RESPIRATORY ANATOMY, PHYSIOLOGY, AND CENTRAL CO_2 CHEMOSENSITIVITY OF THE ARCTIC AIR-BREATHING FISH *DALLIA PECTORALIS*

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A

THESIS

Presented to the Faculty of the University of Alaska Fairbanks

In Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Megan Hoffman

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Abstract

Aerial respiration using a ~~primitive~~ancestral lung, central respiratory rhythm generation, and central CO_2 chemosensitivity arose early in vertebrate evolution prior to the divergence of sarcopterygian and actinopterygian fish. All vertebrate air breathing, however, is not homologous as this trait evolved independently several times among teleost fishes. Two long-standing questions in respiratory physiology are whether air breathing in fish is controlled by a central rhythm generator and whether air breathing and central CO_2 chemosensitivity co-evolved. One means to address these questions is to investigate control of breathing in the brainstem; therefore, we established an isolated brainstem preparation from the Alaska blackfish, *Dallia pectoralis*, a rare example of an arctic air-breathing fish. In blackfish, air breathing consists of gulping and swallowing an air bubble into the~~ir~~ esophagus and holding it in place by ~~there with a sphincter that~~ closing~~es~~ off the esophagus from the buccal cavity with a sphincter. Gulping the air bubble is accomplished by the same opercular and mandibular muscles that draw water into the buccal cavity during gill ventilation. Activation of the opercular and mandibular muscles for ventilation is effected by a central rhythm generator in the brainstem that is spontaneously active in the absence of peripheral input. This central rhythm generator, however, is not modulated by central CO_2 chemosensitivity. Unless central CO_2 chemosensitivity was lost in blackfish, we might conclude that centrally controlled vertebrate air breathing can evolve independent of central CO_2 chemosensitivity.

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I would like to thank my committee members Dr. Amanda E. Rosenberger, Dr. Brian M. Barnes, Dr. Barbara E. Taylor and Dr. Michael B. Harris, for their help and guidance throughout this process. In particular, I would like to thank Barbara for taking me under her wing, even though what I wanted to study was not her main focus area. Her guidance in all aspects of the process, from establishing methodology to writing a succinct and cohesive manuscript, to learning to be more confident in myself and my science, to being able to let go and relax for a couple hours when necessary, was invaluable. I would like to thank Mike for his stimulating conversations and insight.

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Chapter 1 General Introduction

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Respiration is the physiological process of exchanging metabolic gases with the environment (acquiring O_2 and excreting CO_2). Respiration comprises 1) ventilation: movement of the environmental medium to the gas-exchange surface of the organism, 2) diffusion: movement of O_2 and CO_2 across the gas-exchange surface, and 3) perfusion: distribution of the gases throughout the tissues. The mechanics of ventilation vary according to the environmental medium (water or air) and the anatomical structures that serve as the gas-exchange surface (skin, gills, lungs, or another highly vascularized body cavity). Regardless of the mechanics, over the long term, respiration must occur at a rate that matches the metabolic production of CO_2 and demand for O_2. Respiration in vertebrates is controlled by the nervous system. The neural control of the ventilatory component of respiration, breathing, is the central topic of this thesis. Specifically, I~~we~~ am~~are~~ interested in the evolutionary relationship between central CO_2 chemosensitivity, which drives aerial respiration in tetrapods, and air breathing, which evolved independently several times among the fishes, including the progenitors of ~~modern~~derived tetrapods.

Origins of Vertebrate Respiration

The origin of vertebrate ~~air~~ breathing is ascribed to early chordates, and the ~~primitive~~ancestral motor patterns required for pumping water through the pharynx (Wilson et al. 2009). A living example of these ~~primitive~~ancestral chordates is *Amphioxus*, animals that acquire metabolic resources (O_2 and food) from their aquatic environments with rhythmic muscle contractions that pull water through their mouths~~, into pharyngeal structures (where food is obtained)~~, across gills ~~(where gas is exchanged)~~, and out pharyngeal slits. Among these extant non-vertebrate chordates, the gills are not the primary gas-exchange surface, but are used for filter feeding. Nonetheless, the appearance of these gills 570 million years ago (mya) ushered in the evolution of vertebrate gill ventilation.

Gill evolution extends throughout fish evolution. By the emergence of agnathans (jawless fishes like hagfish and lamprey) 520 mya, gills had become specialized as thin, highly vascularized gas-exchange structures (Wilson et al. 2009). By the emergence of osteichthyes (bony fishes) 510 mya, gills were the primary site of O_2 uptake and CO_2 excretion in fish, and they remain so~~to be~~ for extant water-breathing fish (Tufts and Perry 1998). Evolution brought continued refinement of the gill as a ventilatory structure, but the timing of these changes is difficult to establish because gills are a soft tissue ~~and thus less~~ unlikely to be preserved in the fossil record. Bony fishes include sarcopterygians

(lobe-finned fish) and actinopterygians (ray-finned fish), which diverged 430 mya (Graham 1997). In ~~modern~~derived actinopterygian fish (teleosts), the gills are lamellar and filamentous; they serve as the ~~main~~ primary sites of gas exchange because they present a large surface area for diffusion (Hughes 1984). In these ~~modern~~derived gills, water flows over the gill lamellae in a direction opposite to the flow of blood through the lamellae; this countercurrent flow enhances the efficiency of gas exchange via convection and maximized diffusion gradients.

The refinement of fish gills was concomitant with the evolution of air-breathing organs. The precursor to aerial respiration may have been the incidental inspiration of air during aquatic surface respiration, an adaptation to hypoxia used by water-breathers that involves ventilating the gills at the water-air interface where O_2 concentrations are highest (Graham 2006). The ~~primitive~~ancestral lung arose prior to the divergence of lobe- and ray-finned fish (Figure 1.1; Graham 1997). Gills were reduced in the sarcopterygian line, which included the ancestor of ~~modern~~derived tetrapods, and lungs were refined as primary ventilatory structures. The ~~primitive~~ancestral lung (present in Actinopterygii such as *Amia*, gar, and polypterus) lost its respiratory function in more ~~modern~~derived actinopterygians (teleosts) but was retained in many as a buoyancy control organ. Though the ~~primitive~~ancestral lung was lost in teleosts and despite the refinement of actinopterygian gills as gas-exchange organs, several groups have derived secondary ventilatory structures (Graham 1997, Wilson et al. 2009). These include air-breathing organs in the head region and along the digestive tract (Figure 1.2; Graham

1997). Despite the diversity of structure, all these air-breathing organs present a highly vascularized surface where respiratory gases can be exchanged between the environment and blood.

Evolutionary Pressure Favoring Aerial Respiration

There are numerous reasons why the evolution of aerial respiration would be advantageous to an organism. The physical and chemical properties of air make it a better respiratory medium than water due to gas, fluid, and heat-transfer factors (Dejours 1976). T~~Capacitance, t~~he limited availability~~ability~~ of O_2 oxygen in water, primarily due to low solubility, ~~to hold gases,~~ is the main gas factor. ~~The capacitance of water for O_2 is much lower than that of air.~~ At standard temperature and pressure, water equilibrated with air will contain approximately 8 mg O_2/L. The low oxygen content of water is the primary factor limiting gas exchange, with water, in fish. Thus, O_2. ~~Thus, O_2~~ uptake from air is more efficient than from water, ~~which~~ allowing~~s~~ for ~~a~~ reduced frequency of respiratory activity and, consequently, more efficient ventilation. With respect to fluid factors, water is denser and more viscous than air, greatly increasing the energetic cost of moving the respiratory medium~~which renders flow generation~~ across the gas-exchange surface ~~energetically less efficient~~ (Dejours 1976, Scheid and Piiper 1976, Shelton and Croghan 1988).

Unlike atmospheric conditions, which are relatively stable over time and across large areas, aqueous O_2 and CO_2 concentrations can fluctuate seasonally or ~~even~~ daily in fish habitats, often resulting in periodically or chronically inhospitable environments. These fluctuations are, in large part, due to varying activity of photosynthesis and respiration by organisms; at night when photosynthesis ceases, O_2 levels can drop dramatically while CO_2 levels rise (Dejours 1976). The differing heat-transfer properties of water and air are also a factor contributing to the daily and seasonal fluctuations in dissolved gas concentrations. Hypoxic conditions (low O_2) can occur quite readily in aqueous environments, particularly in stagnant waters, either warm or ice-covered (Magnuson et al. 1983, Graham 1997). Warm water holds less O_2 than cooler water; therefore, it follows that warm stagnant waters may become hypoxic. Furthermore, in some ice-covered environments, the lack of photosynthesis coupled with decomposition of decaying matter, can result in hypoxic and/or hypercapnic conditions. A~~Therefore, a~~n organism facing these conditions with the capacity to acquire O_2 from air would have an evolutionary advantage over a purely water-breathing organism (Graham 1997). Whether an animal needs to exercise this advantage occasionally or continuously depends on the specific environment. Most air-breathing fish are bimodal breathers, ~~, they~~ (ventilate with both air and water), ~~,~~ but some fish air breathe continuously (obligate air breathers), and some breathe air only occasionally when aquatic conditions are poor (facultative air breathers; ~~)~~ (Jordan 1976).

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Neural Control of Vertebrate Respiration

The majority of vertebrate gas-exchange surfaces, whether they are ventilated with air or water, are semi-internalized. These surfaces are topographically outside the body but line extensive cavitations of the body walls. The internalized gas-exchange surfaces of all vertebrates require specific ventilation, achieved~~are ventilated as a result of~~by motor patterns that pump the environmental medium across gas-exchange surfaces. ~~in and out of body cavities~~. Fish gills typically are ventilated with water~~ that is~~ drawn across gill surfaces by buccal and opercular pumping. ~~in through the mouth by buccal pumping and pushed out opercular slits after passing over the gills.~~ Fish air-breathing organs are ventilated by pumping air bubbles to and from the site of gas exchange. Motor patterns that accomplish air pumping typically employ the same buccal musculature as those pumping water over the gills but with changes in the timing of muscle activation and the valving of openings to the respiratory cavities.

Aquatic ventilation in fish and amphibians~~,~~ and aerial ventilation in higher tetrapods~~,~~ are typically continuous rhythms of muscle contraction controlled by neural circuits in the brainstem, ~~which is~~ the location of central rhythm generators and associated motor nuclei (Russell 1986, Taylor et al. 1999, Wilson et al. 2009). The rhythm generator for aerial respiration in mammals is ~~made up~~composed of neurons from the pre-Bötzinger complex and parafacial respiratory group, both of which are areas in

the reticular formation of the brainstem (Taylor et al. 1999, Onimaru and Homma 2003). ~~Like~~ As in mammals, respiratory rhythmogenesis in fish occurs in the reticular formation, though specific sites have not been identified (Taylor et al. 1999). Nonetheless, the rhythm generator for aquatic respiration in fish is thought to be homologous with the rhythm generator for suckling in neonatal mammals (Taylor et al. 1999). It is unclear when~~re in evolution~~ separate central rhythm generation for aquatic and aerial respiration evolved~~merged~~. T~~For t~~he ~~purposes~~ focus of this thesis is~~, we focus on~~ aerial respiration and the role of central CO_2 chemosensitivity in driving it~~aerial respiration~~.

Central rhythm generation for air breathing was believed to have emerged somewhere in the sarcopterygian line; aerial ventilation among fish of other evolutionary lines is thought to be a peripherally triggered reflex associated with feeding or aquatic surface respiration (Smatresk 1994). Recently~~, using an isolated brainstem preparation,~~ the presence of a central rhythm generator for air breathing has been established, using an isolated brainstem preparation, in evolutionary diverse actinopterygians, one ~~primitive~~ ancestral (the longnose gar; Wilson et al. 2000) and one ~~modern~~derived (*Betta*; Harris et al. 2001). The generation of aerial respiratory bursts by these isolated brainstem preparations, which receive no peripheral input, is evidence for central respiratory rhythm generation in air-breathing fish (Taylor et al. 1999, Wilson et al. 2000, 2009).

Respiratory Chemosensitivity

Central respiratory rhythm generation must be responsive to, and is therefore modulated by, the concentration of respiratory gases. This respiratory rhythmogenesis can be modulated by peripheral or central chemoreceptors that sense respiratory gases (Smatresk 1994). Depending upon the organism and its mode of ventilation, O_2 and CO_2 chemoreceptors monitor gas concentrations in the external environment and/or the internal environment.

In water-breathing fish, chemosensitivity is highest in the periphery, and respiration is largely driven by O_2 chemosensitivity. The neuroepithelial cells in the gills are thought to be chemosensitive cells that send information to sensory areas in the medulla of the brainstem (Sundin et al. 2007). Environmental O_2 and CO_2 concentrations are sensed via external-facing chemoreceptors located primarily in the gill arches, though ~~and perhaps~~ other chemosensors ~~in the orobuccal cavity~~ have been documented in the orobuccal cavity (Gilmour 2001). The CO_2/pH chemoreceptors found in the gill arches of fish are homologous with those found along gas exchange surfaces of all non-mammalian vertebrates (Milsom 2002; Figure 1.3). With respect to ~~I~~internal-facing ~~peripheral~~ O_2 chemoreceptors, peripheral receptors sense changes in the blood O_2 concentration, ~~of the blood~~ and central receptors ~~central O_2 chemosensitivity~~ ha~~ves~~ not been established in fish (Gilmour 2001). Central CO_2 chemosensitivity has not been generally established in strictly water-breathing fish (Gilmour 2001, Sundin et al. 2007).

However, several air-breathing fish, including lungfish, gar, and *Betta*, have been shown to possess central CO_2 chemosensitivity, though the specific location of chemosensory cells is unknown (Harris et al. 2001, Amin-Naves et al. 2007, Wilson et al. 2009).

In tetrapods, peripheral O_2 and CO_2 chemoreception occurs in the glomus cells of the carotid bifurcation (amphibians and mammals) or the aorta (reptiles and birds), and central CO_2 chemoreception occurs in the nucleus of the solitary tract, ventrolateral medulla, caudal medullary raphe, locus coeruleus, ventral respiratory group, retrotrapezoid nucleus, and the fastigial nucleus of the cerebellum (Milsom 2002, Sundin et al. 2007). Central respiratory O_2 chemoreception has been identified in the brainstem of reptiles (Johnson et al. 1998) and amphibians (Winmill et al. 2005, Fournier et al. 2007), and in the brainstem and midbrain of mammals (Neubauer and Sunderram 2004). Despite the fact that two respiratory gases are sensed in multiple locations in the body/nervous system, it is possible to ~~make the general statement~~generalize that CO_2 and the need to excrete it drives air breathing while O_2 and the need to acquire it ~~drives~~drives water breathing (Gilmour 2001).

The presence of a CO_2-sensitive central respiratory rhythm generator has been demonstrated in tetrapods, ~~primitive~~ancestral lobe-finned fish, ~~primitive~~ancestral ray-finned fish, and a ~~modern~~derived teleost (Smatresk 1994, Harris et al. 2001, Wilson et al. 2009). From this, one may infer that air breathing, central respiratory rhythm generation, and central CO_2 chemosensitivity are necessarily linked and evolved in conjunction with one another. To assess this inference, a survey of air-breathing fish and their central

control of respiration should be undertaken. Specifically of interest is the identification of air-breathing fish that possess both central CO_2 chemosensitivity and central rhythm generation for aerial respiration. Therefore, ~~we~~I begin this survey with an air-breathing species that is likely to encounter hypercapnic (high CO_2) environments, the Alaska blackfish.

Air B~~b~~reathing in Alaska Blackfish

Alaska blackfish, *Dallia pectoralis*, are an ideal candidate for assessing the occurrence of central CO_2 chemosensitivity in air-breathing teleosts. *D. pectoralis* is a facultative air-breathing species that uses a modified, highly vascularized esophagus as its air-breathing organ (Crawford 1971, 1974). After blood flows through the vasculature of the air-breathing organ it passes through the vasculature of the gills. To prevent ~~a~~ loss of O_2 at the gills, the blackfish has a smaller fourth gill arch ~~that is considerably smaller~~ than the first three arches and shunt vasculature to bypass the secondary lamellae of the gills (Crawford 1971). These adaptations, which are seen in many air-breathing fish, dramatically reduce O_2 loss through the gills (Burggren 1979, Hughes ~~et al.~~ 1984~~2~~, Taylor et al. 1999), but may also contribute to a decreased ability to excrete CO_2 at this site.

Blackfish live in shallow ponds and slow moving streams throughout Alaska and northern/eastern Siberia (Blackett 1962; Crawford 1974; Crossman and Ráb 1996). Their

thermal preference is 4-15 °C, and temperatures in excess of 25 °C are lethal (Hanzely 1957). The presence of an arctic air-breathing fish is potentially explained by the occurrence of stagnant, hypoxic aqueous environments during the summer months (Crawford 1974; Crossman and Ráb 1996). Alternatively, winterkill environments, characterized by shallow, stagnant, ice-covered waters that become hypoxic and hypercapnic (Magnuson et al. 1983), may have driven the evolution of an arctic air-breathing fish that expresses central chemosensitivity to CO_2.

~~We~~I investigated whether *D. pectoralis* possesses central CO_2 chemosensitivity using an isolated brainstem preparation as well as a whole-animal acetazolamide-induced tissue-acidosis. The isolated brainstem preparation allowed me~~us~~ to determine whether or not 1) blackfish possess a central respiratory rhythm generator for aerial respiration, and 2) whether or not that generator is CO_2 sensitive. My~~Our~~ first aim was to characterize and validate respiratory structures and the neurocorrelates of both water and air breathing. I~~This was~~ accomplished this using a combination of electromyography, electromyostimulation, and electroneurography. ~~We~~I could then address the overarching aim of this study: to determine whether Alaska blackfish exhibit~~or not~~ central CO_2 chemosensitivity ~~occurred in the Alaska blackfish~~.

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Figures

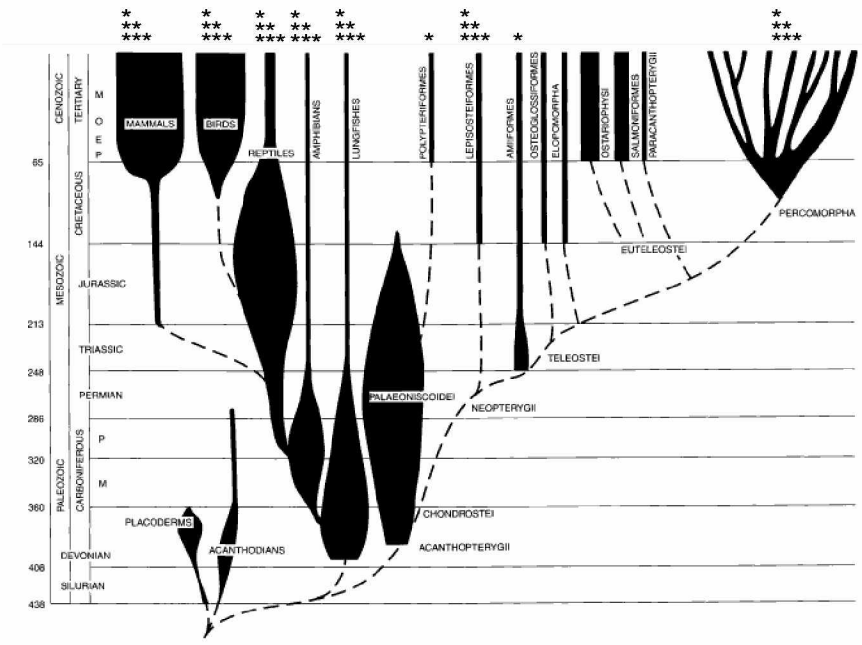

Figure 1.1~~1.1~~ Diagram of vertebrate phylogeny showing extant groups in which one or more representatives exhibit air breathing (*), central CO_2 chemosensitivity (**), and/or central respiratory rhythm generation for air breathing (***). Unmarked groups either lack these traits or have not been investigated. Image modified from Graham (1997).

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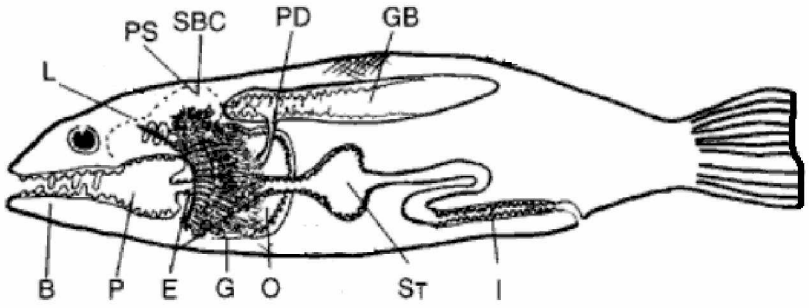

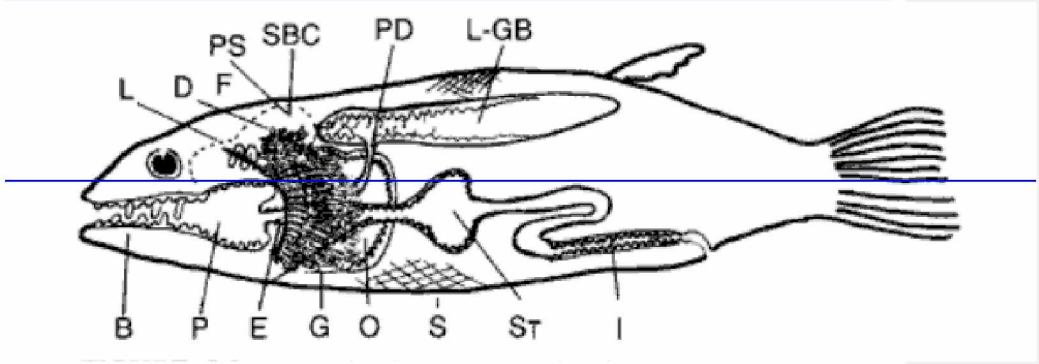

Figure 1.2~~1.2 Lungs/respiratory gas bladder (L-GB) occur in more primitive bony fish. Gas-exchange surfaces~~Air-breathing organs in ~~modern~~derived fish~~es~~ (teleosts) are found in the head region: buccal (B), pharyngeal (P), opercular (O), gills (G), pharyngeal sacs (PS), suprabranchial chamber (SBC), labyrinth (L)~~, dendrites (D), and gill fans (F)~~; and digestive tract: pneumatic duct (PD), esophageal (E), stomach (ST)~~, intestine (I); and the skin (S)~~. Image ~~taken~~ modified from Graham (1997).

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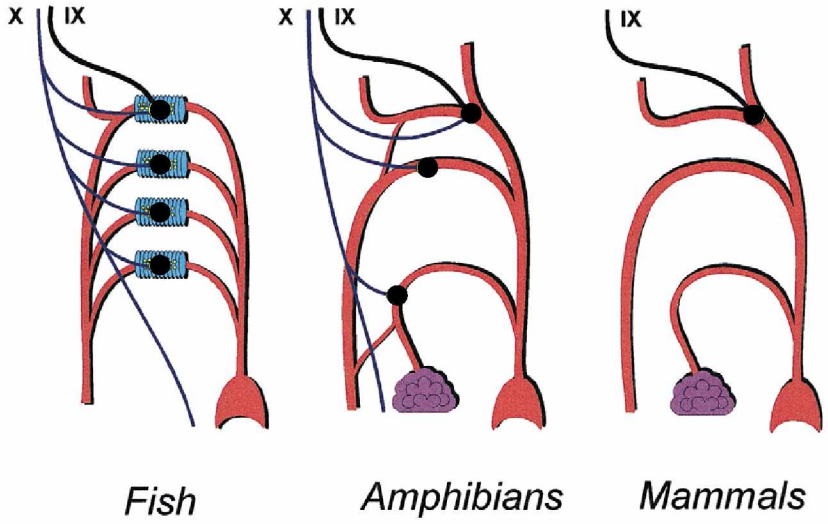
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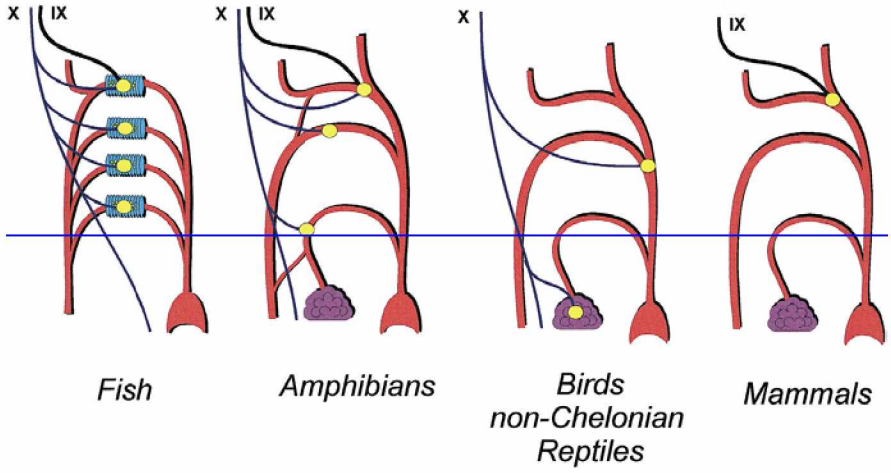
Figure 1.3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~~Figure 1.3~~ Schematic diagram showing the distribution of peripheral arterial and pulmonary CO_2/H^+ -sensitive chemoreceptors (●) in various vertebrate groups. IX and X designate cranial nerves IX and X. Image modified from Milsom (2002).

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Chapter 2 Respiratory Anatomy, ~~and~~ Physiology, and Central CO_2 Chemosensitivity of the A~~a~~rctic A~~a~~ir-breathing F~~f~~ish *Dallia pectoralis*.[1]

Abstract

Aerial respiration using an ~~primitive~~ancestral lung, central respiratory rhythm generation, and central CO_2 chemosensitivity arose early in vertebrate evolution prior to the divergence of sarcopterygian and actinopterygian fish. All vertebrate air breathing, however, is not homologous as this trait evolved independently several times among fish. Two long-standing questions in respiratory physiology are whether ~~or not~~ air breathing in fish is controlled by a central rhythm generator and whether ~~or not~~ air breathing and central CO_2 chemosensitivity co-evolved. T~~One means to address t~~hese questions can be addressed by ~~is to~~ investigating~~e~~ control of breathing in the brainstem~~, and thus, we established an~~ using an isolated brainstem preparation from the Alaska blackfish, *Dallia pectoralis*, a rare example of an arctic air-breathing fish. In blackfish, air breathing consists of gulping and swallowing an air bubble into the~~ir~~ esophagus, which is i~~and holding it there with a sphincter that closes off~~solated ~~the esophagus from~~ from the buccal cavity by a sphincter. Gulping the air bubble is accomplished by the same

[1] Hoffman M., M. B. Harris, B. E. Taylor 2009. Respiratory Anatomy and Physiology and Central CO_2 Chemosensitivity of the Arctic Air-Breathing Species *Dallia pectoralis*. Prepared for Journal of Comparative Physiology B.

opercular and mandibular muscles that draw water into the buccal cavity during gill ventilation. Activation of the opercular and mandibular muscles for ventilation is effected by a central rhythm generator in the brainstem that is spontaneously active in the absence of peripheral input. This central rhythm generator, however, wasis not modulated by central CO_2 chemosensitivity. Thus, unless central CO_2 chemosensitivity was lost in blackfish, we might conclude that centrally controlled vertebrate air breathing can evolve independently of central CO_2 chemosensitivity.

Introduction

Respiration, the physiological process of exchanging metabolic gases with the environment, comprises ventilating a body surface with the environmental medium, exchanging gases by diffusion, and transporting these gases throughout the body via blood circulation. The mechanics of respiration vary according to the environmental medium (water or air), the anatomical structures that serve as the gas-exchange surfaces (skin, gills, lungs, or other highly vascularized body cavities), and the vasculature itself. In all cases, however, respiration is tightly controlled such that itto meets the metabolic demands of CO_2 excretion and O_2 uptake. Neural control and modulation of ventilation is of primary interest in our studies. Here, we are interested specifically in the evolutionary functional relatedness of central CO_2 chemosensitivity, which drives aerial respiration in tetrapods, and air breathing, which evolved independently several times among the fishes, including the tetrapod progenitor.

The ventilatory mode of an organism (aquatic, aerial, or a combination of both) and the pace at which the neural circuits generate ventilatory activity is greatly influenced by the accessibility of respiratory gases in the environment. It is generally accepted that the ~~internal~~ partial pressure of O_2 (P_{O_2}) drives respiration in aquatic vertebrates, while the internal partial pressure of CO_2 (P_{CO_2}) drives respiration in terrestrial vertebrates. Nonetheless, all vertebrate ventilation is influenced to some degree by both P_{O_2} and P_{CO_2}.

Terrestrial vertebrates sense changes in internal P_{O_2} and P_{CO_2} with central and peripheral chemoreceptors. Fish sense changes in environmental O_2 and CO_2 using peripheral receptors located in the gills (Gilmour 2001, Milsom 2002). Internal P_{O_2} is sensed via internal-facing peripheral chemoreceptors of fish, and ~~there is~~ no evidence has indicated ~~for~~ central O_2 chemosensitivity in these lower vertebrates. Central CO_2 chemoreception has been documented in lungfish (sarcopterygians), the remnants of the group that gave rise to tetrapod vertebrates (Graham 1997, Sundin et al. 2007). Recently, central CO_2 chemosensitivity has been ~~generallyHowever, c~~ demonstrated in ~~a number of~~ some actinopterygian air-breathing fish including Siamese fighting fish (*Betta splendens*, Harris et al. 2001) and longnose gar (*Lepisosteus osseus*, Wilson et al. 2000).

Bimodal breathers (i.e., organisms that use aquatic and aerial ventilation) represent an interesting ~~intermediary~~ intermediate between aquatic and terrestrial vertebrates. Many amphibians begin as water-breathing larvae and become air-breathing adults after a period of metamorphosis during which they intermittently breathe air and water. Most air-breathing fish are bimodal breathers and breathe air or water

interchangeably depending on environmental conditions of their aqueous environment. There are two ~~main~~ primary types of air-breathing fish: obligate, those that must breathe air continuously; and facultative, those that breathe air only periodically when conditions warrant (Jordan 1976). Aerial gas exchange surfaces are diverse among bimodal breathers, ranging from well formed lungs in adult frogs to ~~primitive~~ancestral lungs in sarcopterygians and some actinopterygians to highly vascularized regions of the gut and gills in teleosts (Graham 1997).

An important question is what drives ventilation in bimodal breathers: ~~,~~ O_2 or CO_2; and~~,~~ central or peripheral sensation of these respiratory gases. Another is whether central CO_2 chemosensitivity is necessarily linked with aerial ventilation. There is evidence of central CO_2 sensitivity among some air-breathing fishes (Wilson et al. 2000, Harris et al. 2001). We are interested in whether central CO_2 chemosensitivity is common or rare among air-breathing fishes.

Alaska blackfish, *Dallia pectoralis*, are a facultative air-breathing species that uses a modified, highly vascularized esophagus as its air-breathing organ (ABO; Crawford 1971, 1974). There is a large degree of homology of ventilatory pump muscles and innervation patterns in *D. pectoralis* when compared to other vertebrates, air- or water-breathers. The adductor mandibulae, levator hyomandibulae, levator operculi, dilator operculi, and adductor operculi play important roles in ventilation of all teleost fish (Gorlick 1989). Among teleosts, including Alaska blackfish, cranial nerve VII (CN VII; facial nerve) innervates various muscle groups involved in ventilation (levator operculi, dilator operculi; ~~) (~~Gorlick 1989, Kenaley in preparation) and cranial nerve X

(CN X; vagus nerve) innervates the gill arches (Milsom 2002) and, in blackfish, the air-breathing organ (Kenaley in preparation). CO_2/pH chemoreceptors found in the gill arches of fish are homologous with those found along gas exchange surfaces of other vertebrates (Milsom 2002) and analogous to central CO_2 chemoreceptors known to exist in frogs (Taylor et al. 2003) and mammals (Feldman et al. 2003). Thus, it could be ~~we could~~ surmise~~d~~e that air breathing in Alaska blackfish ~~air breathing~~ is controlled by a central neural circuit with activity modulated by central CO_2 chemosensitivity.

An isolated brainstem preparation was used to assess the existence of central CO_2 chemosensitivity in *D. pectoralis* following characterization and validation of the respiratory structures and neurocorrelates of breathing. Validations were accomplished using a combination of electromyography (EMG), electromyostimulation (EMS), and electroneurography (ENG). ~~We~~ We tested the hypothesis that *D. pectoralis* possess a central respiratory rhythm generator and central CO_2 chemosensitivity. Acquiring this understanding of breathing physiology and control in Alaska blackfish will facilitate comparison with other fish and ~~and also~~ may advance our understanding of the ~~evolutionary~~ functional relatedness of air breathing and central CO_2 chemosensitivity.

Comment [MW3]: Rich pointed out in another section to use "I". Is this a thesis requirement? should I change them all to "I"?

Methods

Animals

Adult *D. pectoralis* of either sex were collected from Ballaine Lake, Fairbanks, Alaska and from ponds around Bethel, Alaska under protocols following Alaska Department of Fish and Game (ADFG) and Institutional Animal Care and Use Committee (IACUC) guidelines. Fish (n=32; 2-40g) were held in dechlorinated tap water in 20-L aquaria kept in a refrigerator at 5 ±1 °C and maintained on 5:19 hrs light:dark cycles. Water was changed weekly, and blackfish were fed live blackworms, *Lumbriculus* spp. daily. All animals were acclimated for at least one week prior to experimentation.

Characterization of Air Breathing in *Dallia pectoralis*

Validation of the Air-Breathing Organ by Visual Inspection

The Alaska blackfish, *D. pectoralis,* uses a modified section of its esophagus to exchange oxygen with the atmosphere. Crawford (1971, 1974) described the gross and fine anatomy of the blackfish esophagus and thoroughly documented the histology of this organ. Crawford's description of the gross anatomy of the respiratory section of the esophagus was not accompanied by images to illustrate its high vascularization and dense mucosal folding. In the present study, we performed our own visual inspection of the dissected esophagus and images were captured using a Canon PowerShot A620 digital camera mounted to a dissecting microscope with a camera-specific adapter.

Validation of Air-Breathing Musculature by Electromyostimulation and Electromyography

Gorlick (1989) identified the respiratory pump muscles and their innervation by cranial nerves in *Betta splendens*, another air-breathing teleost fish, using a combination of electromyostimulation (EMS; application of an exogenous myoelectric current) and neural tracing with horseradish peroxidase. To determine respiratory pump muscles in *D. pectoralis*, an exploratory dissection was performed on animals freshly euthanized by immersion in a lethal concentration of tricaine methanesulfonate (MS222; 3.0 g/L dechlorinated tap water). The skin covering the mandibular and opercular muscles was removed and, using EMS, each muscle was stimulated and the resulting movement recorded. The superficial muscles were stimulated first to identify their function and then were removed to gain access to the deeper muscles, which were subsequently stimulated to identify their function. A Grass S44 Stimulator (Grass Technologies, www.grasstechnologies.com) generated a 30 V pulse stimulus at a rate of 3.5 Hz; the stimulus was passed through a Grass SIU5 Stimulus Isolation Unit and into the muscle tissue with a bipolar wire stimulating probe. Tetanus was induced by ~~a~~ 35 Hz ~~signal~~stimulation. Muscles were identified based on the responses to stimuli; nomenclature followed that of Gorlick (1989). The identification of these muscles enabled us to record EMGs from respiratory muscles.

In addition to respiratory pump muscles, air breathing frequently involves valve structures along the air path. It was unclear from Crawford's previous work (1971, 1974)

whether blackfish possess a muscular epiglottis or sphincter to control the movement of water or air in the esophagus. Using EMS, the tissue surrounding the anterior esophageal opening was stimulated and the type of structure was determined based upon the resultant muscle movement.

The function of putative respiratory muscles (pumps and valves) was further assessed using electromyography (EMG) to record endogenous myoelectric activity of selected muscles in *D. pectoralis* during spontaneous ventilation. Fish were anesthetized by immersion for 5-10 min (or until unresponsive to a tail pinch) in a cold (4 °C) solution of MS222 (0.3 g /L dechlorinated tap water, balanced to pH 7.5 with $NaHCO_3$), and two ultra-fine Evanohm EMG electrode wires (0.001″; Wilbur B. Driver Co, Newark, NJ) were inserted into the muscle tissue of mandibular or opercular muscles. Fish were allowed to recover from anesthesia for approximately 30 min, and then electromyograms were recorded using the electroneurogram-recording procedure of Taylor et al. (2008). The electrical activity of the muscles was amplified (100x by a DAM-50 amplifier, World Precision Instruments, www.wpiinc.com; and 1000x by a model 1700 differential AC amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high-pass and 1000 Hz low-pass by the second amplifier). The amplified output was sent to a data acquisition system (Powerlab, ADInstruments, www.adinstruments.com), where data were archived as integrated activity (full-wave rectified and averaged over 50 ms). We were able to correlate the appearance of the recorded electromyogram with the observed ventilatory activity of the intact fish. In this way, we attempted to validated the

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myoelectric correlates of water- and air-breathing events (WBEs and ABEs, respectively).

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Validation of the Neural Correlates of Air Breathing in the Semi-Intact Preparation

A semi-intact (decerebrate with the brain-exposed but otherwise intact) preparation that was modified from Gdovin et al. (1998), was used to combine electroneurography (ENG) and EMG and to fully confirm respiratory function of the putative neuroventilatory activity. Fish were anesthetized as described above. The brain and spinal cord were exposed by opening the dorsal cranium and a portion of the spinal column anterior to the pectoral fins. Under a dissecting microscope, fine scissors were used to cut away dorsal portions of the cranium and spinal column. Once the brain and spinal cord were visible, the fish was decerebrated by resection of the forebrain rostral to the optic tectum and the spinal cord was severed caudal to cranial nerve (CN) X. Then artificial cerebrospinal fluid (aCSF; 104 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2 \cdot 6H_2O$, 10 mM d-glucose, 25 mM $NaHCO_3$, 0.6 mM $CaCl_2$, balanced to pH of 7.5 with HCl) was dripped into the caudal-most end of the exposed spinal column. The choroid plexus was removed to open the fourth ventricle and enhance diffusion of respiratory gases between the aCSF and the brain. CN VII on one side of the brainstem was cut; the contralateral CN VII and all other nerves remained intact. The animal was positioned in a whole-body superfusion

chamber and bathed with aCSF such that the gills were immersed in the bath and the mouth was out of the bath to allow air breathing. The aCSF was equilibrated with hypoxic gas concentrations (99.5% N_2, 0.5% CO_2) and flowed across the exposed brainstem. This was done to bathe the entire fish, and all its possible sites of O_2 chemoreception, in hypoxia thus stimulating air breathing. A suction electrode, fashioned from a pulled glass capillary was attached to the severed CN VII, and EMG wires were inserted into an opercular muscle on the contralateral side. Thus, electroneurograms and electromyograms were recorded from opposing sides of the fish. The fish was allowed to recover from anesthesia for approximately 30 min before recordings were made. Electrical outputs (neural and muscular) were recorded as previously described; concurrent visual monitoring of the semi-intact preparation facilitated description and validation of motor activities.

Characterization of Central Rhythm Generation for Air Breathing and CO_2 Chemosensitivity in *Dallia pectoralis*

Isolated Brainstem Preparation Challenged with High P_{CO_2}

The isolated brainstem preparation was used to confirm the presence of central rhythm generators for water and air breathing, and assess the occurrence of central CO_2 chemosensitivity in *D. pectoralis*. Fish were anesthetized and, using a razor blade, their bodies were transected immediately caudal to the operculum. Under a dissecting

microscope, the cranium and spinal column were opened, the exposed brain decerebrated, and the choroid plexus removed as described above. Then all cranial and spinal nerves were cut leaving roots projecting from the brainstem, and the cerebellum was removed. The brainstem/spinal cord was flipped *en bloc* in the cranium/spinal canal, any loose fragments of meninges were removed, and the nerves were trimmed if necessary.

The isolated brainstem was transferred to a 0.5-mL, Plexiglas, flow-through recording chamber and supported ventral side up between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ± 1 ml/min. A supply of aCSF, equilibrated with O_2-CO_2 mixtures that produced either normocapnia or hypercapnia, flowed through plastic tubing to the recording chamber. To establish normocapnic conditions in the isolated tissue, the aCSF was equilibrated with 0.5-1% CO_2, balance O_2~~,~~. H~~and h~~ypercapnic conditions were established with 3-5% CO_2, balance O_2.

The root of CN VII or X was drawn into a glass suction electrode pulled from 1-mm diameter capillary tube to a tip diameter that fit snugly around the nerve root. Each isolated brainstem was allowed to recover for 1-2 h~~ou~~rs under normocapnia until a ~~stable~~ steady baseline of neural activity was recorded. Whole-nerve output was recorded, as previously described, during a protocol of treatments that consisted of normocapnia for 60 min, followed by hypercapnia for 30 min, and then finally a washout with normocapnia for a minimum of 30 min.

Intact Dallia pectoralis Challenged with Acetazolamide-Induced Acidosis

As a complement to the high P_{CO_2} (hypercapnia) challenge adminstered to isolated brainstem preparations, whole animals were challenged with acetazolamide (ACZ) to induce an internal tissue acidosis similar to the respiratory acidosis caused by high P_{CO_2}. *D. pectoralis* were acclimated for 5-7 days ~~to being housed~~ at 5±1 °C in individual 1-L aquaria separated by visual barriers. Four aquaria were arranged as two stacked pairs, which allowed simultaneous video monitoring of four fish. During acclimation and experimentation, the animals were fasted to ensure similar ~~similar~~ metabolic states for all fish; body mass did not change during the course of these experiments.

An ACZ stock solution of 1.5 g/L dechlorinated water was prepared and buffered to pH 7.8 using HCl and NaOH such that when 21 ml of the stock solution was used to replace 21 ml of the acclimitization water, the final ACZ treatment concentration was 30 mg ACZ/L H_20. After the acclimation period, the treatments were effected by first removing 21 ml aquarium water and replacing it with either 21 ml aquarium water (sham) or 21 ml of ACZ stock solution (treatment acidosis). The first treatment was a sham control, the second treatment was ACZ acidosis, and the final treatment was a recovery control in which 21 ml of aquarium water was added. Between the ACZ-acidosis and recovery-control treatments, however, the water was replaced with ACZ-free dechlorinated water and the fish were given 24 h~~ou~~rs to recover. Treatments were made at the same time each day. The start of each treatment (sham control, ACZ acidosis, and recovery control) coincided with the start of a 12-13 hr video-monitoring period.

Because only four aquaria could be video monitored at a time, the experimental group (n=8) received the treatments in two batches.

Data Analysis and Statistics

Validation

Validation of muscle and nerve activity underlying WBEs and ABEs combined both qualitative and quantitative analyses. Myograms from intact fish, as well as myo- and neurograms from semi-intact preparations, were analyzed for burst frequency (bursts/min) and burst duration (s) measured as burst width at half burst height. During experimentation, the fish were visually monitored such that bursts in the myo- and neurograms could be qualitatively characterized and matched with the ventilatory activity of the animal.

The frequency and duration of putative WBEs were determined and analyzed for either 4~~four~~ or 10~~ten~~-min periods in the adductor and opercular EMG myograms. Our intent was to measure ten consecutive minutes of myogram activity data; however, body movements of the fish disrupted our recordings of the mandibular and opercular muscles. Accordingly, we analyzed the data over 10~~ten~~-min periods or 4~~four~~-min periods ~~for~~

~~wiggly fish~~where mechanical disruption precluded longer duration recordings for especially wiggly fish. Burst frequency and duration between muscle groups were statistically compared using one-way analysis of variance (ANOVA; SigmaStat, Systat Software, www.systat.com). In the semi-intact preparations, additional qualitative data analysis was performed by comparing the synchrony of electrical activity in the muscles and nerves; b~~B~~urst peaks that were within 200 ms were considered synchronous.

Central CO_2 Chemosensitivity

Data collected from isolated brainstem preparations, which had been challenged with high P_{CO_2}, were analyzed to assess the occurrence of central CO_2 chemosensitivity. The neurograms recorded from these preparations were analyzed for burst frequency (bursts/min) and burst duration (s) measured as burst width at half burst height. Each burst was classified as either a putative ABE or a putative WBE. Burst frequency and duration between treatment groups (normocapnia, hypercapnia, and recovery normocapnia) were statistically compared using repeated-measures analysis of variance (RM-ANOVA; SigmaStat, Systat Software, www.systat.com). ~~The frequency and duration of putative ABEs and WBEs were determined and analyzed for the last ten minutes of each treatment in the neurograms and for either four- or ten-min periods in the myograms. In the semi-intact preparations, additional data analysis was performed by comparing the coincident activity in the myo- and neurograms.~~

The number of surfacing events (a surrogate measure of ABEs) was counted for each minute of the video-recording period during sham-control, ACZ-acidosis and sham-recovery treatments. These surfacing frequencies were analyzed via one-way repeated-measures analysis of variance (RM-ANOVA) with pre-/post-period and sham/ACZ treatment as factors. ~~Data on the ACZ-induced increase in surfacing events by intact fish were compared to the hypercapnia-induced increase in ABEs by isolated brainstem preparations.~~

Results

Characterization of Air Breathing in *Dallia pectoralis*

The present study was an investigation of the neural control of ventilation in the Alaska blackfish, *D. pectoralis*. Specifically we sought to determine if neuroventilatory control included a central rhythm generator and central CO_2 chemosensitivity. Toward this end we developed an isolated blackfish brainstem preparation for which the neurocorrelates of ventilation were characterized and validated. Putative neurocorrelates of ventilation, recorded from CN VII and X in isolated brainstem preparations, ~~were characterized by~~consisted of two distinct burst patterns:~~-~~ putative WBEs (low amplitude, high frequency, short duration bursts) and putative ABEs (high amplitude, low frequency, long duration bursts). These burst patterns were confirmed as ventilatory neurocorrelates of

water and air breathing using a combination of electromyostimulation (EMS), electromyography (EMG), and electroneurography (ENG).

~~Validation~~Validation of the Air-Breathing Organ by Visual Inspection

The gross anatomy described by Crawford (1974) was confirmed during dissection of four blackfish. The interior wall of the blackfish esophagus had both respiratory and non-respiratory regions (Figure 2.1). Immediately caudal to the esophageal opening was a non-respiratory region characterized by white-colored tissue with~~and had~~ a lesser degree of mucosal folding. The greater region of the esophagus was the respiratory section, which was posterior to the non-respiratory region, made up the remainder of the esophagus, and ended at the lower esophageal sphincter. Crawford (1974) found this region comprised ~80% of the entire esophagus; this is comparable to our findings that the respiratory region comprised ~67% of the esophagus. This respiratory region was characterized by a reddish color due to the high degree of vascularization. It also had a greater degree of mucosal folding as previously described and documented by Crawford (1974).

Validation of Air-Breathing Musculature by Electromyostimulation and Electromyography

Using EMS, four respiratory muscle groupss were identified. Figure 2.2 is a line drawing of a blackfish head that indicates the sites of stimulation, the resulting movement, and indicates the muscles that generated the movement. The muscles were stimulated and the motor effect determined by observedation. Four muscle groups were identified:. Tthree superficial, (i.e., located just below the skin) and one deep. The superficial muscle groups included the: adductor mandibulae (AM), which lift/retracts the jaw to close the mouth; levator operculi (LO), which lifts the operculum; and dilator operculi (DO), which opens the operculum. The fourth, a deeper identified muscle group,tissue located below the AM, was the levator hyomandibulae (LH), which lifts the mandible to close the mouth.

The esophageal opening was occluded by a sphincter rather than a glottis. A sphincter-like constriction (i.e., simultaneous, circular constriction around the esophageal opening) was observed upon stimulation. This was distinguishable from a wave-like peristaltic contraction and a valve-like glottis closure.

EMGs were used to characterize and validate the neurocorrelates of breathing by comparing neural and muscular activity. WBE frequency and duration were quantified from EMGs recorded from the blackfish; ABEs could not be distinguished in the EMGs because air breathing was always accompanied by gross movements of the fish associated with swimming and surfacing. The recorded muscles were separated into two groups: mandibular (AM) and opercular (LO, DO) muscles. We did not attempt to record activity from the fourth identified muscle, the LH, because being certain of electrode placement in such a deep tissue layer is tantamount to being certain ofwould severely damgeing the

fish. Mandibular muscle firing in the blackfish (n=6) had a mean frequency of 65.6 ± 5.0 breaths/min and a mean duration of 0.4 ± 0.04 s. Opercular muscle firing (n=7) had a mean frequency of 52.0 ± 5.8 burstreaths/min and a mean duration of 0.5 ± 0.04 s. Mandibular and opercular muscle firing were not statistically different from one another in either in frequency (P=0.11) or duration (P=0.06). Therefore, these data were averaged together for our comparison of the myocorrelates of breathing recorded from the respiratory muscles and the neurocorrelates of breathing recorded from the isolated brainstem preparation. Frequencies and durations of burst activities of these two recordings were compared separately. Respiratory muscle activity (mandibular and opercular) had a mean frequency of 58.3 ± 4.2 bursts/min and a mean duration of 0.5 ± 0.04 s.

EMGs were analyzed qualitatively to validate ventilatory activity (Figure 2.3). The mandibular muscles are innervated by CN V, while the opercular muscles are innervated by CN VII (Kenaley in preparation). Qualitatively, these EMGs appear similar, in burst frequency and duration, to the ENGs recorded from CN VII and X. Due to noise artifacts from moving/surfacing, no successful ABEs were recorded. EMG burst frequency and duration were statistically compared to putative respiratory ENG burst frequency and duration for WBEs. EMG mean burst frequency was 58.3 ± 4.2 bursts/min and mean duration was 0.5 ± 0.04 s. Putative ENG mean burst frequency equaled 25.0 ± 2.8 bursts/min; mean duration was 0.7 ± 0.07 s (Table 2.1). Both the frequency (P= <0.001) and duration (P= 0.007) of the bursts were significantly different between myograms and neurograms.

Validation of the Neural Correlates of Air Breathing in the Semi-Intact Preparation

Semi-intact preparations of blackfish were visually monitored while concurrently recording EMMGs and ENMGs. This was done to confirm the relatedness of independently recorded burst patterns in EMGs and ENGs that had been recorded independently to generate the results described above (Figure 2.4). During WBEs the gills were ventilated; the observed activity of the operculum correlated with individual bursts in the myo- and neurograms. Neurograms, however, had double the number of bursts for WBEs (~60 bursts/min); therefore, every other low amplitude, high frequency, short duration burst of the neurogram corresponded with the low amplitude, high frequency, short duration bursts of the myogram (~30 burst/min).

During ABEs, the fish was seen to first release a bubble of air and then gulp air; this action was characterized by a larger recruitment (i.e. greater amplitude and duration of burst activity) of respiratory muscles as evidenced by the myo- and neurograms as well as visual monitoring. Unlike WBEs, ABEs were not denoted by doublet bursts in the ENG recordings. Simultaneous recording of EMG and ENG was limited because the EMG amplifier saturated due to movement artifact anytime the fish "surfaced" for an ABE. Simultaneous recording of EMG and ENG that included a spontaneous ABE were rare; however, we were able to confirm that concomitant bursting in the CN VII ENG and the opercular muscle EMG occurred during the observed ABE (Figure 2.44). Due to the

rarity of recording a putative ABE on the EMG and ENG, we did not generate mean burst parameters (amplitude and duration) for the putative ABE on the EMGs and ENGs.

The mean WBE burst durations were 0.2 ± 0.01 s and 0.4 ± 0.02 s, respectively, for myo- and neurograms. ABE burst duration was more similar on the myo- and neurograms (~1.3 s; ~~)(~~Table 2.1).

Characterization of Central Rhythm Generation for Air Breathing and CO_2 Chemosensitivity in *Dallia pectoralis*

Isolated Brainstem Preparation Challenged with High P_{CO_2}

During normocapnia the mean WBE frequency was 24.8 ± 5.5 bursts/min. During hypercapnia the mean WBE frequency was 24.6 ± 4.3 bursts/min. During recovery normocapnia the mean WBE frequency was 25.6 ± 5.5 bursts/min. There were no statistically significant differences in WBE frequencies during normocapnic, hypercapnic, and recovery normocapnic treatments (Table 2.1, Figure 2.5~~6~~; P= 0.713; n= 6).

During normocapnia the mean WBE duration was 0.7 ± 0.01 s. During hypercapnia the mean WBE duration was 0.7 ± 0.01 s. During recovery normocapnia the mean WBE duration was 0.7 ± 0.01 s. There were no statistically significant differences in WBE durations during normocapnic, hypercapnic, and recovery normocapnic treatments (Table 2.1, Figure 2.6~~7~~; P= 0.587; n= 6).

During normocapnia mean ABE frequency was 0.2 ± 0.1 bursts/min, and during hypercapnia mean ABE frequency was 0.3 ± 0.1 bursts/min. During recovery normocapnia the mean ABE frequency was 0.4 ± 0.2 bursts/min. There were no statistically significant differences in ABE frequencies during normocapnic, hypercapnic, and recovery normocapnic treatments (Table 2.1, Figure 2.7~~8~~; P= 0.383; n= 5).

During normocapnia the mean ABE duration was 2.6 ± 0.7 s. During hypercapnia the mean ABE duration was 2.1 ± 0.8 s. During recovery normocapnia the mean ABE duration was 2.2 ± 0.5 s. There were no statistically significant differences in ABE duration during normocapnic, hypercapnic, and recovery normocapnic treatments (Table 2.1, Figure 2.8~~9~~; P= 0.208; n= 4).

Intact Dallia pectoralis Challenged with Acetazolamide-Induced Acidosis

Frequency of surfacing events was quantified as a proxy for ABEs. Under normocapnic conditions, the mean surfacing frequency was 0.1 ± 0.03 surfacings/min. During ACZ-induced acidosis surfacing frequency was 0.1 ± 0.03 surfacings/min. During recovery normocapnic surfacing frequency was 0.1 ± 0.02 surfacings/min (Table 2.1, Figure 2.9~~10~~). There were no statistically significant differences between treatment groups when analyzed using RM-ANOVA (P= 0.673).

Discussion

RRespiration, the physiological process of excreting CO_2 and acquiring O_2, involves moving the respiratory medium over gas-exchange surfaces to allow for diffusion of gases into or out of the blood, which circulates and distributes the CO_2 generated and the O_2 consumed by metabolic reactions. The ventilatory component of respiration is effected by a pattern of muscle activity that, while likely derived from the muscle activity patterns that underlie feeding movements (Rovainen 1996; Wilson et al. 2009), uniquely serves the respiratory process. Feeding movements in their most primitiveancestral form (filter feeding) are periodic rhythmic events. As complexities of feeding evolved (the advent of sucking, biting, chewing, and swallowing), so too evolved feeding movements driven by periodic non-rhythmic events (e.g. biting) and peripherally driven reflex events (e.g. swallowing). Air breathing is believed to have originated from the incidental ingestion of air bubbles by fish feeding or water breathing at the surface (Smatresk 1994, Graham 2006), and such an origin calls into question whether primitiveancestral air breathing is a peripherally driven reflex or a centrally driven periodic event.

The neural control of primitive air breathing in fish has been investigated for over 230 years. Early studies of primitiveancestral air-breathing fish (*Amia* and gar, McKenzie et al. 1991 and Smatresk, 1987, respectively) indicated that air breathing in these fish was dependent upon afferent input and was a reflex. More recently, however, studies on primitiveancestral and modernderived fish (gar and *Betta*, Wilson et al. 2000. and Harris et al. 2001, respectively) indicated that air breathing in fish is driven by a

central rhythm generator. A confounding factor ~~in this line of investigation~~ is that air breathing has evolved independently several times among fish. Thus, while Smatresk (1987) and Wilson et al. (2000) differ in assigning the type of control for air breathing in gar, one might conclude that air breathing in some fish is a reflex, while in others it is a centrally controlled rhythmic activity. We investigated the neural control of air breathing in the Alaska blackfish, a fish whose air-breathing organ has been evolutionarily derived independently from *Amia*, gar, and *Betta*. We found evidence that air breathing in the blackfish is driven by a central rhythm generator, and we regard this to be indirect evidence that air breathing in all its forms is controlled by a central rhythm generator.

An accepted generalization in respiratory physiology is that the need to excrete CO_2 is paramount in air-breathing vertebrates, while the need to acquire O_2 is paramount in water-breathing vertebrates, though breathing is still modulated to a lesser degree by O_2 and CO_2, respectively (Gilmour 2001, Hedrick et al. 1991, Kinkead et al. 1993). Air breathing in all tetrapod vertebrates is controlled by a neural circuit in the brainstem and modulated by the internal concentration of CO_2 as sensed by the peripheral and central nervous systems (Wilson et al. 2009). This raises the question of whether all air breathing in fish is modulated by CO_2 sensitivity, peripheral and/or central. We found evidence that~~,~~ while air breathing in the Alaska blackfish is controlled by a central neural circuit, th~~eat~~ circuit is not modulated by central sensitivity to CO_2.

Air Bbreathing in Alaska Bblackfish

Air breathing in Alaska blackfish is accomplished by breaking the surface with their mouth and gulping a bolus of air, which is then held in the respiratory region of the esophagus. Expansion of the entire buccal cavity, including operculi, was observed in concurrence with aerial ventilation/gulping. Prior to inspiration, fish have been observed to release a bubble of air, sometimes while still submerged and sometimes at the surface, immediately before inspiration. Blackfish usually take only one breath before resubmerging, but are occasionally seen to take two or more gulps at surface before descending.

We used a combination of electromyography and electroneurography to characterize control of air breathing in Alaska blackfish. Via simultaneous observation and electromyography, we determined that the opercular and mandibular muscles function in pumping water over the gills and aid in the inspiration (and potentially expiration) of air bubbles. Crawford (1974) speculated that the muscular sheath surrounding the esophagus and swim bladder may be involved in the expiration of air bubbles. We were unable to trace nerves to and from the esophagus and swim bladder or to record from nerve branches that innervate these sites. Kenaley (in preparation), however, determined that the muscle sheath is innervated by CN X, a nerve from which we were able to record respiratory-related rhythm in a few preparations. Via simultaneous observation and electroneurography, we determined that rhythmic

contraction and relaxation of the opercular and mandibular muscles is controlled by cranial nerves VII and X.

Through a combination of anatomical observations, electromyography, and electroneurography we investigated air breathing in Alaska blackfish. We confirmed that putative neuroventilatory activity recorded from cranial nerves VII and X is respiratory in nature. We verified the respiratory structure of the esophagus first documented by Crawford (1971, 1974). We know that cranial nerves VII and X innervate buccal musculature and gill arches (Gorlick 1989, Milsom 2002, Kenaley in preparation), which are active during breathing in most fish and gill-bearing tetrapods. We verified that, during respiration, concurrent activity occurs both in ventilatory muscles and in cranial nerves responsible for generating ventilatory movements. All these lines of evidence support that air breathing is centrally controlled in Alaska blackfish.

The frequency and duration of putative WBEs, as measured by neuroventilatory bursts, was significantly different from WBEs as measured by electromyography. It is possible that the electrical signal generated by nerves to activate muscles correlates with, but does not precisely mirror, the resulting electrical activity of the muscle. It is also possible that the difference is due, in part, to the fact that electromyography is performed on whole animals, which- therefore, are subject to peripheral input not seen in the isolated brainstem preparations. An additional possibility is that differences may be due to size differences between the study groups (~30 g for EMG preparations; ~15 g for isolated brainstem experiments). Thus, we conclude that central neural activity and muscular

activity need not be identical for one to conclude that central rhythm generation drives the muscular activity that underlies air breathing.

Differences in the burst patterns of myograms and neurograms were also evident in experiments using the semi-intact blackfish preparation. In the semi-intact preparation, we observed a doubling of WBE bursts in the neurogram compared to the myogram. The doublet burst signal was not seen for ABE neurogram bursts. Every other WBE burst in the neurogram corresponded to the WBE bursts in the myogram. ~~We believe~~Data suggest that this is explained by the recording sites for CN VII. The neurogram was recorded from the base of CN VII immediately lateral from the brainstem. The myogram, on the other hand, was recorded from an opercular muscle far removed from the brainstem. We believe that the neurogram doublets resulted from the CN VII rootlet carrying output intended for multiple muscle groups, where as the myogram was recorded froom a single muscle group with a unified action.

In neurograms recorded from semi-intact and isolated brainstem preparations of Alaska blackfish, WBEs are characterized by high frequency, low amplitude, and short duration bursts; and ABEs are characterized by low frequency, high amplitude, and long duration bursts. This is similar to the distinction between putative gill and lung breaths in neurograms recorded in semi-intact and isolated brainstem preparations of bullfrogs (Gdovin et al. 1998). We are confident in our ability to distinguish the burst activity associated with water breaths, air breaths, and non-respiratory events (e.g. gill-clearing 'coughs' exhibited by many fish). Thus, through our development of the isolated Alaska blackfish brainstem preparation, we have determined that air breathing in these fish,

which occurs through swallowing of air bubbles into a highly vascularized portion of the esophagus, is under the control of a central neural circuit.

Central CO_2 Chemosensitivity

Central neural control of gill breathing among fish and lung breathing among tetrapods is well established (Rovainen 1977; Feldman et al. 1990; Wilson et al. 2009). This leads one to~~, and this leads one to~~ question is whether~~ or not~~ central respiratory chemosensitivity (to O_2 or CO_2) is~~-~~ also common to these vertebrates. All tetrapods demonstrate central CO_2 chemosensitivity, with high CO_2 (hypercapnia) eliciting an increase in the frequency and, for some mammals, the amplitude of breathing. Here we have addressed the question of central CO_2 chemosensitivity in the bimodally breathing Alaska blackfish. There is evidence that CO_2 modulates neuroventilatory output from the brainstem of lamprey, a gill-breathing ~~primitive~~ancestral fish (Rovainen 1977), and the air breathing-related output from the brainstems of longnose gar (Wilson et al. 2000), *Betta* (Harris et al. 2001), and lungfish (Amin-Naves et al. 2007).

Here we present evidence that Alaska blackfish do not possess central CO_2 chemosensitivity. High CO_2/CO_2-related acidosis had no significant effect on the frequency of putative air breathing in the isolated brainstem preparation or the frequency of surfacing in the whole animal. This is not unlike the results of Hedrick et al. (1991), which indicated a lack of central response to hypercapnia in a semi-intact preparation derived from *Amia*. In the present investigation of blackfish, we did not observe an

obvious CO_2-induced change in the amplitude of putative lung bursts, but we did not specifically quantify this parameter of the neuroventilatory bursts. A CO_2-induced change in the amplitude and/or duration of neuroventilatory bursts has not been reported for any bimodally breathing fish with demonstrated central CO_2 chemosensitivity (Wilson et al. 2000; Harris et al. 2001; Amin-Naves et al. 2007) and has been shown not to exist in bimodally breathing tadpoles (Taylor et al. 2003). CO_2-related acidosis had no effect on the surfacing frequency of blackfish or on the frequency of air breathing-related neuroventilation. Our results indicate~~Here we conclude~~ that Alaska blackfish have no central respiratory CO_2 chemosensitivity, despite having a central neural circuit that controls air breathing.

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Air breathing evolved independently several times among ~~the~~ fishes. An ~~primitive~~ancestral lung evolved in fish prior to the divergence of sarcopterygians and actinopterygians. Thus, the ~~primitive~~ancestral lungs of the tetrapod progenitor (lungfish) and non-teleost actinopterygians (*Amia* and gar) are considered homologous with one another and with the tetrapod lung. This ~~primitive~~ancestral lung was lost in the teleost line. Thus, the air-breathing organs that exist among the teleosts are neither homologous with each other nor with the tetrapod lung. Regardless of the homology status of air-breathing organs, most forms of air breathing have been shown to be controlled by neural circuits in the brainstem. It appears that air breathing, regardless of its evolutionary origins, has always evolved as a centrally controlled activity. Air breathing, however, can exist without central CO_2 chemosensitivity, a~~thi~~s was demonstrated previously for *Amia* (Hedrick et al. 1991) and now for Alaska blackfish. Just as centrally controlled air

breathing evolved independently several times among the vertebrates, so perhaps did central CO_2 chemosensitivity. These two traits appear not to be evolutionarily linked. Thus, unless all central CO_2 chemosensitivity is homologous and was lost several times among the fish, we might conclude that central CO_2 chemosensitivity evolved independently, and independent of central control of air breathing, several times among the vertebrates.

Acknowledgements

This work was funded by the National Institute of Health- (NIH-NINDS) 2U54NS041069-06A1. Protocols used in this study adhere to the Institute of Animal Care and Use Committee (IACUC) and Alaska Department of Fish and Game (ADFG) guidelines, as well as local and national ethics standards.

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Figures

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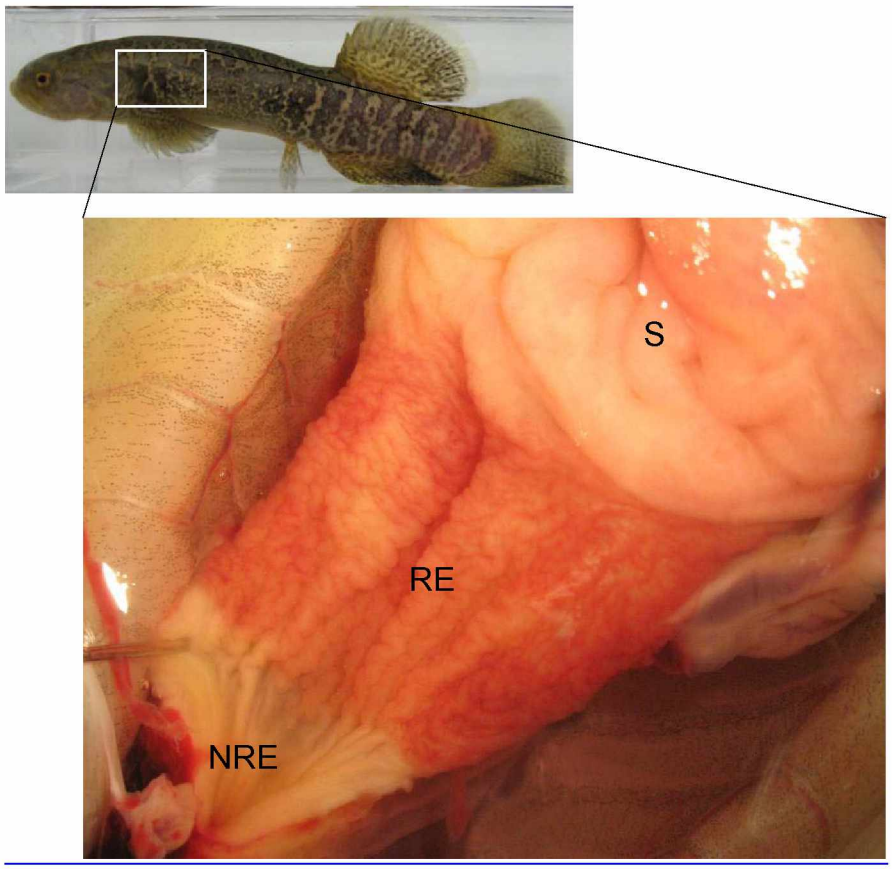
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RE
NRE

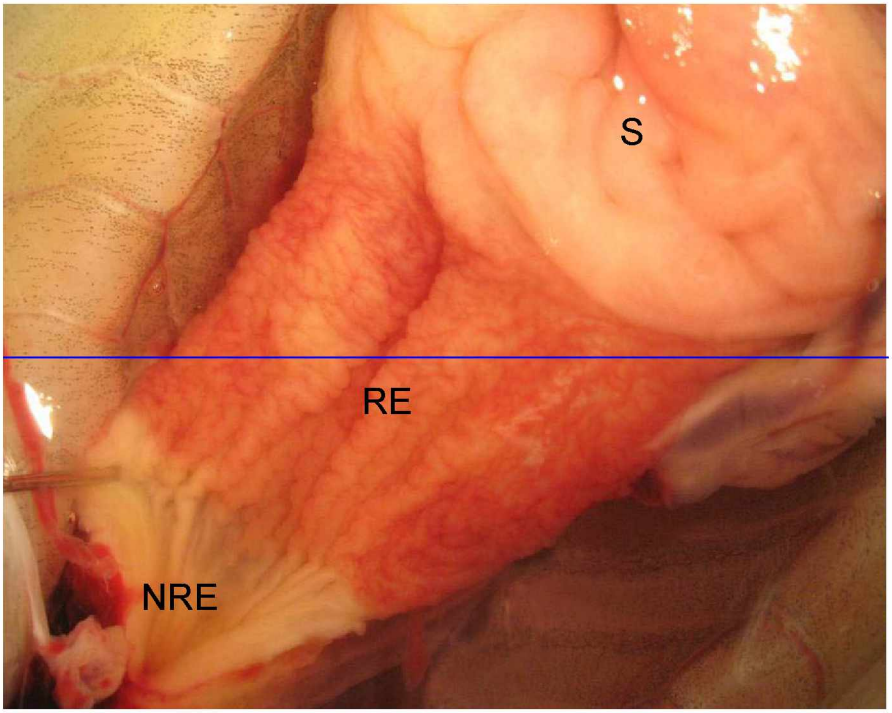


Figure 2.1~~2.1~~ Photomicrograph of the interior structure of the esophagus of the blackfish. NRE= non-respiratory esophagus; RE= respiratory esophagus; S= stomach. 4X magnification

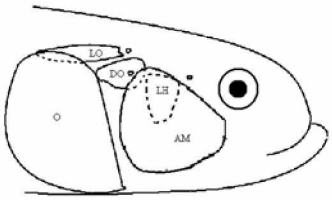
Movement	Muscle	
Lifts operculi	levator operculi (LO)	
Opens operculi	Dilator operculi (DO)	
Closes mandible	adductor mandibulae (AM)	
Lifts/closes mandible	levator hyomandibulae (LH)	

Figure 2.2~~2.2~~ The ventilatory muscles of Alaska blackfish, identified via electromyostimulation, with an indication of location and action. O= operculum. Drawing not to scale.

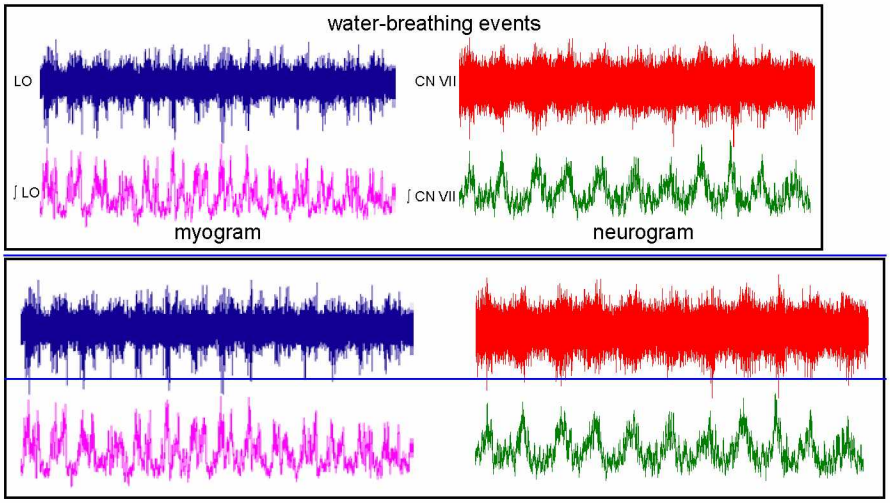
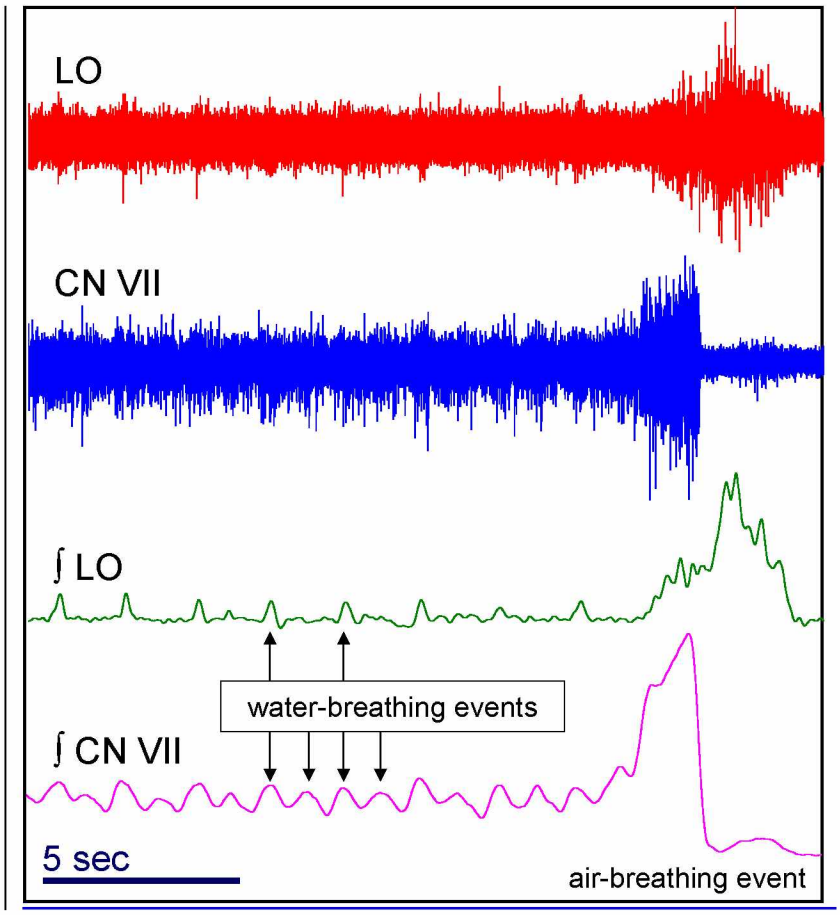


Figure 2.3~~2.3~~ Representative recordings of the electrical correlates of ventilatory activity in Alaska blackfish. An electromyogram (EMG) recorded from the levator operculum of an intact blackfish is depicted on the left with raw activity above and integrated activity below. An electroneurogram (ENG) recorded from cranial nerve VII of an isolated blackfish brainstem is depicted on the right with raw activity above and integrated activity below. Each recording represents 30 s of activity.


LO
CN VII
∫ LO
water-breathing events
∫ CN VII
5 sec
air-breathing event

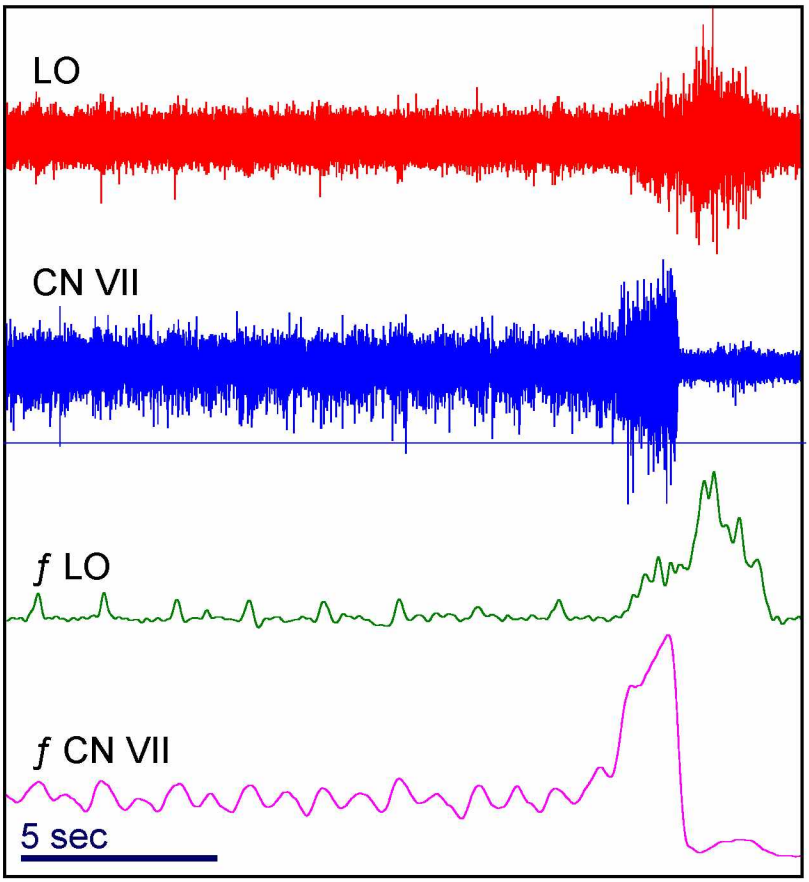
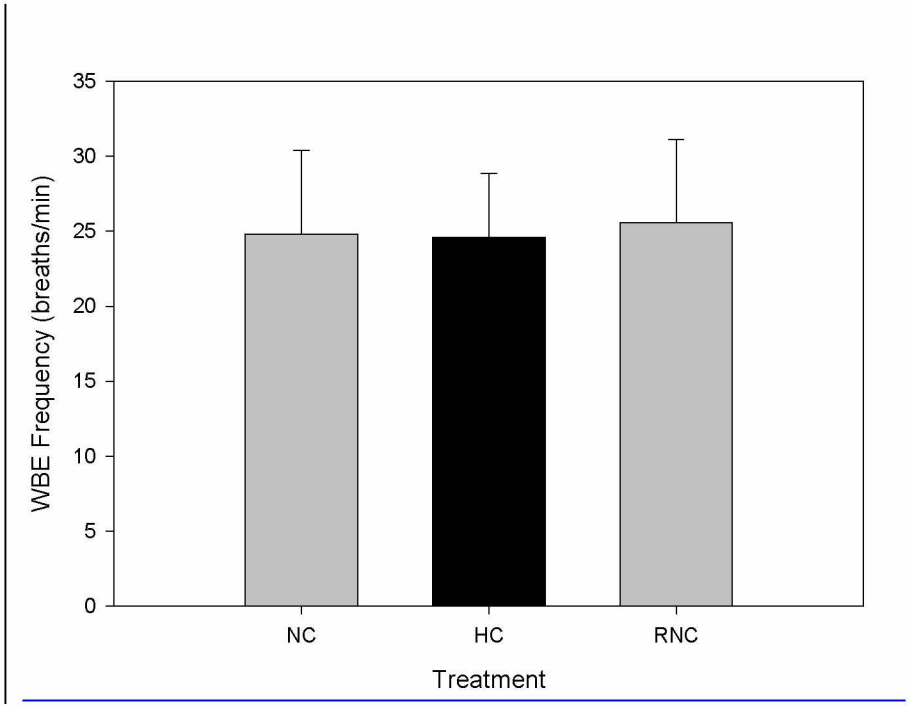


Figure 2.4~~2.4~~ Representative recordings of the electrical correlates of ventilatory activity in Alaska blackfish. Shown are simultaneous recordings from the levator operculum (electromyogram: EMG, raw (LO): top trace, integrated (*f* LO): 3rd trace from top) and cranial nerve VII (electroneurogram: ENG, raw (CN VII): 2nd trace from top, integrated (*f* CN VII): bottom trace). Each recording represents 20 s of activity.


35
30
25
20
15
10
5
0
WBE Frequency (breaths/min)
NC
HC
RNC
Treatment

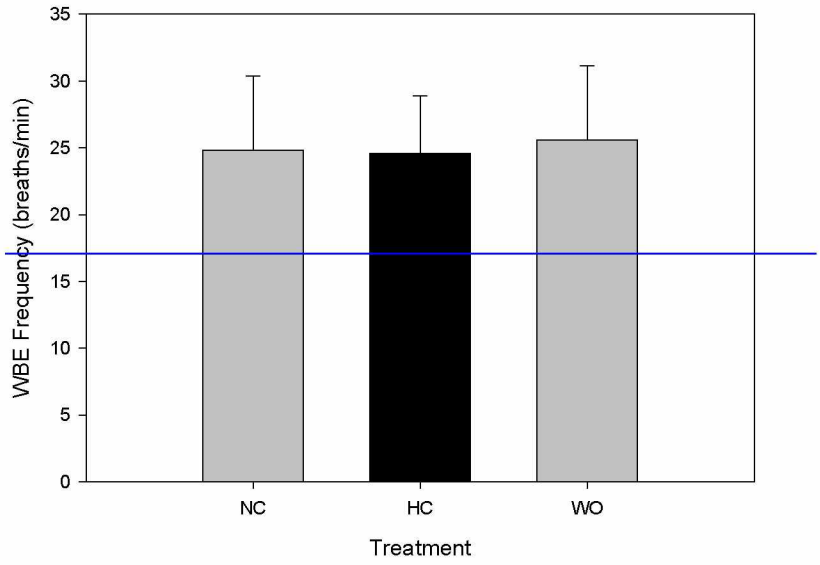
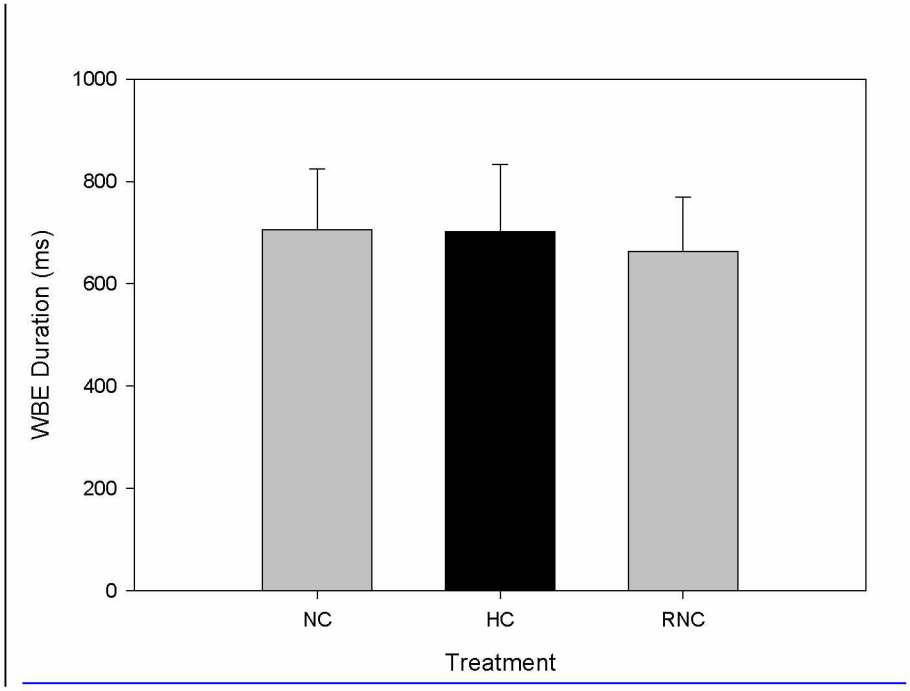


Figure 2.5~~2.5~~ Mean frequency of putative water-breathing events (breaths/min) exhibited by isolated blackfish brainstems during normocapnia (NC), hypercapnia (HC), and recovery normocapnia (RNC~~WO~~). Data are means ± standard errors, n=6. No significant difference among treatments (P= 0.713).


1000
800
600
400
200
0
WBE Duration (ms)
NC
HC
RNC
Treatment

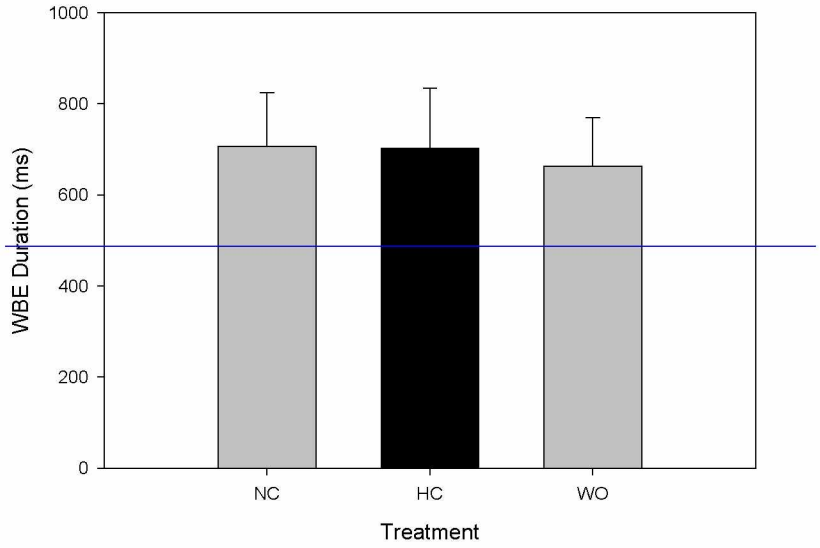
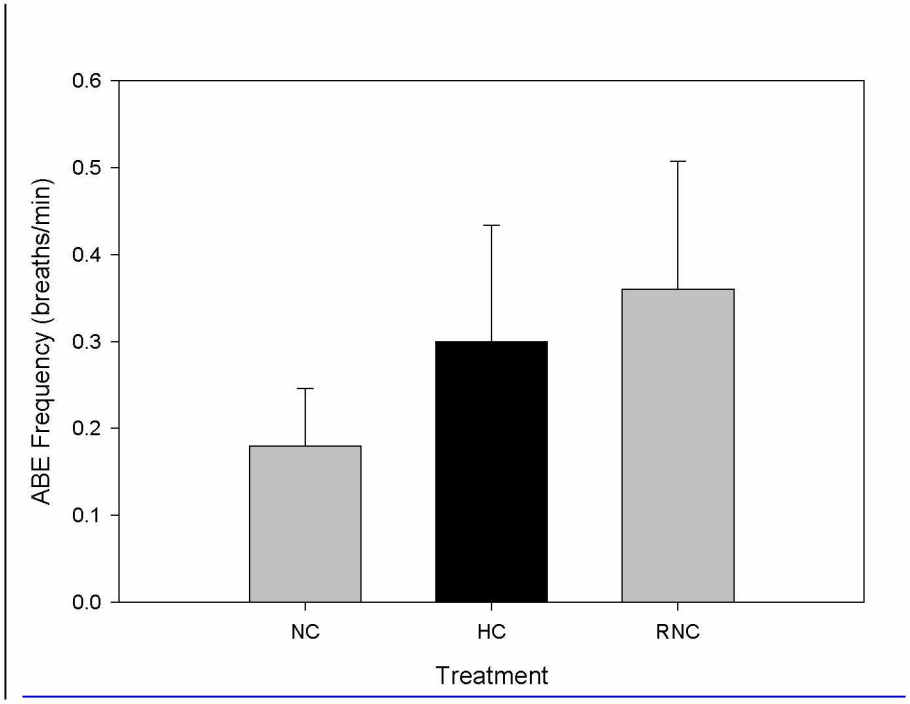


Figure 2.6~~2.6~~ Mean duration of putative water-breathing events (ms) exhibited by isolated blackfish brainstems during normocapnia (NC), hypercapnia (HC), and recovery normocapnia (RNC~~WO~~). Data are means ± standard errors, n=6. No significant difference among treatments (P= 0.587).


ABE Frequency (breaths/min)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NC
HC
RNC
Treatment

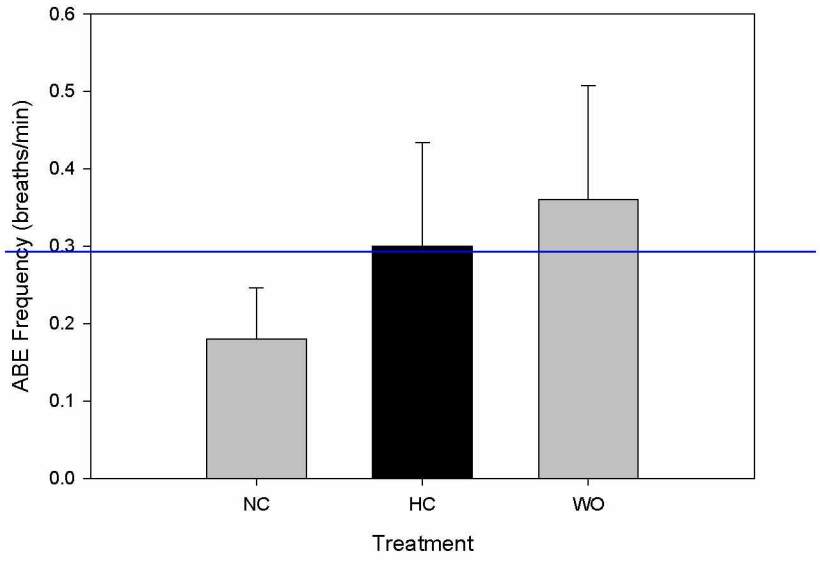
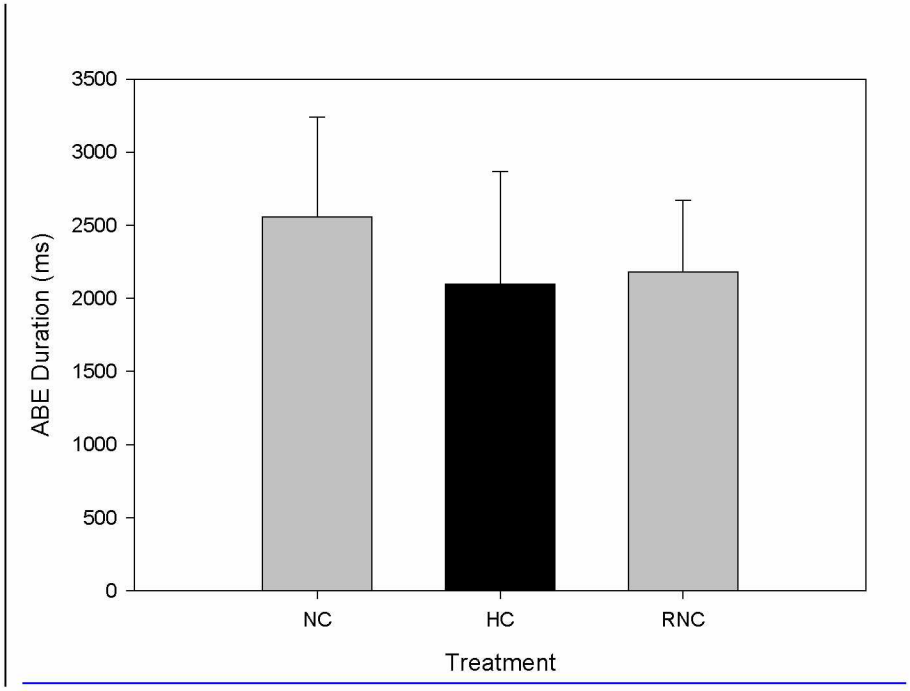


Figure 2.7~~2.7~~ Mean frequency of putative air-breathing events (breaths/min) exhibited by isolated blackfish brainstems during normocapnia (NC), hypercapnia (HC), and recovery normocapnia (RNC~~WO~~). Data are means ± standard errors, n=5. No significant difference among treatments (P= 0.383).


3500
3000
2500
2000
1500
1000
500
0
ABE Duration (ms)
NC
HC
RNC
Treatment

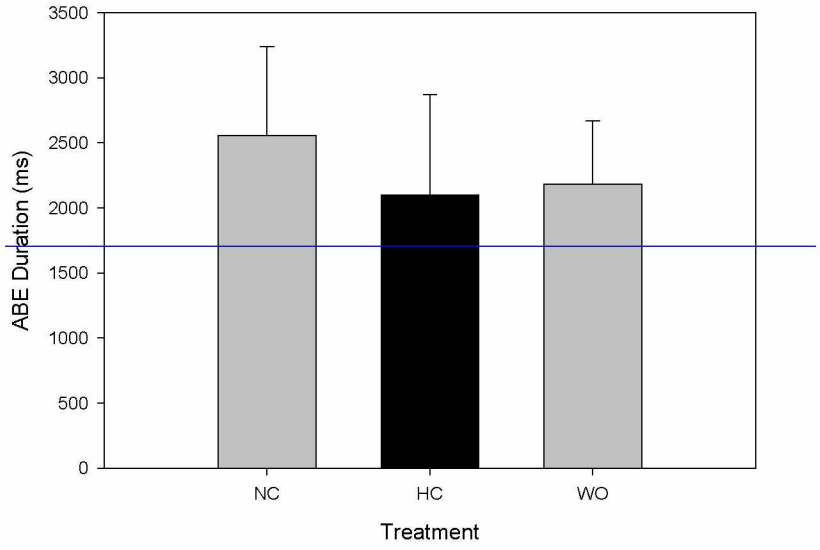
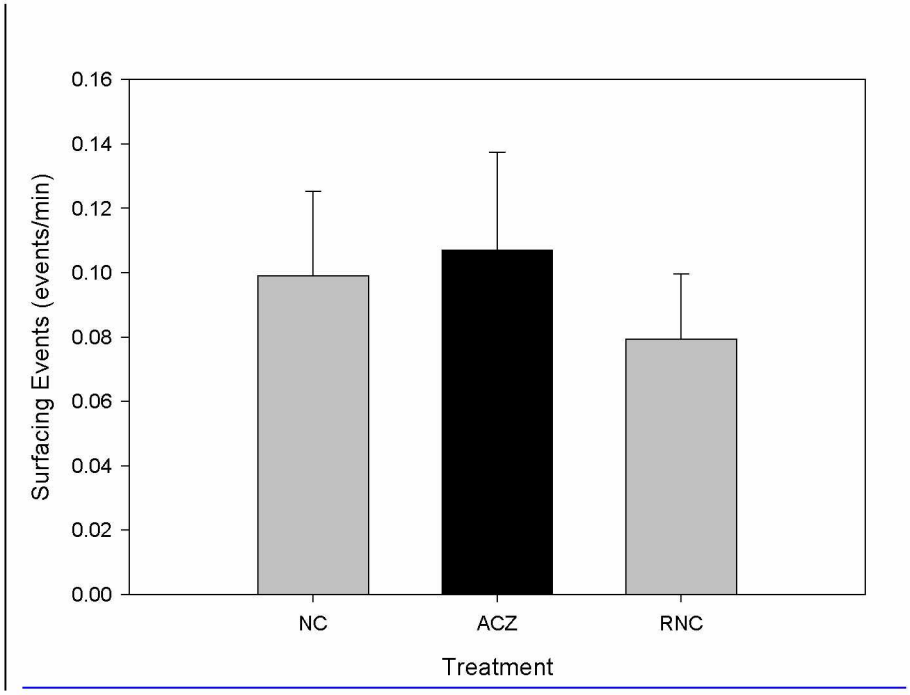


Figure 2.8~~2.8~~ Mean duration of putative water-breathing events (ms) exhibited by isolated blackfish brainstems during normocapnia (NC), hypercapnia (HC), and recovery normocapnia (RNC~~WO~~). Data are means ± standard errors, n=4. No significant difference among treatments (P= 0.208).


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Surfacing Events (events/min)
NC
ACZ
RNC
Treatment

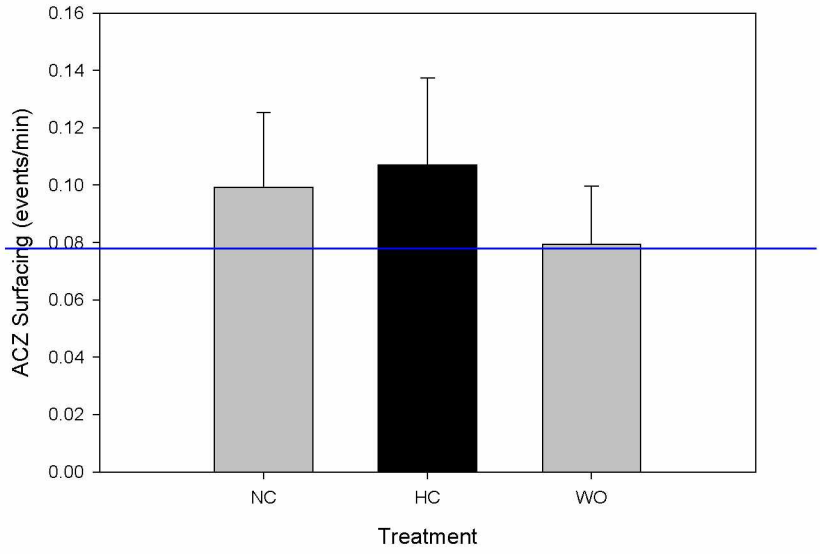


Figure 2.9~~2.9~~ Mean frequency of surfacing events (events/min) exhibited by intact blackfish during normocapnia/sham (NC), hypercapnia/acetazolamide acidosis (ACZ~~HC~~), and recovery normocapnia/sham (RNC~~WO~~). Data are means ± standard errors, n=8. No significant difference among treatments (P= 0.673).

Tables

Table 2.1 Overview of ventilatory-related activity in Alaska blackfish. EMG=electromyography, ENG=electroneurography, ACZ=acetazolamide, NC=normocapnia, HC=hypercapnia, RNC=recovery normocapnia.

Experiment	n	Treatment	Breath type	Frequency (burst/min)	Duration (s)	P value	
						Frequency	Duration
EMG	11	-	WBE	58.3 ± 4.2	0.5 ± 0.04	n/a	n/a
			ABE	n/a	n/a	n/a	n/a
Semi-intact blackfish	1	EMG	WBE	~30	0.2 ± 0.01	n/a	n/a
			ABE	n/a	~1.3	n/a	n/a
		ENG	WBE	~60(30*)	0.4 ± 0.01	n/a	n/a
			ABE	n/a	~1.3	n/a	n/a
ENG	9	NC	WBE	24.8 ± 5.6	0.7 ± 0.01	0.713	0.587
			ABE	0.2 ± 0.1	2.6 ± 0.7	0.383	0.208
		HC	WBE	24.6 ± 4.3	0.7 ± 0.1	0.713	0.587
			ABE	0.3 ± 0.1	2.1 ± 0.8	0.383	0.208
		RNC	WBE	25.6 ± 5.5	0.7 ± 0.1	0.713	0.587
			ABE	0.4 ± 0.2	2.2 ± 0.5	0.383	0.208
Intact blackfish challenged with ACZ-induced acidosis	8	NC	WBE	n/a	n/a	n/a	n/a
			ABE	0.1 ± 0.03	n/a	0.673	n/a
		HC	WBE	n/a	n/a	n/a	n/a
			ABE	0.1 ± 0.03	n/a	0.673	n/a
		RNC	WBE	n/a	n/a	n/a	n/a
			ABE	0.1 ± 0.02	n/a	0.673	n/a

Chapter 3 General Discussion

Respiration

Respiration, or breathing, is the exchange of CO_2 and O_2 with the environment, and is driven by metabolic requirements. Air breathing in terrestrial vertebrates is driven by the need to offload CO_2. Conversely, water breathing in fish is driven by the need to acquire O_2. This difference is largely due to the different physical attributes of CO_2 and O_2 in air and water. Air breathing in fish enables these aquatic organisms to acquire atmospheric O_2 when aquatic concentrations are low. Air breathing likely arose in hypoxic waters where an evolutionary advantage was conferred by the air-breathing trait. Increased availability of O_2, coupled with a reduced ability to excrete CO_2, which often occurs in air-breathing fish due to reduced gill structures and/or shunting vasculature, may have lead to a greater value for central CO_2 chemosensitivity than in strictly water-breathing fish.

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The ventilatory component of respiration is effected by a pattern of muscle activity that is likely derived from feeding movements (Rovainen 1996, Wilson et al. 2009). Feeding movements in their most ~~primitive~~ancestral form (filter feeding) are periodic rhythmic events. As complexities of feeding evolved (the advent of sucking, biting, chewing, and swallowing) so too evolved feeding movements driven by periodic non-rhythmic events (e.g. biting) and peripherally driven reflex events (e.g. swallowing). Air breathing likely originated from the incidental ingestion of air bubbles by fish feeding

or water breathing at the surface (Graham 2006), and such an origin calls into question whether ~~primitive~~ancestral air breathing is a peripherally driven reflex or a centrally driven periodic event.

Some air-breathing fish must breathe air continuously (obligate air-breathers) while others breathe air only when warranted by environmental gas conditions (facultative air-breathers; ~~)(~~Jordan 1976, Graham 1997). It might seem more likely that obligate air breathing is a centrally driven periodic rhythm and that facultative air breathing is a peripherally driven reflex event. However, facultative air breathing in gar and Alaska blackfish is driven by a central rhythm generator. This suggests that all air breathing in fish is driven by a central rhythm generator in the brainstem.

There is much debate (Smatresk 1994, Graham 1997, Wilson et al. 2000, Milsom 2002) over when central CO_2 chemosensitivity evolved in relation to air breathing, whether after or before the divergence of actinopterygians and sarcopterygians (ray- and lobe-finned fishes, respectively) concurrent with evolution of the lung or antecedent or subsequent to it. E~~There is e~~vidence suggests that CO_2 modulates ventilation in the ~~primitive~~ ancestral gill-breathing lamprey (Agnatha; Rovainen 1977), ~~which are agnathans,~~ a line of fish that arose before the sarcopterygian/actinopterygian divergence and prior to the evolution of air breathing. Air-breathing lungfish (sarcopterygians) exhibit central CO_2 chemosensitivity (Amin-Naves et al. 2007). Central CO_2 chemosensitivity has been shown to exist in two air-breathing actinopterygians, a~~one is a~~ non-teleost (gar; Wilson et al. 2000) and~~d one is~~ a teleost (*Betta*; Harris et al. 2001). In contrast however, central CO_2 chemosensitivity is absent from *Amia*, another non-teleost

actinopterygian (Hedrick et al. 1991) and, as we show here, the Alaska blackfish, ~~which is~~ another teleost. Thus, from the phylogenetic distribution of central CO_2 chemosensitivity, it has been difficult to determine if this trait arose in ~~primitive~~ ancestral fish and was lost by many fish during evolution, or if it evolved independently several times among the fish.

One means to address the debate over the phylogenetic origins of central CO_2 chemosensitivity is to conduct an~~d~~ exhaustive survey of the existence of this trait among air-breathing fish. Then, if a majority of air-breathing fish express central CO_2 chemosensitivity, the next step would be to identify and compare the neural mechanisms (cellular phenotypes or ion channels that act as the CO_2 sensors). If most air-breathing fish exhibit central CO_2 chemosensitivity, b~~and b~~y the same or similar mechanisms, then parsimony would argue that central CO_2 chemosensitivity is homologous among ~~the~~ fish and that ~~and that~~ it evolved once ~~as~~ very early ~~as the~~in the evolution of fish (agnathans) and was subsequently lost in fish that do not exhibit central CO_2 chemosensitivity. Conversely, if most air-breathing fish exhibit central CO_2 chemosensitivity, but all or most by different mechanisms, then parsimony would argue that central CO_2 chemosensitivity evolved independently several times among the fish. Evidence gathered in this way would be indirect. ~~,~~ E~~e~~ven if researchers were able to identify the genome elements responsible for central CO_2 chemosensitivity and confirm their similarity or difference among fish, the evolutionary origin of central CO_2 chemosensitivity could remain equivocal. An exhaustive survey of the neural control of breathing in air-breathing fish and the identification and comparison of the mechanisms and genomic

elements of central CO_2 chemosensitivity could only be amassed by the efforts of many researchers;~~,~~ as a single body of work all these investigations are well beyond the scope of a Master's project. The research undertaken here was intended to contribute to the survey of central CO_2 chemosensitivity among air-breathing fish. The Alaska blackfish is a teleost and a rare example of an arctic air-breathing fish that uses an ~~unique~~uncommon- mechanism (a modified esophagus) as its secondarily derived gas-exchange organ.

Validation of R~~r~~espiratory M~~m~~yo- and N~~n~~eurocorrelates

Air breathing in *D. pectoralis* is accomplished by breaking the surface with their mouths and gulping a bolus of air, which is then held in the respiratory section of their esophagus. Expansion of the entire buccal cavity, including operculi, was observed in concurrence with aerial respiration/gulping. Prior to inspiration, fish release a bubble of air, sometime while still submerged, sometimes at the surface immediately before inspiration. Blackfish usually take only one breath before resubmerging, but occasionally take two or more gulps at surface before descending. No report of blackfish air-breathing frequency could be obtained from the literature; here we report a frequency of 6 surfacings/hr at 5±1 °C as a surrogate for air-breathing frequency.

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Before we could determine the role of CO_2 in driving blackfish breathing, we first needed to characterize the anatomical and physiological aspects of breathing in this fish. We identified respiratory muscles recruited during both air- and water-breathing. Using

electromyostimulation, we identified the levator operculum, dilator operculum, adductor mandibulae, and levator hyomandibulae, which, respectively, lift the operculum, open the operculum, close the jaw and lift the jaw. We also identified the upper esophageal sphincter, likely used to occlude the esophagus during water-breathing. Via electromyography, we confirmed that the levator operculum, dilator operculum, and adductor mandibulae are all active during the act of water-breathing. We were unable to test the respiratory function of the deep mandibular muscle (levator hyomandibulae) due to the invasiveness of recording from a deep muscle. We were also unable to confirm, except through observation, the recruitment of these muscles during air breathing due to electronic signal saturation that occurred during whole-body movements associated with air breathing. However, we observed what appeared to be a larger recruitment of the same buccal musculature that was used in water-breathing.

Neuroventilatory bursts recorded from isolated blackfish brainstems appeared very similar (if not identical) to respiratory muscle bursts (the levator operculum, dilator operculum, adductor mandibulae, and levator hyomandibulae). We recorded two types of neuroventilatory bursts;, high frequency, short duration, low amplitude bursts and low frequency, longer duration, higher amplitude bursts. This combination of burst patterns matches the neuroventilation recorded from air-breathing fish (Wilson et al. 2000; Harris et al. 2001) and bullfrog tadpoles, which breathe bimodally with gills and lungs (Gdovin et al. 1998). Thus, one is inclined to ascribe the high frequency bursts to water breathing and the low frequency bursts to air breathing.

Using a semi-intact preparation, we recorded muscle activity and neural activity from contralateral sides of the fish; we were able to record the muscle and neural bursts associated with the same breathing event. We confirmed the endogenous recruitment of opercular muscles during both air and water breathing. Similar to the isolated brainstem experiments, which used only electroneurography, experiments on the semi-intact preparation characterized the neurocorrelates of water and air breathing as high frequency, short duration, low amplitude bursts and low frequency, longer duration, higher amplitude bursts, respectively. There was a notable and interesting difference between the neuro- and myocorrelates of water breathing. The neurogram recorded doublet bursts during water breathing events whereas the myogram recorded single bursts, and every other neural burst was coincident with every muscle burst. We believe this was the result of recording from one muscle group (the levator operculum), which is innervated by a branch of CNVII from which we recorded. CNVII innervates several areas in the oro-branchial cavity as well as other opercular muscles (Reid et al. 2003, Kenaley in preparation). Since our electroneurograms were recorded from the rootlets of CNVII at the brainstem surface, we believe we recorded neural output traveling to multiple respiratory-related muscles.

Myocorrelates of water breathing, whether recorded from intact or semi-intact fish, and neurocorrelates of water breathing, whether recorded from isolated brainstems or semi-intact fish, differed significantly in frequency and duration. Water-breath muscle burst durations were always shorter than water-breath neural burst durations whether they were compared between myograms and neurograms recorded concomitantly from semi-

intact fish or when recorded separately from intact fish and isolated brainstems, respectively. Furthermore, water-breath burst durations, whether recorded in myo- or neurograms, were always shorter in semi-intact fish than in the intact fish or isolated brainstems. This is contrary to neuroventilatory burst durations (water and air breathing) recorded for gar (Wilson et al. 2000), which were greater in the semi-intact fish than isolated brainstem preparations. Wilson et al. (2000) considered the difference to be a secondary effect resulting from a greater burst frequency in isolated brainstem preparations, which they attributed to a lack of inhibition in the isolated brainstem. We attribute the shorter duration of water breathing bursts in the semi-intact fish to the hypoxia we imposed on these preparations, which was intended to promote air breathing. Aerial respiration in all air-breathing fish is well known to be increased by hypoxia (Graham 1997).

The differences in water-breath burst frequencies in myo- and neurograms recorded from the three preparations were far more complex. Water-breath burst frequencies were greater when recorded as muscle bursts from intact fish than when recorded as neural bursts from isolated brainstems, but the opposite was true when both were recorded from semi-intact fish. Thus, our blackfish data differ from the gar data (Wilson et al. 2000) in that blackfish neuroventilatory water bursts were more frequent in semi-intact fish than in isolated brainstems, but the opposite was true for gar, where all neuroventilatory bursts were more frequent in isolated brainstems than in semi-intact fish. Another anomaly is that the myocorrelates of water breathing occurred at a much lower frequency in semi-intact fish than in intact fish, but neurocorrelates of water breathing

were more frequent in semi-intact fish than in isolated brainstems. Again, we attribute the difference to the hypoxic condition of the semi-intact fish. Hypoxia typically elicits an initial response of increased gill ventilation in fish (Gilmour 2001). We recorded d~~In summary, the d~~ifferences in frequency and duration ~~of ventilatory bursts:~~ among the myo- and~~or~~ neurocorrelates of blackfish ventilation~~,~~ when we~~ther~~ recorded from either the intact or semi-intact blackfish~~,~~, or from their isolated brainstems. The only comparable data from other studies are f~~, and as compared with data f~~or gar~~-~~ (~~the~~ only air-breathing fish~~, for~~ which exhibited similar differences. These differences, those we measured for blackfish and those measured for gar (Wilson et al. 2000 ~~we could find such data~~), seem to be best explained by our imposition of hypoxia on the semi-intact blackfish and normoxia on the other two preparations.

Through combinations of observation, electromyostimulation, electromyography, and electroneurography, we identified and validated the neurocorrelates of water and air breathing in Alaska blackfish. This facilitated use of the isolated blackfish brainstem preparation to assess the existence of central CO_2 chemosensitivity in this arctic air-breathing teleost, which uses a modified esophagus as a gas-exchange organ.

Central CO_2 C~~c~~hemosensitivity

Intact blackfish and isolated preparations of their brainstems were used to assess their sensitivity to internal and/or central CO_2. An acidosis akin to respiratory acidosis was induced in intact fish with the carbonic anhydrase inhibitor, acetazolamide.

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Acetazolamide is taken up by the gills and inhibits the conversion of carbon dioxide and bicarbonate, which results in a respiratory-acidosis-like state. In a companion series of experiments, isolated brainstem preparations were directly exposed to high P_{CO_2}. The results of these two experimental approaches led us to conclude that Alaska blackfish are relatively insensitive to central and general internal hypercapnia-related acidosis.

Alaska blackfish showed no significant increase in surfacing in response to whole-animal acetazolamide-induced tissue acidosis. However, a caveat to assessing central CO_2 chemosensitivity by quantifying whole-animal surfacing events in response to systemic acetazolamide treatment is that in addition to air breathing, fish surface for other purposes. In video recordings of surfacing blackfish, it was sometimes difficult to determine if air breathing occurred or if the fish had surfaced for another purpose.

Fish surface to feed, to conduct aquatic surface respiration, and adjust the volume of their swim bladder. Blackfish feed on invertebrates and, though we have never observed them surface feeding, invertebrates that land on the water surface might serve as blackfish prey; therefore surfacing to look for prey could be an important foraging behavior among blackfish. Many fish exhibit aquatic surface respiration, gill ventilation near the surface where the water is more oxygenated (Chapman et al. 1995). In captivity, blackfish were observed ventilating their gills at the surface, but this has not been documented in the wild. Many actinopterygians, including blackfish, have swim bladders. The swim bladder is an internal gas-filled organ that contributes to the ability of a fish to control its buoyancy, to maintain depth without having to expend energy swimming. The blackfish is a physostomous species, meaning that adjusting buoyancy

involves surfacing, gulping air, and passing it to the swim bladder (Barton 2007). Given that air breathing and buoyancy control are both accomplished by gulping air, the only way to determine the purpose of a fish surfacing to gulp air would be to determine the gas content of air bubble expelled after the surfacing event. With such complexities involved in using surfacing as a surrogate measure of air breathing, we deemed it easier to develop and validate the isolated blackfish brainstem preparation. Nonetheless, we persisted with the acetazolamide study so that we would have a complement for the hypercapnic isolated brainstem study.

Even considering these potential caveats, we did not appear to have an overestimate of air-breathing events in the acetazolamide study (~6 surfacings/hour) when compared with neuroventilatory air breathing in the hypercapnic isolated brainstem study (~18 ABEs/hour). This is within the range of air-breathing frequencies (0-150 ABEs/hour) reported for several species of air-breathing fish under various conditions (Graham 1997). Air-breathing frequency depends greatly on O_2 content of the water and the metabolism of the fish (Graham 1997); both of these are greatly influenced by the water temperature (Saksena 1975; Dejours 1976; Graham 1997). An air-breathing fish in cold water has less aerial respiratory drive than a similar fish in warm water. Furthermore, not only do blackfish live at colder temperatures than many air-breathing fish, but they also have a lower metabolic rate, a lower O_2 demand, than other arctic fish (Hanzely 1957). Based on observations of Hanzely (1957), Crawford (1974), and those presented here, blackfish may utilize a combination of adaptations to the arctic environment: low metabolism, aquatic surface respiration, and air breathing.

Isolated brainstems from Alaska blackfish showed no significant increase in neuroventilatory frequency in response to hypercapnia/high P_{CO_2}. There is a possibility that blackfish neuroventilatory CO_2 response manifests as an increase in burst amplitude, because we did not quantify this parameter. An amplitude-based CO_2 response, however, is unlikely because in other vertebrates that breathe water and air the central CO_2 chemosensitivity is manifest as an increase in air-breathing-related neuroventilatory frequency (gar, Wilson et al. 2000; *Betta*, Harris et al. 2001, tadpoles, Taylor et al. 2003). We have concluded from our studies that the isolated blackfish brainstem was insensitive to the hypercapnia we imposed.

Studies utilizing acetazolamide-induced systemic acidosis and CO_2-induced brainstem acidosis indicate that Alaska blackfish have no ventilatory response to internal or central hypercapnia. This suggests that they have experienced no ~~evolutionary~~ selective pressure to maintain or secondarily derive central CO_2 chemosensitivity~~. This is~~, despite the fact that blackfish habitats are prone to winterkill (ice-covered stagnant waters develop low O_2 and high CO_2 resulting in the death of most fish species; Magnuson et al. 1983). However, we have already indicated that blackfish ~~are~~ likely exhibit low metabolism and air breathing as adaptations to the arctic environment. A low metabolism would reduce the production of endogenous CO_2~~. and a~~. Air breathing, potentially from gas pockets trapped under the ice~~,~~ as has been shown in some mudminnows (Magnuson et al. 1983), could provide some opportunity to acquire O_2 and excrete CO_2. CO_2 excretion via fish air-breathing organs is limited except in fish whose air-breathing organs are derived from epibranchial tissue and ~~so~~ produce carbonic

anhydrase (Burggren 1979). Carbonic anhydrase catalyzes the reversible reaction that converts CO_2 and H_2O to HCO_3^- and H^+, and its presence on the gills aids in CO_2 excretion. It seems unlikely that the modified esophagus of blackfish has carbonic anhydrase, but this has not been investigated. Regardless of whether or not blackfish are capable of CO_2 excretion via air breathing, a marked metabolic reduction could lower endogenous production of CO_2 enough to protect blackfish from internal hypercapnia.

General Ceonclusion

Alaska blackfish are facultative air-breathers that have a modified esophagus as their secondarily derived gas-exchange organ. In these fish, air breathing consists of gulping and swallowing an air bubble into their esophagus and holding it there with a sphincter that closes off the esophagus from the buccal cavity. Gulping the air bubble is accomplished by the same opercular and mandibular muscles that draw water into the buccal cavity during gill ventilation. Activation of the opercular and mandibular muscles for ventilation is effected by a central rhythm generator in the brainstem that is spontaneously active in the absence of peripheral input. This central rhythm generator, however, is not modulated by central CO_2 chemosensitivity. Thus, unless central CO_2 chemosensitivity was lost in blackfish, we might conclude that centrally controlled vertebrate air breathing can evolve independently of central CO_2 chemosensitivity.

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