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The Metabolic Cost of Electric Signalling in Weakly Electric Fish

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The Metabolic Cost of Electric Signalling in Weakly Electric Fish

By

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ABSTRACT

Wave-type weakly electric fish emit a highly regular electric discharge using a specialized electric organ. This electric organ discharge (EOD) forms the basis for an electric sense used for navigation, prey detection and communication. The metabolic cost of the EOD is not fully understood, but recent research suggests that it constitutes a significant portion of the fish's energy budget. In the current study, manipulation of metabolic rate via exposure to hypoxia did not significantly alter EOD frequency. Changes in metabolic rate through swimming resulted in EOD frequency increase. To manipulate EOD frequency directly in individual fish, the jamming avoidance response (JAR) and long term frequency elevation (LTFE) were used. EOD frequency elevation and jamming stimulation resulted in an increased $\dot{M}O_2$ possibly associated with increases in sensory processing. Taken together, these data indicate that electric signalling in wave-type weakly electric fish is a not a major contributor to whole-animal energetic cost.

RESUMÉ

Les poissons électriques à faible décharges ondulatoires émettent une décharge électrique très régulière à l'aide d'un organe électrique spécialisé. Cette décharge d'organe électrique (EOD) constitue la base d'un sens électrique utilisé pour la navigation, la détection des proies et la communication. Le coût métabolique du EOD n'est pas entièrement compris mais des recherches récentes proposent qu'il constitue une partie importante du bilan d'énergie du poisson. Dans la présente étude, la manipulation du taux de métabolisme par l'entremise de l'exposition à l'hypoxie n'a pas modifié significativement la fréquence du EOD. Les variations du taux de métabolisme lors de la nage ont augmenté la fréquence du EOD. Afin de manipuler directement la fréquence du EOD d'un poisson individuel, le “jamming avoidance response” (JAR) et le “long term frequency elevation” (LTFE) ont été utilisées. L'élévation de la fréquence du EOD et la stimulation de brouillage ont entraîné l'augmentation du $\dot{M}O_2$ qui est éventuellement associée à une augmentation du traitement sensoriel. En somme, ces données indiquent que les signaux électriques des poissons électriques à faible intensité ne contribuent pas de façon majeure au coût énergétique de l'animal.

CONTRIBUTIONS

Respirometry experiments on hypoxic brown ghost knifefish (fig. 3-1 and 3-2 A) were completed by Melanie Nguyen. Initial hypoxia experiments in brown ghost knifefish (percentage change in EOD frequency in brown ghost knifefish (fig. 3-3A) and ventilation amplitude and frequency (fig. 3-4)) were completed during my undergraduate Honour's thesis and included in this work for completeness.

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LIST OF ABBREVIATIONS

ANOVA – analysis of variance

ATP –adenosine triphosphate

BCF – body-caudal fin

Df – frequency difference

EOD – electric organ discharge

EOD_{amp} – EOD amplitude

EOD_f - EOD frequency

EOD_{cv} – coefficient of variation of the EOD cycle period

fMRI – functional magnetic resonance imagery

GABA – gamma-aminobutyric acid

Hb - haemoglobin

JAR – jamming avoidance response

LTFE – long-term frequency elevation

$\dot{M}O_2$ – mass specific rate of oxygen consumption

MPF – median paired fin

MS222- ethyl-*p*-aminobenzoate

N – number of animals

NMDA – N-methyl-D-aspartate

NMR – nuclear magnetic resonance

O_2 – Oxygen

P_{crit} – critical oxygen tension

PPnc- prepacemaker nucleus

PwO_2 – partial pressure of oxygen in water

RBC – red blood cell

RM – repeated measures

RMR – resting metabolic rate

RNA – ribonucleic acid

SEM – standard error of mean

SPPn – sublemniscal prepacemaker nucleus

V_{amp} – ventilation amplitude

V_f – ventilation frequency

V_w – ventilation rate

CHAPTER ONE:

General Introduction

1.1 The cost of neuronal processing

The metabolic costs associated with neural processing are thought to represent a significant portion of an animal's energy budget. The human brain, for example, represents just 2% of body mass, yet by some estimates is responsible for approximately 20% of whole animal metabolic rate (Clarke and Sokoloff, 1999). The photoreceptor system of the blowfly retina is responsible for approximately 8% of whole animal resting metabolic rate, with a mass specific metabolic rate exceeding that of some striated muscle (Laughlin et al., 1998). This disproportionate level of energy consumption suggests that neural signalling is an energetically expensive process and should pose a significant constraint on nervous system evolution. In addition, the long held assumption that increases in neural activity are associated with local increases in oxygen consumption and cerebral blood flow is the basis for fMRI technology (Heeger & Ress, 2002). However, thus far it has been difficult to directly measure the metabolic cost of neural activity.

Every signalling event that takes place in the brain requires energy. The cost of an action potential, or spike, comes from restoring ionic balances perturbed by voltage-dependent ion channels, synaptic potentials, and the release and reuptake of neurotransmitters at synapses (Lennie, 2003). The dominant energy consumer in the nervous system is the Na^+/K^+ ATPase, which is continuously active to maintain ion gradients and is concentrated in neurons, synapses and glial cells (Wong-Riley et al., 1998). Thus, neural signalling is thought to represent 50-80% of neural metabolism in

mammalian systems, with less than 10% of this signalling budget allocated for recycling neurotransmitters and second messengers (Laughlin, 2001). The remainder of the neural energy budget is involved in maintaining resting potentials and turnover of macromolecules (Laughlin, 2001). The metabolic rate of individual neurons is highly variable, with activity levels ranging from rest states, involving house-keeping functions such as protein trafficking, to maximal firing states, involving large fluxes of ions (Barros, 2010). The high estimated cost of neural activity may limit to approximately 1% the number of neurons that can be substantially active concurrently, suggesting that nervous systems might evolve mechanisms for coding and transfer of information that involve as few active neurons as possible (Lennie, 2003).

Experimental data on brain metabolism are limited and incomplete. The lack of research in this area is largely due to the difficulties inherent in devising experiments to directly examine metabolism. The skull restricts physical access to the brain, while the blood-brain barrier restricts chemical access. *In vitro* experiments can be limited by the inability to preserve the conditions that are present *in vivo.* Current techniques for measuring metabolite concentration and metabolic flux have limited spatiotemporal resolution (Barros, 2010). NMR spectroscopy offers temporal resolution on the order of seconds, and shows that synaptic activity is followed by a decrease in interstitial glucose levels and an increase in interstitial lactate concentrations (Barros et al., 2007; Mangia et al., 2009). Currently, the best temporal resolution is obtained by single-photon and multi-photon microscopy recording of NAD(P)H and flavoprotein autofluorescence (indicators of the redox potential in the mitochondria; Shuttleworth,

2010). These techniques suggest that within hundreds of milliseconds, synaptic activity is followed by changes in mitochondrial metabolism (Shuttleworth, 2010). These time-scales are still much longer than a single action potential (~1 ms) or typical fast synapses (5-10 ms). A complementary approach to such cellular-level studies is to explore the metabolic costs of neural processing in animals with unique neural processing systems for which the energetic demands are expected to be exaggerated. In the following paragraphs, we describe one such case, the weakly electric fish, as an ideal model system for examining the cost of sensory processing at the whole animal level.

1.2 Weakly electric fish

Weakly electric fish generate an electric field in the surrounding water using a specialized electric organ. The ability to generate and use electric fields as a sensory modality evolved in two distinct families of weakly electric fish: the Neotropical Gymnotiforms and the African Mormyriforms (Julian et al., 2003). Within these two families, EODs are categorized as either pulse-type or wave-type. Pulse-type weakly electric fish emit intermittent pulse-like electrical discharges (Bennett, 1971), whereas wave-type weakly electric fish, such as the brown ghost knifefish (*Apteronotus leptorhynchus*) or the glass knifefish (*Eigenmannia virescens)*, emit a continuous, quasi-sinusoidal discharge that is maintained at a nearly constant frequency (Mills and Zakon, 1987).

This electric organ discharge (EOD) is used for navigation and prey detection (electrolocation) as well as communication with conspecifics (electrocommunication) (Hopkins, 1998). Distortions in the electric field generated by the animal (the so-called electric image), for example caused by objects in the animal's environment, create a voltage change that is detected by electroreceptors in the skin. In this way, the electric image provides cues that allow the fish to determine the size, location and electrical properties of nearby objects (Caputi and Budelli, 2006).

The EOD also serves as a communication tool for weakly electric fish. Modulations of the EOD are produced in social situations, including courtship and aggressive interactions, allowing the EOD to encode information about species and sex, as well as behavioural and physiological states throughout the lifetime of the fish (Silva et al., 2006: Zakon et al., 2002; Hupé and Lewis, 2008). The types of EOD modulation vary widely, but perhaps the best-characterized EOD modulation is the jamming avoidance response (JAR) exhibited by some wave-type fish (Heiligenberg, 1991). When a fish detects the EOD of a conspecific with a frequency nearly identical to its own, the fish with the slightly higher EOD frequency will increase its EOD frequency whereas that with the lower EOD frequency will decrease its EOD frequency: the net effect of these responses is to increase the EOD frequency difference and avoid jamming of the two signals (Oestreich and Zakon, 2005). Such longer-term modulations of EOD frequency will be discussed in more detail below.

1.3 EOD generating machinery

A pacemaker nucleus in the brain is responsible for the generation and timing of the EOD in weakly electric fish (fig. 1-1). The pacemaker nucleus comprises approximately 120 pacemaker neurons that make electrotonic synapses (via gap junctions) on each other, and on a second group of about 30 neurons within the pacemaker nucleus called relay cells. The relay cells project out of the pacemaker nucleus and down the spinal cord, synapsing onto specialized spinal electromotor neurons, which innervate cells in the electric organ called electrocytes (Bennett, 1961). Pacemaker and relay neurons fire in synchrony at highly regular rates in wave-type species (Moortgat et al 1998, 2000), and at lower, less precise rates in pulse-type species (Spiro, 1997). The EOD then fires in a one-to-one fashion with the pacemaker nucleus output (Bennett, 1961). Depending on the species, the electric organ is derived from either nervous or muscle tissue, and is correspondingly termed neurogenic or myogenic. In a neurogenic species, such as the brown ghost knifefish, the electromotor neurons projecting from the spinal cord form a direct electrical synapse with electrocytes in the electric organ; activation of electrocytes results in current flow through the fish body and into the surrounding water, creating the electric field. In the case of a myogenic species, such as the glass knifefish, the spinal electromotor neurons form a chemical synapse with myogenic electrocytes; acetylcholine released by the electromotor neurons causes electrocytes to fire and generate the EOD (Bennett, 1961).

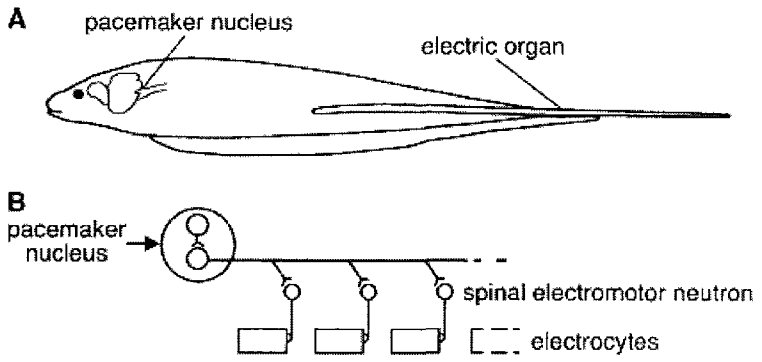


Figure 1-1. The EOD generating machinery in a weakly electric fish. (A) The pacemaker nucleus responsible for EOD generation and timing projects to the electric organ in the tail responsible for EOD production. (B) The pacemaker nucleus activates electrocytes in the electric organ via spinal electromotor neurons (modified from Stoddard and Markham, 2006).

Wave-type fish exhibit EOD frequencies as high as 1800 Hz (Bennett, 1971; Crampton, 1998a). To generate this high frequency electrical signal, all of the EOD generating machinery must be continuously active for the lifetime of a wave-type fish, i.e. thousands of neurons and electrocytes fire continuously (Bennett, 1971). Moreover, in at least some species, the amplitude of the fish's signal is controlled by the number of Na^+ channels present in the electrocytes of the electric organ (Markham et al., 2009). EOD frequencies and hence the firing frequency of the EOD-generating machinery are orders of magnitude higher than those predicted for the average cortical neuron (Lennie 2003). Therefore, it would seem that there could be a considerable energetic cost to EOD production.

1.4 Is the EOD metabolically costly?

The observation of Crampton (1998a) that EOD type is correlated with habitat type provided support, albeit indirect, for the hypothesis that EOD production is associated with a significant metabolic cost. Pulse-type electric fish, in which EOD is intermittent and thus low in average frequency, are able to survive in persistently hypoxic habitats and are tolerant of experimental hypoxia (Crampton, 1998a). Wave-type weakly electric fish, by contrast, appear to be less hypoxia tolerant than pulse-type fish according to their habitat distribution (Julian et al., 2003). Wave-type fish are found in well-oxygenated waters of rivers and streams (Julian et al., 2003). The glass knifefish, a wave-type fish, appears to be somewhat exceptional in this regard, in that it is found in more hypoxic habitats than other wave-type species (Crampton, 1998a). Hypoxia poses physiological challenges; perhaps most importantly, a reduction in oxygen available to fuel oxidative metabolism. The apparent hypoxia intolerance of wave-type electric fish may indicate a metabolic restriction on EOD production in hypoxic waters.

Further indirect evidence supporting the hypothesis of a significant metabolic cost to EOD production has come from studies that have documented systematic variations in the EOD. For example, Salazar and Stoddard (2008) examined EOD day-night cycle variations in non-breeding and breeding *Brachyhypopomus pinnicaudatus* (a pulse-type weakly electric fish). Male *B. pinnicaudatus* displayed higher amplitude EODs than females during the breeding season, and increased EOD amplitude further

at night when socially active (during spawning and courtship periods) (Franchina and Stoddard, 1998). EOD amplitude is proportional to the number of Na^+ channels present in the electrocytes of the electric organ (Markham et al., 2009). Larger amplitude signals allow the fish to send its signal farther, allowing for communication with conspecifics and navigation and prey detection over a larger range (reviewed by Hopkins, 1998). Higher amplitude signals, that require a larger flow of current, should be more metabolically costly to produce (Salazar and Stoddard, 2008) and may attract electroreceptive predators (Stoddard, 1999). Pulse-type fish have the ability to modulate their EOD frequency and amplitude to reduce metabolic cost and the likelihood of attracting predators. Thus, this circadian rhythmicity in EOD amplitude may have evolved to allow the animal to balance the costs and benefits of the electrosensory system (e.g. males produce potentially costly high amplitude signals only when benefits are greatest) (Curtis and Stoddard, 2003). The enhanced EOD of males leads to greater reproductive success; females prefer larger males with higher pulse amplitudes and durations (in pulse-type fish) (Salazar and Stoddard, 2008). Such EOD modulations are mediated by circulating melanocortin peptides (Markham and Stoddard, 2005; Markham et al., 2009).

A recent study by Markham et al. (2009) examined circadian and social cues that may regulate ion channel trafficking in the electric organ of *Sternopygus macrurus,* a pulse-type weakly electric fish that also displays a circadian rhythm in EOD amplitude. This study found that EOD amplitude is modulated in a matter of minutes by rapid recruitment of voltage-gated Na^+ channels into the membrane of electrogenic cells via

peptide hormones and intracellular second messenger pathways (Markham et al., 2009). Markham et al. (2009) estimated that the increase in Na^+ conductance associated with a high-amplitude EOD would result in an up to 300% increase in ATP expenditure to remove accumulated sodium via active transport in the electrocyte. However, direct measurements of the metabolic cost associated with these amplitude modulations have yet to be made.

The findings of the aforementioned studies imply that EOD production is metabolically expensive, yet the metabolic cost of electric signalling in weakly electric fish has been directly studied in only a few cases. Salazar and Stoddard (2008) used respirometry and pharmacological manipulations to examine whether daytime reductions in EOD amplitude resulted in significant reductions in energy consumption. Oxygen consumption was measured under resting conditions when fish were emitting a basal EOD, after treatment with the anaesthetic metomidate-HCl (a $GABA_A$ binding enhancer that inhibits swimming but not EOD), and after treatment with flaxedil (a curare analog that inhibits motor activity and silences the myogenic electric organ). By comparing the data from these different conditions, it was estimated that male fish expended approximately 11-22% of their energy budget on electric signal generation whereas females spent only 3%. Both sexes reduced the energy spent on electric signals during daylight hours through modulation of EOD amplitude, repetition rate and duration. Salazar and Stoddard (2008) concluded that electric signalling constitutes a significant portion of the fish's energy budget, making the circadian rhythm of the EOD an energy-saving adaptation.

A limitation of the approach used by Salazar and Stoddard (2008) is the inhibition of motor activity during respirometry periods. Although the pharmacological techniques used by Salazar and Stoddard (2008) allowed comparison of oxygen consumption with and without the EOD, inhibition of all motor activity required the fish to be artificially ventilated throughout the respirometry periods, a concern in interpreting metabolic data. In his review of respirometry techniques, Cech (1990) points out that use of anaesthetics during respirometry is not recommended because they may depress overall metabolism. However, in this case, comparisons were made between anaesthetized fish and anaesthetized fish in which the EOD was silenced. Perhaps more importantly, anaesthetized fish were artificially ventilated with a flow of 30 $mlmin^{-1}$ of water (containing anaesthetic). Their fish ranged in size from 6.6 g for the smallest female to 17.3 g for the largest male. The constant ventilation rate therefore translates into mass-specific ventilation rates (V_w) of 5000 $mlmin^{-1}kg^{-1}$ (for the 6.6 g female) to 1734 $mlmin^{-1}kg^{-1}$ (for the 17 g male). "Typical" V_w values for fish are around 250 $mlmin^{-1}kg^{-1}$ (Perry et al., 2009). Ventilation flow rates were high, and should impact on $\dot{M}O_2$. Also, ventilation flow rates were not matched to fish size, which means small fish were hyperventilated to a greater extent than large fish. Since females were mostly smaller than males, this factor may account for some of the male-female differences reported. An added issue is the extent to which pumping water into the mouth can mimic the flow of water over the gills generated by the fish's pumping mechanism, and how this might affect $\dot{M}O_2$ values. Nonetheless, the study indicated that electric signal production in pulse-type weakly electric fish was associated with a high metabolic cost. Previous

studies have found that pulse-type fish seem to be more hypoxia tolerant than wave-type fish, possibly because of their intermittent, rather than continuous, EOD production (Crampton, 1998a; Julien et al., 2003). This observation suggests that the energetic cost associated with signalling in a wave-type fish may be even higher than that of the pulse-type fish estimated by Salazar and Stoddard (2008).

1.5 Hypothesis and predictions

We aimed to test the hypothesis that the production of an EOD is metabolically costly. Our first objective was to examine the effects of hypoxia on metabolic rate and EOD frequency. When oxygen availability is limited, metabolic rate falls (Boutilier, 2001; Hochachka and Sommero, 2002; Nilsson and Lutz, 2004; Richards, 2010). Under the hypothesis that EOD production is metabolically costly, EOD frequency should be reduced during hypoxia to match metabolic demand with oxygen availability. In an alternate approach, oxygen consumption and EOD were examined during exercise. We predicted that the increased metabolic demand of active skeletal muscle (Videler, 1993) would constrain energy availability for EOD production, resulting in a reduction in EOD frequency. Effects on EOD frequency were the main focus of our studies due to the ease of measurement, though EOD amplitude and frequency variability (cycle to cycle variation) were measured when practical. A third, complementary approach involved manipulating EOD frequency using the jamming avoidance (JAR) and long-term frequency elevation (LTFE) responses and examining

the effects of these EOD frequency modulations on oxygen consumption. In short, our experimental approach essentially involved manipulating oxygen availability, metabolic rate or EOD frequency of the fish while examining the effects of these manipulations on metabolism and EOD characteristics.

1.6 Manipulating $\dot{M}O_2$ via Hypoxia

Two approaches are typically observed in response to hypoxic exposure. Initially, fish try to defend O_2 uptake and delivery by increasing ventilation and increasing the capacity of the gill and blood to transfer oxygen (Gilmour, 2001; Perry et al., 2009; Perry and Gilmour, 2009). Increases in ventilation amplitude (V_{amp}) as well as ventilation frequency (V_f) are used to enhance O_2 delivery to the gill (Gilmour, 2001; Perry et al., 2009). Many species respond to hypoxia by primarily or solely increasing V_{amp}. This strategy of employing large changes in V_{amp} coupled with modest changes in V_f is thought to be an energetically favourable strategy, given the viscosity and density of water (Perry et al., 2009). When hypoxia is prolonged, a suite of responses aimed at increasing O_2 uptake and delivery to tissues is employed. The affinity of hemoglobin for O_2 increases via the Bohr effect and decreases in organic phosphates, a response that benefits loading of O_2 into the tissues in an O_2-limited environment. Hematocrit levels increase as well, resulting in an increase in blood O_2 carrying capacity. Increases in haematocrit are achieved primarily by recruitment of RBCs from the spleen as well as erythropoietin stimulated generation of new RBCs. Cardiovascular changes are also

recruited during hypoxia, including bradycardia, induced by increased activity of cardiac parasympathetic nerves (reviewed by Perry and Gilmour, 2009).

As environmental oxygen levels decline further, it becomes impossible and/or too expensive to defend O_2 uptake. The lowest PwO_2 level at which a fish can maintain its $\dot{M}O_2$ is termed the critical oxygen tension (P_{crit}). The P_{crit} is commonly used as an indicator of hypoxia tolerance (Ultsch et al., 1978; Ott et al., 1980; reviewed by Nilsson, 2009). Considering the apparent hypoxia intolerance of wave-type weakly electric fish (Crampton, 1998a), we predict that knifefish P_{crit} values will be relatively high compared to more hypoxia tolerant, non-electric fish. As PwO_2 falls below P_{crit}, ventilation amplitude and/or frequency are expected to fall off as the animal adopts the approach of lowering the metabolic demand for O_2 (Hochachka and Lutz, 2001). At the transition from defending $\dot{M}O_2$ to conforming to environmental O_2, reorganization of the animal's energy budget is observed (Hochachka and Lutz, 2001; Richards, 2010). Behavioural, physiological and biochemical adaptations occur to reduce ATP and O_2 demands and prolong cellular survival (Hochachka et al., 1996; Richards, 2010). At the behavioural level, decreases in muscular activity, reproduction and courtship, feeding and other activities provide energetic savings by reducing metabolism. As hypoxia persists, physiological adaptations occur, including reductions in growth and gonadal development, and energy is reserved for the maintenance of essential physiological functions. Biochemical adjustments at the cellular level include reductions in Na^+/K^+ ATPase activity, protein synthesis, RNA synthesis and urea synthesis (reviewed by Richards, 2010).

Studies on mammalian cerebro-cortical neurons exposed to hypoxia have shown that primary neurons in culture reduce energy utilization in response to hypoxia (Munns et al., 2003). Malthankar-Phatak et al. (2008) found that neurons that survive hypoxic exposure adapt to prolonged hypoxia by up-regulation of glycolysis and down-regulation of oxidative metabolism. Exposure of brown ghost and glass knifefish to graded hypoxia will give insight into the metabolic trade-offs that occur during hypoxia exposure. We predict that at oxygen tensions beyond P_{crit}, EOD frequency will be compromised in an effort to match oxidative metabolism to O_2 supply in hypoxic waters. The overall energy budget could be reorganized in additional ways as well, involving for example a switch towards higher rates of glycolysis (anaerobic metabolism) to sustain metabolic activity.

1.7 Manipulating $\dot{M}O_2$ via Swimming

Exercise is a metabolically costly process; increases in muscle activity during exercise can result in large increases in metabolic rate (Videler, 1993). Fast streamlined fish can increase $\dot{M}O_2$ approximately 10-fold with increasing speed (Brett, 1972). During swimming, skeletal muscle activity increases to propel the body through water. The incompressibility and high density of water have played an important role in the evolution of swimming in fish (Lindsey, 1978; Videler, 1993). Because water is an incompressible fluid, any movement generated by the fish sets the surrounding water in motion. The high density of water (800 times that of air) is comparable to that of the

body of the fish, acting to nearly counterbalance the force of gravity, and allowing the development of propulsive swimming as weight support is not an issue (Lindsey, 1978). For a fish to swim at a constant speed, the total thrust it exerts on the water must exceed the resistance it encounters in moving forward (reviewed by Stafiotakis et al., 1999).

To achieve this movement, skeletal muscle is recruited to propel the body forward via fin and/or body movements. As the speed of movement increases, faster muscle fibres are recruited in addition to slower fiber types (Gillner, 1981; Armstrong, 1981; Videler, 1993). Red muscle fibres are used to power slow to medium speed movement, while a combination of both slow (red) and faster (white) muscle fibres are used to power high speed movements in fish (Jayne et al., 1990). White muscle is recruited at or immediately before the maximal activation of red muscle is reached (Jayne et al., 1990). Red muscle activity is fuelled by aerobic metabolism and red muscle fibres are characterized by a well developed blood supply, low activity myosin ATPase, large numbers of mitochondria and high enzyme activity associated with aerobic metabolism. In contrast, white muscle activity relies largely on anaerobic glycolysis, and white muscle fibres have high myosin ATPase activity, few mitochondria, and lower capillary density (Crabtree and Newsholm, 1972, reviewed by Altringham and Ellerby, 1999). Short periods of burst swimming result in mobilization of muscle glycogen stores and accumulation of lactic acid. Removal of lactic acid from white muscle can take 12-14 hours, making anaerobic glycolysis a short-term strategy with a costly recovery period (Black et al., 1962; Kieffer, 2000).

Weakly electric knifefish are elegant swimmers that are able to swim as easily backwards as forwards, and are able to rapidly change direction. Indeed, the common name "knifefish" comes from the knife-like way in which the fish slice through the water as they swim. The main propulsive agent is an elongated anal fin termed the ribbon fin, located ventrally (Blake, 1983; reviewed by Colgate and Lynch, 2004). When knifefish swim, the body is generally held straight, and sinusoid-like travelling waves pass along the ribbon fin (Blake, 1983; Shirgaonkar et al., 2009). This type of swimming with undulatory anal fin propulsion is termed gymnotiform mode (Breder, 1926). With this swimming mode, movement and propulsion are dissociated from electrosensing. In other words, the body can be moved to optimize sensory performance while eliminating the potentially large perturbations in the electric field cause by undulating tail movements (Nelson and MacIver, 1999). Swim direction can be easily and rapidly changed by reversing the direction of the wave passing down the ribbon fin (MacIver et al., 2001).

Whereas pulse-type fish alternate between periods of swimming and rest with their body in or on the substrate, wave-type fish swim continuously and maintain scan-swimming as a part of normal foraging behaviour (Lannoo and Lannoo, 1993). Scan swimming is specific to wave-type fish and involves moving forwards and backwards near the substrate, creating motion-related changes in voltage (distortions in the electric image) that are detected by electroreceptors on the body surface (MacIver et al., 2001). This back-and-forth swimming may be important for increasing spatial acuity during prey detection or when investigating novel objects (Babineau et al., 2007).

Generally, teleost fish swimming falls under two main classes based on the principal fins used; median-paired fin (MPF) swimming, where paired fins (pectoral or pelvic fins) or median fins (dorsal and anal fins) are the primary means of locomotion, and body-caudal fin (BCF) swimming, where the body is used to generate waves that drive the caudal fin as the primary propulsor (Webb, 1984). The majority of research on swimming fish has focused on BCF swimming. The Salmonids are an example of BCF-style swimmers. Rainbow trout (*Onchorhynchus mykiss*), for example, increase $\dot{M}O_2$ by 1.8-fold, from 5.6 $mmolkg^{-1}h^{-1}$ at rest to approximately 10 $mmolkg^{-1}h^{-1}$ at maximum swimming speed (Kieffer et al., 1998). Similarly, a study on sockeye (*Onchorhynchus nerka)* and coho salmon (*Onchorhynchus kisutch*) revealed 3-4 fold increases in $\dot{M}O_2$ from resting metabolic rate (RMR) to swimming at maximum speed (approximately 130 cms^{-1}) (Lee et al., 2003).

One style of MPF locomotion is 'labriform' swimming, in which fish make use of pectoral fin oscillation with almost no body movement. Although labriform swimming is thought to be a less efficient swimming style for high-speed swimming, it provides higher efficiency and manoeuvrability at low speeds (reviewed by Colgate and Lynch, 2004). Species in the family Embiotocidae exhibit swimming gait transitions from exclusively pectoral fin propulsion to a combination of pectoral and caudal fin propulsion as swimming speed increases (Webb, 1973). Pectoral fin movements are powered by and controlled by pectoral girdle muscles, while undulatory movements are powered by the segmented myotomal musculature (Kendall et al., 2007). In *Embiotoca lateralis* (striped sunperch), $\dot{M}O_2$ rates increased by approximately 2-fold when swimming speed

increased from approximately 15 cms^{-1} to 40 cms^{-1} (speeds powered by pectoral propulsion), indicating an increase in O_2 consumption by active skeletal muscle (Cannas et al., 2006).

Despite the very different biomechanics of swimming in weakly electric fish, swimming is expected to increase metabolic rate in a swimming-speed dependent fashion due to increases in skeletal muscle activity. Julien et al. (2003) noted an approximately 2-fold increase in oxygen consumption from resting to scan-swimming in *Apteronotus albifrons* (the black ghost knifefish). In the present study, the O_2 consumption associated with swimming against a water current at low to medium speed was examined in brown ghost and glass knifefish. Though the muscle fibre type devoted to powering ribbon fin movement is currently unknown, this approach provided a means of manipulating metabolic rate and examining the resultant effects on EOD production under conditions where aerobic metabolism was largely devoted to powering skeletal muscle.

1.8 Manipulating EOD frequency via JAR and LTFE

Although weakly electric fish typically maintain a highly regular EOD frequency (Moortgat et al., 1998, 2000), the EOD may be modulated in social situations and in response to stressors such as changes in temperature and pH (Silva et al., 2007). EOD frequency is dependent on temperature, with a Q_{10} value of 1.62 (Dunlap et al., 2000). Manipulating EOD frequency by exposing fish to different temperatures provides an

interesting means of examining the effects of EOD frequency elevation on $\dot{M}O_2$ However, changes in temperature would confound measures of $\dot{M}O_2$, as metabolism is also temperature dependent. Although the Q_{10} value for $\dot{M}O_2$ in knifefish has not yet been determined, the mean Q_{10} value for resting metabolism in a teleost fish is approximately 1.83 (calculated across 69 species) (Clarke and Johnston, 1999). For example, a tropical fish at 30°C requires approximately 6 times more O_2 for resting metabolism than a polar fish at 0°C (Clarke and Johnston, 1999). A second point of concern is the dependence of EOD amplitude on temperature. At temperatures over 25°C, the Q_{10} value for EOD amplitude is 1.15 (Dunlap et al., 2000). Therefore, one could use temperature to manipulate EOD frequency, but difficulties arise in examining the effect of frequency elevation alone on $\dot{M}O_2$.

The jamming avoidance response (JAR) and long-term frequency elevation (LTFE) exhibited by these fish may provide an alternate approach to examining the metabolic cost of electric signalling: experimentally manipulating EOD frequency via the JAR or LTFE, while examining the effect of this manipulation on metabolic rate. Effective electrosensory processing requires that the fish monitor small amplitude modulations of its stable EOD. When two fish with nearly identical EOD frequencies (within 2-10 Hz) meet, a "beat" frequency arises at the frequency difference (Df) between the two conspecifics; this beat masks small perturbations in the fish's electric field, essentially jamming each fish's electrosensory system (Heiligenberg, 1973). In this instance, the fish with the slightly higher EOD frequency will raise its frequency further to increase the Df and restore electrosensory capabilities. The JAR can be

elicited in the laboratory by playing to the fish a sinusoidal wave with a Df of approximately 3-4 Hz (Heiligenberg, 1991) (fig. 1-2).

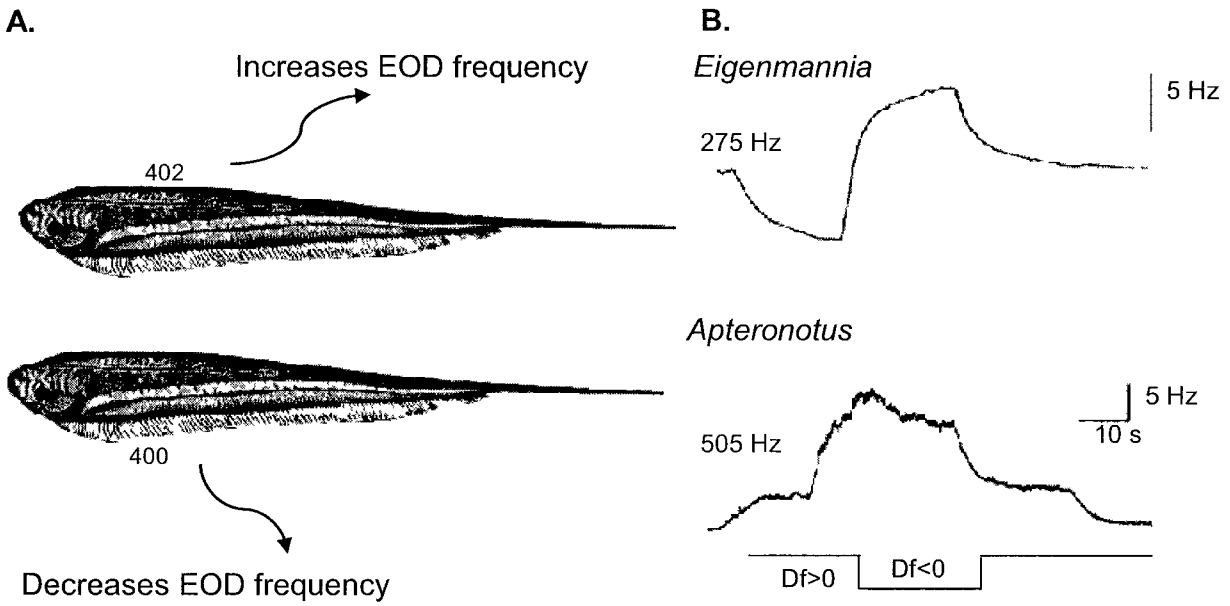


Figure 1-2. The jamming avoidance response in weakly electric fish. (A) When conspecifics with nearly identical EOD frequencies meet, the fish with the lower frequency will reduce its frequency further, while the fish with the higher frequency will increase its frequency further to avoid electrosensory jamming. (B) Traces of EOD frequency in *Eigenmannia* and *Apteronotus* during jamming. The basal EOD frequency of the unstimulated fish is indicated to the left. The bottom trace indicates the presence of a jamming stimulus of approximately one-third the amplitude of the fish's EOD, frequency-clamped at 4Hz above (+ Df) and then below (- Df) the fish's EOD frequency.

At the onset of the + Df jamming stimulus, *Eigenmannia* respond by lowering its frequency below the basal level. When the Df is reversed, the fish raises its EOD frequency to a point above the basal frequency. The JAR of *Apteronotus'* is asymmetrical. The fish gives an initial small rise, known as a non-selective response, at the onset of the +Df jamming stimulus followed by a larger rise after the switch to –Df jamming. Both species lower frequency towards resting value after removal of the stimulus (Modified from Heiligenberg et al., 1996; photograph taken by Eric Fortune, http://www.psy.jhu.edu/~fortune/).

This behavioural response may provide a more direct means of examining the metabolic cost of changes in EOD frequency. Input to the pacemaker nucleus is controlled by two upstream nuclei, the mesencephalic sublemniscal prepacemaker nucleus (SPPn) involved in the JAR and the diencephalic prepacemaker nucleus (PPn-c) involved in chirping, a communication signal used during courtship and agonistic interactions (Dye et al., 1987; Heiligenberg et al., 1996; Zakon et al., 2002; Hupé and Lewis, 2008). The SPPn controls the JAR via NMDA (N-methyl-D-aspartate) receptor synapses with relay neurons of the pacemaker nucleus, which project to the electromotor neurons in the electric organ (Dye et al., 1987; Heiligenberg et al., 1996). Longer term activation of these synapses elicited by presenting a jamming stimulus for at least 30 min results in a form of sensorimotor adaptation referred to as long-term frequency elevation (LTFE). Sensorimotor adaptation is used by nervous systems to fine tune motor responses, generating lasting motor output in response to stable sensory input (Oestrich and Zakon, 2002). One example of sensorimotor adaptation is

the manipulation of sensory input in humans using prism goggles; distortion of visual input through the prism goggles leads to initial disorientation of subjects, however, within minutes the subject learns to compensate for the distortion (Held and Freedman, 1963). In weakly electric fish presented with a stable jamming stimulus, the firing frequency of the pacemaker nucleus changes to minimize the effect of the jamming stimulus, through activation of NMDA receptors in the pacemaker nucleus (Oestrich and Zakon, 2002). EOD frequency remains elevated for up to 9 h after stimulus presentation has ceased, gradually returning to baseline frequency (Oestrich and Zakon, 2002). Thus, LTFE provides an additional means of examining the effects of EOD frequency elevation on $\dot{M}O_2$ without the potential effects of an ongoing stimulus to the fish, which may result in metabolic changes due to activation of a stress response or the detection and processing of the signal. Weakly electric fish engage in social and/or agonistic encounters (Hupé and Lewis, 2008). If the jamming signal is interpreted as a conspecific fish, the subject may experience a change in metabolic rate as it prepares to encounter a conspecific. This response would be independent of the adjustment of EOD frequency but could affect metabolic rate. In addition, stimulus presentation in itself may result in more synaptic input due to detection and processing of the signal, which could also be reflected in a change in metabolic rate.

1.9 Summary

Weakly electric fish provide an interesting model in which to evaluate the metabolic cost of sensory signalling at the whole animal level. The electrosense is vital for the lifestyle and survival of these fish, and provides an ideal system for examining the metabolic cost of neural activity. The objective of this study was to examine the energetic requirements of the electrosensory system in two wave-type species of weakly electric fish. In particular, we aimed to test the hypothesis that EOD production requires considerable metabolic expenditure. These experiments will provide insight into the energy requirements of the electrosensory system, and the energetic trade-offs that may occur when metabolic constraints are present.

CHAPTER TWO:

Materials and Methods

2.1 Experimental Animals

Wild caught brown ghost (*Apteronotus leptorhynchus)* and glass knifefish (*Eigenmannia virescens)* were purchased from a tropical fish supplier and maintained in large community aquaria at the University of Ottawa Aquatic Care Facility in a climate-controlled room (28 ± 1.5°C) supplied with dechlorinated city of Ottawa tap water and a 12D:12L photoperiod. All fish were fed frozen bloodworms *ad libitum* three times per week. All experiments were performed at a water temperature of 28 ± 0.5°C.

In a subset of trials, fish were equipped with impedance electrodes consisting of small diameter plastic-coated wires to allow measurement of ventilation frequency (V_f) and amplitude (V_{amp}). Fish were anaesthetized in an aerated solution of 0.56 gL^{-1} MS-222 (ethyl-*p*-aminobenzoate; Sigma-Aldrich, Oakville, ON) adjusted to neutral pH with $NaHCO_3$ (1.1 gL^{-1}) and were then placed in a groove cut into a piece of foam so that a constant flow of aerated anaesthetic solution could be passed across the gills by means of a peristaltic pump throughout surgery. Electrodes were passed through a small hole made in each operculum with a 23 G needle and were held in place using two sutures. Fish were revived post surgery by irrigating the gills with fresh water and allowed to recover overnight. Animal holding and experimental protocols were approved by the University of Ottawa animal care committee (BL-229) and conformed to the guidelines of the Canadian Council on Animal Care.

2.2 Experimental protocols

Series I: Hypoxia

The effects of reduced environmental O_2 availability on EOD frequency (EOD_f), EOD variability (coefficient of variation of the EOD cycle period, EOD_{CV}), EOD amplitude (EOD_{amp}) ventilation frequency and amplitude (V_f, V_{amp}) and, in separate trials, metabolic rate ($\dot{M}O_2$) were examined by exposing knifefish acutely to graded hypoxia. Brown ghost knifefish (5.70 ± 0.54g, N=12) fitted with impedance leads were placed in a respirometer for an overnight acclimation period. The respirometer consisted of a clear plexiglass cylinder, 22.5 cm long and 2.5 cm in diameter (103.5 ml volume), that was supplied with aerated, flowing water at 28 ± 0.5°C and contained in a water bath of the same temperature. Experiments commenced with measurements under normoxic conditions. Once stable EOD, ventilatory and water O_2 partial pressure (PwO_2) readings were obtained (~20 min), PwO_2 was lowered to ~50 Torr over 30 min. Acute hypoxia was achieved by bubbling a water equilibration column supplying the respirometer with appropriate gas mixtures provided by a gas mixing flowmeter (Cameron Instruments, model GF-3/MP, Port Arnasas, TX, USA). Continuous recordings of PwO_2 and the changes in impedance between the electrodes attached to the opercula (from which ventilation parameters were calculated; see below) were collected using a data acquisition system linked to a PC. Recordings of EOD (10s) were collected at 10 Torr intervals. To monitor activity levels during experimental trials, fish were recorded using an overhead Sony video camera (model DCR-TRV 260). Control experiments were carried out on a separate group of brown ghost knifefish (6.57

± 0.44g, N=6) in which the P_wO_2 of the water was maintained at normoxia for the duration of the trial and measurements were collected continuously or at time intervals matched to those used during hypoxic exposure, as appropriate.

Following acute hypoxia exposure, fish were terminally anaesthetized. Tissue samples were collected from each fish; axial muscle powering ribbon fin movement, and axial muscle from the dorsal mid-body. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until later analysis of lactate levels.

In a separate experiment, brown ghost (8.14 ±1.73g, N=6) or glass knifefish (3.90 ± 0.86g, N=6) were exposed to graded hypoxia for the measurement of EOD and $\dot{M}O_2$. Following an overnight acclimation period in which the respirometer was supplied with water on a flow-through basis, $\dot{M}O_2$ was measured by closed-system respirometry under normoxic conditions (P_wO_2 = ~150 Torr), and at 20 Torr intervals as P_wO_2 was gradually reduced to ~50 Torr over 1 h. Water circulation during the period of closed-system respirometry was achieved using a peristaltic pump that passed water through a short tubing loop that included the P_wO_2 electrode.

Series II: Exercise

A Blaschko-type swim respirometer (total volume 3.0685 L) consisting of a variable speed submersible pump (Little Giant Pump Co., model 4E–34NR, Oklahoma City, USA) connected to a custom-built cylindrical plexiglass chamber was used to assess the impact of exercise on EOD and $\dot{M}O_2$. The swim respirometer was partially submerged in water of 28 ± 0.5°C to maintain a constant temperature and was covered

to provide the low light levels preferred by knifefish. Three types of experiments were carried out. In all cases, fish were acclimated to the swim chamber overnight, with continuous water flow. The first experiment assessed EOD and $\dot{M}O_2$ at increasing water velocities in brown ghost knifefish (7.30 ±1.86g, N=8). Once stable baseline EOD and resting $\dot{M}O_2$ were established, water velocity was increased to approximately 7 cms^{-1} (0.5 BL/S) and EOD and $\dot{M}O_2$ were measured. This protocol was repeated for water velocity increments of $1cms^{-1}$ until a velocity of 15 cms^{-1} (1 BL/S) was achieved; this velocity was the maximum achievable with the pump used. Fish swam readily but to prevent fish from resting against the end of the chamber, a 7 V electrical stimulus was delivered across two wires at the end of the swim chamber in some trials (pulse duration, 500 ms; frequency, 1Hz); although effective, this stimulus was relatively weak and did not have an effect on EOD. No differences were observed between trials where the stimulus was used and where it was not used. $\dot{M}O_2$ was measured by closed-system respirometry; the respirometer was sealed for ~20 minutes during each speed interval, followed by 5 min of open circulation to flush the chamber with aerated water. Small increases in water temperature (+0.5°C) were measured during closed system trials owing to heat produced by the pump.

The second experiment also utilized brown ghost knifefish (8.57 ± 0.83g, N=12) and followed a procedure similar to the first experiment with the exception that once stable baseline EOD and $\dot{M}O_2$ were achieved, water velocity was increased to either $7cms^{-1}$ (low-velocity group) or 15 cms^{-1} (high-velocity group) for 60 minutes. EOD was recorded every 10 min and $\dot{M}O_2$ was measured every 20 min.

A final experiment was designed to control for the effects of the pump on water temperature. This experiment employed brown ghost (7.16 ± 0.99g, N=6) and glass knifefish (2.41 ± 0.48g, N=6) and utilized the protocol of the first experiment except that water flow was maintained throughout the swimming period so $\dot{M}O_2$ was not measured.

Series III: Jamming avoidance response and long term frequency elevation

Glass knifefish (1.72 ± 0.34g, N=9) were acclimated to the respirometry chamber overnight; the respirometry chamber was contained in a 28 ± 0.5°C water bath to ensure that water temperature remained constant throughout the experiment. Experiments commenced once baseline EOD frequency and $\dot{M}O_2$ measurements had been collected. Once a sustained increase in EOD frequency had occurred, usually within 5 min of stimulation, $\dot{M}O2$ measurements were carried out using closed system respirometry. The jamming stimulus was played to the fish for 30 min to evoke a long term frequency elevation (LTFE). $\dot{M}O_2$ was measured by closed system respirometry immediately after cessation of the jamming stimulus. Trials were recorded using a Canon video camera (model NTSC-2R70 MC) to monitor activity level of the fish during JAR/LTFE trials. Only fish deemed to exhibit low activity (hovering with intermittent ribbon fin movement) were included in the final analysis.

In a separate control experiment, glass knifefish (1.38 ± 0.23g, N=6) were exposed to a neutral JAR stimulus, alternating +3Hz and -3Hz every 2 minutes, for 30 minutes. $\dot{M}O_2$ measurements were taken at rest (before stimulus presentation), and during neutral JAR stimulus presentation. The objective of the neutral JAR stimulus

was to examine the effect of stimulus presentation on metabolic rate without eliciting EOD frequency modulations. As in the JAR and LTFE experiments, only low activity fish were included in the final analysis.

2.3 Analytical techniques

P_wO_2 and *$\dot{M}O_2$*

For both the standard respirometer and the Blaschko-type swim respirometer, a small peristaltic pump was used to move water past a polarographic O_2 electrode (Analytical Sensors E-101, Sugarland, Texas, USA) housed within a temperature-controlled (28 ± 0.5°C) cuvette before returning it to the respirometer. The O_2 electrode was connected to a blood gas analyzer (Cameron Instruments BGM 200, Port Aransas, Texas, USA) linked to a data acquisition system (Biopac Systems Inc) running AcqKnowledge data acquisition software on a PC. This system enabled continuous measurements of water P_wO_2 to be recorded. The O_2 electrode was calibrated with solutions of sodium sulfite (20 mg ml^{-1}; P_{O_2}=0 Torr) and air-saturated dechlorinated Ottawa tap water (P_{O_2}=153 Torr).

To measure $\dot{M}O_2$ by closed-system respirometry, the respirometer was sealed and the water was recirculated for ~20 min or until P_wO_2 had declined by 10 Torr. $\dot{M}O_2$ was calculated from the slope of the relationship between P_wO_2 and time over the interval that the water in the respirometer was recirculated, taking into account chamber volume, and fish mass. The solubility coefficient of O_2 in fresh water at 28°C was obtained from Boutilier et al. (1984).

Videotapes were reviewed to rate the activity level of each fish on a scale of 0-3 every 10 s for approximately 40 s at every 10 Torr drop in PwO_2. The activity scale was based on movement of the ventral ribbon fin. No ribbon fin movement received a score of 0. Intermittent ribbon fin movement received a score of 1. Constant ribbon fin movement (while hovering, no swimming) was categorized as 2, and constant ribbon fin movement together with swimming was categorized as 3 (the highest activity level).

EOD

EOD was recorded using Teflon-coated silver-wire electrodes (diameter: 0.38mm, insulated to the tip; WPI, Inc. Sarasota Florida, USA) placed at each end of the respirometer or Blaschko-type swim respirometer, i.e. at the head and tail of the fish. EOD was amplified (WPI DAM 50 Differential Amplifier; 10X amplification, low frequency cut off 10Hz, high frequency cut off 3kHz) and recorded via a Toshiba laptop sound card (44kHz sample rate). Stimulus signals (sine waves) used in the JAR experiments were generated and delivered through the sound card using custom Labview code (National Instruments). EOD signals were analyzed using spectrograms (Matlab, The Mathworks Inc) with the following parameters: window size 5000, NFFT 5000, and overlap 2000. EOD amplitude was analyzed using custom Labview code (National Instruments). Cycle variability of the EOD was calculated as the CV (coefficient of variation, SD/mean) of the cycle period (Matlab, The Mathworks Inc.). During swimming trials, a grounded silver-wire electrode was placed inside the swim respirometer to reduce interference.

In JAR and LTFE experiments, a sine-wave signal was delivered across the fish's body using two Teflon-coated silver-wire electrodes (diameter 0.38 mm, insulated to the tip; WPI, Sarasota, Florida, USA). As is the convention, the perpendicular arrangement of stimulating electrodes and recording electrodes prevented interference between the EOD and stimulus signal. The stimulus signal was maintained at a difference frequency (Df) of -3 Hz (i.e. 3 Hz below the EOD frequency of the fish) and an amplitude close to the EOD amplitude of the fish to evoke the jamming avoidance response (Heiligenberg et al., 1996).

Ventilation

The frequency and amplitude of opercular displacements were assessed as indices of ventilation using a custom-built impedance converter (University of Ottawa Electronics workshop) that recorded the changes in impedance between the wire electrodes attached to the opercula (Peyraud and Piquemal, 1962; Vulesevic et al., 2006). Analog signals were converted to digital and stored on a PC (AcqKnowledge data acquisition software, Biopac Systems Inc.). Ventilation frequency was determined from the impedance traces by the number of opercular displacements averaged over five 10s intervals.

To estimate ventilation amplitude, the minima and maxima of the impedance trace were identified; the differences between successive minimum-maximum pairs were then calculated and averaged over 10 breathing cycles. Opercular deflection (in

mm) was then obtained directly from the impedance amplitude through a predetermined calibration factor.

Tissue lactate concentration

Frozen axial muscle and liver tissue were individually ground to a fine power in liquid nitrogen using a mortar and pestle. Aliquots of muscle (20-130 mg) were then homogenized in 1 $mlmg^{-1}$ 8% PCA on ice using a hand held homogenizer (Omni TH Homogenizer, Omni International, Marietta Georgia). The homogenate was then centrifuged (10000 *g*) for 5 min. Tissue lactate levels were determined enzymatically on 100 µl of deproteinized extract using Sigma lactate assay reagents as per the protocol of Milligan and Girard (1993).

Statistical Analysis

Data are presented as means ± standard error of the mean (SEM) of at least six observations. Statistical analysis of $\dot{M}O_2$ consumption vs. EOD_f in individual fish was performed using linear regression. Hypoxia and swimming experiments were statistically evaluated using one and two-way RM-ANOVA as appropriate with Holm-Sidak multiple pairwise comparison. Statistical evaluations in JAR and LTFE data were performed using Student's one-sample *t*-tests. All statistical analyses are performed on raw data, though percentage data is presented in some cases. Data were considered to be statistically significant at $p < 0.05$. All statistical analysis was performed using SigmaStat v3.5 (SPSS, Inc.) and SigmaPlot v10.0 (SPSS, Inc.).

CHAPTER THREE:

Results

As an initial step in examining the relationship between electric signalling and metabolic cost, resting mass specific oxygen consumption ($\dot{M}O_2$) was measured in individual brown ghost and glass knifefish with a range of baseline EOD frequencies (fig. 3-1). Regression analysis of these data indicated that there was no significant relationship between $\dot{M}O_2$ and EOD frequency under resting conditions in either species (brown ghost, R^2=0.004, p=0.849; glass knifefish, R^2=0.097, p=0.195). Glass knifefish typically exhibited higher $\dot{M}O_2$ (4.6 ± 0.9 $mmolkg^{-1}h^{-1}$; N=6) at the basal EOD (200-600 Hz) than did brown ghost knifefish (3.7 ± 0.7 $mmolkg^{-1}h^{-1}$; N=6) (basal EOD 700-1100 Hz) (student's *t*-test, p=0.015) (mean $\dot{M}O_2$ values were calculated from 6 individuals under identical conditions for each species).

3.1 Series I: Hypoxia

To examine the effect of reduced O_2 availability on electric signalling, brown ghost and glass knifefish were exposed to graded hypoxia while $\dot{M}O_2$, EOD_f, EOD_{amp} and EOD_{cv} were measured. Both species responded to hypoxia by significantly reducing $\dot{M}O_2$ (fig. 3-2). At the most severe level of hypoxia (PwO_2 = ~50 Torr), $\dot{M}O_2$ was reduced by 32% and 43% in brown ghost and glass knifefish, respectively. Both species of fish were able to maintain stable EOD frequencies throughout the hypoxic exposure (fig. 3-2). Mean amplitude (0.67 mV ± .027) and cycle variance (3.5×10^{-4} ± 6.6×10^{-6}) of the EOD also remained constant throughout hypoxic exposure in glass knifefish (two-way RM ANOVA; p=0.70 and p=0.515 respectively; data not shown).

Although fish exposed to hypoxia were more active, even under normoxic conditions, than control fish, no significant changes in activity were observed in either hypoxic or control groups during hypoxic exposure (two-way RM ANOVA; p=0.006 for group, p=0.748 for P_wO_2, p=0.348 for interactions between group and P_wO_2) (data not shown).

Changes in ventilation are one means through which fish attempt to match oxygen uptake to oxygen demand. Brown ghost knifefish exhibited a moderate ventilatory response to hypoxic exposure, consisting of an increase in ventilation amplitude, V_{amp} (fig. 3-4); ventilation frequency, V_f, did not change significantly during hypoxic exposure (data not shown; two-way RM ANOVA; p=0.164 for group, p=0.175 for P_wO_2, p=0.751 for the interaction between group and P_wO_2).

Axial muscle tissue samples collected from brown ghost knifefish immediately after hypoxic exposure were analyzed for lactate concentrations. Lactate levels in axial muscle tissue collected from the dorsal mid-body of the fish, and in axial muscle responsible for powering ribbon fin movement, were not significantly elevated in fish exposed to hypoxia compared to fish maintained under normoxic conditions (fig. 3-5).

3.2 Series II: Exercise

$\dot{M}O_2$ increased significantly as a function of swimming speed during trials in which water velocity was progressively increased (fig. 3-6). Although we had predicted that EOD frequency would be reduced during exercise, EOD frequency increased significantly as a function of swimming speed in brown ghost knifefish, reaching a peak increase of approximately 4% at top speeds (fig. 3-7). A trend for increased EOD

frequency during swimming was observed in all individuals tested. To investigate whether the observed increase in EOD frequency during swimming was an effect of swimming time, or swimming speed, brown ghost knifefish were swum for 1 h at a constant velocity of either 7 (low velocity) or 15 cms^{-1} (high velocity). EOD frequency increased rapidly upon commencement of swimming in the high velocity group, reaching a maximum after approximately 20 min of swimming (fig. 3-8). No significant increase in EOD frequency was observed in the low velocity group. In the high velocity group, $\dot{M}O_2$ increased rapidly upon commencement of swimming from the routine value of 10.1 ± 1.3 $mmolkg^{-1}h^{-1}$ (N=6) to 16.1 ± 2.9 $mmolkg^{-1}h^{-1}$ (N=6) and remained constant over the course of the 60 min swim period (one-way RM ANOVA; p=0.527). A similar pattern was observed in the low velocity group, with $\dot{M}O_2$ increasing from the routine value of 11.2 ± 1.8 $mmolkg^{-1}h^{-1}$ (N=6) to 18.8 ± 2.3 $mmolkg^{-1}h^{-1}$ (N=6) and remaining at this value throughout the swim trial (one-way RM ANOVA; p=0.249).

During closed system respirometry trials, water temperature tended to increase (by 0.5°C) at the highest water velocities (10 to15 cms^{-1}). Although EOD frequency (and $\dot{M}O_2$) increased much more abruptly than did temperature, an additional experiment was carried out to assess the effects of swimming speed on EOD frequency under constant temperature conditions (i.e. ± 0.2°C). Both brown ghost and glass knifefish displayed a significant increase in EOD frequency with water velocity during trials in which open water circulation was maintained to prevent water temperature increases (fig. 3-9). The increase in EOD frequency measured in brown ghost knifefish during open circulation trials was significantly lower, at 3.0 ± 0.3% (at top speed), than

that observed during the closed-system respirometry trials (3.7 ± 1.1%) in which the 0.5°C increase in temperature occurred (two-way RM ANOVA; $p=0.763$ for treatment, $p<0.001$ for swimming speed, $p<0.001$ for treatment vs. swimming speed).

3.3 Series III: Jamming avoidance response and long-term frequency elevation

The jamming avoidance response (JAR) and long-term frequency elevation (LTFE) are examples of modulations of the EOD that can be used to examine the metabolic cost associated with changes in EOD frequency. Glass knifefish were used for this experiment because they produce a robust JAR (Bullock et al., 1972). Exposure to the JAR stimulus or LTFE protocol increased EOD frequency significantly (fig. 3-10). During the period of increased EOD frequency associated with the JAR, $\dot{M}O_2$ was also significantly elevated above the resting value (fig. 3-10). However, $\dot{M}O_2$ did not differ from baseline values during LTFE even though EOD frequency remained elevated (fig. 3-10). In a control experiment carried out on a separate group of fish, glass knifefish were exposed to a neutral jamming stimulus (alternating Df, ±3 Hz, see Methods) designed to examine the effect of stimulus presentation itself on $\dot{M}O_2$, while EOD frequency remained constant (neutral JAR). Exposure to the neutral JAR stimulus did not elevate EOD frequency significantly. Although $\dot{M}O_2$ responses of the fish exposed to the neutral jamming stimulus were highly variable ($\dot{M}O_2$ increased in 4 of 6 fish and decreased in 2 of 6 fish), overall a significant increase in $\dot{M}O_2$ was measured during neutral JAR trials (fig. 3-10). Figure 3-11 presents $\dot{M}O_2$ rates for individual fish as a

function of EOD frequency during JAR, LTFE and neutral JAR. In some cases, $\dot{M}O_2$ decreased while EOD increased.

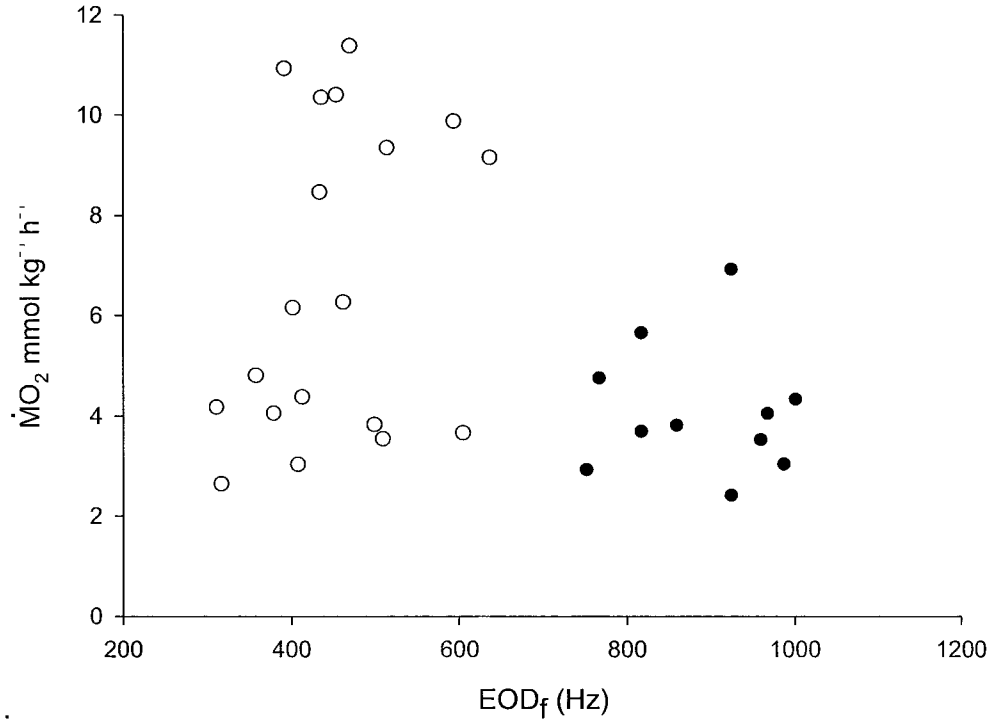
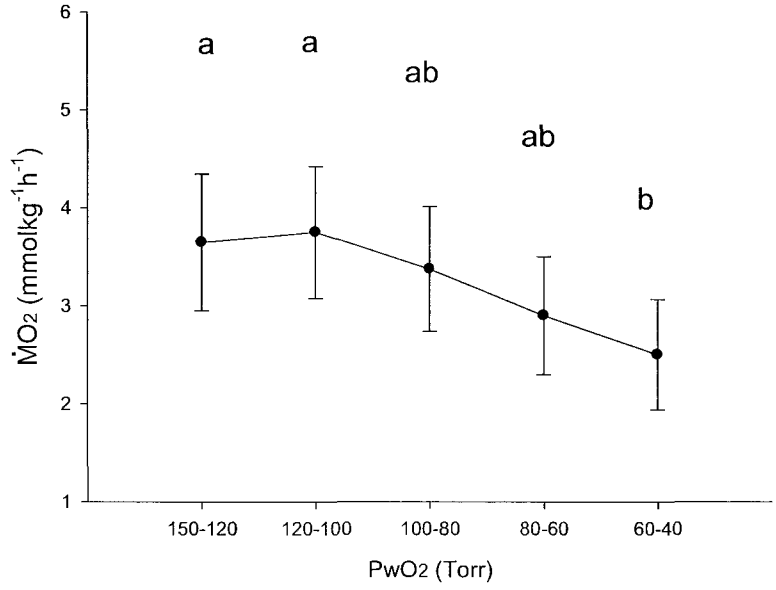

12
10
8
6
4
2
0
200
400
600
800
1000
1200
$\dot{M}O_2$ mmol kg^{-1} h^{-1}
EOD_f (Hz)

Figure 3-1. Mass specific oxygen consumption rate ($\dot{M}O_2$) and EOD frequency in brown ghost (*A. leptorhynchus*) and glass (*E. virisciens*) knifefish. Brown ghost knifefish are represented by the filled symbols (N=11; R=0.065, p=0.849), and glass knifefish are represented by the unfilled symbols (N=19; R=0.311, p=0.195) (linear regression analysis). Data presented are for individual fish emitting a basal EOD frequency under normoxic, resting conditions.

A.


a
a
ab
ab
b
6
5
4
3
2
1
$\dot{M}O_2$ (mmolkg^{-1}h^{-1})
150-120
120-100
100-80
80-60
60-40
PwO2 (Torr)

B.

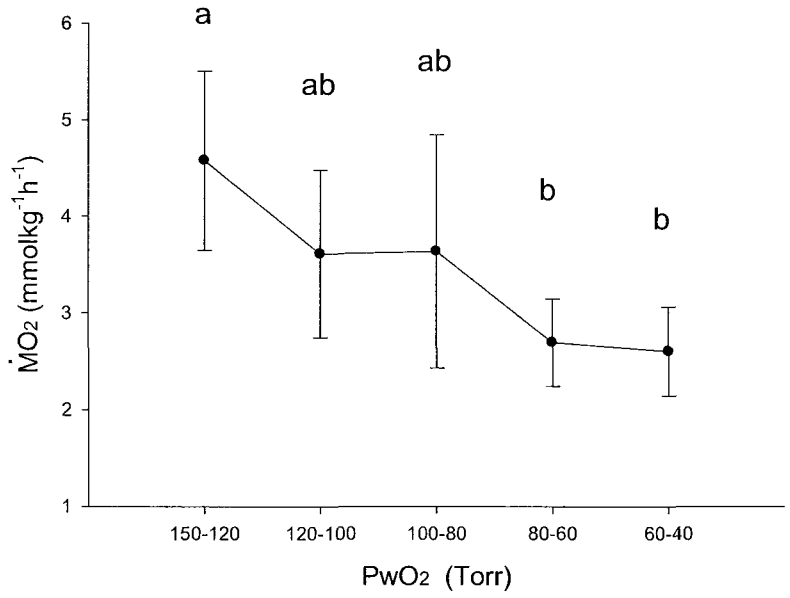
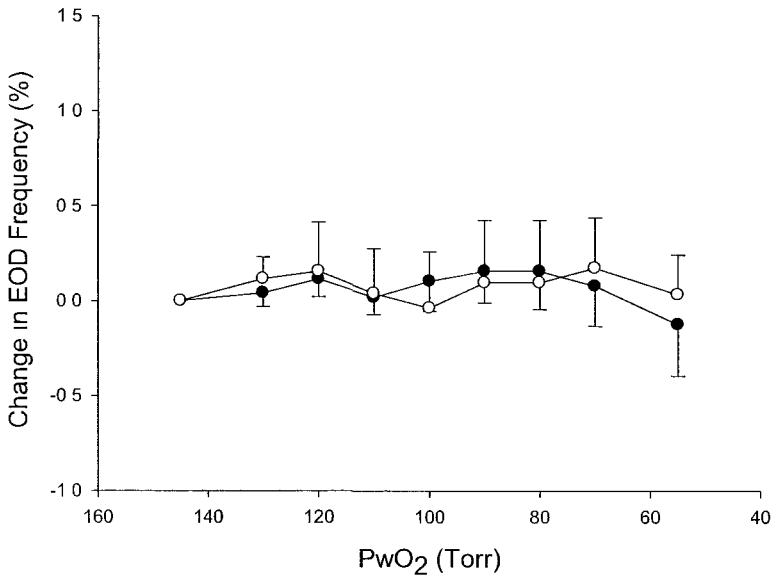

a
ab
ab
b
b
6
5
4
3
2
1
$\dot{M}O_2$ (mmolkg^{-1}h^{-1})
150-120
120-100
100-80
80-60
60-40
PwO2 (Torr)

Figure 3-2. $\dot{M}O_2$ as a function of water PO_2 (PwO_2) for (A) brown ghost (*A. leptorhynchus*) and (B) glass (*E. virisciens*) knifefish during exposure to graded hypoxia. The PwO_2 ranges for each measurement point were 150-120, 120-100, 100-80, 80-60, and 60-40 Torr. Data presented are means ± SEM (N=6 for each species). Data points that do not share a letter are significantly different from one another [one-way RM ANOVA, $p<0.001$ for (A) and $p=0.002$ for (B)].

A.


Change in EOD Frequency (%)
1 5
1 0
0 5
0 0
-0 5
-1 0
160
140
120
100
80
60
40
PwO2 (Torr)

B.

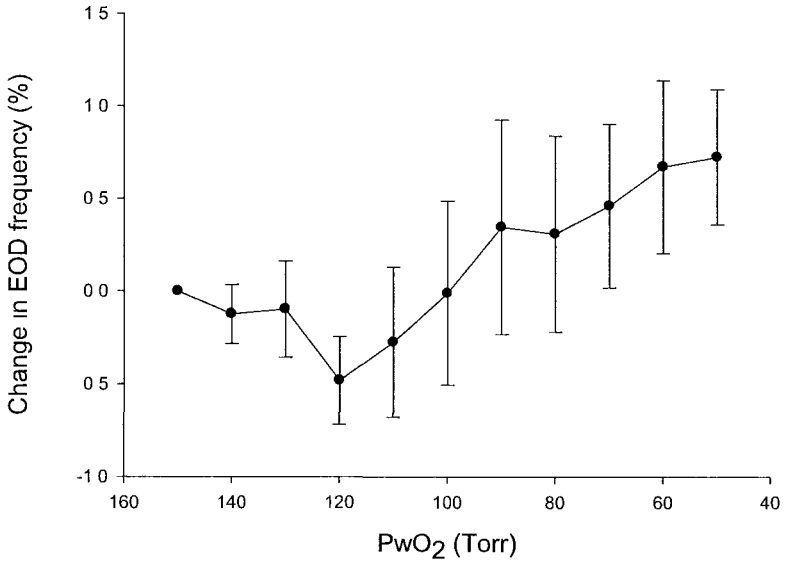

Change in EOD frequency (%)
1 5
1 0
0 5
0 0
0 5
-1 0
160
140
120
100
80
60
40
PwO2 (Torr)

Figure 3-3. Percentage change in EOD frequency during exposure to graded hypoxia. (A) Brown ghost knifefish (*A. leptorhynchus*) exposed to graded hypoxia maintained a nearly constant EOD frequency. Fish exposed to hypoxia (unfilled circles) were compared to those maintained under normoxic (filled circles) conditions for an equivalent period. No significant difference was observed between hypoxic (N=12 for 150-70 Torr, N=8 for 60-50 Torr) and control (N=6) groups (two-way RM ANOVA; $p=0.930$ for the effect of group, $p=0.865$ for the effect of PwO_2, $p=0.741$ for the interaction between group and PwO_2). (B) Glass knifefish (*E. virisciens*) (N=6) exposed to graded hypoxia also maintained a near constant EOD frequency (one-way RM ANOVA; $p=0.066$). Change in EOD frequency was calculated by subtracting from each data point the value of EOD frequency under normoxic conditions; the change was then expressed as a percentage of the normoxic EOD frequency. Data are mean values ± SEM.

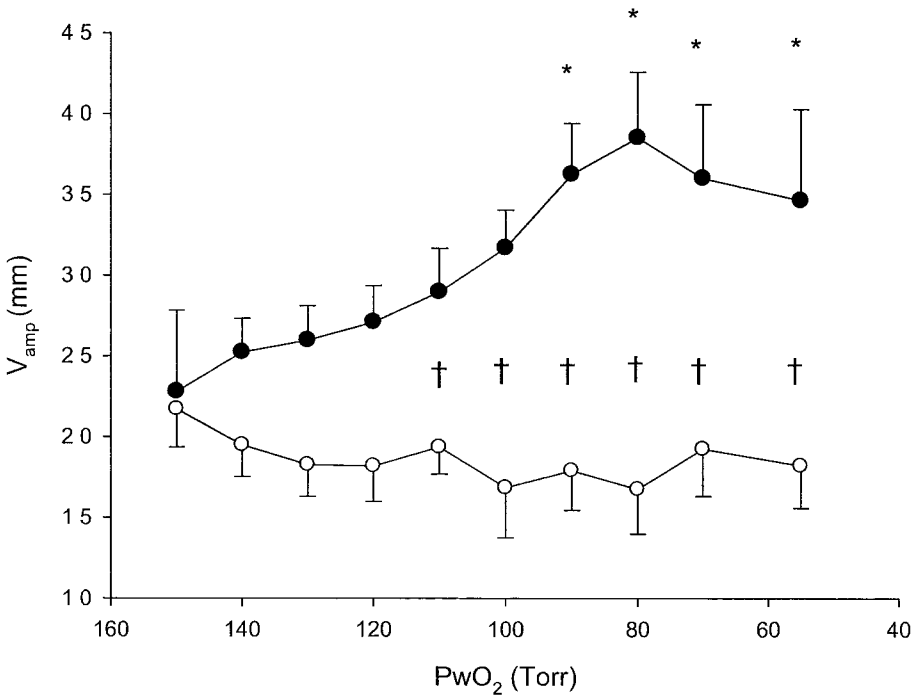

45
40
35
30
25
20
15
10
V_{amp} (mm)
160
140
120
100
80
60
40
PwO_2 (Torr)
*
*
*
*
†
†
†
†
†
†

Figure 3-4. Ventilation amplitude, V_{amp}, during hypoxic exposure in brown ghost knifefish (*A. leptorhynchus*). Ventilation amplitude increased significantly in hypoxic fish (N=6, closed circles) compared to control fish maintained under normoxia (N=6, open circles). An asterisk indicates a significant difference from the normoxic time point within a group. A dagger indicates significant differences between the hypoxic and control groups (two-way RM ANOVA; $p=0.005$ for the effect of group, $p=0.028$ for the effect of PwO_2, $p<0.001$ for the interaction between group and PwO_2). Data are mean values ± SEM.

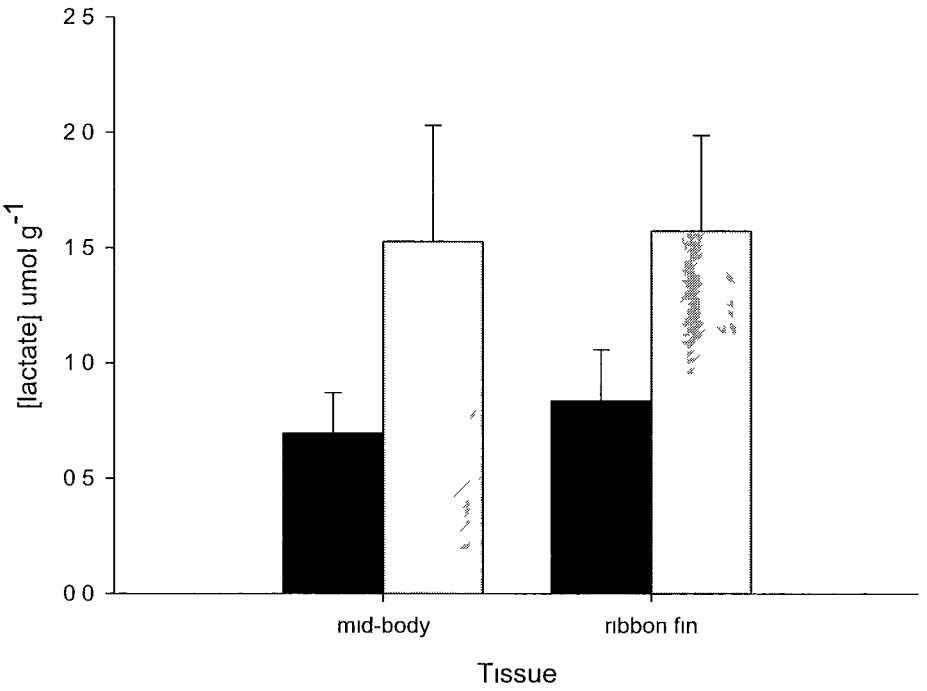

25
20
15
10
05
00
[lactate] umol g-1
mid-body
ribbon fin
Tissue

Figure 3-5. Lactate concentration ([lactate]) in axial muscle tissue samples collected from hypoxic (N=6; grey bars) and normoxic (N=6; black bars) brown ghost knifefish (*A. leptorhynchus)*. No significant differences in lactate concentration were observed (two-way RM ANOVA; $p=0.110$ for the effect of group, $p=0.415$ for the effect of tissue, $p=0.560$ for the interaction between group and tissue). Data presented are means ± SEM.

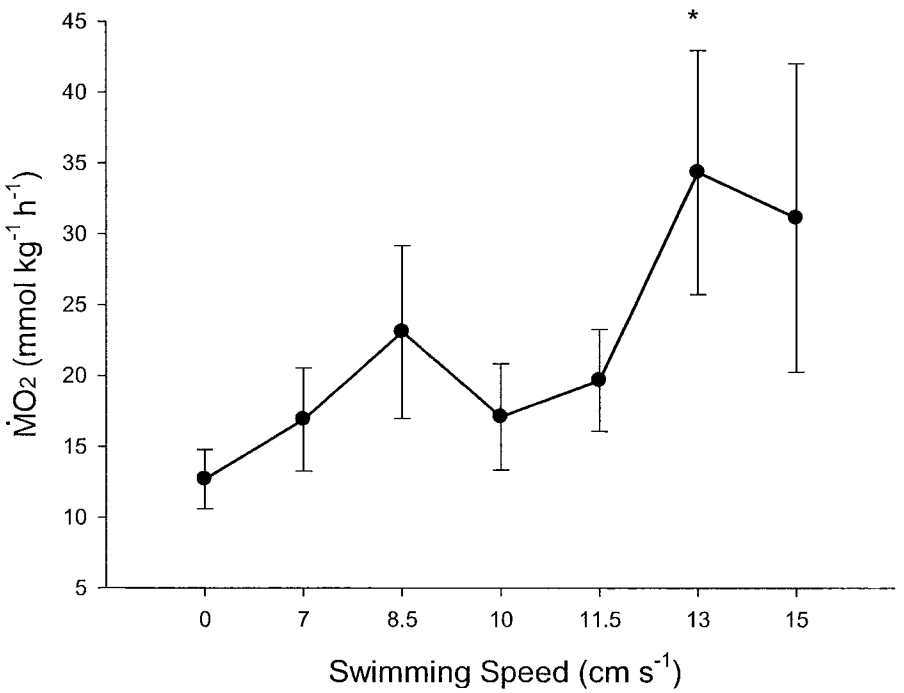

*
45
40
35
30
25
20
15
10
5
$\dot{M}O_2$ (mmol kg^{-1} h^{-1})
0
7
8.5
10
11.5
13
15
Swimming Speed (cm s^{-1})

Figure 3-6. $\dot{M}O_2$ as a function of swimming speed in brown ghost knifefish (*A. leptorhynchus)*. Oxygen consumption increased significantly as swimming speed increased but post hoc comparisons were unable to identify the points of difference (one-way RM ANOVA on ranks; p=0.045). Data presented are means ± SEM (N=8).

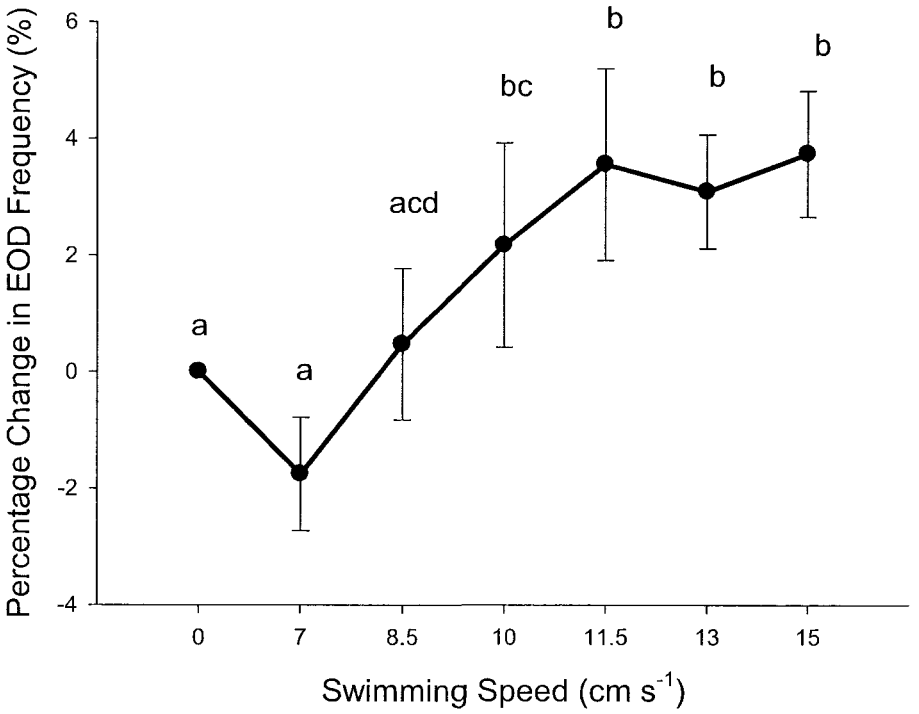

Percentage Change in EOD Frequency (%)
6
4
2
0
-2
-4
a
a
acd
bc
b
b
b
0
7
8.5
10
11.5
13
15
Swimming Speed (cm s-1)

Figure 3-7. Percentage change in EOD frequency as a function of swimming speed in brown ghost knifefish (*A. leptorhynchus*). EOD frequency increased significantly as swimming speed increased. Data points that share a letter are not significantly different from one another (one-way RM ANOVA; $p<0.001$) (N=6). Change in EOD frequency was calculated by subtracting from each data point the value of EOD frequency at rest; the change was then expressed as a percentage of the resting EOD frequency. Data are mean values ± SEM.

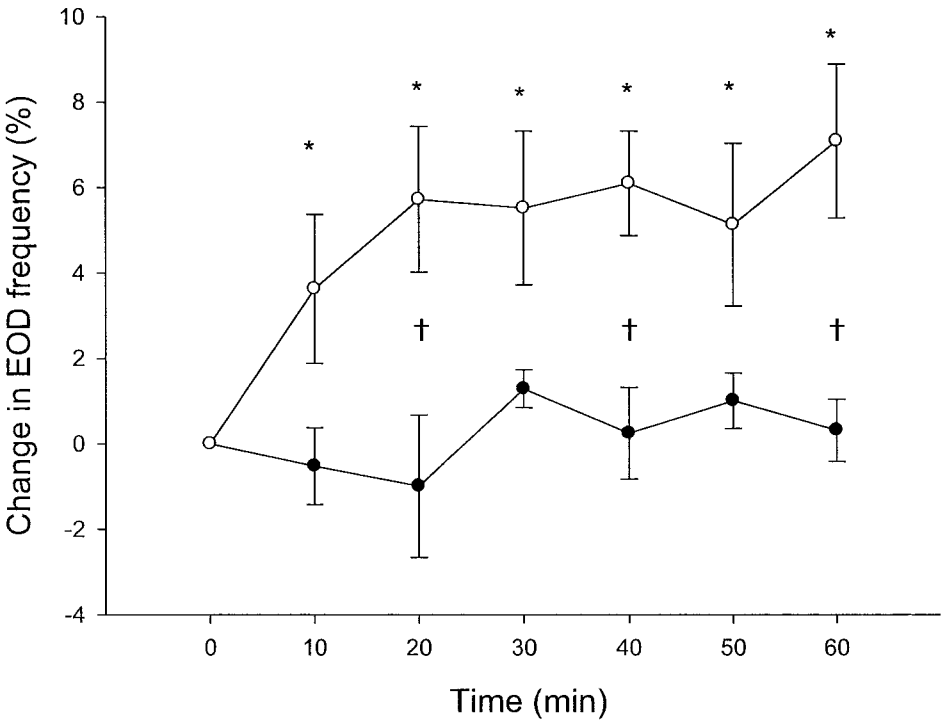
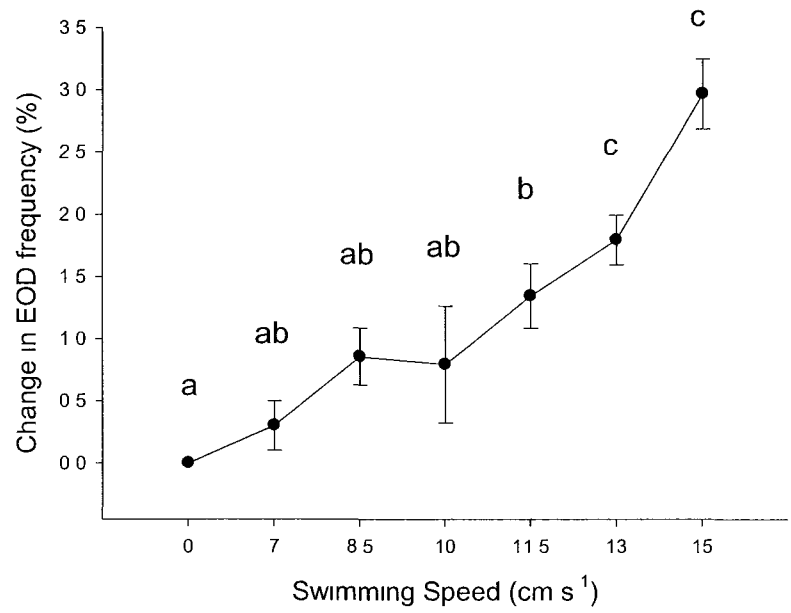

Change in EOD frequency (%)
10
8
6
4
2
0
-2
-4
0
10
20
30
40
50
60
Time (min)
*
*
*
*
*
*
†
†
†

Figure 3-8. Percentage change in EOD frequency during high (unfilled symbols; N = 6) or low–velocity (filled symbols; N=6) swimming in brown ghost knifefish (*A. leptorhynchus*). EOD frequency increased significantly in the high velocity group but remained nearly constant in the low velocity group. An asterisk indicates significant differences from resting values within a group. A dagger indicates a significant difference between groups (two-way RM ANOVA; $p=0.05$ for the effect of group, $p<0.001$ for the effect of time, $p=0.004$ for the interaction between group and time). Change in EOD frequency was calculated by subtracting from each data point the value of EOD frequency at rest; the change was then expressed as a percentage of the resting EOD frequency. Data are mean values ± SEM.

A.


Change in EOD frequency (%)
35
30
25
20
15
10
05
00
a
ab
ab
ab
b
c
c
0
7
8 5
10
11 5
13
15
Swimming Speed (cm s 1)

B.

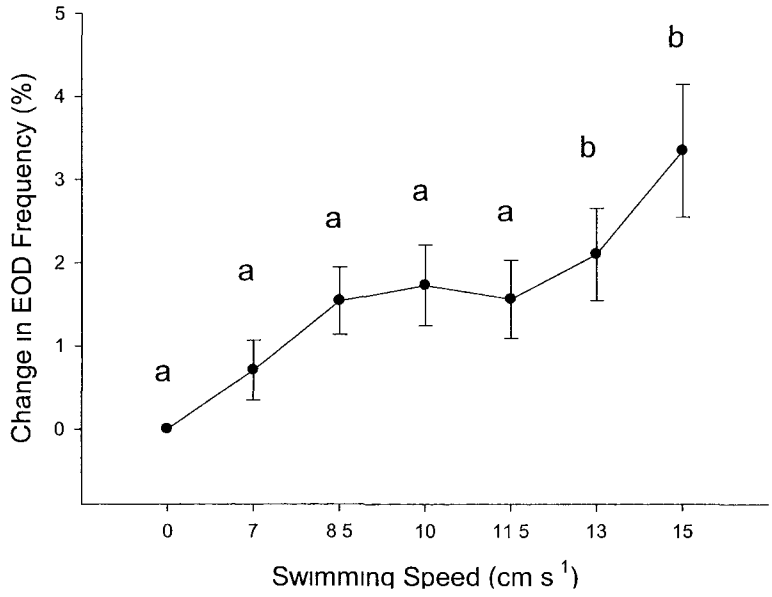
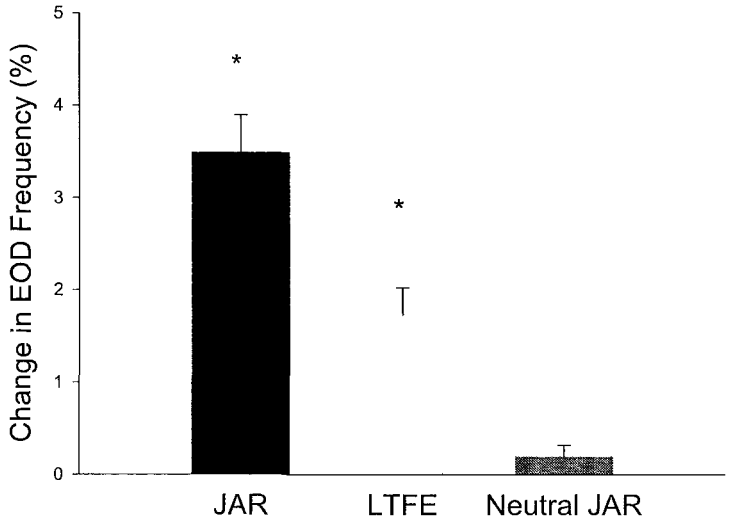

Change in EOD Frequency (%)
5
4
3
2
1
0
a
a
a
a
a
b
b
0
7
8 5
10
11 5
13
15
Swimming Speed (cm s 1)

Figure 3-9. Percentage changes in EOD frequency during open circulation swimming trials for (A) brown ghost knifefish (*A. leptorhynchus*) and (B) glass knifefish (*E. virisciens*) (N=6 for each species). EOD frequency increased significantly in both species as swimming speed increased. Data points that share a letter are not significantly different from one another (one-way RM ANOVA; $p<0.001$ for each species). Change in EOD frequency was calculated by subtracting from each data point the value of EOD frequency at rest; the change was then expressed as a percentage of the resting EOD frequency. Data are mean values ± SEM.

A.


Change in EOD Frequency (%)
5
4
3
2
1
0
*
*
JAR
LTFE
Neutral JAR

B.

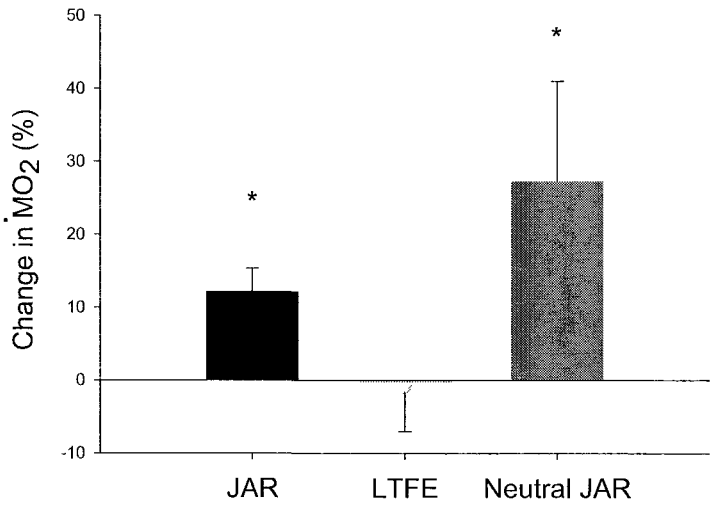

Change in $\dot{M}O_2$ (%)
50
40
30
20
10
0
-10
*
*
JAR
LTFE
Neutral JAR

Figure 3-10. Percentage changes in $\dot{M}O_2$ associated with increases in EOD frequency in glass knifefish (*E. virisciens*) during EOD frequency modulations. $\dot{M}O_2$ was measured during exposure to a jamming stimulus (JAR), following a 30 min exposure to the jamming stimulus (LTFE), or during exposure to a neutral jamming stimulus (neutral JAR). (A) Percentage change in EOD frequency. EOD_f was significantly elevated in JAR ($p< 0.001$) and LTFE ($p=0.002$) conditions, but not neutral JAR ($p=0.202$). (B) Percentage change in $\dot{M}O_2$. $\dot{M}O_2$ was significantly elevated in JAR ($p=0.016$) and neutral JAR ($p<0.001$) but not LTFE ($p=0.359$) conditions. An asterisk indicates that the change was significantly different from zero (one sample Student's *t*-test; p values as noted above). Change in EOD frequency and $\dot{M}O_2$ were calculated by subtracting from each data point the value of EOD frequency and or $\dot{M}O_2$ at rest; the change was then expressed as a percentage of the resting EOD frequency or $\dot{M}O_2$. Data are mean values ± SEM.

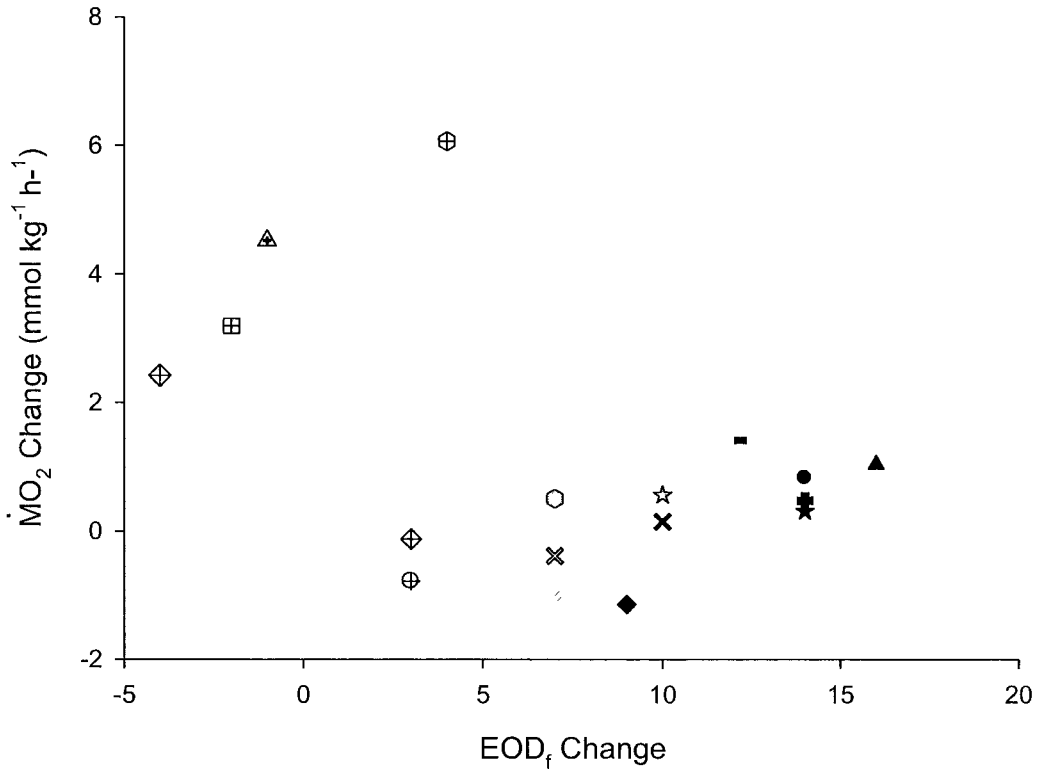

8
6
4
2
0
-2
-5
0
5
10
15
20
$\dot{M}O_2$ Change (mmol kg^{-1} h^{-1})
EOD_f Change

Figure 3-11. The difference in $\dot{M}O_2$ as a function of EOD change in glass knifefish (*E. virisciens*) during JAR (black symbols), LTFE (grey symbols) and Neutral JAR (white crossed symbols). $\dot{M}O_2$ was measured while fish were emitting a basal EOD (resting), when EOD frequency was elevated during JAR and/or LTFE, and when EOD was relatively constant during a neutral JAR. Measurements collected during LTFE and JAR in an individual fish share the same symbol. $\dot{M}O_2$ and EOD frequency change were calculated by subtracting data collected during JAR, LTFE and Neutral JAR from resting values. Absolute change data for individual fish are presented (N=15).

CHAPTER FOUR:
Discussion

The current study aimed to test the hypothesis that production of an EOD is metabolically costly. The metabolic cost of electric signalling was assessed by manipulating $\dot{M}O_2$ and/or EOD and examining the effects on EOD or $\dot{M}O_2$ as appropriate using hypoxia exposure, exercise and direct frequency modulation (JAR, jamming avoidance response, and LTFE, long-term frequency elevation). Hypoxia exposure resulted in a significant decrease in $\dot{M}O_2$; however, EOD frequency, amplitude and timing variability remained constant. $\dot{M}O_2$ increased significantly during swimming trials, as did EOD frequency, contrary to our prediction, and the idea that electric signalling is metabolically costly. Experiments aimed at eliciting EOD frequency elevation via the JAR and LTFE revealed a significant increase in $\dot{M}O_2$ during JAR, with values returning to baseline during LTFE despite frequency elevation. It is not clear whether the increase in $\dot{M}O_2$ observed during JAR is a result of EOD frequency elevation, or simply an effect of jamming stimulus presentation. Our results indicate that the electric organ discharge does not represent a major fraction of the overall energy budget of weakly electric fish, and thus may not be as metabolically costly as previously thought.

4.1 $\dot{M}O_2$ as a function of EOD frequency

If EOD production is metabolically costly, then routine $\dot{M}O_2$ might be expected to vary with EOD frequency both within and across species. However, the results of the present study do not support this prediction. Within species, fish with higher EOD

frequencies did not in turn have an elevated mass specific $\dot{M}O_2$, particularly in the brown ghost, where some of the lowest EOD frequencies recorded in the group corresponded to the highest $\dot{M}O_2$ values. In comparing species, the lack of relationship becomes even clearer. Glass knifefish, with a lower EOD frequency range, have higher $\dot{M}O_2$ values than brown ghosts, which have a much higher EOD frequency range. This difference in basal $\dot{M}O_2$ could reflect differences in electric organ structure, as the glass knifefish has a myogenic electric organ while the brown ghost has a neurogenic electric organ (Bennett, 1961). Perhaps there is an elevated cost associated with EOD production in a muscular electric organ, owing to the chemical synapse required for activation. However, energetic cost did not depend on EOD type in other studies. For example, Julian et al., (2003) found that $\dot{M}O_2$ did not differ between pulse- and wave-type Gymnotiforms during EOD generation; a finding that is inconsistent with the idea that pulse- and wave-type weakly electric fish have different habitat distributions (i.e. hypoxic vs. well-oxygenated water) due to differences in metabolic costs of EOD production (Crampton, 1998a). Observed differences in routine $\dot{M}O_2$ could be caused by a number of interspecies differences beyond EOD frequency.

4.2 Manipulation of $\dot{M}O_2$: Hypoxia

Two metabolic phases were observed during hypoxic exposure. $\dot{M}O_2$ was maintained in the initial phase of hypoxic exposure (approximately 150 to 80 Torr, fig. 3-2). Several compensatory mechanisms could be employed by knifefish to defend

oxygen uptake during mild to moderate hypoxia (Gilmour, 2001; Perry et al., 2009; Perry and Gilmour, 2009). Ventilatory adjustments are typically employed to match gas exchange with the demands of oxidative metabolism (Gilmour, 2001; Perry et al., 2009). Consistent with this strategy, brown ghost knifefish increased V_{amp} significantly while maintaining constant V_f during the initial phase of hypoxia. These findings are comparable to those of previous studies which suggest that increases in V_{amp} are more efficient than increases in V_f for increasing O_2 uptake and are therefore usually the dominant ventilatory adjustment observed during hypoxia (Gilmour, 2001; Perry et al., 2009).

As hypoxia became more severe (approximately 80 to 50 Torr, fig. 3-2), significant decreases in $\dot{M}O_2$ were observed despite hyperventilation (increased V_{amp}). As the cost of ventilation increases during hyperventilation and consumes more of the overall $\dot{M}O_2$, many fish reduce ventilation because the cost-benefit ratio is no longer in their favour (Perry et al., 2009). $\dot{M}O_2$ remained stable until a PwO_2 of approximately 80 - 100 Torr was reached for both species, but was reduced significantly as hypoxia became more severe; a response consistent with matching metabolic demands to O_2 availability in an O_2-limited environment (Hochachka and Lutz, 2001; Richards, 2010). Although P_{crit} was not measured in this study, the point at which the animal changed strategies from defending O_2 uptake (by ventilatory adjustments, for example) to reducing metabolic demand, appeared to occur between approximately 100 Torr and 80 Torr in both brown ghost and glass knifefish. As predicted, the estimated P_{crit} range for wave-type gymnotiform fish in these experiments was much higher than published

values for other teleost species. For example, the P_{crit} for the rainbow trout (*Onchorhynchus mykiss*), a hypoxia intolerant species, at 20°C is approximately 27 Torr (Ott et al., 1980). The P_{crit} for a hypoxia tolerant species, the zebrafish (*Danio rerio)*, is approximately 20 Torr at 28°C (Ott et al., 1980). In the Crucian carp (*Carrassius carrassius*), a hypoxia tolerant species, the P_{crit} value at 25°C is approximately 20 Torr (Ott et al., 1980). P_{crit} values for 10 species of teleost that exhibit ventilation patterns similar to that of the brown ghost (increases in V_{amp} amplitude accompanied by modest increases in V_f) range from approximately 20 Torr (*Hoplias malabaricus, Scpohthalmus maximus*) to approximately 70 Torr in *Anguilla anguilla* and *Platichthys flesus.* The Japanese eel (*A. Japonica)* is an example of a water-breathing teleost with an exceptionally high P_{crit}, approximately 115 Torr (reviewed by Perry et al., 2009). Our estimated P_{crit} range lies in the high end of P_{crit} values for teleost fish. Future experiments aimed at determining formal P_{crit} values for gymnotiform species would be useful.

Behaviour monitoring during hypoxia experiments indicated that the brown ghost knifefish did not significantly alter activity level during exposure to graded hypoxia. Therefore, reducing activity was not employed as a means of O_2 conservation during hypoxic exposure. Increasing reliance on anaerobic metabolism is often employed as a means of meeting metabolic demand during hypoxic exposure. Muscle lactate concentrations in hypoxic brown ghost knifefish tended to be higher but were not significantly different from controls, suggesting a limited contribution of anaerobic metabolism to meet metabolic demands despite reduced O_2 availability. Analysis was

carried out on axial muscle tissue thought to be powering the ribbon fin, and from the mid-body. Ideally, lactate concentration could be measured in the electric organ itself; difficulties in collecting and analyzing small amounts of tissue prevented measurement of electric organ lactate levels in the present study. Measuring lactate levels in the tail of the fish (where the electric organ is the predominant tissue) may provide a more direct means of examining whether anaerobic metabolism is involved in the maintenance of EOD frequency during hypoxia.

The brown ghost and glass knifefish maintained a nearly constant EOD frequency during hypoxic exposure despite reduced environmental O_2 availability and a significant reduction in $\dot{M}O_2$. The small changes in frequency observed were not statistically significant. This finding was not in agreement with the prediction that EOD frequency would be reduced during hypoxic exposure. The timing of successive EOD cycles (timing variability) and EOD amplitude also remained stable during hypoxic exposure. Since $\dot{M}O_2$ decreased significantly during hypoxic exposure in both brown ghost and glass knifefish, EOD production must have occupied a larger fraction of available $\dot{M}O_2$. The maintenance of EOD frequency, amplitude, and timing variability during hypoxic exposure perhaps points to the importance of this sensory system in knifefish, but is difficult to reconcile with the hypothesis that EOD generation is a metabolically costly process.

A second conclusion from these experiments is that these knifefish appear to be more hypoxia tolerant than expected (Crampton, 1998a). Increases in ventilation amplitude in the brown ghost, although statistically significant, were employed to a

lesser degree than that observed in other hypoxic teleost fish. V_{amp} increased by approximately 70% in brown ghost, relative to an average V_{amp} increase of approximately 143% calculated from 15 teleost species exposed to acute hypoxia (reviewed by Perry et al., 2009). Although ventilation is just one of the many possible responses to hypoxia, this relatively blunted ventilatory response may indicate that a PwO_2 level of 50 Torr is not a severe hypoxic challenge for the brown ghost knifefish. The minimum PwO_2 value used in this experiment (50 Torr) was chosen conservatively to elicit hypoxia responses without having detrimental effects on the fish. Although significant increases in ventilation amplitude and decreases in $\dot{M}O_2$ were elicited at this level of hypoxia, perhaps exposing the fish to lower PwO_2 levels would result in a greater hypoxic response, including effects on EOD characteristics as the EOD begins to account for more and more of the fish's energy budget.

Aquatic surface respiration (ASR) is another strategy used by fish that live in hypoxic environments to overcome limitations on aquatic O_2 availability. During ASR, the gills are ventilated with water from a thin zone of relatively oxygen rich water at the air-water interface (Perry et al., 2009). Although gymnotiform species live in hypoxic habitats (mainly pulse type fish), the respiratory behaviour of only three species has been documented (Crampton, 1998b). The electric eel, *Electrophorus electricus*, is an obligate air breather, relying on a hypervascularized lining of the buccal cavity as a respiratory organ (Farber and Rahn, 1970; Garey and Rahn, 1970; Johansen, 1970). Two species of pulse-type fish, *Gymnotus carapo* and *Brachyhypopomus pinnicaudatus,* are facultative air-breathers. *G. carapo* uses a modified swim bladder as

an accessory respiratory organ (Liem et al., 1984), while *B. pinnicaudatus* absorbs oxygen across its gill by trapping bubbles of air in its gill chamber (Carter and Beadle, 1931; Hopkins, 1991). The brown ghost and glass knifefish are known to rely on ASR (Reardon et al., personal communication; Crampton, 1998a). It is possible that knifefish are more hypoxia tolerant than our data suggest, as respirometry measurements and hypoxia exposure took place in a closed chamber that did not allow ASR. Further experiments may be useful in assessing the hypoxia tolerance levels of the brown ghost. It is possible that knifefish are more hypoxia tolerant than was previous thought based on habitat distribution studies (Crampton, 1998a).

4.3 Manipulation of $\dot{M}O_2$: Swimming

Locomotion is an energetically expensive activity. $\dot{M}O_2$ increased significantly during swimming in brown ghost knifefish; however, the approximately 2-fold increase in $\dot{M}O_2$ observed during increasing swimming speed trials was more variable than is typical of a swimming teleost. Brett (1964) reported that $\dot{M}O_2$ tends to increase linearly with swimming speed, but this typical, strong linear relationship was not observed in the present study. This discrepancy could be due to a number of factors. One possibility is non-laminar flow in the swim chamber. If some turbulence is present, fish may reduce their workload by taking advantage of small eddies in the chamber. A variable $\dot{M}O_2$ response could also be in part due to the relatively low range of swimming velocities studied. For example, the bluegill sunfish (*Lepomis macrochirus*), a labriform swimmer,

must swim at speeds of 1.7-1.9 BL/S before a measurable increase in $\dot{M}O_2$ is achieved (Drucker and Lauder, 1999). Maximum sustained swimming speeds (velocities that can be maintained for 200 mins) range from approximately 2.2 BL/S (*Pollachius virens)* to 7.1 BL/S (*Chromis punctipinnis)* in teleost fish (Videler and Wardle, 1999). The 2-fold increase in $\dot{M}O_2$ observed in swimming fish in the current study is similar to the 2-fold increase in $\dot{M}O_2$ observed by Julian et al. (2003) during scan swimming, supporting the suggestion that fish were not pushed to their swimming limit in this study.

Knifefish swimming is driven by a propulsive anal fin that passes a sinusoid-like wave down its length, while the body can be moved independently for electrosensing (Shirgaonkar et al., 2008; MacIver et al 2010). In addition to being elegant swimmers, previous research on ribbon-finned swimmers has also suggested that they are highly efficient for movement at low velocities (Blake, 1983; Lighthill and Blake, 1990). The sleek rigid body may allow knifefish to experience significantly less viscous drag when swimming than fish of equivalent size swimming at the same speed using other swimming modes (Blake, 1983). A recent examination of knifefish swimming used an accurate urethane model of a black ghost knifefish, *Apteronotus albifrons*, a species with a similar body plan to both the brown ghost and glass knifefish, and digital particle image velocimetry to calculate the drag force acting on the fish's body while swimming at velocities of 10-15 cms^{-1} (Shirgaonkar et al., 2008). The drag force at these velocities ranged between 1 and 2 mN respectively (Shirgaonkar et al., 2008). Similar techniques were used to examine the effect of drag on the body of species that uses labriform swimming (powered by the pectoral fins), the bluegill sunfish (*L. macrochirus*)

(Drucker and Lauder, 1999). This species uses labriform swimming at low velocities, and a combination of caudal and pectoral fins and oscillatory swimming at higher velocities. The calculated drag force acting on the fish's body was 10.58 mN at velocities of 10 cms^{-1} where swimming was strictly labriform (Drucker and Lauder, 1999), approximately 10 times higher than that of the black ghost knifefish at the same velocity. Recent research also suggests that the knifefish body shape maximizes forward propulsion from the anal fin, making the knifefish body plan a desirable model for highly maneuverable underwater vehicles for application in environmental monitoring (Epstein et al., 2006; MacIver et al., 2004).

EOD frequency was monitored at different water velocities throughout the swimming trial. We predicted that EOD frequency would be reduced during exercise as a strategy to minimize energy costs other than those associated with powering skeletal muscle. Somewhat surprisingly, EOD frequency increased significantly with swimming speed in both brown ghost and glass knifefish, in 25 of 26 individuals tested. EOD frequency seemed to plateau at the highest speeds tested, indicating that increases in EOD frequency are not unlimited. $\dot{M}O_2$ and EOD frequency did not increase in parallel; EOD increased more rapidly, indicating that EOD generation was occupying a larger proportion of the overall energy budget during swimming. Because the EOD is temperature dependent with a Q_{10} value for EOD frequency of 1.62 (Dunlap et al., 2000), the small increases in water temperature (+0.5°C) that occurred during closed respirometry trials due to the swim pump heating the water may have contributed to the elevation of EOD frequency during swimming. However, additional control experiments

where water temperature remained constant confirmed that the increase in EOD frequency was not completely explained by increases in water temperature. Further experiments forcing fish to swim to their maximum capacity may give insight into whether this increase in EOD frequency is maintained, or whether metabolic restraints cause a reduction in EOD frequency under more extreme conditions. EOD amplitude was not monitored during swimming trials owing to technical difficulties associated with measuring signal amplitude in free-swimming fish in an approximately 3 L chamber with stationary recording electrodes. Markham et al. (2009) found that EOD amplitude can be rapidly modulated by recruitment of Na^+ channels in the electrocytes of *S. macrurus*, potentially as a means of reducing metabolic cost. Thus, it is possible that EOD amplitude is reduced during swimming as a means of reducing metabolic cost.

Although the increase in EOD frequency during swimming was not consistent with our predictions (or with the idea that EOD generation is metabolically expensive), it raises interesting questions about the mechanisms involved as well as the implications of an elevated EOD frequency during swimming. One possible explanation involves the maintenance of accurate sensory perception in fast moving waters. If one assumes that the EOD frequency is related to the rate at which the fish samples its environment, a higher EOD frequency would allow for higher resolution sampling. This assumes, of course, that downstream sensory networks can encode information at these rates. Thus, it is possible that the increase in EOD frequency observed is employed by the fish to maintain sensory acuity in fast moving waters. In support of this hypothesis, Lissman (1961) noted that pulse type species increase their EOD rate when they require more

temporal acuity for foraging. He speculated that fishes in fast moving water may require higher EOD frequencies in order to track moving objects. The large scale habitat distribution study of Crampton (1998a) did not note any relationship between EOD frequency of wave-type species and habitat selection. However, some correlations between lifestyle and EOD frequency have been previously noted, such as the type of prey captured by fish with high or low EOD frequencies (Crampton, 1998a). Lissman (1961) similarly proposed that fish feeding on mobile prey items should require a high EOD frequency. Among families with myogenic EODs, *Sternopygidae* and *Eigenmannidae*, the highest EOD frequencies are found in the genus *Rhabdolihops* (600-900 Hz), which feeds on mobile or planktonic prey items (Crampton, 1996; Lundberg et al., 1987). *Eigenmannia* and *Sternopygus*, both of which have EOD frequencies in the lower range (200-600 Hz), feed on stationary food items such as chironomids (Crampton, 1998a).

Although fast flowing rivers are typically dominated by wave-type species, some pulse species (e.g. *Steatogenys elegans* and *Rhamphichthys sp.*) also are successful in such rivers (Crampton, 1998a). Pulse-type fish living in fast flowing rivers exhibit EOD frequencies of approximately 60-110Hz, indicating that frequencies in this range provide sufficient temporal resolution to meet sensory demands (Crampton, 1998a). An important question is whether the approximately 3% increase in EOD frequency observed in our experiments would provide any sensory advantage in fish already firing at such high frequencies, particularly in the case of the brown ghost knifefish. The typical brown ghost increased EOD frequency from 813 to 837 Hz. In addition, from a

central processing perspective, it is not yet known whether central processing occurs on the same temporal scale as the external EOD (Chacron et al., 2001; Crampton, 1998a). Clearly, the relationship between swimming speed/water velocity and EOD parameters warrants further investigation.

4.4 Manipulation of EOD frequency: JAR and LTFE

In contrast to hypoxia or swimming in which $\dot{M}O_2$ was manipulated to observe the effect on EOD frequency, the JAR and LTFE provided a means of examining the metabolic cost of EOD frequency elevation directly. The relationship between EOD frequency elevation and $\dot{M}O_2$ was not, however, clear cut. We hypothesized that $\dot{M}O_2$ would increase with increases in the firing rate of electrocytes in the electric organ owing to the metabolic cost of neuronal activation. In agreement with our prediction, an EOD frequency elevation during JAR of 3-4% resulted in a significant, 12% increase in $\dot{M}O_2$. During LTFE conditions, EOD frequency elevation was maintained, albeit at a level slightly lower than during the JAR (1-2 %), in the absence of stimulation of the fish. However, $\dot{M}O_2$ did not remain significantly elevated. This experiment served as a control for the effect of the jamming stimulus itself on $\dot{M}O_2$. The lack of $\dot{M}O_2$ elevation during LTFE may suggest that the metabolic cost associated with an increase in EOD frequency is only incurred during the initial frequency increase; once the higher EOD frequency has been achieved, there is no longer an additional metabolic cost, allowing $\dot{M}O_2$ to return to baseline values. An alternate explanation is that the increase in $\dot{M}O_2$ observed during the JAR is simply a response to the jamming stimulus, independent of

EOD frequency elevation. Although activity levels remained low during JAR and LTFE conditions, it is possible that increases in sensory reception and processing (during the stimulus), or a metabolic response to the perception of a conspecific, caused an increase in $\dot{M}O_2$.

The neutral JAR experiments were designed to test whether the increase in $\dot{M}O_2$ observed during the JAR but not the LTFE was a result of EOD frequency elevation, or as mentioned above, a result of increases in neural processing or the perception of a conspecific. These experiments involved a repeated switch from increasing to decreasing EOD frequency, with an average increase of zero. If the metabolic cost of the JAR reflects the activation of mechanisms to raise EOD frequency, then $\dot{M}O_2$ should not increase during the neutral JAR because EOD frequency will not change. On the other hand, if the metabolic cost of the JAR is a response to the stimulus itself (synaptic cost and/or response to a potential conspecific), then $\dot{M}O_2$ should increase during the neutral JAR even though EOD frequency does not. Although EOD frequency remained stable during neutral JAR experiments (maximum fluctuations of ±4Hz were observed, with mean changes <0.5%), $\dot{M}O_2$ was significantly elevated (approximately 27%); note that $\dot{M}O_2$ was elevated approximately 12% during JAR when EOD frequency was elevated. These data point to the possibility that the increase in $\dot{M}O_2$ observed in JAR and neutral JAR conditions is due to an increase in synaptic cost or the perception of a conspecific elicited during stimulation, and is not related to EOD frequency elevation itself. Perhaps an additional experiment presenting a neutral stimulus to the fish (i.e. a non-jamming stimulus that would not be detected as a conspecific) while examining the

effect on $\dot{M}O_2$ would help to distinguish whether the increase in $\dot{M}O_2$ observed was due to an increase in synaptic firing or the perceived detection of a conspecific.

4.5 Methodological critique

Our experimental approach was aimed at exploring the metabolic cost of electric signalling at the whole animal level in intact animals. The benefit of the whole animal approach is the ability to conduct experiments in fully functional animals, where measurements have relevance to the whole animal. Cellular experiments can be somewhat limited as it is difficult to replicate natural conditions. When measurements are made on reduced preparations, the costs must be scaled up to the whole animal level, an approach than can introduce a host of approximations and errors. However, the difficulty of the whole animal approach lies in the complex interactions of physiological systems. During hypoxia for example, there are many responses that could be recruited to allow the maintenance of EOD frequency despite reduced $\dot{M}O_2$ and low oxygen availability. It can therefore be difficult to pinpoint the relationship of interest. The respirometry study of *B. pinnicaudatus* by Salazar and Stoddard (2008) aimed to solve this problem by using pharmacological manipulations to partition the energy budget. To distinguish the cost of EOD production from the whole animal metabolic cost, respirometry was carried out on intact fish (i.e. those emitting an EOD), fish in which the myogenic electric organ was silenced (i.e. baseline $\dot{M}O_2$ in the absence of EOD cost), fish in which the motor system was inhibited (i.e. EOD production but no locomotion), and finally fish in which both the motor system and the electric organ were

silenced (i.e. no locomotion, no EOD). By comparing these treatment groups, Salazar and Stoddard (2008) concluded that EOD production is responsible for approximately 11-20% of the fish's energy budget. One difficulty in interpreting the results of this study lies in the reliability of $\dot{M}O_2$ data collected by respirometry on paralyzed, artificially-ventilated fish. Differences between the results of the present study and those of Salazar and Stoddard (2008) may lie in the different experimental approaches employed as well as physiological strategies used by the fish. For example, the pulse-type species examined by Salazar and Stoddard (2008) rely heavily on amplitude modulations during social interactions (Franchina and Stoddard, 1998). Perhaps there is a cost associated with amplitude and changes in amplitude in pulse-type fish that is not prevalent in the wave-type fish used in the current study.

Our data suggests that the cost of electric signalling in wave-type fish is not detectable at the whole animal level, indicating that the production of EOD is not as metabolically costly as previously suggested (Crampton, 1998a; Julian et al., 2003; Salazar and Stoddard, 2008). It is possible, however, that energy budget trade-offs are occurring that mask the potential high cost associated with electric signalling. Although electric signalling may be costly, overall $\dot{M}O_2$ may not be higher than a typical teleost due to metabolic savings from energy budget trade-offs. In addition, an essential sensory system like electric signalling might be protected by energy budget reorganization in cases where the energy budget is limited.

4.6 Future directions

Although the current study has focused on exploring the metabolic cost of electric signalling at the whole animal level, weakly electric fish also provide an excellent model for exploring this question at the cellular level, focusing specifically on the pacemaker nucleus responsible for EOD generation and timing. The pacemaker nucleus is a discrete group of cells that continue to fire when isolated and removed from the animal (Heiligenberg, 1996). In principle, the pacemaker preparation provides an opportunity for tissue respirometry (Gnaiger, 2001) that would allow tissue $\dot{M}O_2$ to be compared directly with AP firing frequency, thereby providing, for the first time, a direct quantitative measure of the cost of action potential generation in a neural system. As mentioned previously, LTFE arises from plasticity at the level of the pacemaker nucleus via NMDA receptor mediated synapses owing to long-term jamming stimulus presentation (Oestreich and Zakon, 2002). Our study focused on LTFE at the whole animal level; however, this form of sensorimotor plasticity can be examined at the cellular level by isolating the intrinsically active pacemaker nucleus, allowing observation of the activity of these cells alone while eliciting LTFE *in vitro* (Oestreich and Zakon, 2002). NMDA agonists and antagonists can be used to increase or decrease the firing rate of the pacemaker nucleus (Oestrich and Zakon, 2002) while tissue respirometry is used to measure $\dot{M}O_2$ of the pacemaker nucleus at different firing frequencies. This preparation would provide a direct observation of the relationship between frequency elevation and metabolic rate at the cellular level.

4.7 Summary

Previous studies have suggested that electric signalling by weakly electric fish is a metabolically expensive process (Crampton, 1998a; Julien et al., 2003; Salazar and Stoddard, 2008). By contrast, metabolic restrictions associated with hypoxia and exercise did not in the present study result in any limitation on EOD frequency. Examination of the effects of EOD frequency elevation on $\dot{M}O_2$ also yielded results that were inconsistent with the hypothesis that electric signalling is a metabolically costly process. Clearly there must be a cost to signalling at the cellular level, i.e. ATP is consumed by the individual electrocytes of the electric organ, but the collective cost of active electrocytes was not detectable at the whole animal level when set against a background of the metabolic demand associated with fuelling the whole animal and/or skeletal muscle during exercise. Our results indicate that electric signalling may not impose a metabolic cost beyond that of any other sensory system, at least when examined at the whole animal level.

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