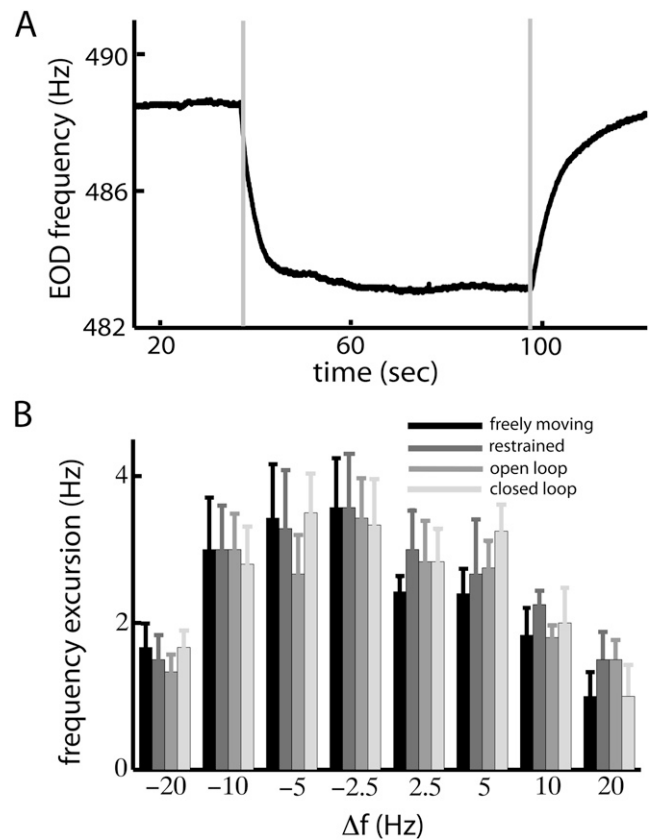


Figure 6 Effects of restraint and immobilization on the jamming avoidance response (JAR) in *Eigenmannia virescens* (see text for a description of "open-" and "closed-loop" conditions). (A) Sample trace showing the animal's EOD frequency as a function of time. The gray bars indicate the onset and offset of the sinusoidal stimulus with a frequency 10 Hz below the animal's EOD frequency. (B) Population-averaged ($n = 7$) frequency excursion as a function of Δf for all four conditions. EOD, electric organ discharge; Δf , difference between the fish's EOD frequency and the stimulation frequency.

naturalistic because the fish has control over the electric field in the water, but it is possible to elicit a JAR under both open- and closed-loop conditions as the S1 frequency need not be exactly equal to the animal's EOD frequency (Heiligenberg 1991). In fact, any S1 frequency within ± 50 Hz of the fish's EOD frequency is effective to elicit this electrosensory behavior. Stimuli a few Hz above the animal's EOD frequency led to a drop in the EOD frequency, whereas those below increased it. Figure 6A shows an example where the stimulus frequency was 2.5 Hz above the EOD frequency under freely moving conditions.

We characterized the JAR in *Eigenmannia* using the same measures as for *Apteronotus*. Overall, we found that the frequency excursion had a similar dependency on Δf under the following conditions: freely moving, restrained, immobilized open loop, and immobilized closed loop. We found no statistically significant difference between the four conditions (Figure 5B). We also quantified the rise and decay time constants and overall found no statistically significant

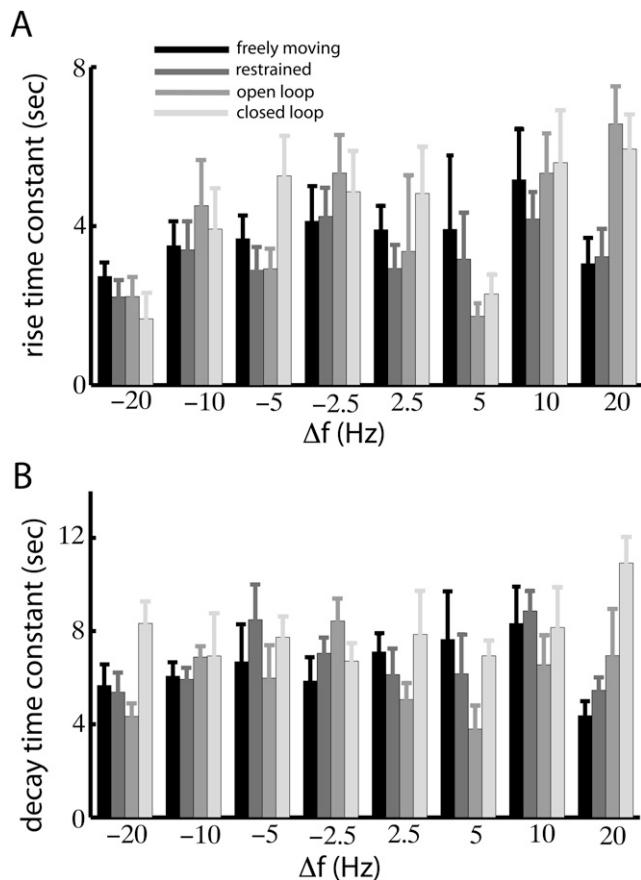


Figure 7 Effects of restraint and immobilization on rise and decay time constants of the jamming avoidance response (JAR) in *Eigenmannia virescens* (see text for a description of “open-” and “closed-loop” conditions). (A) Population-averaged ($n = 8$) rise time constants as a function of Δf for all four conditions. (B) Population-averaged ($n = 8$) decay time constants as a function of Δf for all four conditions. Δf , difference between the fish’s electric organ discharge (EOD) frequency and the stimulation frequency.

differences across all four conditions (Figure 7): all t-values using pairwise t-tests were >1.5 , with p values of 0.1 to 0.9.

Experiment 2: Social Signaling—The Production of Electrical Chirps

Many species of weakly electric fishes make stereotyped brief changes in their electric field properties, using different types of chirps as a form of communication in mate choice, reproduction, and aggressive displays (Hupé and Lewis 2008). Chirping behavior has been most studied in male *A. leptorhynchus* (Zakon et al. 2002; Zupanc and Maler 1993), which reliably display transient (10–20 ms long) frequency increases in their discharge rates during agonist and courtship behavior. These chirps can also be reliably elicited through appropriate sinusoidal electrical stimulation. We used low Δf s and observed mostly chirps that occur during agonistic encounters (Figure 8A).

We compared the rates of chirping under the three conditions in *A. leptorhynchus* and found that restraint caused a

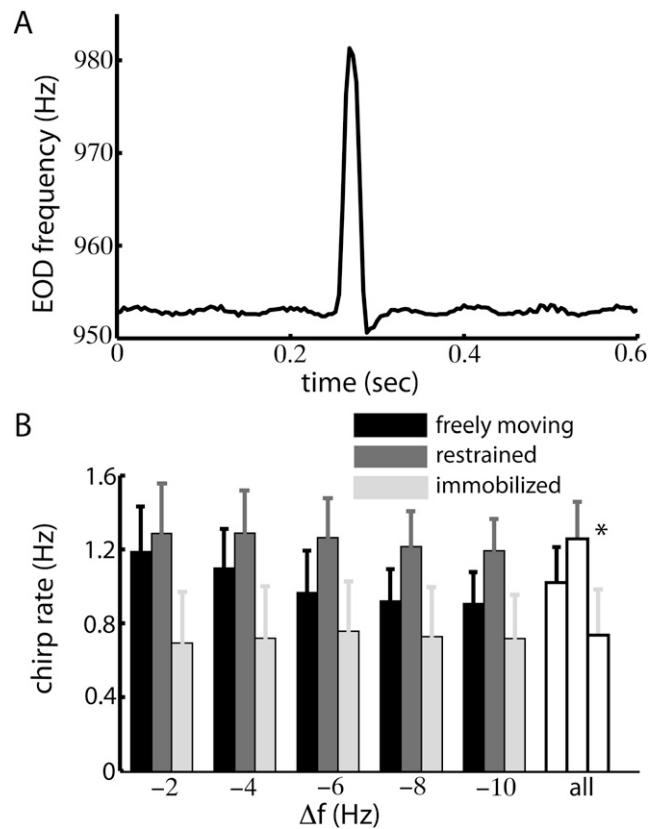


Figure 8 Effects of restraint and immobilization on chirping in *Aptereronotus leptorhynchus*. (A) Sample chirp waveform showing the electric organ discharge (EOD) frequency as a function of time. (B) Chirp rate as a function of Δf for all three conditions. Δf , difference between the fish’s EOD frequency and the stimulation frequency; * indicates a statistically significant difference (paired t-test, $p < 0.05$) between immobilized and freely moving conditions.

small increase in chirp rate that was significant only when we pooled all the data across Δf s. Immobilization, on the other hand, led to a 33% decrease in chirp rate that was significant for all values of Δf s tested ($p < 0.04$, paired t-tests, $n = 8$).

Experiment 3: Novelty Responses

In pulse-type species, fish transiently increase their EOD frequencies in response to novel stimuli and this behavior, known as the novelty response, rapidly habituates (Lopes Correa and Hoffmann 1998; Post and von der Emde 1999) (Figure 9A).

We investigated the effects of restraint and immobilization on the novelty response in the pulse gymnotiform species *B. brevirostrus*. We quantified this behavior by the frequency excursion (i.e., the maximum EOD frequency increase), the decay time constant obtained by fitting an exponential function, and the latency to peak frequency. Overall, restraint caused increases in the frequency excursion (Figure 9B), the decay time constant (Figure 9C), and the latency to peak (Figure 9D), but only the increases in frequency excursion

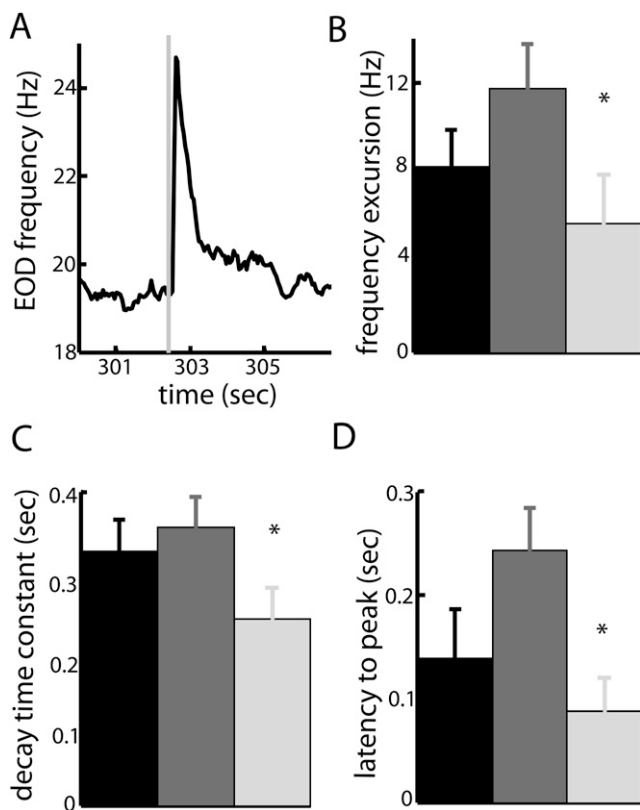


Figure 9 Effects of restraint and immobilization on the novelty response in *Brachyhyopomus brevirostrus*. Black column = free movement; dark gray = restraint; light gray = immobilization. (A) Sample novelty response under freely moving conditions. The gray bar indicates delivery of mechanical stimulation. (B) Maximum frequency rise for all three conditions. (C) Latency for peak for all three conditions. (D) Decay time constant for all three conditions. EOD, electric organ discharge; * indicates statistical significance at the $p = 0.05$ level using a paired t-test.

and latency to peak were significant. Immobilization had the opposite effect of restraint in this species and caused a significant decrease in all three quantities ($p < 0.04$, paired t-tests, $n = 8$).

Experiment 4: Evidence of Pain and Distress

In three experiments with *Eigenmannia*, the fish exhibited signs of pain and distress. In one experiment, the mouth of the animal was just a little too small for the breathing tube, and excessive handling was necessary in order to place the animal on the tube. This animal had irregular and attenuated JARs (Figure 10A) that were statistically different from unconstrained responses (Figure 10B). This fish also did not maintain its EOD frequency and so was not included in the JAR measurements above. In two other animals, water flow to the gills became unexpectedly restricted during the experiment, resulting in rapid anoxia, rapid degradation of the JAR, and a drop in the amplitude of the residual EOD. Data from these animals prior to the anoxia were used in the JAR measurements above. Restoration of water flow over the gills

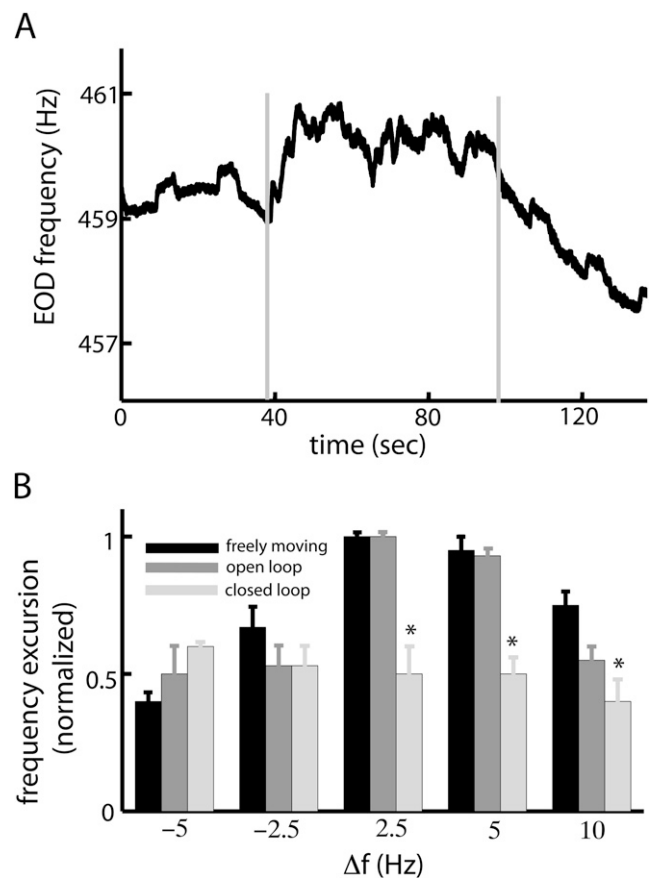


Figure 10 Effects of stressful conditions on the jamming avoidance response (JAR) in *Eigenmannia virescens*. (A) Sample JAR under stressful conditions and under closed loop stimulation showing a weaker frequency excursion. (B) Normalized frequency excursion for freely moving, prestress, and poststress conditions. The stressed animal produced significantly lower-frequency excursion JARs.

within 1 minute usually leads to the recovery of the residual EOD amplitude and the JAR, although this was not the case for these two animals.

We have also seen similar drops in EOD amplitude from brief anoxia in *Apteronotus* during electrophysiological experiments. EOD amplitudes often recover to preanoxic levels within a minute of the resumption of proper respiration.

Discussion

We performed a quantitative comparison of various electrical behaviors in four species of weakly electric fishes under freely moving, restrained, and immobilized conditions and found generally small differences in the responses of immobilized versus freely moving fish. The fact that natural electrical behaviors such as the JAR persist in immobilized weakly electric fish has been known for quite some time (Heiligenberg 1991). Indeed, it is the primary reason that these animals are used as a model system in behavioral neuroscience: sophisticated neurophysiological analyses can be achieved in awake, behaving but motionless animals. The data presented here confirm these results. But fish that clearly

experienced pain and distress while immobilized showed dramatic changes in electrosensory behavior, and brief anoxia resulted in rapid reductions in EOD amplitudes.

Effects of Immobilization on the JAR in Wave-Type Fishes

JAR behavior, which occurs in the wave-type weakly electric fishes *E. virescens*, *A. albifrons*, and *A. leptorhynchus*, was qualitatively similar in all three conditions. Although immobilization did not have any significant effect on quantitative measures of the JAR in *E. virescens*, it did lead to significant changes in the frequency excursion and rise and decay time constants in both *A. leptorhynchus* and *A. albifrons*. These changes in responses in *Apteronotus* could be due to small differences in stimulus delivery that necessarily occur in immobilized conditions—in other words, the changes may be solely due to details of stimulus delivery and not to the state of the fish. The fact that we did not observe any statistically significant differences in *E. virescens* was perhaps more surprising. In *Eigenmannia*, the stimulus regime when the fish is immobilized is quite different from that of untreated fish, which produce their own electric field, whereas immobilized fish require an artificial replacement. In our experiments the artificial stimuli were clearly effective and were well matched to the normal stimuli that the fish experienced in freely moving conditions.

Finally, we observed dramatic correlates of pain and distress in the electric behaviors of *E. virescens*. A stressed fish exhibited changes in JAR behavior, including a significant decrease in frequency excursion of its JAR, and a failure to maintain its EOD frequency. We also observed that decreases in the amplitude of the residual EOD is a rapid and reliable indicator of asphyxiation in these animals.

Effects of Immobilization on the Novelty Response in Pulse-Type Fishes

Our results show that immobilization leads to smaller changes in pulse rates during the novelty response of the gymnotiform pulse species *B. brevirostrus* as compared to those of freely moving and restrained fish. Our results are consistent with those of Post and von der Emde (1999), who showed that immobilization had similar effects on the novelty response of the mormyriiform pulse-type species *Gnathonemus petersii*. Both of these species have myogenic electric fields that are attenuated by curare-like drugs. In these experiments, therefore, the animal's own electric discharge is absent. One way to remedy this situation is to provide artificial pulses to replace the fish's own EOD pulses as in the experiments with *G. petersii* (Post and von der Emde 1999). However, these artificial pulses differ from the normal EOD pulses in a number of ways. First, the artificial pulses are delivered through stationary electrodes, whereas the natural pulses have a moving dipole along the electric organ. Second, the artificial pulses do not have the precise

temporal features of the fish's natural pulses. The fact that curare had the same qualitative effect on the novelty responses of these two species suggests that the EOD pulse shape might play an important role in determining the overall magnitude of the novelty response. Another hypothesis is that the depression in the novelty response relative to the unconstrained condition is due to pain and distress. At this time, it is impossible to say which is true. Nevertheless, the novelty response does not exhibit significant changes over time in immobilized fish that are in good condition.

Effects of Immobilization on Chirping in *Apteronotus leptorhynchus*

We observed that the stimuli used to elicit the JAR in *A. leptorhynchus* also elicited chirping. We found that restraint and immobilization had opposite effects on chirp rate: the former led to a small increase while the latter produced a significant 33% decrease in chirp rate. The decrease in the immobilized fish clearly represents a change in their behavior, but whether it is a result of pain and distress is unlikely. There is dramatic variation between fish in chirp production under normal conditions, and immobilization did not affect the chirp waveform itself.

Chirping is frequently accompanied by aggressive behavior that requires movement (e.g., physical attacks) under natural conditions (Hupé and Lewis 2008); in fact, some fish repeatedly attacked the electrodes under freely moving conditions. Aggressive behavior triggers the release of steroid hormones that have been shown to modulate chirp rate (Bastian et al. 2001) and other EOD properties (Schaefer and Zakon 1996; Zakon 1993). It is therefore possible that the lower chirp rate of immobilized fish results from lower concentrations of steroid hormones, as the animal cannot engage in aggressive behavior that requires movement.

Detecting Pain and Distress in Immobilized Weakly Electric Fishes

Changes in electrical behaviors that are known to be correlated with pain and distress are readily observable. First and foremost, the amplitude of the EOD or residual EOD rapidly reflects the oxygenation of the fish. It is therefore advisable to continuously monitor this amplitude in immobilized fishes. Next, electric behaviors such as the JAR can become more erratic, with lower amplitudes, in distressed fish. Furthermore, since chirping is a high-level social behavior, it seems reasonable to assume that complete cessation of chirping indicates pain and distress. Finally, studies have shown that the introduction of toxic chemicals in the water reliably leads to changes in the EOD frequency of weakly electric fish, suggesting the use of EOD as an early warning for changes in water quality (Thomas et al. 1996).

For all of these reasons it is advisable to monitor the behavioral performance of weakly electric fish during experiments. Reduction in the reliability and consistency of

electrical behavioral responses should be considered a sign of pain and distress in all species of these fish.

Conclusions

Weakly electric fishes produce certain electrical behaviors that persist under restraint and under immobilization by drugs that block the neuromuscular junction. We performed the first quantitative comparison of these behaviors in animals under three experimental conditions—free swimming, restraint, and immobilization—and in general found only small changes in their electrosensory behaviors in the three conditions. These data suggest that immobilization itself is likely not a source of pain and distress in these animals. However, dramatic changes in electrosensory behaviors resulted from rough handling and anoxia, and these changes are likely good indicators of pain and distress in these animals.

Although electrosensory behaviors appear to be a reliable indicator of pain and distress in these animals, future studies might examine the expression of stress hormones in restrained and immobilized fish. Similarly, it may be useful to monitor the heart rate of these fish. Future studies should also include other species of weakly electric fish, particularly mormyrids from Africa.

Acknowledgments

This research was supported by a National Science Foundation grant (IOB-0543985; ESF) and funding from the Canadian Institutes of Health Research, Canada Foundation for Innovation, and Canada Research Chairs (MJC).

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