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Context-dependence of physiological systems: environment-physiology interactions in the respiratory control system

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CONTEXT-DEPENDENCE OF PHYSIOLOGICAL SYSTEMS: ENVIRONMENT-PHYSIOLOGY INTERACTIONS IN THE RESPIRATORY CONTROL SYSTEM

A dissertation in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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2017
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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION
BY Joseph M. Santin ENTITLED Context-dependence of physiological systems: environment-
physiology interactions in the respiratory control system BE ACCEPTED IN PARTIAL
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ABSTRACT

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Context-dependence of physiological systems: environment-physiology interactions in the respiratory control system

We know that animals are tuned to survive different environmental conditions or else life would not exist. Unfortunately, this is often forgotten or ignored when designing experiments. As Marsh Tenney articulated, "The physiologist keeps the whole always in mind. He accepts the tactical necessity of reductionism to understand parts, but, once done, for him it is only the beginning, never the end" (from Remmers, 2005). In an era when it is all too common to take environmental complexity out of the organism to understand physiology, my work puts some of that complexity back in the study of organisms. I take advantage of the plasticity and robustness of the bullfrog, *Lithobates catesbeianus*, as my model system to determine environment-physiology interactions in the regulatory systems that control breathing. Given that breathing is a critical process for sustaining life, under most circumstances, I determine how the respiratory control circuit operates on background of changing environmental conditions, specifically temperature and seasonal fluctuations. My major findings include 1. CO₂ chemosensory neurons that regulate breathing frequency in bullfrogs do not act as chemosensors at cold temperatures (Santin et al., 2013), 2. CO₂
chemosensory neurons have firing frequencies inversely proportional to temperatures \( i.e. \), they are cold-activated) (Santin et al., 2013), 3. activation of a hyperpolarization-activated cation current \( I_h \), paradoxically, leads to reversal of neuronal cold-activation (Santin and Hartzler, 2015a), 4. lung ventilation can be measured in bullfrogs (Santin and Hartzler, 2016c), 5. breathing motor processes operate normally after months without use, but lose \( \text{CO}_2 \) chemosensitivity (Santin and Hartzler, 2016a), and 6) after overwintering, the chemosensory system within the brainstem of adult bullfrogs resembles that of predominately water-breathing tadpoles (Santin and Hartzler, 2016b). Overall, these findings have major implications for interpreting well-studied physiological problems. First, \( \text{CO}_2 \) sensing in ventilatory control may be more or less important depending on the environmental context of the animal. Second, factors that regulate neuronal excitability exert different effects depending on whether environmental factors are stable or changing. Third, bullfrogs may have unique physiological adaptations that allow normal motor function after months of respiratory inactivity. Finally, phenotypes described as developmental may, in fact, be a plastic response to changing environmental conditions that overlap with a developmental time course. Although it is common to take environmental complexity (internal and external) out of experiments when studying biological problems, all animals live in environments that alter the function and need for specific physiological processes. My work highlights that only when physiological systems are tested
across a range of environmental situations can organismal function be ascribed to specific mechanisms.
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Lastly, all of the work I did could not have been done without the financial support from the Biomedical Sciences PhD program.
CHAPTER I.

INTRODUCTION
Animals operate over a continuum of “normal” internal and external environmental contexts (e.g., fed-starved, sleep-awake, hot-cold, etc.). Early physiologists such as Claude Bernard, Walter Cannon, and August Krogh embraced understanding environmental challenges faced by animals, and that led to understanding key principles of modern physiology like homeostasis and redundancy (Cannon, 1929; Joyner, 2013; Joyner and Pedersen, 2011; Krogh, 1929). Unfortunately, in most of the past ~20 years, classical physiology approaches (i.e., systems level and integrative approaches across multiple scales of biological organization) to understanding physiological adaptations of animals have waned. Although the specific origin of this decline is debated, it presumably occurred after funding priorities shifted from “classic” physiology to “gene-centric” physiology when molecular tools became accessible to physiologists (Joyner and Prendergast, 2014; Noble, 2008). The salient message from recent arguments for “classic” physiology is that “gene-centric” approaches that emerged with much promise have provided disappointingly little useful information regarding predicting or treating disease. Yet, understanding how animals function in complex environments that challenge homeostasis has yet to fail to provide novel and insightful information as to how physiological systems overcome organismal challenges (Joyner, 2011; Kaiser, 2012; Noble, 2010). It is clear that ignoring the challenges encountered by animals in their environments will lead to a loss of novel information and destruction of the potential for discovery of new physiological principles that could facilitate biomedical discoveries (Marder, 2002).
Despite a consensus trend away from the basic study of physiological adaptation, comparative and evolutionary physiologists have always defined and continue to define adaptive mechanisms of animals that experience and thrive in unusual/ extreme environmental situations. Given the apparent observation that all organisms experience environmental challenges, prominent voices in physiology like Dr. Gary Sieck from the Mayo Clinic and editor of the review journal *Physiology* have become enthusiastic advocates for the comparative approach because many animals live in environments that necessitate interesting and novel adaptations. He and others appreciate that many of these adaptations used by animals in extreme or unique environments can directly or may indirectly lead to biomedical discoveries and principles that cannot be studied in conventional biomedical approaches (e.g., rats, mice, cell culture, etc.) (Joyner et al., 2011; Krogh, 1929; Sieck et al., 2013). This renewed impetus toward encouraging studying environmental physiology of animals is immediately apparent as ~20% of the articles in *Physiology* since 2013 contain reviews directly dealing with integrative environmental challenges addressed by comparative physiology. In contrast, from 2003-2005 and 1993-1995, *Physiology* published only 2% of articles containing reviews associated with environmental physiology of animals (http://physiologyonline.physiology.org).

Elucidating integrative mechanisms used by animals to cope with or circumvent environmental disturbances to homeostasis can have far-reaching benefits that surpass the hype associated with immediate translation of information gained from consensus models or preparations (Joyner and
Pedersen, 2011; Marder, 2002; Sieck et al., 2013). Therefore, in my dissertation work I fully embraced an integrative approach to physiology in an environmental context. Here I focus on aspects of breathing control system in bullfrogs (*Lithobates catesbeianus*) that allow this system to regulate breathing when it is needed and to not regulate breathing when it is not needed. Anuran amphibians (frogs and toads) use lung ventilation and cutaneous gas exchange to satisfy metabolic demands and acid-base regulation; however, all gas exchange requirements can be met by the skin at cold temperatures without lung ventilation. Therefore, frogs and other anuran amphibians experience an unusual scenario where an entire neuromotor system dedicated to maintaining homeostasis is only required at warm, but not cold, temperatures. To make matters more complex for the respiratory control system, body temperature and even the ability to breathe varies drastically and rapidly depending on time of day, season, microhabitat, and geography. Based on these observations, two basic questions arise: 1.) is the respiratory control system of frogs tuned to determine when breathing is or is not necessary, and 2.) does the respiratory system remain functional when breathing has been absent during the winter? In contrast to the conventional view that temperature acts on the respiratory control system (and other physiological systems as well) through “simple” Q_{10} effects, I hypothesize that the respiratory control system is appropriately tuned to and by the environment to ensure breathing occurs when needed and does not when not needed to adequately satisfy gas exchange requirements in *frogs*. Understanding “built in” characteristics of the respiratory control system
that allow physiology to match environmental demands of frogs provides an ideal platform to gain mechanistic insight into how physiological scales are integrated to generate appropriate behavior in the background of a varying context (in the case of my work, changing body temperatures).

In addition to uncovering some of the integrative mechanisms and processes important for satisfying a variable need to breathe in bullfrogs, my dissertation work highlights that accounting for seemingly small differences in physiological context (using temperature as the example) is imperative when attempting to interpret basic mechanisms of physiological processes. This is not only a problem for frogs, but a problem for all animals. As I and others have pointed out, this problem is often intentionally ignored during molecular/genetic approaches to physiology (Joyner and Prendergast, 2014). Therefore, a major theme throughout my work is that “context matters” when drawing conclusions about physiological processes. This is not a new idea, but it is often forgotten or ignored when using reductionist approaches and/or molecular and genetic tools in physiology.

My dissertation includes data from five of my first-author, published manuscripts. For clarity I will separate these five manuscripts into two complimentary parts. Part I (aims 1 and 2) investigates how acute temperature changes affect the function of cellular chemoreceptors in the respiratory control system (i.e., how does respiratory system determine when to and when not to drive breathing?). Part II (aims 3-5) investigates the consequences of chronic cooling that mimics overwintering conditions on the function of the respiratory
control system in the spring after overwintering (i.e., how well does the respiratory control system work after long-term disuse?).
CHAPTER II

BACKGROUND
**Temperature and the Organism:** Temperature has a profound role in shaping the physiology of cells, necessitating that organisms either respond to or cope with temperature change. As a reflection of molecular kinetic energy within a system, temperature has the ability to influence all aspects of physiology owing to temperature-sensitivity of subcellular processes (Sengupta and Garrity, 2013). Biochemical reactions that determine the organismal metabolic rate (Hochachka and Somero, 1968), ion channel properties essential to nervous system function (Katz and Miledi, 1965), and ligand-receptor binding properties critical for intracellular signaling (Isorna et al., 2005) all operate with a characteristic temperature coefficient ($Q_{10}$) following the equation

$$Q_{10} = \left( \frac{R_2}{R_1} \right)^\frac{10}{T_2-T_1} \quad \text{(Equation 1).}$$

In this equation, $R_1$ represents the rate of the reaction or process at the lower temperature ($T_1$) and $R_2$ represents the rate at the higher temperature ($T_2$). Most enzymatic reactions have a $Q_{10}$ value near 2, reflecting a rate change of 2-fold with a 10°C temperature difference (Hochachka and Somero, 1984) and biophysical properties of many ion channels have $Q_{10}$s that greatly exceed doubling with 10°C (Maingret et al., 2000; Peloquin et al., 2008; Tang et al., 2010; Voets et al., 2004). If the physicochemical basis of virtually every biological process displays temperature-sensitivity, mechanisms must be in place to allow organisms to endure a vast temperature range while maintaining the ability to precisely regulate physiological processes and behaviors to support requirements for survival.
Anuran amphibian respiratory control: regulating breathing with variable convective requirements: Although ectothermic vertebrates can thermoregulate by behavioral mechanisms (Beitinger and Fitzpatrick, 1979; Lillywhite, 1970; Lillywhite et al., 1973), these animals routinely experience variable and even rapid temperatures changes throughout a single day. Factors including ambient temperature, amount of available shade, microhabitat, and water temperature all influence body temperature (Stevenson, 1985b). The American bullfrog, *Lithobates catesbeianus*, is one example of a poikilothermic ectotherm (*i.e.*, its body temperature changes because heat is obtained from the environment). Although their native range is mainly the eastern United States, bullfrogs inhabits the majority of North America, from northern Canada to Mexico, and experiences widely varied daily and seasonal temperature changes (Lannoo, 2005). As a result of this poikilothermic life history, physiological systems must be equipped with mechanisms to promote survival over a large range of temperatures. Among a host of unique physiological adaptations of anuran amphibians (Burggren and Warburton, 2007), mechanisms of gas exchange have received extensive attention with respect to body temperature changes.

Amphibians are unique in that, depending on the developmental stage or environmental context, oxygen and carbon dioxide may be exchanged using a combination of lungs, skin, and/or gills. I will focus on adult frogs in which the lungs are the actively controlled sites for gas exchange, while passive gas exchange occurs across the skin. As bimodal breathers, adult bullfrogs and other amphibians use cutaneous gas exchange for a large proportion of CO₂
elimination (Gottlieb and Jackson, 1976) and, to much a lesser extent, O$_2$ uptake (Pinder and Burggren, 1986). Despite the use of skin as a respiratory surface, lung breathing is obligatory to eliminate CO$_2$ for maintenance of acid-base balance (specifically above ~12°C (Wang et al., 1998)) and to acquire O$_2$ for aerobic metabolism (above ~5°C). The control of lung breathings occurs through a negative feedback loop. At temperatures where breathing is required, amphibians presumably regulate blood gases at temperature-specific set-points through a negative feedback loop. Changes in blood gas composition are detected by central (CO$_2$/pH and to a lesser extent, O$_2$) (Noronha-de-Souza et al., 2006; Taylor et al., 2003b; Winmill et al., 2005) and peripheral (O$_2$ and CO$_2$/pH) (Van Vliet and West, 1992) chemoreceptors. Through uncharacterized anatomical connections of neurons, chemoreceptor information is transmitted to influence the pattern produced by, at least 1 of 3, respiratory rhythm generators located in the medulla (Baghdadwala et al., 2015; Wilson et al., 2002). The altered respiratory rhythm then activates pools of motor neurons (cranial nerves V, VII, X, and spinal nerve II) that cause contraction of respiratory muscles used to ventilate the lungs and regulate blood gases.

Based on the variable convective requirements of bullfrogs depending on the temperature, frogs and other anurans experience a unique scenario where an entire neuromotor system dedicated to maintaining organismal homeostasis (control of lung breathing) is required at warm, but not cold, temperatures. To make matters more complex for the respiratory control system, body temperature varies drastically and rapidly depending time of day, season, microhabitat, and
geography. Because adult frogs can be considered obligate air breathers, facultative air breathers, or obligate water breathers depending on their environmental context and body temperature, two basic, unanswered questions arise: 1.) how is the respiratory system of frogs tuned to know when breathing is needed? 2.) does the respiratory control system remain fully functional after prolonged disuse associated with overwintering. In Part I of this dissertation, I use electrophysiology to understand how important respiratory modulating neurons function across a range of temperatures and correlate these changes in activity with respiratory activity in the whole-animal. In Part II, I use a multi-pronged approach to understand which aspects of the respiratory control system are maintained or lost following long-term cessation of breathing due to cold-acclimation mimicking overwintering. All data I have collected support my central hypothesis that the respiratory control system of frogs is tuned to and by the environment to adequately satisfy gas exchange demands. Furthermore, each series of experiments provides examples highlighting the premise that physiological problems considered in an environmental context do not have one “solution”, but rather “solutions”; therefore, interpreting any one piece of physiological data presented here would be impossible without understanding the environmental context or life history of the animal.
Chapter III

SPECIFIC AIMS
**Specifics Aims:**

**Part I:** Define the effects of temperature on central respiratory chemoreceptive neurons from the locus coeruleus of bullfrogs. Is there temperature-dependent tuning in the respiratory network?

**Aim 1:** Determine the influence of temperature on neuronal activity and chemosensitivity of putative respiratory chemosensors in the locus coeruleus (LC).

**H$_{1-1}$:** I hypothesize that CO$_2$/pH chemoreceptors in the LC will increase chemosensitivity at warm temperatures and decrease chemosensitivity at cold temperatures.

**H$_{1-2}$:** I hypothesize that temperature *per se* will have a proportional influence on firing frequency in the LC chemoreceptors.

**Aim 2:** Determine the role of the hyperpolarization-activated current ($I_h$) in the regulation of paradoxical cold-activation of central chemoreceptive LC neurons.

**H$_{5-1}$:** I hypothesize that $I_h$ acts as a physiological brake on cold-activation.

**H$_{5-2}$:** I hypothesize that modulation of $I_h$ can influence the magnitude of cold-activation.

**Part II:** How well does the respiratory control system work after it has not been used for 2 months?
Aim 3: Adapt previously existing methods to directly measure ventilation and metabolism in bullfrogs and reassess chemical control of breathing. To assess respiratory physiology in bullfrogs following overwintering, I needed to measure ventilation. This has been estimated, but not directly quantified in bullfrogs. Thus, aim 1 is dedicated to technique development, validation, and reassessment of chemical control of breathing in bullfrogs. Admittedly, this aim is not hypothesis driven; however it is necessary for directly measuring ventilation and was not previously possible in this species.

Aim 4: Understand how conditions that mimic overwintering affect breathing control (ventilation, breathing pattern, and metabolism during rest, hypercarbia, and hypoxia).

\( H_1 \): I hypothesize that despite long-term disuse the respiratory control system will remain functional after overwintering conditions in bullfrogs.

Aim 5: Determine how lack of breathing during overwintering influences brainstem respiratory motor output.

\( H_{2,1} \): I hypothesize that overwintering conditions will reduce CO\(_2\)/pH, but not hypoxia, chemosensitivity.

\( H_{2,2} \): I hypothesize that cold-acclimation will reduce CO\(_2\)/pH sensitive firing.
CHAPTER IV
MANUSCRIPT I

Temperature influences neuronal activity and CO\textsubscript{2}/pH sensitivity of locus coeruleus neurons in the bullfrog, \textit{Lithobates catesbeianus}.

Joseph M. Santin, Kayla C. Watters, Robert W. Putnam, and Lynn K. Hartzler

ABSTRACT

The locus coeruleus (LC) is a chemoreceptive brainstem region in anuran amphibians and contains neurons sensitive to physiological changes in CO₂/pH. The ventilatory and central sensitivity to CO₂/pH is proportional to the temperature in amphibians, i.e., sensitivity increases with increasing temperature. We hypothesized that LC neurons from bullfrogs, Lithobates catesbeianus, would increase CO₂/pH sensitivity with increasing temperature and decrease CO₂/pH sensitivity with decreasing temperature. Further, we hypothesized that cooling would decrease, while warming would increase, normocapnic firing rates of LC neurons. To test these hypotheses, we used whole-cell patch clamp electrophysiology to measure firing rate, membrane potential (V_m), and input resistance (R_in) in LC neurons in brainstem slices from adult bullfrogs over a physiological range of temperatures during normocapnia and hypercapnia. We found that cooling reduced chemosensitive responses of LC neurons as temperature decreased until elimination of CO₂/pH sensitivity at 10°C. Chemosensitive responses increased at elevated temperatures. Surprisingly, chemosensitive LC neurons increased normocapnic firing rate and underwent membrane depolarization when cooled and decreased normocapnic firing rate and underwent membrane hyperpolarization when warmed. These responses to temperature were not observed in non-chemosensitive LC neurons or neurons in a brainstem slice 500μm rostral to the LC. Our results indicate that modulation of cellular chemosensitivity within the LC during temperature changes may influence temperature-dependent respiratory drive during acid-base disturbances in amphibians. Additionally, cold-activated/warm-inhibited LC
neurons introduce paradoxical temperature sensitivity in respiratory control neurons of amphibians.
INTRODUCTION

The bullfrog, *Lithobates catesbeianus* (formerly *Rana catesbiena*) inhabits much of North America and experiences a broad range of daily and seasonal changes in temperature. Activity during high and low temperatures can cause substantial disturbances to acid-base status in amphibians (Andersen and Wang, 2003; Tattersall and Boutilier, 1999b). Unlike the endothermic mammals and birds, which regulate constant arterial pH (pH$_a$), amphibians and other ectothermic vertebrates preserve acid-base balance by maintaining temperature-dependent pH$_a$. This acid-base regulatory strategy is achieved by allowing pH$_a$ to vary inversely with body temperature (T$_b$; for recent review see (da Silva et al., 2013)). Although extensive work has attempted to illuminate the precise physiological role of an inverse pH-temperature relationship, it is still unclear as to which intracellular or extracellular variable(s) are regulated (Burton, 2002). Reeves (Reeves, 1972) proposed that an inverse pH-temperature relationship (-ΔpH$_a$/ΔT) defends the fractional dissociation (α) of imidazole moieties on histidine residues (α-stat hypothesis); however, Cameron (Cameron, 1989) asserted that maintenance of protein net charge during changes in T$_b$ may be a more appropriate model. Regardless of the defining component that dictates -ΔpH$_a$/ΔT, ectothermic vertebrates maintain a temperature-specific pH$_a$. For example, bullfrogs regulate an arterial pH of~8.3 at 7°C, ~7.9 at ~20°C, and ~7.75 at ~30°C (Howell et al., 1970; Mackenzie and Jacson, 1978; Reeves, 1972; Tattersall and Boutilier, 1999a).
Anuran amphibians utilize both skin and lungs for CO₂ elimination. Although each plays a role in the maintenance of acid-base balance, the lungs become increasingly important at elevated temperatures. Gottlieb & Jackson (Gottlieb and Jackson, 1976) showed that pulmonary elimination of CO₂ contributes 30-75% of the total CO₂ loss at 20°C and varies directly with metabolic rate. Additionally, when bullfrogs in this study were made apneic at 20°C, arterial Pco₂ increased 3-fold, while pHₐ decreased from ~8 to 7.6.

Cutaneous gas exchange has been shown to be predominately diffusion limited and independent of Tₘ in amphibians (Mackenzie and Jacson, 1978; Moalli et al., 1981). These experiments demonstrated that cutaneous CO₂ conductance is not regulated and does not take part in the active regulation of Pco₂. At low Tₘ (≤10 °C), however, bullfrogs utilize the skin as the primary mode of CO₂ elimination (Mackenzie and Jacson, 1978). Consequently, preservation of acid-base homeostasis does not seem to require pulmonary ventilation at low Tₘ (Tattersall and Boutilier, 1999a) since submerged bullfrogs without access to air maintained at 7 °C (pHₐ ~8.4) can fully compensate a metabolic acidosis following exhaustive exercise (pHₐ 7.6 returned to ~8.4 in 8 hours (Tattersall and Boutilier, 1999b).

Ventilatory sensitivity to acid-base disturbances in amphibians and other air-breathing vertebrates is derived primarily from CO₂/pH sensitivity that occurs at various locations within the brainstem (Feldman et al., 2003; Gargaglioni and Milsom, 2007; Milsom, 2010). Since pulmonary control of acid-base homeostasis increases in importance as Tₘ rises in amphibians, it is not surprising that the
bullfrog CO$_2$/pH sensory system can drive ventilation at elevated $T_b$, while concurrently, become insensitive to changes in CO$_2$/pH at lower temperatures that allow complete exchange of CO$_2$ across the skin. For example, the respiratory frequency of bullfrogs is highly sensitive to inhalation of 5% CO$_2$ at 20˚C and 30 °C, while at 10 °C, respiratory frequency is completely insensitive to this stimulus (Bicego-Nahas and Branco, 1999). Further, the modulation of respiratory CO$_2$ sensitivity appears to be of central origin because breathing-related nerve activity from the bullfrog brainstem is sensitive to acidification at 20˚C and 25˚C, but exposure of the brainstem to 15˚C reduces sensitivity to large, non-physiological pH changes (Morales and Hedrick, 2002). Despite extraordinarily high-levels of respiratory drive at lower temperatures, bullfrogs lose chemoreceptive drive to breathe at cool temperatures. Additionally, central CO$_2$/pH sensitivity exhibits similar temperature dependence in toads (Branco et al., 1993). These examples provide strong evidence that CO$_2$/pH sensitive neurons within chemosensitive areas in the brainstem of anuran amphibians may be equipped with mechanisms to up or down regulate cellular chemosensitivity during changes in temperature depending on the necessity for pulmonary ventilation to regulate acid-base balance.

The locus coeruleus (LC) is a chemosensitive area within the brainstem that is used for ventilatory control in amphibians (Gargaglioni et al., 2010). Focal acidification of the LC increases minute ventilation in vivo, while ablation attenuates the hypercapnic ventilatory response by abolishing increases in tidal volume (Noronha-de-Souza et al., 2006). We recently found that neurons within
the LC of bullfrogs are highly sensitive to physiological levels of acidification by hypercapnic acidosis (HA) \textit{in vitro}. Additionally, we demonstrated that LC neurons are intrinsically CO$_2$/pH sensitive (Santin and Hartzler, 2013a). Studying the influence of temperature on CO$_2$/pH sensitivity of LC neurons provides an outlet to investigate temperature-dependent tuning of the anuran amphibian respiratory control system. We hypothesized that neurons within the bullfrog LC would exhibit reduced sensitivity to CO$_2$/pH changes at lower temperatures, while exposure to warmer temperatures would enhance their responsiveness. Further, we hypothesized that neurons of the bullfrog LC would decrease firing rates with cooling and increase firing rates with warming. To investigate these hypotheses we used the whole-cell patch clamp technique to measure changes in membrane potential ($V_m$), firing frequency, and input resistance in LC neurons from brainstem slices during normocapnic and hypercapnic exposures over a physiological range of temperatures. In order to identify whether chemosensitive and temperature sensitive responses were intrinsic \textit{versus} driven by chemical or electrical neurotransmission, we performed experiments with neural networks intact and blocked. Some of these data have been previously published in the form of a meeting abstract (Santin and Hartzler, 2013b).
METHODS

Brainstem slice preparation

Adult bullfrogs, *Lithobates catesbeianus*, (N=44; 117±26g) were housed in tanks containing 22°C water with access to wet and dry areas, exposed to 12:12 light: dark cycles, and fed crickets *ad libitum*. Experiments were approved by the Wright State University Institutional Animal Care and Use Committee. After rapid decapitation the head was submerged in 4°C artificial cerebral spinal fluid (aCSF; see solutions for composition) equilibrated with 97.5% O₂ and 2.5% CO₂ and the brainstem was dissected. Following dissection, the brainstem was adhered to an agar block using cyanoacrylate glue then sectioned into ~400μm transverse slices using a Vibratome tissue slicer (Leica Microsystems Inc., Buffalo Grove, IL, USA). The locus coeruleus (LC) has been identified in several anuran amphibians including *Xenopus laevis* (González and Smeets, 1993), *Rana ridibunda* (González et al., 1994), *Rana perezi* (Sánchez-Camacho et al., 2003), *Bufo schneideri* (Noronha-de-Souza et al., 2006), *Hyla versicolor* (Endepols et al., 2004), and *Lithobates catesbeianus* (Fournier and Kinkead, 2008) using tyrosine hydroxylase immunoreactive staining. Brainstem slices containing the locus coeruleus were given one hour to recover at room temperature in control aCSF equilibrated with 80% O₂, 1.3% CO₂, balance N₂ (pH ~7.9). Following recovery from slicing, a transverse slice containing the LC was transferred to the polyethylene recording chamber with a glass coverslip base, immobilized with a nylon grid, and superfused with aCSF at a rate ~2.5 ml/minute.

Temperature Control
Temperature of the chamber was controlled with a Warner Instruments bipolar temperature controller (Model CL-100, Hamden, CT, U.S.A.). The measured temperature of the bath varied ±1°C depending on location of the thermocouple. To ensure precise temperature measurements at the exact location of the slice, the thermocouple was placed where the slice would be positioned during experiments and the experimental temperatures were determined under identical conditions prior to experiments.

Solutions

aCSF was composed (in mM) of 104 NaCl, 4 KCl, 1.4 MgCl$_2$, 7.5 glucose, 40 NaHCO$_3$, 2.5 CaCl$_2$, and 1 NaH$_2$PO$_4$ (Taylor et al., 2003b). Gases were mixed using an infrared gas mixer (MFC-4 Mass Flow Controller, gas mixer, Sable Systems International, Las Vegas, NV, USA) and continuously bubbled in solution. Control aCSF at 10°C, 15°C, 20°C, 26°C, and 30°C was equilibrated with 80% O$_2$, 1.3% CO$_2$, balance N$_2$ (pH 7.83, 7.85, 7.87, 7.91, 7.93, respectively). The solubility of gases is dependent on temperature. To maintain consistency and enable comparability of chemosensitivity in LC neurons at different temperatures, we kept the control gases constant at each experimental temperature and allowed pH to vary slightly. 1.3% CO$_2$ was chosen as the control because this is the percentage of CO$_2$ that bullfrogs typically maintain during rest at 20°C (Gottlieb and Jackson, 1976; Howell et al., 1970; Reeves, 1972). Hypercapnic aCSF was identical to control aCSF except the CO$_2$ was elevated to 3% (ΔpH from control aCSF= 0.29), 5% (ΔpH=0.49), or 10% (ΔpH=0.82) depending on the experiment. The pH changes during HA were
similar at all temperatures. In order to reduce neurotransmission due to chemical synapses and gap junctions, synaptic blockade media (SNB) was produced by lowering the CaCl\(_2\) and raising the MgCl\(_2\) of aCSF to 0.2 mM and 11.4 mM, respectively (Dean and Boulant, 1989) and adding 100 µM carbenoxolone (CAR; Sigma–Aldrich Co., St. Louis, MO, USA) to block gap junctions (Davidson and Baumgarten, 1988). NaCl was adjusted to maintain the osmolarity of SNB+CAR at ~317mOsm.

**Electrophysiological Recordings**

Whole-cell patch clamp recordings were obtained as previously described (Santin and Hartzler, 2013a). Briefly, micropipettes with resistances of 4–7 MΩ were back-filled with mock-intracellular solution (composition (in mM): 110 K-gluconate, 2 MgCl\(_2\), 10 HEPES, 1 Na\(_2\)-ATP, 0.1 Na\(_2\)-GTP, 2.5 EGTA; pH 7.2) (Martini et al., 2009) and placed over a AgCl\(_2\)-coated Ag wire connected to Axon instruments CV 203BU headstage (Molecular Devices, Sunnyvale, CA, USA). The slice was visualized at 4X magnification with a Nikon FN1 fixed stage microscope (Nikon, Elgin, IL, USA) using NIS Elements Imaging Software (Nikon, Elgin, IL, USA). The LC was identified on the slice by its bilateral location adjacent to the fourth ventricle (for anatomy see references presented in Brainstem Slice Preparation). Individual neurons of interest were visualized at 60X magnification prior to recording. A 10 mL syringe was connected to the headstage which was used to apply positive pressure through the pipette tip in order to keep the tip free of debris. A micromanipulator (Burleigh PCS 5000, Thorlabs, Newton, NJ, USA) was used to position the pipette adjacent to the
neuron of interest and the pipette offset was zeroed prior to entering the on-cell configuration to ensure accurate $V_m$ measurements. Positive pressure was removed and suction was applied until a $\geq 1$ GΩ seal existed between the pipette tip and cell membrane in the on-cell configuration. Further negative pressure was applied to rupture the seal and obtain whole-cell electrochemical access to the neuron. In several experiments, neurons located on a slice ~500 μm rostral to the LC slice were recorded for negative controls. Changes in $V_m$ were recorded in ‘current-clamp mode’ (Axopatch 200 B integrating patch clamp amplifier, Molecular Devices, Sunnyvale, CA, USA) and collected using Molecular Devices P10 Clampex software (Molecular Devicies, Sunnyvale, CA, USA). Current-clamp recordings were analyzed off-line using pCLAMP software (Molecular Devices, Sunnyvale, CA, USA).

**Experimental Procedures**

**Effect of Cold Temperatures on Firing Rate and Chemosensitivity of LC Neurons**

Upon entering whole-cell current-clamp mode, at least 5 minutes of baseline firing was recorded from 39 LC neurons ($n=34$ chemosensitive; $n=5$ non-chemosensitive) and 7 negative control neurons (~500 μm rostral to LC) in control aCSF (1.3% CO$_2$) at 20°C. Since ~85% of neurons within the bullfrog LC have been shown to increase firing rates and undergo membrane depolarization during exposure to 5% CO$_2$ (Santin and Hartzler, 2013a), this stimulus at 20°C was used as a positive control for chemosensitivity in experiments investigating
the influence of reduced temperatures on CO₂/pH sensitivity in LC neurons. Once control firing rates and V_m were established, the LC neuron was exposed to 5% CO₂ for 3-5 minutes. Only neurons that recovered firing rates and V_m after the return to control aCSF were used in subsequent experiments and included in this data set. Once firing rates of LC neurons returned to near-initial values, a slow cooling ramp to either 15°C (n=10) or 10°C (n=24) was applied. The chamber temperature was cooled to 15° in ~2 minutes and to 10°C in ~4 minutes. Baseline firing at the new temperature was established for ~2 minutes and then the neuron was exposed to 5% CO₂ for 3-5 minutes at the lower temperature. The time required for 5% CO₂ to induce increases in firing rates varied depending on individual neuronal sensitivity to CO₂/pH and depth of neuron in the slice. This interneuron variability necessitated that the exposure time of 5% CO₂ vary between 3-5 minutes; however, HA exposures on the same neuron during control and cool temperatures were identical in length. For example, if a neuron was exposed to 5% CO₂ for 4 minutes at 20°C the same neuron was also exposed to 5% CO₂ for 4 minutes at 10°C. In additional experiments, this procedure was also performed in the presence of SNB+CAR (n=6).

Further experiments were completed in LC neurons following the previously described protocol with the addition that after HA (5% CO₂) exposure was completed at 15°C, a subset of LC neurons (n=7) were then cooled to 10°C in order to make repeated-measures comparisons of CO₂/pH sensitivity at 20°C, 15°C, and 10°C. Following CO₂ exposures at 10°C three further experiments
were performed in a subset of chemosensitive LC neurons. First, nine LC neurons were returned to 20°C and then challenged with 5% CO₂ to ensure reversibility of the changes in chemosensitivity observed at 10°C. Second, since cooling evoked large increases in firing rates and membrane depolarization (see Fig. 1) at 10°C, four chemosensitive LC neurons were injected with -16±8 pA of current at 10°C to control the initial firing rate to resemble the firing rates measured at 20°C. After ~2 minutes of controlled baseline firing at 10°C, HA (5% CO₂) was administered, and then removed after 3-5 minutes. Lastly, six chemosensitive LC neurons were exposed to 10% CO₂ at 10°C following the washout of 5% CO₂ from the chamber.

*Effect of High Temperatures on Firing Rate and Chemosensitivity of LC Neurons*

The influence of warming on firing rate and chemosensitivity was investigated in 27 LC neurons (n= 21 chemosensitive; n=6 non-chemosensitive). The procedure for determining the effect of warming on firing rate and V_m of LC neurons was identical to the procedure for cooling except the chamber temperature was warmed to either 26°C (n=17) or 30°C (n=4). The bath temperature was warmed to 26°C in ~2.5 minutes and to 30°C in ~ 3.5 minutes. The protocol for establishing the influence of warming on LC activity and chemosensitivity was also repeated in the presence of SNB+CAR (n=5).

Similar to the cooling experiments, a subset of chemosensitive LC neurons (n= 5) were returned to 20°C following determination of chemosensitivity.
at 26°C to ensure reversibility of the changes in CO₂/pH sensitivity observed at 26°C. To assess changes in the sensitivity to CO₂/pH, rather than the magnitude of the response during 5% CO₂, another group of chemosensitive LC neurons (n=5) were subjected to both 3 and 5% CO₂ to elicit increases in firing rate at 20°C and 26°C.

*Measurement of Input Resistance (R_{in}) at Various Temperatures*

Measuring the resistance that the headstage encounters during a negative current injection (input resistance; R_{in}) can provide insight into net channel opening or closing caused by different treatments. For example, excitatory chemosensitive LC neurons from bullfrogs undergo small increases in R_{in} during exposure to 5% CO₂, which suggests that increased excitability may be due to a net closure of (possibly K⁺) channels (Santin and Hartzler, 2013a). In contrast, some LC neurons in mice have been shown to increase firing rates, but decrease R_{in}, during 8% CO₂ exposure. These results suggest that these LC neurons become more excitable during HA because of a net opening of non-selective cation channels (Cui et al., 2011). To measure R_{in} at different temperatures, two -25pA and two -50 pA current pulses (800ms/current pulse) were injected into LC neurons at 10°C, 15°C, 20°C, and 26°C. These negative current injections elicited hyperpolarization of the membrane. In most chemosensitive LC neurons, negative current injection resulted in depolarizing “sag” which indicates the presence of the hyperpolarization-activated current (I_{h}) (Pape, 1996). Average ΔV_m (V_m during plateau of the hyperpolarization following the depolarizing “sag”
− control Vm) from the four current injections was used in Ohm’s law (ΔVm = IR) to calculate R_{in}.

**Data Analysis**

All values are reported as means ± SEM. The mean firing rate and Vm during control, 5% CO2, and the return to control (washout) are reported at 10°C, 15°C, 20°C, and 26°C. Mean firing rate and Vm were calculated as described previously (Santin and Hartzler, 2013a). Washout of the response to HA typically took < 5 minutes. Percent change in firing rate \(((FR_{HA} - FR_{Initial})/FR_{Initial} \times 100)\) was used to quantify relative change in firing rate. The effect of temperature on firing rate during normocapnia was assessed by comparing the average integrated firing rate one minute before cooling or warming with the first minute of average integrated firing once the new temperature was achieved. The effect of SNB and CAR on firing rate and Vm have previously been described (Santin and Hartzler, 2013a) and therefore were not reported in this study. Action potential properties were analyzed according to the diagram in the inset of Fig. 3. The height of the action potential was measured from the threshold to the maximum height. The rate of depolarization was calculated as the height of the action potential divided by the time from the threshold to the peak. The half-width was analyzed by measuring the time halfway between threshold and peak during depolarization until halfway between threshold and peak during repolarization. The rate of repolarization was calculated as the height of the action potential divided by the time from peak to threshold. The after hyperpolarization (AHP) was measured as the threshold subtracted from the lowest point following the action potential.
Statistical Analysis

Unless otherwise specified, comparisons among three means were analyzed using a two-tailed repeated measures analysis of variance (RM-ANOVA) with Tukey’s post hoc test for multiple comparisons, while comparisons between two means were made using a two-tailed paired $t$ test. Frequencies of presence or absence of depolarizing “sag” during -25 and -50pA current injections at different temperatures were analyzed using Fisher’s Exact test. A sigmoidal dose response curve was fitted to a graph of temperature vs. average percent change in firing rate to quantify temperature at 50% maximal chemosensitivity. Significance was accepted in all statistical analyses when $P<0.05$ (indicated by *). All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego CA, U.S.A).
RESULTS

Influence of Temperature on Chemosensitive and Non-chemosensitive LC Neurons in aCSF

The majority of LC neurons in bullfrogs 54/65 (83%) exhibited increases in firing rate and membrane depolarization during exposure to HA in aCSF at 20°C. This is consistent with previously reported percentages of chemosensitivity (47). Fig. 1A illustrates the firing response of a chemosensitive LC neuron during cooling of aCSF under normocapnic conditions. Surprisingly, firing rates of all chemosensitive LC neurons increased during cooling. At 20°C chemosensitive LC neurons (n=24) had control firing rates of 0.60±0.13 Hz and after a ~4 minute cooling ramp to 10°C firing rates increased to 2.11±0.18Hz (Q\textsubscript{10}=0.28; \textit{P}<0.05; Fig. 1B). Additionally, chemosensitive LC neurons (n=24) underwent membrane depolarization during cooling (-43.54±1.35 mV at 20°C vs. -33.83±1.88 mV at 10°C; \textit{P}<0.05). In non-chemosensitive LC neurons, however, cooling from 20°C to 10° (n=3) did not affect firing rates (20°C: 1.81±0.58 Hz vs.10°C: 1.53±0.89 Hz \textit{P}>0.05; Q\textsubscript{10}=1.18; Fig. 1C). The membrane potentials recorded at 20°C and 10°C did not differ in non-chemosensitive LC neurons (-49.13 mV vs. -47.38 mV; \textit{P}>0.05). Furthermore, non-chemosensitive neurons in a brainstem slice 500μm rostral to the slice containing the LC (n=7) had slowed firing rates during cooling from 20°C to 10°C (20°C=0.87±0.18 Hz vs. 10°C= 0.19±0.06; Q\textsubscript{10}=14.5 \textit{P}<0.05) and underwent membrane hyperpolarization (20°C= -47.86±1.32 mV vs. 10°C= -56.03±2.70 mV; \textit{P}<0.05)
Figure 1.

A

Time (min)

Temp. (°C)

20
10
0

Firing Rate (Hz)

2
3
1

20 mV

B

Firing Rate (Hz)

Chemosensitive LC

20°C 10°C

C

Firing Rate (Hz)

Non-Chemosensitive LC

20°C 10°C

D

0 1 2 3 4 5 6

E

0 1 2 3 4 5

F

0 1 2 3

Temp. (°C)

Time (min)

0 1 2 3

20 mV
**Figure 1** Cooling increases and warming decreases firing rates of chemosensitive LC neurons under normocapnic (1.3% CO$_2$) conditions, but does not increase firing rates of non-chemosensitive LC neurons. (A) shows the integrated firing rate (top) and firing trace (below) from a chemosensitive LC neuron during a slow cooling ramp to 10°C. (B) shows matched firing rates of individual chemosensitive neurons at 20°C and 10°C. The matched point bolded in red represents the neuron used as the example in (A). All chemosensitive LC neurons increased firing rate and underwent membrane depolarization during cooling (n=24; paired t test; $P<0.05$), but non-chemosensitive LC neurons (C) did not demonstrate a consistent firing trend during cooling (n=3; paired t test; $P>0.05$). (D) shows the integrated firing rate (top) and representative trace (below) of a chemosensitive LC neuron during a warming ramp to 26°C. The sample size shown in D reflects the rarity of non-chemosensitive neuron. This makes it difficult to make statistically meaningful comparisons. (E) shows matched firing rates of individual chemosensitive LC neurons during warming. The matched point bolded in red represents the neuron used as the example in (D). All chemosensitive LC neurons decreased firing rates and $V_m$ during warming (n=17; paired t test; $P<0.05$); however, non-chemosensitive LC neurons (F) increased firing rates in response to warming (n=6; paired t test; $P<0.05$). The larger sample of non-chemosensitive neurons during warming enhances our confidence that NCS neurons have temperature proportional firing frequencies.
Fig. 1D illustrates the firing response of a chemosensitive LC neuron during warming over a ~2.5 minute period. At 20°C, chemosensitive LC neurons (n=17) fired at a rate of 0.73±0.15 Hz, but decreased firing rates (0.30±0.09 Hz) during warming to 26 °C (Q_{10}=0.23; P<0.05; Fig. 1E). Warming from 20°C to 26°C also induced hyperpolarization of the membrane of LC neurons (20°C: -37.39±1.71 mV vs. 26°C: -40.01±1.70 mV, respectively (P<0.05). Additionally, in all cases (n=4) warming chemosensitive LC neurons to 30°C resulted in a cessation of firing that could be reversed by injection of < 10pA positive current. In contrast to chemosensitive LC neurons, non-chemosensitive LC neurons (n=6) increased firing rates during warming (20°C: 0.73±0.25 Hz vs. 26°C 1.50±0.22 Hz; Q_{10}=3.1; P<0.05: Fig. 1F) and underwent membrane depolarization (20°C: -41.21±1.71 mV vs. 26°C: -36.97±1.91 mV ; paired t test; P<0.05).

In order to assess whether these firing responses of chemosensitive LC neurons were due to modulation of temperature-sensitive ionic conductances intrinsic to the neuron or temperature sensitive-synaptic transmission, cooling and warming experiments were performed in the presence of low Ca^{2+}/ high Mg^{2+} and the gap junction blocker, carbenoxolone. Similar to the firing responses in aCSF, cooling from 20°C to 10°C (n=6) in the presence of SNB+CAR resulted in increases in firing rates (0.89±0.17 Hz vs. 3.11±0.45 Hz; P<0.05). During warming from 20°C to 26°C (n=5) firing rates decreased from 1.60±0.26Hz to 0.81±0.36 Hz, respectively (paired t test; P<0.05). Fig. 2 summarizes the influence of temperature on the firing rates of chemosensitive LC neurons in aCSF, SNB+CAR, and non-chemosensitive LC neurons in aCSF.
Figure 2

A

Time (min)

Temp. (°C)

Firing Rate (Hz)

20mV

B

Temp. (°C)

C

Firing Rate (Hz)

Temperature (°C)

Chemosensitive LC Neurons (aCSF)

Chemosensitive LC Neurons (SNB+CAR)

Non-chemosensitive LC Neurons

D

Resistance (MΩ)

Temperature (°C)
Figure 2 Summary of the effects of temperature on firing rates of chemosensitive LC neurons in aCSF (black circles) and SNB+CAR (red circles) and non-chemosensitive LC neurons in aCSF (grey circles). There is an inverse relationship between firing rate and temperature in chemosensitive LC neurons with synapses intact and blocked which provides evidence that temperature sensitivity is an intrinsic property of chemosensitive LC neurons. In non-chemosensitive LC neurons, temperature does not have an inverse relationship with firing rate.
Figure 3

A

Resistence (MΩ)

0 1000 2000 3000 4000 5000

0 10 20 30

Temperature (°C)

B

20 °C 10 °C

20 mv

20 ms

5 mv

100 ms

C

20 mx

10 ms

100 ms
Figure 3 Influence of cooling to 10°C on input resistance ($R_{in}$) and action potential properties of chemosensitive-LC neurons. (A) $R_{in}$ of chemosensitive LC neurons decreases as temperature increases ($R^2=0.94; \ P<0.05; \ 10°C: \ n=12$, $15°C: \ n=11$, $20°C: \ n=25$, $26°C: \ n=10$). Cooling increased the half-width of action potentials and decreased the rate of repolarization. (B) illustrates an action potential at 20°C (black) overlaid with an action potential at 10°C (grey). (C) emphasizes the reduction in after-hyperpolarization (AHP) by showing a truncated action potential at 20°C (black) overlaid with a truncated action potential at 10°C (grey). The inset illustrates the action potential properties analyzed: 1.) rate of depolarization 2.) action potential height 3.) action potential half-width 4.) rate of repolarization 5.) AHP.
### Table 1.

*Influence of cooling on action potential properties*

<table>
<thead>
<tr>
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<th>20°C (n=9)</th>
<th>10°C (n=9)</th>
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</thead>
<tbody>
<tr>
<td>Rate of Depolarization (mV/ms)</td>
<td>35.33 ± 7.09</td>
<td><em>12.29 ± 3.10</em></td>
</tr>
<tr>
<td>Height (mV)</td>
<td>60.22 ± 6.36</td>
<td>54.61 ± 5.54</td>
</tr>
<tr>
<td>Half-Width of Action Potential (ms)</td>
<td>2.03 ± 0.38</td>
<td><em>6.84 ± 2.06</em></td>
</tr>
<tr>
<td>Rate of Repolarization (mV/ms)</td>
<td>-28.54 ± 8.50</td>
<td><em>-8.70 ± 2.54</em></td>
</tr>
<tr>
<td>After Hyperpolarization (mV)</td>
<td>-15.25 ± 2.06</td>
<td><em>-8.90 ± 1.05</em></td>
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Input resistance ($R_{in}$) was measured in several chemosensitive LC neurons in aCSF at 10°C (1274±153.5 MΩ; n=12), 15°C (833.6±54.78 MΩ; n=11), 20°C (668.5±34.75 MΩ; n=25), and 26°C (427.8±27.5 MΩ; n=10). There was an inverse linear relationship between $R_{in}$ and temperature in chemosensitive LC neurons ($R^2=0.94; P<0.05$; Fig. 3A). In the presence of SNB+CAR cooling increased $R_{in}$ (601.4±42.2 vs. 801.4±80.2; paired t test; $P<0.05$). The relative increase in $R_{in}$ during cooling (expressed as percent increase compared to baseline $R_{in}$) was greater in aCSF than in SNB+CAR (91.86±16.6% vs. 34.8±11.6%; two-tailed t test; $P<0.05$). There was a decreasing trend in $R_{in}$ during warming (20°C: 507.6±59.3 vs. 26°C: 471.8±73.6; $P>0.05$). These results suggest that cooling intrinsically increases input resistance in chemosensitive LC neurons, but inhibition of synaptic transmission or gap junctions due to cooling may account for a portion of the increased $R_{in}$ observed in control aCSF (Weight and Erulkar, 1976).

Since temperature-sensitive firing and $R_{in}$ responses of chemosensitive LC neurons have an apparent intrinsic component, we measured changes in action potential properties at 20°C and 10°C (n=9) in order to gain insight into the possible temperature-sensitive ionic conductance mediating the cold-induced increases in firing rates of chemosensitive LC neurons. These results are summarized in Table 1. Fig. 3B illustrates the cooling-induced increase in action potential half-width in addition to the slower repolarization rate, while Fig. 3C demonstrates the decrease in after hyperpolarization during cooling.
During the measurement of $R_{in}$ we observed depolarizing “sag” upon negative current injection in the majority of chemosensitive LC neurons. The presence of depolarizing “sag” in response to hyperpolarizing current in whole-cell current clamp configuration is the signature of expression of hyperpolarization-activated cyclic nucleotide gated (HCN) channels which mediate the hyperpolarization-activated current ($I_h$) (Biel et al., 2009). The presence or absence of depolarizing “sag” in chemosensitive LC neurons depended on the magnitude of the current injection as well as temperature. Few chemosensitive LC neurons exhibited “sag” during negative current injection at 10°C (Fig. 4A); however, the majority of neurons demonstrated the presence of sag at 15°C (Fig. 4B), 20°C (Fig. 4C), and 26°C (Fig. 4D). The proportion of neurons exhibiting “sag” during -25 and -50pA at each temperature did not occur by chance (two-tailed Fisher Exact test; P<0.05), suggesting that increasingly negative current injections were able to activate $I_h$. In addition, the proportion of neurons exhibiting “sag” differed at 20°C and 10°C with -50pA injections (100% vs. 33%; two-tailed Fisher Exact test; P<0.05), suggesting that cooling may alter the conductance of $I_h$ or shift the voltage dependence of activation to more negative potentials. These results are summarized in Fig. 4A-D.

**Influence of Cooling on Chemosensitivity in LC Neurons**

In this set of experiments 34/39 (87%) LC neurons exhibited increased firing rate when exposed to 5% CO$_2$ at 20°C. Chemosensitivity was then measured either at 15°C (n=10) or 10°C (n=24) in these chemosensitive neurons. At 20°C and 15°C, exposure to 5% CO$_2$ resulted in reversible firing
Figure 4.
Figure 4 Cooling alters the percentage of chemosensitive LC neurons exhibiting depolarizing “sag” (characteristic of hyperpolarization-activated current; $I_h$) upon -25 pA and -50 pA current injection. The presence of depolarizing “sag” is indicated by an arrow under each example. $a'$ and $a''$ (identical labeling for all temperatures) are representative examples demonstrating the most frequent occurrence of depolarizing “sag” at each temperature during -25 pA ($'$) and -50 pA ($''$) at 10°C. To illustrate the influence of temperature on the presence of depolarizing “sag,” A-C were obtained from the same neuron; however, the raw trace used in D was acquired during a separate experiment. (A) shows that most neurons do not exhibit depolarizing “sag” with -25 pA or -50 pA current injection at 10°C. (B), however, shows that sag becomes prevalent at 15°C with -50 pA current injection (90% of neurons express “sag”). (C) and (D) illustrate that >90% of neurons demonstrate depolarizing “sag” during -50 pA current injection, while 40% of neurons exhibit “sag” during -25 pA injection. These data show that increasingly negative current injection increases the likelihood of observing depolarizing “sag” (Fisher Exact test; $P<0.05$ for all temperatures). Additionally, the probability of observing “sag” during -50 pA current injection were greater at 20°C (100% of neurons exhibited “sag”) compared to 10°C (33% of neurons exhibited “sag”; Fisher Exact test; $P<0.05$).
rate increases (RM-ANOVA; \( P<0.05 \); Fig. 5 A and B); however, 5% \( \text{CO}_2 \) did not elicit any change in firing rate at 10°C (RM-ANOVA: \( P>0.05 \); Fig. 5C). Transitioning LC neurons from control to 5% \( \text{CO}_2 \) led to depolarization of the membrane at 20°C (-45.50±0.99 mV vs. -41.48±0.89 mV) and 15°C (-42.57±2.18 mV vs. -39.27±1.9mV) \( (P<0.05 \) for both temperatures); however at 10°C, transitioning from control to 5% \( \text{CO}_2 \) did not result in depolarization (-33.92±2.59 vs. -34.65±1.50mV; \( P>0.05 \)). In all neurons tested (n=10), percent increase in firing rates during exposure to 5% \( \text{CO}_2 \) was greater at 20°C compared to 15°C (20°C: 168.1±20.58% vs. 15°C: 58.11%±7.94%; \( P<0.05 \)), while cooling to 10°C (n=24) eliminated the relative increase in firing rate during HA (20°C: 253.0±38.95 vs. 10°C: 6.07%±2.68%; paired t test; \( P<0.05 \)).

In order to make a repeated measures comparison of chemosensitive firing responses at all three temperatures, 7 LC neurons were exposed to 5% \( \text{CO}_2 \) at 20°C, 15°C, 10°C. In each neuron, firing rate increased during 5% \( \text{CO}_2 \) exposure at 20°C, exhibited a smaller increase in firing rate at 15°C, and showed no change in firing rate at 10°C (Fig. 5 D). Additionally, to assess whether the elimination of \( \text{CO}_2 \) sensitivity at 10°C was intrinsic vs. synaptically-driven, 5% \( \text{CO}_2 \) exposures were performed in the presence of chemical and electrical synaptic blockade (SNB+CAR) at 20°C and 10°C. The elimination of \( \text{CO}_2/pH \) sensitivity at10°C persisted in the presence of chemical and electrical synaptic blockade \( (P<0.05; \) Fig. 5E), suggesting that cooling reduces sensitivity to \( \text{CO}_2/pH \) through a mechanism intrinsic to LC neurons.
Figure 5

A  $20^\circ C$

B  $15^\circ C$

C  $10^\circ C$

D

E  SNB + CAR

$\Delta$ Firing Rate (% of Control)

Control  5% CO$_2$  Washout

0.0  0.5  1.0  1.5  2.0  2.5

0.0  0.5  1.0  1.5  2.0  2.5

0.0  0.5  1.0  1.5  2.0  2.5

0.0  0.5  1.0  1.5  2.0  2.5

0.0  0.5  1.0  1.5  2.0  2.5

$\Delta$ Firing Rate (% of Control)

20$^\circ C$  15$^\circ C$  10$^\circ C$

-50  0  50  100  150  200

-50  0  50  100  150  200

-50  0  50  100  150  200

-50  0  50  100  150  200
**Figure 5** Cooling reduces firing rate increases during HA (5% CO₂). A-C control aCSF (1.3% CO₂; white bars), HA (5% CO₂; black bars), washout (1.3% CO₂ patterned bars). (A) illustrates the firing rate increase during exposure to HA in LC neurons with recovery upon washout at 20°C (n=34; RM-ANOVA; P<0.05). At 15°C, neurons increased firing rates during HA and fully recovered upon washout with control aCSF (n=10; RM-ANOVA; P<0.05); however, neurons at 10°C did not increase firing rates during HA (n=24; RM-ANOVA; P<0.05). (D) shows that cooling reduced chemosensitive responses in a stepwise manner (n=7; percent change in firing rate during HA at 20°C >15°C>10°C; RM-ANOVA; P<0.05). To assess whether or not cold-induced depression of chemosensitivity was intrinsic vs. the result of altering synaptic input, LC neurons were exposed to HA at 20°C and then 10°C in the presence of chemical and electrical synaptic blockade (SNB+CAR). (E) shows percent change in firing rate of chemosensitive LC neurons during HA at 20°C and 10°C in the presence of SNB+CAR. Like the responses observed in aCSF, in SNB+CAR, LC neurons retained chemosensitive responses at 20°C and lost chemosensitive responses at 10°C (paired t test; P<0.05).
Fig. 1B shows that cooling resulted in increases in firing rate of LC neurons. In order to ensure that the elimination of CO$_2$/pH sensitivity was a result of cooling to 10°C and not the consequence of elevated initial firing rates, a subset of LC neurons (n=4) were injected with -16±8 pA in order to slow firing rate to control values at 20°C following completion of an exposure to 5% CO$_2$ at 10°C (Fig. 6). As demonstrated in Fig. 6, exposure to 5% CO$_2$ at 20°C caused significant, reversible increases in firing rate (RM-ANOVA; $P<0.05$), which were not present at 10°C (RM-ANOVA; $P>0.05$). Slowing the firing rate at 10°C to the control firing rate observed at 20°C did not result in increases in firing rate during exposure to 5% CO$_2$. This demonstrates that cooling *per se* eliminates chemosensitivity in LC neurons and the removal of LC chemosensitivity during cooling is not dependent on baseline firing rate.

To ensure that elimination of chemosensitivity in LC neurons upon cooling to 10°C is reversible, 10 neurons were returned to 20°C and exposed to 5% CO$_2$ following testing of chemosensitivity at 10°C (Fig. 7A). Exposure of LC neurons to 5% CO$_2$ resulted in a 259.7±74.7% increase in firing rate at 20°C, but not at 10°C (0.54±5.5% increase). Upon the return to 20°C, increases in firing rates during 5% CO$_2$ exposure were restored to near-initial values (212.0±63.66% greater than baseline firing) (Fig. 7B).

Santin and Hartzler (Santin and Hartzler, 2013a) found that 10% CO$_2$ results in a 3x greater increase in firing rate compared to 5% CO$_2$ in chemosensitive LC neurons. We reasoned that cooling may cause a “right-shift” in the sensitivity to CO$_2$/pH, requiring a larger ΔCO$_2$/pH to observe an increase in
Figure 6

Firing Rate (Hz)

- 1.3% CO₂
- 5% CO₂
- Washout

Conditions:
- 20°C
- 10°C
- 10°C FR Controlled

Statistical Analysis:
- * indicates significant difference.
Figure 6 To ensure that elimination of CO\textsubscript{2}/pH induced firing responses at 10°C was the result of cooling and not a greater initial firing rate, four chemosensitive LC neurons at 20°C were cooled to 10°C and then exposed to HA. At 20°C, each neuron increased its firing rate during HA (5% CO\textsubscript{2}) and showed no increase firing rates during exposure to 5% CO\textsubscript{2} (expressed as percent change in baseline during HA). Upon washout of 5% CO\textsubscript{2} at 10°C, -16±8 pA of current was injected to slow the initial firing rate (10°C FR Controlled). LC neurons did not increase firing rates during 5% CO\textsubscript{2} exposure with firing rate controlled.
Figure 7

A

B

C

D
Figure 7 (A) shows integrated firing rate for a neuron exposed to 5% CO$_2$ at 20°C, 10°C, and after the return to 20°C. Switching from normocapnic to hypercapnic aCSF (5% CO$_2$) caused increased firing at 20°C. After cooling to 10°C, firing rate was controlled by injecting negative current (indicated by downward arrow) to maintain initial firing rate near control firing used at 20°C. This neuron does not increase firing rate at 10°C during HA. Follow washout of HA at 10°C, the bath temperature was returned to 20°C. Positive current (indicated by upward arrow) was injected to stabilize control firing rate. This neuron had a larger absolute increase in firing rate during HA upon return to 20°C. This example demonstrates that chemosensitivity recovers when the neuron is returned to 20°C. (B) shows percent change in firing rate of 10 chemosensitive LC neurons during HA at 20°C, 10°C, and return to 20°C. All neurons exhibiting chemosensitive responses at 20°C lost chemosensitivity at 10°C and fully recovered chemosensitive responses when returned to 20°C (RM-ANOVA; $P<0.05$). (C) illustrates integrated firing rate of a chemosensitive LC neurons during exposure to 5% and 10% CO$_2$ at 10°C. First, the neuron was exposed to 5% CO$_2$ at 20°C to determine baseline chemosensitivity. The neuron was then cooled to 10°C (downward arrow indicates negative current used to control initial firing rate) and exposed to 5% and 10% CO$_2$. This neuron did not increase firing rate during 5% CO$_2$ ($\Delta$pH=0.49) or 10% CO$_2$ ($\Delta$pH=0.82) exposure. (D) shows that all five neurons exhibiting chemosensitive responses
at 20°C did not increase firing rates during exposure to either 5% or 10% CO₂ (RM-ANOVA; P<0.05).
firing rate at 10°C. Therefore, LC neurons (n=6) were exposed to 10% CO₂ at 10°C following washout of 5% CO₂ (Fig. 7C). In these neurons, 5% CO₂ at 20°C caused a 161.7±43.45% increase in firing rate compared to baseline, while 5% and 10% CO₂ at 10°C resulted in a 7.25±2.55% and 4.81±9.99 increase in firing rate, respectively (Fig. 7D). Even with an extraordinarily high partial pressure of CO₂ (76 mmHg) and acidification (ΔpH=0.82), LC neurons do not alter firing rates when exposed to HA at 10°C.

**Influence of Warming on Chemosensitivity in LC Neurons**

In this set of experiments, 17/23 (74%) LC neurons were found to be chemosensitive at 20°C. Since warming caused firing rates to fall below 0.2Hz (shown in Fig. 1), neurons were injected with depolarizing current (~5pA) until firing reached ~0.5Hz to ensure similarity between control firing rates at 20°C and 26°C. Exposure to HA (5% CO₂) at 26°C induced larger changes in firing rate compared to 20°C in all neurons (20°C: 168.5±17.52% increase vs. 26°C: 362±43.53% increase; P<0.05) (Fig. 8A). In addition to chemosensitive LC neurons having a >2-fold increase in the relative firing rate change during HA, 10/17 chemosensitive neurons increased absolute firing rate during HA (paired t test; P<0.05; Fig. 8A). The membrane depolarized during HA at 20°C (control: -41.52±1.05 vs. 5% CO₂: -36.70±1.01) and 26°C (control: -38.90±1.68 mV vs. CO₂: -35.37±1.61 mV). In order to determine whether increases in relative firing rate due to HA were reversible following exposure of the neuron to 26°C, LC neurons (n=6) were returned to 20°C (Fig. 8C). 5/6 neurons increased the magnitude of their firing responses during 5% CO₂ at 26°C and returned to near-
Figure 8

A

B

20 °C

26 °C

5% CO₂

5% CO₂

2 Minutes

Firing Rate (Hz)

Firing Rate (% of Control)

1.5% CO₂

5% CO₂

Washout

20°C

26°C

10/17

* 

20°C

26°C

20°C

26°C

SNB+CAR

D
Figure 8 Warming enhanced CO₂/pH sensitive responses in LC neurons. (A) Integrated firing of a chemosensitive LC neuron at 20°C and 26°C. Changing aCSF from 1.3% to 5% CO₂ caused increase firing rate at 20°C. Warming slowed firing of chemosensitive LC neurons; therefore, depolarizing current was injected to counteract reduction in firing (indicated by upward arrow). This particular neuron had a larger change in firing rate during exposure to 5% CO₂ at 26°C compared to 20°C. (B) mean absolute firing change during HA at 20°C (black bars) and 26°C (white bars) in the 10/17 neurons with elevated firing rates during HA. Warming to 26°C increased firing responses during HA in chemosensitive LC neurons (paired t test; P<0.05). (C) 5/6 chemosensitive LC neurons experienced increases the magnitude of the firing response to HA (expressed as percent change in firing rate during HA) and returned to near-initial chemosensitive responses when returned to 20°C (RM-ANOVA; P<0.05). To assess whether or not warm-induced increases in chemosensitivity was intrinsic vs. the result of altering synaptic input, LC neurons were exposed to HA at 20°C and then 26°C in the presence of chemical and electrical synaptic blockade (SNB+CAR). (D) shows percent change in firing rate of chemosensitive LC neurons during HA at 20°C and 26°C in the presence of SNB+CAR. Unlike the responses observed in aCSF, in SNB+CAR, these LC neurons did not exhibit increases in chemosensitive responses at 26°C (paired t test; P>0.05).
control chemosensitivity upon the return to 20°C ($P<0.05$). Unlike the increases in chemosensitivity observed with chemical and electrical networks intact, exposure of LC neurons to 5% CO$_2$ in the presence of chemical and electrical synaptic blockade did not result in increased chemoresponsiveness (20°C: 97.61±21.39% vs. 26°C: 105.6±10.20; $P>0.05$; Fig. 8D). These results demonstrate the possibility that increases in chemosensitivity of LC neurons at 26°C may be mediated by synaptic/gap junction -driven mechanisms.

In order to assess whether increasing temperature caused elevated CO$_2$/pH sensitivity vs. an increase in the magnitude of the response to HA, 5 LC neurons were exposed to 3 and 5% CO$_2$ (Fig. 9A). Both CO$_2$ percentage and temperature increased the relative change in firing rate. (Two-Way ANOVA; $P<0.05$) (Fig. 9B). The slopes of the firing response during exposure to 3 and 5 CO$_2$ are nearly parallel (ANCOVA; $P>0.05$ 20°C: 74±14% increase in firing rate per 1% CO$_2$ increase vs. 26°C: 96±35% increase in firing per 1% CO$_2$ increase) (Fig. 9B), suggesting temperature influences the magnitude of the firing response at a given temperature and not CO$_2$/pH sensitivity.

Fig. 10 shows pooled data for relative increases in firing rate during 5% CO$_2$ exposure at 10°C, 15°C, 20°C, 26°C, and 30°C. The firing response to CO$_2$ increases sigmoidally with temperature (Hill coefficient= 7.59). Interestingly, the temperature at half maximal firing during elevated CO$_2$ occurs at ~20°C, which is near the mean preferred body temperature of many anuran amphibians (Hutchison and Dupre, 1992).
Figure 9

A  

20 °C

26 °C

Firing Rate (Hz)

3% CO₂  5% CO₂

3% CO₂  5% CO₂

5 Minutes

B

Δ Firing Rate (% of Control)

26 °C

20 °C

CO₂ (%)
Figure 9 Warming from 20°C to 26°C increased the magnitude of the firing response.  (A) Integrated firing rate of a chemosensitive LC neuron exposed to 3% and 5% CO$_2$ at 20°C, followed by exposure to 3% and 5% CO$_2$ at 26°C (upward arrow indicates depolarizing current injection to maintain firing rate). The horizontal dashed grey line matches the firing response to 3% CO$_2$ at 20°C and 26°C and the horizontal solid grey line matches the firing response to 5% CO$_2$ at 20°C and 26°C to illustrate that warming increased the magnitude of the firing response to HA.  (B) mean percent change in firing rate during HA of five chemosensitive LC neurons exposed to 3% and 5% CO$_2$ at 20°C, followed by exposure to 3% and 5% CO$_2$ at 26°C. Increasing temperature and CO$_2$ increased in the percent change in firing rate (two-way ANOVA; $P<0.05$). The slopes of the lines ($\Delta$firing rate/ $\Delta$CO$_2$) at 20°C and 26°C were not different (ANCOVA; $P>0.05$), demonstrating that temperatures increased the magnitude of the firing response, rather than the sensitivity, to changes in CO$_2$/pH.
Figure 10

![Graph showing temperature (°C) vs. firing rate (% of control) with data points and error bars.]

Delta Firing Rate (% of Control) vs. Temperature (°C)
Figure 10 Summary of the percent change in firing rate during 5% CO$_2$ exposure at different experimental temperatures. Data points for 10°C, 15°C, and 20°C are taken from Fig. 5; therefore, these relative changes in firing rate were calculated with increasing control firing rate. At 26°C and 30°C initial firing rate was adjusted to ~0.5Hz to counteract decreases in firing rate due to increased temperature. Chemosensitive LC neurons did not increase firing rates during exposure to 5% CO$_2$ at 10°C, and increased CO$_2$ responsiveness sigmoidally as temperature increased until reaching a plateau at ~26°C ($R^2$= 0.52). The temperature at half maximal CO$_2$ responsiveness occurred at 19.5°C.
DISCUSSION

This study generated several interesting findings. Strikingly, cooling increased, while warming decreased, the firing rates of chemosensitive LC neurons under normocapnic conditions. These unexpected responses to changes in temperature were not observed in non-CO$_2$/pH sensitive LC neurons and neurons located ~500μm rostral to the LC. As anticipated, cooling reduced CO$_2$/pH sensitivity of LC neurons in a temperature dependent manner. Surprisingly, chemosensitive responses were completely eliminated by cooling to 10°C even in response to relatively large hypercapnic acidotic stimuli (10% CO$_2$; ΔpH from control = 0.82). Warming also increased chemosensitivity in LC neurons. Over half of the chemosensitive LC neurons tested had larger increases in absolute firing rates during exposure to 5% CO$_2$ at 26°C compared to 20°C, while most neurons had greater relative increases in firing rate at 26°C compared to 20°C in response to hypercapnia.

Modulation of Cellular Chemosensitivity in Anuran Respiratory Control

CO$_2$ is a powerful stimulator of breathing in terrestrial air-breathing tetrapods (Milsom, 2010). The magnitude of the increase in breathing during hypercarbia is proportional to body temperature in anuran amphibians (Bicego-Nahas and Branco, 1999; Branco et al., 1993). The relationship between temperature and ventilatory sensitivity to hypercarbia is, at least partly, due to modulation of central chemosensitivity because cooling the brainstem reduces respiratory-related nerve activity during acidification (Morales and Hedrick, 2002). Additionally, cooling blunts the ventilatory response to central acidification.
(Branco et al., 1993). In order to gain insight into a possible cellular mechanism responsible for modulation of CO₂/pH sensitivity within the respiratory network of amphibians, we studied chemosensitivity of LC neurons from bullfrogs over a physiological range of temperatures. Like breathing in the intact animal and respiratory-related nerve activity in the brainstem, we demonstrated that chemosensitivity of individual LC neurons exhibits temperature dependence (Fig. 10).

Exposure to 4.5% CO₂ acidifies pHₐ from 7.9 to ~7.6 and results in a ~3-fold increase in breathing at 20-22°C in bullfrogs (Kinkead and Milsom, 1996). In contrast, Tattersal and Boutlier (Tattersall and Boutilier, 1999b) found that bullfrogs can use cutaneous gas exchange to compensate a metabolic acidosis (pHₐ= 8.4 to 7.6) when submerged without access to air at temperatures ≤10°C, suggesting that lung ventilation is neither used for acid-base regulation nor stimulated by acidosis at low temperatures. These two examples illustrate that body temperature dictates whether or not bullfrogs increase breathing during arterial acidification. Given the overall importance of the LC as a respiratory chemoreceptive site in anuran amphibians (Noronha-de-Souza et al., 2006), our data suggest that reduction in cellular chemosensitivity of LC neurons may play a role in inhibiting respiratory-drive during acid-base disturbances at low temperatures where the skin is sufficient to maintain temperature-dependent acid-balance. As temperature increases, however, lung ventilation becomes increasingly critical for regulation of acid-base balance in amphibians (Jackson and Braun, 1979a; Mackenzie and Jackson, 1978; Wang et al., 1998). In the
toad, *Bufo paracnemis*, the ventilatory sensitivity to inspired CO₂ increased from 15°C to 25°C, but increased only slightly from 25°C to 35°C (Branco et al., 1993). We posit that enhanced chemoreceptor sensitivity with increasing temperature may be necessary to detect changes in acid-base status and elicit ventilatory compensation as lung ventilation increases in importance. In direct comparison to ventilatory sensitivity to elevated CO₂ in toads, we found that LC neurons from bullfrogs increase the magnitude of their chemosensitive responses during temperature increases from 10°C until a plateau at ~26°C (Fig. 10) where half maximal chemosensitivity of LC neurons of bullfrogs occurred at ~20°C. The chemosensitive response vs. temperature plot follows a sigmoidal curve and has a Hill slope of 7.6, which demonstrates that small changes in temperature deviating from 20°C will have a profound impact on the magnitude of neuronal output from LC neurons during changes in CO₂/pH. The response of LC neurons during changes in temperature clearly parallels the overall chemosensitive drive to breathe in anuran amphibians (Bicego-Nahas and Branco, 1999; Branco et al., 1993), at least in bullfrogs acclimated to ~20°C. Unlike other ectotherms (da Silva et al., 2013), Wang et al. (Wang et al., 1998) showed that anuran amphibians are able to maintain −ΔpH/ΔT_b with no change in ventilation relative to metabolic rate; therefore, lung ventilation is less important at low temperatures and essential at high temperatures during acid-base disturbances. Wang et al. speculate that this may occur because lung ventilation functions in concert with cutaneous gas exchange. This respiratory control strategy appears to be mediated by reducing central sensitivity to CO₂/pH with cooling and increasing
sensitivity to CO₂/pH with warming (Branco et al., 1993). Given that LC neurons in bullfrogs exhibit similar changes in sensitivity with temperature, our data provide correlative evidence for the mechanistic role of the LC in regulation of $-\Delta \mathrm{pH}/\Delta T_b$ in anuran amphibians.

Changes in CO₂/pH-sensitivities of LC neurons during changes in temperature corroborate findings from the brainstem and whole-animal ventilatory responses in bullfrogs. Minute ventilation and breathing related-nerve activity typically decrease with decreasing temperature in ectothermic vertebrates (Morales and Hedrick, 2002; Stinner, 1982). Paradoxically, we found that warming decreased, while cooling increased the control firing rates of chemosensitive LC neurons. In order to put these findings into context, it is useful to identify aspects of anuran respiratory control that either increase with cooling, decrease with warming, or remain unaffected by temperature. Kruhøffer et al. (Kruhøffer et al., 1987) demonstrated that the toad, *Bufo paracnemus*, increased minute ventilation by ~4-fold during an increase in temperature 15°C to 32°C. Strikingly, tidal volume was unchanged during this temperature increase, while respiratory frequency increased by 6-fold. In the bullfrog brainstem preparation, temperature did not influence the tidal volume correlates of fictive breaths, burst duration or amplitude, despite proportional changes in frequency with temperature (Morales and Hedrick, 2002). Noronha de Souza et al. (Noronha-de-Souza et al., 2006) showed that ablation of the LC in amphibians resulted in the inability to increase tidal volume during exposure to hypercarbia without influencing respiratory frequency. Therefore, the LC appears to mediate
increases in minute ventilation through increases in the tidal volume. Since we found that cooling stimulates and warming depresses firing in LC neurons, it is plausible that altering LC output in this manner may regulate tidal volume during changes in temperature. The physiological function of cold-activated/ warm-inhibited chemosensitive LC neurons in amphibians must be confirmed in vivo to demonstrate a role for temperature sensitivity of LC neurons in respiratory control during body temperature changes. Further, the amphibian LC is considered to be homologous to the LC of mammals based on its anatomical location, noradrenergic content, and its axonal projections to the telencephalon (Marin et al., 1996). Given the anatomical homology and its analogous role in respiratory control, the functional consequences, i.e., learning and memory, stress response, cardio-respiratory control (Berridge and Waterhouse, 2003), of a cold activated/warm inhibited noradrenergic system on amphibians physiology will be interesting to explore.

*Temperature Modulation of LC Chemosensitivity*

The relationships between the cellular components that “sense” changes in CO$_2$/pH (termed, chemosensory transducer) and respiratory control remain speculative (Huckstepp and Dale, 2011); however, in vitro electrophysiological approaches have been incredibly useful in generating putative mechanisms of CO$_2$/pH sensitivity involved in respiratory control (Putnam et al., 2004). Our results from the LC of bullfrogs yield novel insights into potential mechanisms responsible for altering the magnitude of the chemosensitive response during changes in temperature. Cooling reduced CO$_2$/pH sensitivity and to our surprise,
completely eliminated chemosensitive responses at 10°C. These responses persisted in the presence of chemical and electrical blockade suggesting that reduction in chemosensitive responses with cooling were intrinsic rather than mediated by changes in chemical or electrical synaptic transmission. Three approaches to investigating this loss of chemoresponsiveness at 10°C all suggest that cooling to 10°C causes cessation of cellular CO₂/pH sensitivity. First, chemosensitive responses of LC neurons ceased when increases in firing rate due to cold-activation (~0.5 Hz to 2 Hz firing rate; Fig. 6) were compensated by negative current injection to control the initial firing rate (~0.4 Hz firing rate) at 10°C. Maintained reduction in chemosensitivity at 10°C with the initial firing rate controlled shows that elimination of chemosensitivity is not caused by a “ceiling effect,” which would prevent the neuron from further increasing firing during excitatory stimuli such as HA. Second, upon return to 20°C, all neurons restored chemosensitive responses to near-initial values. Restoration of sensitivity to CO₂/pH sensitivity demonstrates that chemosensitive responses did not rundown over time (Fig. 7B). Lastly, exposure of LC neurons to 10% CO₂ (ΔpH=0.82) did not evoke increases in firing rate (Fig. 7D). At ~20°C, 10% CO₂ induces firing rate increases that are ~3-fold larger than those observed using 5% CO₂ (Santin and Hartzler, 2013). Given that such a large chemosensory stimulus did not evoke firing responses from LC neurons at 10°C, cooling appears to completely eliminate chemotransduction. In direct contrast to cooling, warming to 26°C caused reversible increases in firing responses to 5% CO₂ (Fig 8C). Warming appears to influence the magnitude of the firing response at any given CO₂/pH
change because exposure of LC neurons to 3% and 5% CO\textsubscript{2} did not reveal increases in the slopes of the firing responses, \textit{i.e.}, sensitivity (Fig. 9). The changes in the magnitude of the response to HA did not persist in the presence of chemical and electrical synaptic blockade suggesting that increases in chemosensitivity at higher temperatures are network-driven. Since cooling appears to intrinsically reduce the magnitude of the firing responses, we argue that our data provide evidence towards a mechanism by which cooling modifies the number of chemosensory transducer molecules available to elicit increases in firing rate during HA. Although incompletely understood at this time, our data imply that warming increases firing in responses to CO\textsubscript{2}/pH through a separate, synaptic-driven mechanism.

Neuronal chemosensitivity is generally considered to arise by expressing pH-activated, inward-conducting cation channels (\textit{i.e.}, ASIC1a and TRP channels) or CO\textsubscript{2}/pH-inhibited, outward-conducting K\textsuperscript{+} channels (Cui et al., 2011; Huckstepp and Dale, 2011; Putnam et al., 2004; Ziemann et al., 2009). Although the cellular mechanism(s) that mediate chemosensitivity in LC neurons of bullfrogs are unknown, our data demonstrate that temperature changes modify the \textit{magnitude}, \textit{i.e.}, the size of the firing response induced by a given CO\textsubscript{2}/pH change, rather than the \textit{sensitivity}, \textit{i.e.}, how much CO\textsubscript{2}/pH is required to elicit a chemosensory response. Interestingly, temperature has a profound influence on the conductances of ion channels implicated in mediating cellular pH sensitivity. Protons inhibit conductance through the tandem-pore (K\textsubscript{2}P) K\textsuperscript{+} leak channel TASK-1 (Duprat et al., 1997) which has a Q\textsubscript{10} of 2 (Wechselberger et al., 2006),
i.e., this channel is inhibited by both low pH and decreasing temperature.

Additionally, ASIC1a is a proton-activated Na⁺ channel which has an inactivation $Q_{10}$ of 2.25; therefore, these channels are opened/available to be opened by protons and cold temperatures (Blanchard and Kellenberger, 2011). The reduction in CO₂/pH sensitivity at low temperatures may be mediated by changes in ion channel open/closed probabilities.

Increases in the chemosensitive response to 5% CO₂ when the temperature was elevated from 20°C to 26°C did not persist in all neurons during chemical and electrical synaptic blockade (Fig. 8D; 3/5 neurons; means not statistically different), suggesting that chemical and gap-junction mediated transmission may play a role in increased CO₂/pH responsiveness at higher temperatures. Chemical and electrical blockade did not influence the magnitude of the chemosensitive response of bullfrog LC neurons at ~20°C (Santin and Hartzler, 2013a). Many factors could lead to enhanced neurotransmission (e.g., enhanced neurotransmitter release, decrease neurotransmitter reuptake, etc.). Although glutamate release has a $Q_{10}$ of 3.6-5.5 (Nakashima and Todd, 1996), we are unaware of a mechanism that explains how increases in temperature could enhance neurotransmitter release specifically during HA to enhance the magnitude of the firing responses to HA compared to 20°C. Additionally, low pH has been shown to enhance inward current through a subtype of the kainate (glutamate) receptor (Mott et al., 2003); however, the modulatory role of temperature on pH-dependent activity of neurotransmitter-gated ion channels has not been investigated.
Effect of Temperature on Chemosensitive LC Neurons

Contrary to our hypothesis, we observed increases in firing frequency with cooling and decreases in firing frequency of chemosensitive LC neurons with warming (Fig. 1). Cold-activated neurons have been studied in the dorsal root and the trigeminal ganglia for cold-sensory transduction (McKemy et al., 2002; Reid and Flonta, 2001) and in the preoptic area of the hypothalamus (POAH) for thermoregulation (Boulant, 2000). Hippocampal neurons have also been shown to depolarize during cooling (de la Peña et al., 2012; Shen and Schwartzkroin, 1988). Strikingly, all chemosensitive neurons in the LC of bullfrogs (83% of the LC neurons studied) displayed either cold-activation or warm-inhibition, while only ~15% of neurons studied within the trigeminal ganglion, ~7% of neurons in the dorsal root ganglion (McKemy et al., 2002), and ~5% of neurons in the POAH (Wechselberger et al., 2006) demonstrate cold-activation. The large proportion of neurons exhibiting cold-activation/ warm- inhibition within the LC of bullfrog makes them an excellent model system in which to study mechanisms of neuronal temperature sensitivity. Interestingly, these firing responses during acute changes in temperature were not present in non-chemosensitive LC neurons and neurons in a slice ~500μm rostral to the LC, suggesting that cold-activated responses were unique to chemosensitive LC neurons. We are unsure of the functional implication of cold-activated/ warming-inhibited chemosensitive LC neurons (discussed above in Modulation of Cellular Chemosensitivity in Anuran Respiratory Control); however, our results provide evidence supporting the role of thermosensitive K⁺ channels in cold-activated responses. We
observed increases in $R_{in}$ at 10°C in addition to action potential broadening, reduced rate of repolarization, and reduced after-hyperpolarization (AHP).

Increases in $R_{in}$, wider action potentials, and reduced AHP coupled with increases in neuronal excitability are consistent with inhibiting $K^+$ conductance (Mathie et al., 1998). As mentioned in the Temperature Modulation of LC Chemosensitivity section, TASK-1 in addition to TREK-1 have $Q_{10}$s that favor reduced conductance during cooling (Maingret et al., 2000; Wechselberger et al., 2006). Inhibition of $K^+$ conductance during cooling would cause depolarization and increased firing rate. Such a mechanism involving TREK-1 has been suggested to cause increased excitability in neurons of the hippocampus (de la Peña et al., 2012). Further, given that input resistance decreased in neurons that decreased firing rate with warming, the mechanism responsible for cold-activation is also probably responsible for warm-inhibition of chemosensitive LC neurons, e.g., closing a $K^+$ channel during cooling and opening the same $K^+$ channel during warming. We also gathered evidence that cooling may decrease the hyperpolarization-activated current ($I_h$; Fig. 4) in chemosensitive LC neurons. Delineating the physiological role of $I_h$ in the function of chemosensitive LC neurons will clearly require further study. Overall, our data provide evidence that multiple temperature-sensitive ionic conductances may shape the firing and $V_m$ responses to temperature changes in chemosensitive LC neurons.

Perspectives and Significance

Bullfrogs have a broad geographical distribution within North America and experience grossly different temperatures depending on their locations and the
season. Under different environmental conditions, respiratory and acid-base regulation requirements vary dramatically. Over the course of a day, ambient temperature fluctuates significantly; therefore, acute changes in body temperature impose many physiological challenges to bullfrogs. Given the relationship between temperature, central chemoreception, breathing, and acid-base regulation, compensatory mechanisms within LC neurons of bullfrogs at warm and cold temperatures provide mechanistic insight into how the brain can modify respiratory drive to satisfy homeostatic requirements under variable environmental conditions.

Mechanisms of neuronal thermosensitivity are well delineated in the peripheral nervous system; however, mechanisms and functional consequences of neuronal temperature sensitivity in the central nervous system of vertebrates are poorly understood. To our knowledge, our data are the first to suggest a role for intrinsically cold-activated and warm-inhibited brainstem neurons in central control of physiological systems in vertebrates. Given that only chemosensitive LC neurons demonstrated cold-activation/ warm inhibition, a relationship may exist between temperature sensitivity and respiratory control. Uncovering the causes of thermosensation in chemosensitive LC neurons, in addition to their functional significance, will provide new insight into cellular mechanisms of the central nervous system required to combat unavoidable changes in brain temperature in order to control systems level output.
CHAPTER V.

MANUSCRIPT II

Activation state of the hyperpolarization-activated current modulates temperature-sensitivity of firing in locus coeruleus neurons from bullfrogs


Santin, J.M. & Hartzler, L.K.
ABSTRACT

Locus coeruleus (LC) neurons of anuran amphibians contribute to breathing control and have spontaneous firing frequencies that, paradoxically, increase with cooling. We previously showed that cooling inhibits a depolarizing membrane current, the hyperpolarization-activated current (I_h) in LC neurons from bullfrogs, Lithobates catesbeianus. This suggests an unlikely role for I_h in generating cold-activation, but led us to hypothesize that inhibition of I_h by cooling functions as a physiological brake to limit the cold-activated response. Using whole-cell electrophysiology in brain slices, we employed 2mM Cs+ (an I_h antagonist) to isolate the role of I_h in spontaneous firing and cold-activation in neurons recorded with either control or I_h agonist (cyclic AMP)-containing artificial intracellular fluid. I_h did not contribute to the membrane potential and spontaneous firing at 20°C. Although voltage-clamp analysis confirmed that cooling inhibits I_h, its lack of involvement in setting baseline firing and membrane potential (V_m) precluded its ability to regulate cold-activation as hypothesized. In contrast, neurons dialyzed with cAMP exhibited greater baseline firing frequencies at 20°C due to I_h activation. Our hypothesis was supported when the starting level of I_h was enhanced by elevating cAMP because cold-activation was converted to more ordinary cold-inhibition. These findings indicate that situations leading to enhancement of I_h facilitate firing at 20°C, yet the hyperpolarization associated with inhibiting a depolarizing cation current by cooling blunts the net V_m response to cooling to oppose normal cold-depolarizing factors. This
suggests that the influence of $I_h$ activation state on neuronal firing varies in the poikilothermic neuronal environment.
INTRODUCTION

The American bullfrog, *Lithobates catesbeianus*, has a broad geographical distribution within North American and undergoes rapid and variable temperature changes throughout a single day (Hutchison and Dupre, 1992; Stevenson, 1985a). Owing to the temperature sensitivity of neurophysiological mechanisms, changes in temperature pose a challenge to regulating neurally controlled behaviors, like breathing, in the bullfrog and other poikilothermic animals. Despite rate increases of neurally controlled, rhythmic behaviors typically associated with temperature (Robertson and Money, 2012), aspects of the respiratory pattern are maintained across temperatures in amphibians and other ectothermic vertebrates. Specifically, breathing frequency has low temperature dependence (Q_{10}~1.7) during changes at higher temperatures (Bicego-Nahas and Branco, 1999; Rocha and Branco, 1997), and tidal volume (*i.e.*, the volume of air consumed in a breath) is insensitive to variations in temperature (Kruhøffer et al., 1987; Stinner, 1982). Consistent with these observations *in vivo*, respiratory-related cranial nerve activity of the isolated bullfrog brainstem *in vitro* maintains burst frequency across higher temperatures, and the duration and amplitude of each burst (*i.e.*, neural correlate of tidal volume) does not depend on temperature (Morales and Hedrick, 2002). Therefore, the central respiratory control system may be equipped with mechanisms that offset the effects of temperature to preserve respiratory effort across temperatures relevant for lung ventilation in amphibians.
The locus coeruleus (LC) is one brainstem region involved in control of breathing (Gargaglioni et al., 2010) that contains neuronal activity consistent with a role in temperature compensation of respiratory parameters. Thus far, the LC of adult amphibians is the only discrete brain nucleus described with an unequivocal role in modulation of breathing in vivo (Noronha-de-Souza et al., 2006); specifically, as a central chemoreceptor. Stimulation of the LC by focal acidification leads to increases in breathing, while ablation results in reduced ventilatory response to hypercarbia (Noronha-de-Souza et al., 2006). Further, LC neurons of bullfrogs appear to directly sense CO$_2$/pH (Santin and Hartzler, 2013a). We recently demonstrated that CO$_2$/pH chemoreceptive neurons within the LC (~90% of neurons) counterintuitively exhibit intrinsic spontaneous firing rates that vary inversely with temperature (i.e., neurons increase firing frequencies when cooled and decrease firing frequencies when warmed) (Santin et al., 2013). Intriguingly, only chemoreceptors show these responses to temperature changes, but CO$_2$/pH insensitive neurons within or outside the LC do not. Because chemosensitive LC neurons are part of the respiratory network of amphibians (Noronha-de-Souza et al., 2006), firing frequencies that vary inversely with temperature present a plausible neuronal mechanism to oppose temperature-proportional rate effects that presumably act on other aspects of the respiratory control system. Understanding the mechanisms that underlie and/or modulate cold-enhanced/warm depressed LC activity could provide insight into how temperature interacts with the respiratory control system to regulate breathing across temperatures in amphibians and other ectothermic vertebrates.
Two ionic mechanisms have been proposed to determine cold-activation in LC neurons from bullfrogs which include the activation of a putative cold-induced depolarizing current ($I_{\text{cold}}$) and the inhibition of a hyperpolarization activated current ($I_h$) (Santin et al., 2013). Here we investigate $I_h$. $I_h$ is an inward (depolarizing) Na$^+$/K$^+$ current carried by hyperpolarization-activated cyclic nucleotide gated (HCN) channels. Consistent with evidence that cooling inhibits $I_h$ (Gambardella et al., 2012; Orio et al., 2009; Pena et al., 2006), we found cooling eliminated the depolarizing voltage “sag” caused by activation of $I_h$ during negative current injection (Santin et al., 2013). Inhibition of an excitatory membrane current associated with pacemaking is seemingly at odds with our findings that LC neurons intrinsically increase spontaneous discharge at cold temperatures. This suggests that a role for $I_h$ in the generation of cold-activation is unlikely, but implies that inhibition of this depolarizing current by cooling may function to dampen or limit the magnitude of cold-activation in LC neurons. For this hypothesis to be supported, $I_h$ would have to, first, be inhibited by cooling and, second, contribute to spontaneous firing in LC neurons under resting conditions. We therefore tested three specific hypotheses: 1. cooling inhibits $I_h$ in LC neurons from bullfrogs, 2. $I_h$ contributes to spontaneous firing, and 3. inhibition of $I_h$ due to cooling negatively regulates the magnitude of cold-induced firing. To address hypothesis one, we used whole-cell voltage clamp to characterize the electrical properties, pharmacology (antagonists and agonists), and temperature sensitivity of $I_h$. Using whole-cell current clamp combined with a pharmacological inhibitor approach, we next tested hypothesis two by elucidating...
the role of $I_h$ in generation of spontaneous firing by measuring action potential firing frequency in two experimental groups of LC neurons; one group recorded with control artificial intracellular fluid in the patch pipette and the other containing elevated cAMP to study $I_h$ in the activated state. Finally, we tested hypothesis three using the same approach to assess the ability of $I_h$ to influence cold-activation.
METHODS

Preparation of Brainstem Slices

Adult bullfrogs, *Lithobates catesbeianus*, (N=38) of either sex were kept in plastic tanks containing 22°C water with access to wet and dry areas, exposed to 12:12-h light-dark cycles, and consumed a diet consisting of crickets. All experiments performed were approved by the Wright State University Institutional Animal Care and Use Committee. Bullfrogs were euthanized by rapid decapitation posterior to the tympanic membranes and the head was placed in ice-cold artificial cerebral spinal fluid (aCSF; see Solutions section for composition) bubbled with 97.5% O₂ and 2.5% CO₂. Following removal of the frontoparietal bone, the brainstem was carefully dissected. Dissection time ranged between 5 and 10 minutes. Half of the forebrain was removed and then the brain was then attached to an agar block (ventral surface attached to block; rostral side facing down) and cut into ~400μm cross sections using a Vibratome tissue slicer (Leica Microsystems, Buffalo Grove, IL). Brain stem slices containing the locus coeruleus (Sánchez-Camacho et al., 2003) were given ~1h to recover from slicing in aCSF equilibrated with 80% O₂, 1.3% CO₂, balance N₂ (pH=7.9) at room temperature. Prior to electrophysiology experiments, the slice containing the LC was transferred to the 1mL recording chamber, stabilized with a nylon grid, and superfused with 20°C aCSF at rate of ~1-2ml/min.
Temperature of the chamber was manipulated using a Warner Instruments bipolar in-line temperature controller (model CL-100; Hamden, CT). Because we controlled the temperature with an in-line heater-cooler, the temperature of the solution varied up to 1°C along the longitudinal axis of the chamber. Temperatures (including kinetics of temperature changes) at the location of the slice within the recording chamber were, therefore, determined prior to experiments under identical experimental conditions, but without the slice present in the chamber to ensure accurate experimental temperatures.

Solutions

Artificial cerebral spinal fluid (aCSF) was composed of (in mM) 104 NaCl, 4 KCl, 1.4 MgCl₂, 7.5 glucose, 40 NaHCO₃, 2.5 CaCl₂, and 1 NaH₂PO₄ and equilibrated with 80% O₂, 1.3% CO₂, and balance N₂ (pH=7.9 at 20°C and 7.8 at 10°C). Although cooling resulted in a minor acidification, firing increases during cooling are not explained by this small pH decrease (Santin and Hartzler, 2013a; Santin et al., 2013). This pH and CO₂/HCO₃⁻ combination was chosen because it closely mimics arterial composition in vivo (Gottlieb and Jackson, 1976). We added either 2mM CsCl (Sigma-Aldrich, St. Louis, MO) or 50 μM ZD7288 (Tocris, Bristol, United Kingdom) to the aCSF to inhibit the hyperpolarization-activated current (Ih). H-89 dihydrochloride hydrate (10 μM) (H-89; Sigma-Aldrich, St. Louis, MO) was included in the aCSF to inhibit protein kinase A and possibly several other protein kinases (Lochner and Moolman, 2006). Inhibitors H-89 and ZD7288 were reconstituted in water and stored in 20mM and 40mM stock.
solutions, respectively, at -20 °C. For experiments using ZD7288 and H-89, stock solutions were diluted to final concentrations in aCSF.

**Electrophysiological Recordings**

Whole-cell current and voltage clamp recordings were acquired as previously described (Santin et al., 2013). Briefly, 4-7MΩ pipettes were backfilled with mock intracellular fluid containing (ICF; in mM) 110 K-gluconate, 2 MgCl₂, 10 HEPES, 1 Na₂-ATP, 0.1 Na₂-GTP, 2.5 EGTA; pH 7.2 with KOH and placed over an AgCl₂-coated, Ag wire. For experiments that assessed the effects of increased cAMP on whole cell currents and voltage, the artificial ICF included 100μM cAMP. cAMP was provided to the neurons by dialysis through the pipette to ensure that a near-uniform amount of cAMP entered each neuron across experiments. The slice was visualized at 4X magnification using a Nikon Cool Snap camera and NIS Elements Imaging Software (Nikon, Elgin, IL). The LC was identified by its bilateral location adjacent to the 4th ventricle (Sánchez-Camacho et al., 2003). Neurons within the LC were then visualized and selected for recording at 60X magnification. A syringe was connected to the headstage by a tube to apply positive pressure through the pipette to keep the tip free of debris. The pipette was positioned adjacent to soma of the neuron of interest using a micromanipulator (Burleigh PCS 5000; Thorlabs, Newton, NJ). Before entry into the on-cell configuration the pipette offset was zeroed. Negative pressure was applied until a >1GΩ seal formed. Rapid, but light suction then was applied by mouth to rupture the seal and enter the whole-cell configuration. Changes in membrane potential ($V_m$) were measured in “current-clamp mode” using an
Axopatch 200B amplifier, Digidata 1440A A/D converter, and Molecular Devices P10 Clampex software (Molecular Devices).

**Voltage Clamp Protocols and Analysis**

Whole-cell voltage clamp recordings were performed to measure properties of $I_h$ and outward $K^+$ currents also using an Axopatch 200B amplifier and Digidata 1440A A/D converter. To study properties of $I_h$, a hyperpolarizing step protocol was applied from either -52mV or -72mV, depending on the experiment, to -132 or -142mV ($\Delta$= -10mV). To measure outward $K^+$ currents, $V_m$ was held at -82mV and stepped from -92 to +28mV ($\Delta$= 10mV). The reversal potential of $I_h$ ($E_h$) was estimated as previously described (Mayer and Westbrook, 1983). Briefly, we plotted the current-voltage (I-V) relationship of the instantaneous component of $I_h$ elicited from holding potentials where $I_h$ is relatively activated (-112mV) and less activated (-72mV). The instantaneous component of the current elicited from holding potentials of -72mV and -112mV was fitted with a linear regression. The membrane potential where these extrapolated linear regressions of the instantaneous components of $I_h$ intersect indicates the membrane potential where no current passes through the hyperpolarization-activated conductance, and thus represents $E_h$ (see Figs.1 C&D).

Similar to the methods of others (Horwitz et al., 2014; Okamoto et al., 2006; Rodrigues and Oertel, 2006), steady-state voltage dependence of $I_h$ activation and maximal conductance was determined by eliciting tail currents.
from a series of prepulse steps (-52 to -142 mV) for 3 seconds and then measured immediately following a step to -92 mV (see Fig. 3). Measurement of the tail current at the instant of the step to -92 mV was selected because it is near the predicted Nernst potential for K⁺ \( (E_k \approx -86\text{mV}) \) and negative to activation voltages of other voltage-sensitive channels; therefore, voltage-dependent activation and conductance of \( I_h \) is presented with little contamination from other ion channels. Maximal steady-state conductance \( (G_{h\text{max}}) \) under each condition was determined by converting the tail current recorded immediately following the transition to -92mV from a conditioning pulse at -142mV into a conductance value using the equation:

\[
G_{h\text{max}} = I_{\text{max tail}} - I_{\text{min tail}} / (V_m - E_h) \quad (1)
\]

where \( I_{\text{max tail}} \) and \( I_{\text{min tail}} \) are the maximum and minimum tail currents, respectively, measured at -92mV immediately following the conditioning pulse at -142 mV; and \( V_m \) (-92 mV) subtracted from the average reversal potential (-40.75mV; determined in this study) of \( I_h \) is the driving force. All tail currents were then normalized according to the equation:

\[
I_{\text{normalized tail}} = (R - R_{\text{min}}) / (R_{\text{max}} - R_{\text{min}}) \quad (2)
\]

where \( R \) is the tail current measured at -92 mV following the step from the prepulse potential; \( R_{\text{min}} \) is the minimum inward tail current; and \( R_{\text{max}} \) is the maximum inward tail current. Normalized tail currents were then fitted to the Boltzmann equation using Clampfit software in the form:
where $G(V_m)$ represents the fraction of activated $G_h$ at membrane potential, $V_m$; $V_{0.5}$ is the voltage at which the steady state conductance is half-maximal, and $k$ is the Boltzmann slope factor.

Series resistance ($R_s$) was typically ~20MΩ and was routinely compensated by 50-70% using the circuitry of the Axopatch 200B amplifier. The largest steady-state $I_h$ current recorded at -142 mV was ~ -400 pA; therefore, in our study, a recording with an $R_s$ of 20 MΩ that underwent 50-70% compensation ($i.e.$, effective $R_s$ is 6-10MΩ) and contained a steady-state $I_h$ current of -400pA will produce a maximum voltage error of 2.4-4 mV. These small voltage errors that occurred as a result of the uncompensated $R_s$ were not corrected. In contrast, maximum outward ($K^+$) currents were larger (1-6nA recorded at a command potential of +28mV) and will therefore suffer from large voltage errors as a result of the $R_s$. The goal of these experiments was to qualitatively assess the effect of 2mM Cs$^+$ or 50 μM ZD7288 on outward currents to select the most suitable antagonist of $I_h$ in our preparation. Since $R_s$ underwent only small and generally acceptable increases throughout the experiment (mean $\Delta=1.68\pm0.33M\Omega$ or $14.06\pm2.75\%$ after compensation), voltage errors as a result of $R_s$ were consistent across the control and antagonist treatments in the same neuron; therefore, we did not correct for errors due to $R_s$. To accurately represent these data in the face of probable voltage errors due to $R_s$, normalized outward currents are presented as a function of the command potential ($V_{\text{command}}$) rather than membrane potential. All neurons used in experiments had an

$$\text{Normalized } G(V_m) = \frac{1}{1+e^{(V_m-V_{0.5})/k}}$$

(3)
interspike $V_m$ more negative than -50mV and action potential amplitudes >60mV upon entry into the whole-cell configuration. Data were filtered at 2kHz and collected at 10kHz. Current and voltage-clamp recordings were analyzed offline using pCLAMP software (Molecular Devices). All voltages from voltage and current clamp experiments were corrected for a liquid junction potential of 12mV.

**Current Clamp Protocols and Analysis**

Action potential firing frequency from current-clamp recordings was analyzed by integrating the trace into 10 second bins. Firing rates for control (n=24) and 100μM cAMP (n=24) pipette solutions were determined by averaging 1 minute of integrated firing after a ~3 minute stabilization period upon entry into the whole-cell configuration. The three minute window was provided to allow adequate diffusion of the cAMP into the neuron. Action potential properties of neurons containing control and elevated-cAMP pipette solution were analyzed as follows- threshold voltage ($V_{\text{threshold}}$): the membrane potential at the instant of rapid depolarization, depolarization rate: the slope of the rising phase of the action potential, from the threshold voltage to the peak, excluding the top and bottom 10% of the upstroke, repolarization rate: the slope of the falling phase of the action potential, from the peak to the threshold, excluding the top and bottom 10% of the downstroke, half-width: the time elapsed at the membrane potential halfway between threshold and peak during depolarizing and repolarization, afterhyperpolarization (AHP): the most negative membrane potential reached following repolarization subtracted from the interspike membrane potential, and height: voltage difference from the threshold to the peak. After recording the
initial firing frequency at 20°C the in-line heater-cooler was used to change the temperature of the bath from 20°C to 10°C. Temperature changes took ~4 minutes and the firing rate of each neuron at 10°C was measured for 1 minute once the bath temperature reached 10°C.

Recordings of control (n=8) and cAMP-containing (n=6) neurons upon exposure to Cs⁺ was measured for one minute after ~6 minutes of exposure. Each neuron exposed to 2mM Cs⁺ was then cooled from 20°C to 10°C. In current and voltage clamp experiments that used Cs⁺, neurons were not returned to control aCSF. Although the effects of Cs⁺ are often reported to washout following the return to control solution, pilot experiments revealed that even 20 minutes after washout of Cs⁺ with control aCSF, Iₜₜ amplitude measured in voltage clamp only recovered to ~50% of initial values. This reduction was not a result of rundown because Iₜₜ amplitude remained stable for at least 20 minutes (data not shown). A series of experiments was also conducted using the PKA inhibitor, H-89, to determine any Iₜₜ-independent effects of cAMP on the firing responses we observed. These experiments assessed both firing rates at 20°C and firing rates after the transition to 10°C in three separate neurons undergoing different treatments from the same slice preparation (n=5 slices): neuron 1- control pipette solution with control aCSF, neuron 2- elevated intracellular cAMP in the absence of the PKA inhibitor, and neuron 3- elevated cAMPᵢ following ~20-30 minute incubation of the slice with the PKA inhibitor, 10μM H-89, dissolved in the aCSF.

Membrane potential (Vₘ) was determined by measuring the average Vₘ of the interspike interval. Input resistance (Rᵢᵣᵣ) was determined by injecting a series
of hyperpolarizing current injections (-15pA to -60pA; Δ= -15pA) into the soma of the neuron for 500ms and measuring the voltage deflection. Since LC neurons contain $I_{h}$, depolarizing “sag” occurred during negative current injection; thus, we measured the voltage change at steady-state and used Ohm’s law to calculate $R_{in}$ for each current injection. We averaged the $R_{in}$ determined by each of the four current injections as an estimation of the $R_{in}$ for the neuron under each condition.

**Statistical Analysis**

Data are presented as mean± SEM. Statistical analyses were run and figures were constructed using Graph-Pad Prism 6.01 (GraphPad Software, San Diego CA). Unless otherwise indicated, n represents the number of cells included in each analysis. Means between two groups collected from separate neurons were analyzed using an unpaired, two-tailed t-test. If comparisons were made between treatments on the same neuron, a paired t test was used. These statistical tests assume equal standard deviations. Some analyses in this study had F statistics that rejected the null hypothesis that populations were heterogeneous. These situations are indicated in the results and had a Welch’s correction applied to the t test. In some situations, as indicated in the results section, a two-way ANOVA was used to determine the effect of treatment group (e.g., control, cAMP-dialyzed) on firing rate under different conditions (e.g., changing temperature or application of Cs$^+$). Within group comparisons were then made using Holm-Sidak’s multiple comparisons test. Means of three or more groups were analyzed using a one-way ANOVA with Tukey’s post hoc test.
for multiple comparisons. Pertinent statistical information including, $p$ values, $T$ values, $F$ values, and degrees of freedom are presented in the results section. Statistical significance was accepted when $p<0.05$. 
RESULTS

Characterization of the hyperpolarization-activated current ($I_h$) in bullfrog LC neurons

These experiments were undertaken to characterize $I_h$ and to confirm its similarity to $I_h$ carried by HCN channels as described in other excitable cells. Fig. 11A shows that voltage steps from -52 mV to -142 mV elicited slowly activating, non-inactivating, inward currents. The current voltage-relationship of the steady-state component (black circle in Fig. 11A) shows inward rectification as the membrane becomes increasingly hyperpolarized (Fig. 11B). The membrane potential where $I_h$ will be at steady-state equilibrium (reversal potential ($E_h$)) was estimated by plotting the instantaneous component of the current elicited from holding potentials of -72 mV (less $I_h$) and -112 mV (activated $I_h$). The circles under the current recordings evoked from different holding potentials in Fig. 11C shows the instantaneous portion of the current that is plotted to determine $E_h$. The membrane potential at the intersection of the instantaneous components elicited from these holding potential presented in Fig. 11C provides an estimate of $E_h$ (Fig. 11D) (Mayer and Westbrook, 1983). In LC neurons $E_h$ was $-40.7\pm3.2$ mV ($n=12$). This mean for $E_h$ is similar to values obtained for $I_h$/HCN channels in mammalian cells (Bayliss et al., 1994; Biel et al., 2009; Rodrigues and Oertel, 2006).

The next series of experiments were performed to determine if blockers of $I_h$ also inhibit $I_h$ in LC neurons of bullfrogs. $I_h$ is typically characterized by its
Figure 11

A

Membrane Potential (mV)

-140 -120 -100 -80 -60 -40 -20

n = 11

200 pA

1s

B

Membrane Potential (mV)

-140 -120 -100 -80 -60 -40 -20

n = 11

Current (pA)

-100

-500

-300

500

100

Eh = -40.7 mV

C

Membrane Potential (mV)

-72 mV

-112 mV

Vhold = -72 mV

Vhold = -112 mV

200 pA

1s

D

Membrane Potential (mV)

-140 -120 -100 -80 -60 -40 -20

n = 12

Vhold = -72 mV

Vhold = -112 mV

Eh = -40.7 mV
**Figure 11** Characterization of the hyperpolarization-activated current (I_h). A (representative I_h recording) and B (mean steady-state I-V data; n=11) show that LC neurons from bullfrogs contain a slowly activating, non-inactivating, inwardly rectifying current that slowly activates upon a 3 second steps from -52 to -142 mV in voltage-clamp. The black circle in A. indicates the time point where the steady-state current was measured for each voltage as plotted in B. C shows two families of current recordings elicited from a more positive (-72 mV) and more negative (-112 mV) holding potential to determine the reversal potential (E_h) of I_h. E_h can be estimated by extrapolating the intersection of the instantaneous component of the current evoked from each holding potential. The time point of the instantaneous component used for estimation of E_h is indicated by a black circle for currents elicited from -72 mV (●) and open circle for currents elicited from -112 mV (○) holding potential. D. shows the mean E_h (-40.7±3.2 mV) as determined by the described method.
sensitivity to ZD7288 and low millimolar concentrations of cesium chloride (CsCl). Fig. 12A shows representative current recordings from a voltage-clamp experiment that applied a hyperpolarizing step protocol to activate $I_h$ in the absence and presence of 2 mM Cs$^+$ (top panel) and 50 μM ZD7288 (bottom panel). Figs. 12B and C show summary I-V relationships of $I_h$ before and after exposure to Cs$^+$ and ZD7288, respectively. In these figures, $I_h$ is presented as the instantaneous component of $I_h$ at the moment of the voltage step subtracted from the steady-state component at the end of the 3s voltage step. Application of 2mM Cs$^+$ inhibited $I_h$ by 97.4±2.17%, while ZD7288 also reduced a substantial portion of $I_h$ by 86.06±2.55%; however, Cs$^+$ achieved slightly greater inhibition compared to ZD7288 (Fig. 12G; p=0.0069; $T_{10}$= 3.387; two-tailed unpaired t test). Collectively, these electrophysiological and pharmacological properties indicate that LC neurons from bullfrogs contain a hyperpolarization-activated current carried by HCN channels as described in mammalian neurons.

We wanted to use these antagonists to determine the role of $I_h$ in spontaneous firing and cold-sensitivity in current-clamp experiments. Unfortunately, in addition to its block of $I_h$, 2 mM Cs$^+$ may also inhibit certain K$^+$ channels (Mermelstein et al., 1998). Additionally, ZD7288 inhibits T-type Ca$^{2+}$ channels (Sánchez-Alonso et al., 2008), voltage-gated Na$^+$ channels (Wu et al., 2012), and outward currents (unpublished observation) (Do and Bean, 2003). To assess whether or not Cs$^+$ and ZD7288 inhibited outward (K$^+$) currents and select the best blocker of $I_h$ in LC neurons, we measured their effects on outward currents with depolarizing steps from -92 mV to +28 mV in voltage-clamp. As
Figure 12

A. Control 2 mM Cs⁺ 2mM Cs⁺

B. Membrane Potential (mV)

C. Membrane Potential (mV)

D. Control 2mM Cs⁺

E. Normalized Inward Current

F. Normalized Inward Current

G. Inhibition of Ih max (%)

H. Inhibition of Ih max (%)
**Figure 12** $I_h (I_{\text{steady state}}-I_{\text{instantaneous}})$ is sensitive to 2 mM Cs$^+$ and ZD7288, but ZD7288 reduces outward currents. A. shows a representative family of $I_h$ traces ($V_{\text{hold}}$= -72 mV; -72 mV to -132 mV; $\Delta$=-10mV) before and after application of 2 mM Cs$^+$ ($n=7$; top panel) and 50 μM ZD7288 ($n=5$; bottom panel). B&C. shows summary I-V data of the steady-state $I_h$ recorded in control aCSF ($\bullet$; Cs$^+$ experiments/$\square$; ZD7288 experiments), 2 mM Cs$^+$ or 50 μM ZD7288($\bullet$/$\square$), and isolation of the Cs$^+$ or ZD7288-sensitive current ($\circ$/□). D. representative family of outward (K$^+$) current traces ($V_{\text{hold}}$=-92 mV; -92 mV to +28 mV) before and after application of 2 mM Cs$^+$ ($n=4$; top panel) and 50 μM ZD7288 ($n=4$; bottom panel). E&F. shows summary I-V data of the peak outward currents at each voltage at the time point marked by the black circle in D. The near superimposed I-V relationship recorded in control aCSF ($\bullet$) and 2 mM Cs$^+$ ($\bullet$) shown in E. demonstrates that there is no 2 mM Cs$^+$-sensitive outward current ($\circ$). In contrast, F. shows that outward currents elicited in ZD7288 (□) are reduced compared to currents evoked in aCSF (■), leaving a large ZD7288-sensitive current (□). G. Summary of mean percent inhibition of the maximum $I_h$ current. 2 mM Cs$^+$ achieves slightly greater block of $I_h$ compared to ZD7288 ($p=0.0069$; two-tailed unpaired t test). H. Summary of mean percent inhibition of the maximum outward current. 2 mM Cs$^+$ does not inhibit outward currents, while ZD7288 reduces the maximum outward current by ~50% ($p=0.0003$; two-tailed unpaired t test). *$p<0.05$; **$p<0.001$
demonstrated in the representative trace, Fig. 12D (top panel; representative trace) and Fig. 12E (mean data), 2mM Cs\(^+\) did not reduce outward currents which corroborates previous findings from mammalian neurons where Cs\(^+\) applied at low millimolar concentrations effectively blocked I\(_h\), but did not reduce outward K\(^+\) currents (Thoby-Brisson et al., 2000). In contrast, Fig. 12D (bottom panel; representative recording) and 12F (mean data) shows that ZD7288 reduced outward currents; thus resulting in greater reduction of outward currents compared to Cs\(^+\) (Fig. 2H; p=0.0003; \(T_6\)= 7.267; two-tailed unpaired t test). Similar to the observation of Do and Bean (2003), this result suggests that ZD7288 may directly inhibit or indirectly lead to the inhibition of K\(^+\) channels responsible for the measured changes in outward currents. However, we cannot exclude the possibility that ZD7288 specifically blocked I\(_h\) and as a result, improved the space clamp conditions in the slice preparation which led to artifactual reductions in outward current. This possibility, however, seems less likely because we did not observe a similar decrease in outward current after application of Cs\(^+\). We therefore decided to use 2 mM Cs\(^+\) to block I\(_h\) in subsequent current clamp experiments.

**I\(_h\) is reduced by cooling and activated by cAMP**

Although we have shown that cooling reduces the hyperpolarization-activated depolarizing “sag” in LC neurons (Santin et al., 2013), we wanted to directly measure the effects of cooling on the conductance and voltage dependence of I\(_h\) activation. Additionally, we intended to study how I\(_h\) activation state can influence spontaneous firing and cold sensitivity; therefore, we also
assessed the sensitivity of the conductance and voltage dependent activation to cAMP to validate cAMP as an agonist of \( I_h \) in LC neurons. For these experiments, neurons recorded with microelectrodes filled with either control pipette solution \((n=11)\) or 100 μM cAMP pipette solution \((n=12)\) were stepped to prepulse potentials from -52 mV to -142 mV (-72mV holding potential) (Fig. 13A). Following the 3s prepulse that sufficiently activated \( I_h \), neurons then were stepped to the test potential of -92 mV to elicit tail currents. After the measurements at 20°C, the neurons were cooled to 10°C. Consistent with the notion that cAMP alters the open-closed probability without influencing single channel conductance (Thon et al., 2013), Fig. 13B shows that presence of cAMP does not influence \( G_{h_{\text{max}}} \) at either 20°C or 10°C (Two-way ANOVA; \( p=0.7511; F_{(1,42)}=0.1021 \)). In contrast, cooling reduced \( G_{h_{\text{max}}} \), regardless of cAMP concentration (Holm-Sidak’s multiple comparisons test; \( p<0.0001 \) for control and cAMP; \( T_{(42)}=4.898 \) (control); \( T_{(42)}=4.654 \)).

cAMP typically leads to \( I_h \) activation by shifting the voltage-dependence of activation to more depolarized potentials (Biel et al., 2009). A normalized conductance-voltage relationship in Fig. 13C estimates the fraction of activated \( I_h \) at a given \( V_m \). To determine the voltage at half maximal activation \( (V_{0.5}) \), we fit a Boltzmann function to the normalized conductance curve as shown in Fig. 13C. \( V_{0.5} \) values indicate that in control pipette solution at 20°C, \( I_h \) is half activated \( (V_{0.5}) \) at -89.1±3.0 mV and increasing cAMP depolarizes the \( V_{0.5} \) to -74.9±2.4 mV (Fig. 13D; one-way ANOVA; \( p<0.0001; F_{(3,42)}=21.27 \)). Cooling to 10°C hyperpolarized the \( V_{0.5} \) to -107.1±2.4 mV (from -89.1±3.0 mV at 20°C). In the
**Figure 13**

**A**

- A graph showing membrane potential (mV) against time (ms) with peak values of Rmax and Rmin.
- Peak values are indicated as -52mV, -142mV for Rmin and 92mV for Rmax.
- Current values are marked at 100pA, 50pA, and 100pA.

**B**

- A bar graph showing Gmax (nS) with different conditions:
  - Control 20°C
  - 100µM cAMP 20°C
  - Control 10°C
  - 100µM cAMP 10°C
- ns indicates no significant difference.

**C**

- A graph showing the fraction of activated Ih against membrane potential (mV) with different conditions.
- Different lines represent control 20°C, control 10°C, 100µM cAMP 20°C, and 100µM cAMP 10°C.

**D**

- A graph showing V0.5 (mV) with control 20°C, control 10°C, cAMP 20°C, and cAMP 10°C.
- ns indicates no significant difference.
Figure 13 $I_h$ is reduced by cooling and activated by cAMP. A. shows an example of $I_h$ current traces evoked by 3 second prepulse steps to various potentials from -52 mV to -142 mV. Following the 3 second prepulse, tail currents were elicited by a step to -92 mV. The right portion of A. shows the same recording with a magnified time scale to visualize the tail currents recorded at -92 mV and normalization protocol used for construction of the voltage-dependent activation curve. B. summary data for maximum $G_h$ in the presence (☐ - 20°C; □; 10°C; n=12) and absence (■-20°C; ●-10°C; n=11) of elevated cAMP. All results in B-D represent $G_{max}$ and voltage-dependent activation from this population of neurons. Temperature, independent of cAMP, reduced the magnitude of $G_{max}$ (p<0.0001; two-way ANOVA with Holm-Sidak’s multiple comparisons test). C. summary data of normalized tail currents recorded immediately following a step to -92 mV from a prepulse at -52 mV to -142 mV in the presence (☐-20°C; C-10°C; n=12) and absence (●-20°C; ●-10°C; n=11) of elevated cAMP fitted with a Boltzmann function. C. highlights that cAMP shifts voltage-dependent activation to more positive potentials at 20°C and 10°C. D. summary data of the voltage at half $I_h$ activation ($V_{0.5}$) obtained from normalized tail currents illustrated in C. showing that cAMP shifts voltage-dependent activation by ~+15 mV at 20°C and by ~+19 mV at 10°C. Additionally, cooling to 10°C results in a hyperpolarizing shift in the $V_{0.5}$ by ~-20 mV with and without cAMP. **p<0.01; ***p<0.001; ns= not significant.
presence of cAMP at 10°C, $V_{0.5}$ was half maximal at -88.32±3.89 mV, as compared to -74.9±2.4 mV at 20°C. Consistent with our hypothesis and previous observation that cooling eliminates the depolarizing voltage “sag” (Santin et al., 2013), we confirm that cooling inhibits $I_h$ by reducing the maximal conductance and hyperpolarizing voltage-dependent activation with or without cAMP present. In addition, cAMP shifted the $V_{0.5}$ by ~+15 mV, indicating that cAMP activates $I_h$ in LC neurons of bullfrogs. These results suggested that $I_h$ would be more active in neurons dialyzed with cAMP at 20°C, but similarly inhibited at 10°C.

$I_h$ is not active at rest, but influences firing frequency and $V_m$ when activated

These next experiments used whole cell current-clamp recordings to characterize and compare spontaneous firing in control pipette solution and in the presence of the $I_h$ agonist, cAMP. Summary data are presented in Fig. 14A. Mean firing rates were greater in cAMP-containing neurons (Fig. 14A; p<0.0001; $T_{27.27}=4.752$; unpaired, two-tailed t test with Welch’s correction). Most LC neurons fired action potentials spontaneously at 20°C with a mean frequency of 0.72±0.12 Hz (n=23; Fig. 14B). Consistent with enhanced activity of a hyperpolarization-activated depolarizing current (Chu and Zhen, 2010), Fig. 14B shows that cAMP eliminated most of the action potential undershoot (afterhyperpolarization; AHP) in neurons dialyzed with cAMP. Further, the input resistance ($R_{in}$) that the headstage encounters during a negative current injection allows qualitative insight into whether addition of cAMP increased firing through net channel opening or closing. If cAMP acted predominately through $I_h$
**Figure 14**

**A**

![Graph showing firing rate (Hz) for Control and 100μM cAMP](image)

**B**

**B1**

Control ~1 Hz

![Control response with 20mV, 20s, -55 mV, 1s](image)

**B2**

cAMP ~3 Hz

![cAMP response with 20mV, 20s, 20mV, 1s](image)
Figure 14 cAMP enhances spontaneous firing frequency in majority of LC neurons from bullfrogs. A: Summary data showing that cAMP enhances firing, leading to statistically significant increases in firing frequency compared to control (p<0.0001; unpaired, two-tailed t test). B: Example action potential traces illustrating 1 minute of firing from control neurons (B₁; ○), neurons dialyzed with cAMP (B₂; ○). The bottom panel in 4B shows the action potential traces in an expanded time scale, while the bottom insets illustrate the action potential waveform under each condition (scale bars indicate 20 mV and 10 ms for each action potential). ****p<0.0001
activation, we would expect to observe decreases in $R_{in}$ compared to control. $R_{in}$ was measured in some of the neurons in each group, and was reduced in the presence of cAMP [control (n=20): 530.1±34.1 MΩ vs. cAMP (n=12): 383.4±26.1 MΩ; $p=0.0051$; $T_{30}=3.02$; two-tailed t test]. Other action potential and membrane properties are included in Table 2 and the waveforms are presented at the bottom of Fig. 14.

The previous experiments suggest that $I_h$ may increase firing frequency when activated. To remove ambiguity associated with population studies and account for the fact that cAMP is not a specific agonist of $I_h$, we isolated the contribution of $I_h$ to spontaneous firing and $V_m$ in control and elevated cAMP using 2 mM Cs$^+$ at 20°C. If $I_h$ contributed to $V_m$ and spontaneous firing, we expected that blocking $I_h$ with Cs$^+$ would reduce firing frequency and hyperpolarize the membrane. Fig. 15A shows an example of an LC neuron before and after the application of 2 mM Cs$^+$. Exposing LC neurons containing control pipette solution to 2 mM Cs$^+$ had no net effect on firing rate and $V_m$; however, as demonstrated in Fig. 15B, this response was variable (Fig. 15B; n=8; $p=0.40$; $T_7=0.7969$; two-tailed paired t test). Cs$^+$ also did not alter $V_m$ (Fig. 15C; n=8; $p=0.4938$; $T_7=0.7218$; two-tailed paired t test).

In contrast to neurons containing control pipette solution, Fig. 15D illustrates that all neurons containing elevated cAMP underwent reductions in firing (Fig. 15E; $p=0.015$; $T_5=3.611$; two-tailed paired t test) and slight membrane hyperpolarization (Fig. 15F; $p=0.03$; $T_5=2.983$; two-tailed paired t test) in response to Cs$^+$ application. We conclude that $I_h$ makes little to no contribution to
Table 2. **Membrane and Action Potential Properties**  
***p<0.001; ****p<0.0001

<table>
<thead>
<tr>
<th></th>
<th>Control (n=23)</th>
<th>cAMP (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Potential (mV)</td>
<td>-54.43±0.93</td>
<td>-53.61±1.12</td>
</tr>
<tr>
<td>V\text{\textsubscript{threshold}} (mV)</td>
<td>-44.95±1.14</td>
<td>-44.16±1.14</td>
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<tr>
<td>Depolarization Rate (mV/ms)</td>
<td>120.70±5.84</td>
<td>80.04±8.08</td>
</tr>
<tr>
<td>Repolarization Rate (mV/ms)</td>
<td>-91.66±4.64</td>
<td>-60.54±5.98</td>
</tr>
<tr>
<td>Half-width (ms)</td>
<td>0.77±0.03</td>
<td>1.11±0.09</td>
</tr>
<tr>
<td>Afterhyperpolarization (mV)</td>
<td>-13.59±0.83</td>
<td>-4.82±1.01</td>
</tr>
</tbody>
</table>
**Figure 15** $I_h$ does not contribute to spontaneous firing and $V_m$ unless pharmacologically activated by cAMP. A. representative whole-cell recording of an LC neuron before and after application of 2 mM Cs$^+$, indicating that inhibition of $I_h$ does not alter firing rate and $V_m$ in neurons recorded using control pipette solution. B. summary data of the firing frequency and C. membrane potential ($V_m$) of LC neurons at 20°C before and after inhibition of $I_h$ with 2 mM Cs$^+$ (n=8) (p=0.40; two-tailed paired t test) and $V_m$ (p=0.4938; two-tailed paired t test). The data point indicated by the red hexagon was used to generate the representative recording for neurons recorded with control pipette solution. D. representative whole-cell recording of an LC neuron before and after exposure to 2 mM Cs$^+$ in the presence cAMP. In contrast to neurons recorded using control pipette solution, when cAMP is added to the pipette solution inhibition of $I_h$ reduces firing rate and induces a slight hyperpolarization of the membrane E. summary data of the firing frequency and F. $V_m$ of LC neurons at 20°C in the presence of 100 μM cAMP before and after inhibition of $I_h$ with 2 mM Cs$^+$. The data point indicated by the blue hexagon was used to generate the representative recording for neurons recorded with cAMP pipette solution. Decreases in firing frequency (p=0.015; paired, two-tailed t test) and membrane hyperpolarization (p=0.030; paired, two-tailed t test) upon inhibition of $I_h$ indicate that activation of $I_h$ by cAMP contributes to enhanced firing frequencies of LC neurons at 20°C.*p<0.05; **p<0.01
the spontaneous firing and $V_m$ under control conditions. Consistent with our voltage clamp experiments, cAMP activates $I_h$ to enhance firing presumably through small depolarization of $V_m$.

**Enhancing $I_h$ reverses cold-activation**

The next series of experiments were conducted to determine if the combination of elevated cAMP and $I_h$ influences cold-activation. Consistent with previous reports (Santin et al., 2013), LC neurons from bullfrogs increase firing frequency during cooling (Fig. 16A). In 23/24 neurons containing control ICF firing frequency increased from $0.56\pm0.08$ Hz to $2.19\pm0.30$ Hz ($Q_{10}=0.37\pm0.06$; mean and $Q_{10}$ represent all neurons cooled) (Fig. 16B; $p<0.0001$; $T_{46}=4.978$; two-way ANOVA with Holm-Sidak’s multiple comparisons test) upon transition from 20°C to 10°C. To test the hypothesis that $I_h$ activation would reduce the magnitude of the cold-activated response, neurons containing 100 µM cAMP were exposed to the cooling ramp. In direct contrast with neurons containing control pipette solution, 20/24 (83%) neurons with elevated cAMP decreased firing rates from $1.82\pm0.33$ Hz to $0.72\pm0.15$ Hz during cooling from 20°C to 10°C ($Q_{10}=3.81\pm0.87$; mean and $Q_{10}$ represent all neurons cooled containing cAMP) (Fig. 16C & D; $p<0.01$; $T_{46}=3.357$; two-way ANOVA with Holm-Sidak’s multiple comparisons test). Thus, cAMP qualitatively changes the response to cooling compared to control ($p<0.0001$; two-way ANOVA; $F_{(1,46)}=34.73$). Cooling also resulted in a smaller membrane depolarization in neurons dialyzed with cAMP (Fig. 16E; control: $n=23$; $6.50\pm0.69$ mV vs. cAMP: $n=20$; $1.55\pm0.65$ mV;
p<0.0001; $T_{41}=5.266$; unpaired, two-tailed t test). Cooling increased steady-state $R_{in}$, but $R_{in}$ was not different in neurons.
Figure 16 $I_h$, agonist, cAMP, reverses normal responses to cooling in LC neurons. A. shows bath temperature (top), integrated firing rate (middle), and action potentials (bottom) from an LC neuron undergoing a cooling ramp in the presence of control ICF. The gray line represents the interspike membrane potential at -56 mV. Action potentials are shown from the numbered time points indicated in the integrated firing rate trace to highlight frequency and $V_m$ changes induced by cooling. Notice that cooling from 20°C to 10°C induces increases in firing frequency that can be reversed upon return to 20°C. B. summary data of firing rates from neurons at 20°C and 10°C. Each point at 20°C is connected by a line to the firing rate of the same neuron at 10°C, showing that LC neurons undergo, at least, small increases in firing during cooling ($n=23; p<0.0001; p<0.0001$; two-way ANOVA with Holm-Sidak’s multiple comparisons test). The data point indicated by the red hexagon was used to generate the representative recording for neurons recorded with control pipette solution. C. illustrates bath temperature, integrated firing rate, and action potentials from an LC neuron that contains elevated (100 μM) cAMP. The gray line represents the interspike membrane potential at -56 mV. Unlike control ICF, neurons with elevated cAMP undergo reversible decreases in firing and considerably smaller depolarization during cooling from 20°C to 10°C. D. summary data of firing rates from neurons at 20°C and 10°C containing elevated cAMP. The data point indicated by the blue hexagon was used to generate the representative recording for neurons recorded with cAMP pipette solution. Each point at 20°C is connected by a line to the firing rate of the same neuron at 10°C, showing that LC neurons with
elevated cAMP undergo decreases in firing during cooling (n=20; p<0.01; two-way ANOVA with Holm-Sidak’s multiple comparisons test). E. shows mean change in $V_m$ in LC neurons containing control and elevated cAMP ICF (p=0.0002; unpaired, two-tailed t test). **p<0.01; ***p<0.001; ****p<0.0001
containing elevated cAMP (n=7) compared to control (n=8) (control: 892±149 vs. 100 μM cAMP: 825±30 MΩ; p=0.6739; $T_{7.672}=0.4374$; unpaired, two-tailed t test with Welch’s correction), suggesting that $I_h$ was equally inhibited by cooling whether or not neurons were dialyzed with cAMP. Overall, these results demonstrate that elevated cAMP, potentially through increasing active $I_h$ before cooling, leads to reversal of the typical cold response in LC neurons.

Like the experiments conducted at 20°C, we next used Cs⁺ to isolate the contribution of $I_h$ to cold-induced firing responses in control and in the presence of the $I_h$ agonist, cAMP. Since $I_h$ does not contribute to $V_m$ and firing frequency in control conditions, unsurprisingly, inhibition of $I_h$ using Cs⁺ did not influence cold-activation (0.75±0.27Hz increase during cooling in control vs. 1.12±0.72 Hz in Cs⁺; n=6; p=0.403; $T_5=0.912$; two-tailed paired t test). However, in support of our hypothesis that $I_h$ could negatively regulate cold-activation, Fig. 17A depicts an example integrated firing rate trace of a neuron containing 100 μM cAMP during two cooling ramps; one in control aCSF followed by one in the presence of 2mM Cs⁺. A repeated measures two-way ANOVA revealed an interaction between temperature and group (presence/absence of Cs⁺) (p=0.0024; $T_{(1,100)}=16.14$) indicating that response to cooling is qualitatively different when comparing neurons dialyzed with cAMP with and without Cs⁺. Cooling neurons dialyzed with cAMP (n=6) resulted in reduced firing rates (Fig. 17B; left; 20°C: 1.42±0.34Hz vs. 10°C: 0.63±0.28Hz; p<0.05; $T_{10}=3.078$; Holm-Sidak’s multiple comparisons test). In direct contrast, in the presence of Cs⁺, all neurons that
Figure 17

A

Temp (°C)

[Graph showing temperature change over time with 100μM cAMP and 100μM cAMP + 2mM Cs⁺]

B

Firing Rate (Hz)

[Graph showing firing rate at different temperatures for cAMP and cAMP + Cs⁺]

C

Δ Firing Rate Induced by Cooling (Hz)

[Graph showing change in firing rate induced by cooling for cAMP and cAMP + Cs⁺]

D

ΔV during Cooling (mV)

[Graph comparing ΔV during cooling for 100μM cAMP and 100μM cAMP + 2mM Cs⁺]

E

Control for Repeated Exposures

[Graph showing control for repeated exposures with n=4]

F

Δ Firing Rate (%)

[Graph showing change in firing rate with control and 100μM cAMP + 2mM Cs⁺ with n=6]
Figure 17 Inhibition of $I_h$ rescues cold-activation in the presence of cAMP. A. shows a representative trace containing bath temperature and integrated firing rate of an LC neuron containing elevated cAMP during two cooling ramps before (left panel) and after (right panel) inhibition of $I_h$ with 2 mM Cs$^+$. Notice that prior to inhibition of $I_h$, the neuron reversibly decreased firing frequency during cooling in the presence of elevated cAMP; however, after application of 2 mM Cs$^+$, the same neuron reversibly increased its firing frequency during the second cooling ramp. B. shows summary data of firing rates from neurons containing elevated cAMP at 20°C and 10°C in the absence (left) and then in the presence (right) of 2 mM Cs$^+$. Each point at 20°C is connected by a line to the firing rate of the same neuron at 10°C. B. shows that all neurons reduced firing rates during cooling with 100 μM cAMP (p<0.05; Holm-Sidak’s multiple comparisons test); however in the presence of 2 mM Cs$^+$ the same neurons increased firing frequency during cooling (p<0.05; Holm-Sidak’s multiple comparisons test). C. illustrates paired comparisons of the same neurons dialyzed with cAMP before and after exposure to Cs$^+$, highlighting negative and then positive changes in firing induced by cooling (p=0.0040; paired, two-tailed t test). D. shows that each neuron containing elevated cAMP in the absence of Cs$^+$ underwent significantly smaller membrane depolarization during cooling prior to the Cs$^+$ exposure (p=0.0063; two-tailed paired t test). E. shows summary data of neurons containing 100 μM cAMP (n=4) that underwent two cooling ramps to control for possible changes that could occur during the second cooling ramp independent from the actions of Cs$^+$. In these experiments both the first and second cooling exposure resulted in
reduced firing frequency as indicated by a negative percent change in E. (p=0.50; paired, two-tailed t test). This experiment negates the possibility that a time or multiple exposure-dependent artifacts caused increased firing frequency during cooling after application of 2 mM Cs+ as shown in A.-D. F. summary data of firing increases during cooling in neurons containing control ICF recorded in control aCSF (□) and neurons containing 100 μM cAMP recorded in 2 mM Cs+ (□). Percent increases in firing rate during cooling did not differ between control and 100 μM cAMP/ 2 mM Cs+, indicating that activation of Ih before transitioning to 10°C is sufficient to reduce excitability during cooling. *p<0.05; **p<0.01; ***p<0.001
previously decreased firing rates during cooling when dialyzed with cAMP responded with increased firing rates during the second cooling ramp (Fig. 17B; right; 20°C: 0.27±0.50 Hz vs. 10°C: 0.94±0.33 Hz; p<0.05; $T_{10}=2.604$; Holm-Sidak’s multiple comparisons test). Reduced firing frequencies induced by cooling that was converted to increased firing frequencies following inhibition of $I_h$ is summarized in Fig. 17D by highlighting the differences in the change in firing rate evoked by cooling ($p=0.0040$; $T_5=5.041$; two-tailed paired t test). The depolarization due to cooling in these neurons was also significantly greater in the presence of 2mM Cs$^+$ (Fig. 17E; $p=0.0007$; $T_5=7.395$; two-tailed paired t test).

To eliminate the possibility that the increase in firing rate during the second cooling ramp in the presence of Cs$^+$ is result of dialysis time and not the inhibition of $I_h$, four additional neurons containing elevated cAMP$_i$ underwent two cooling ramps (Fig. 17F). For each neuron, the first and second cooling ramp both led to decreases in firing rate. This control experiment indicates that restoration of cold-activation during exposure to Cs$^+$ was, presumably, the result of $I_h$ inhibition and not an artifact due to time or multiple cold exposures. Additionally, neurons (n=6) were also recorded from each slice with normal pipette solution as a positive control for cold-activated responses. Comparing cold-activated responses of cAMP + Cs$^+$ (i.e., the recovered cold-activated response) with control revealed that $I_h$ inhibition was, evidently, sufficient to restore cold-activation to near-control levels (Fig. 17G; n=6; $p=0.102$; $T_{10}=1.801$; unpaired two-tailed t test).
To summarize, these results show that $I_h$ is likely to be closed under normal conditions in LC neurons which precludes its ability to influence spontaneous firing and cold-sensitivity. We expected that inhibition of $I_h$ by cooling could dampen cold-sensitivity when $I_h$ was initially contributing to firing rate and $V_m$, but to our surprise, neurons underwent a completely opposite response to cooling when $I_h$ was initially activated.

**$I_h$ density correlates with responsiveness to cAMP**

Most neurons (20/24) exhibited cAMP sensitivity (as represented in Fig. 18A1), but four neurons, represented in Fig. 8A2, appeared insensitive to increased cAMP. Given that $I_h$ inhibition reversed the effects of cAMP, we reasoned that neurons dialyzed with cAMP resembling the control condition may result from lower $I_h$ density. In the presence of cAMP, 6 “cAMP-sensitive” neurons (*i.e.*, reduced firing rate with cooling) and 4 “cAMP-insensitive” neurons (*i.e.*, neurons that, like in the control condition, increased firing with cooling), $I_h$ density was measured. We found that cAMP sensitive neurons had greater $I_h$ densities compared to cAMP insensitive neurons (Fig. 18B; $p=0.019$; $T_8=2.915$; unpaired two-tailed t test). Interestingly, $I_h$ density correlated with the $Q_{10}$ (*i.e.*, fold-change over 10°C) of firing frequency (Fig. 18C; $r^2=0.6459$; $p=0.0051$; $F_{(1,8)}=14.59$; linear regression). Fig. 18C illustrates that neurons with greater $I_h$ densities have firing frequency $Q_{10}$s $>1$, indicating that neurons with greater $I_h$ density were most affected by cAMP (*i.e.*, they had larger decreases in firing rate with cooling). Conversely, when neurons with low $I_h$ densities are exposed to cAMP, they retain neuronal response to cooling as if cAMP was not
Figure 18

A1

\[ \text{cAMP - Sensitive} \]

\[ \text{Ih Density @ } -142 \text{ mV (pA/pF)} \]

A2

\[ \text{cAMP - Insensitive} \]

\[ \text{cAMP Sensitive} \]

\[ \text{cAMP Insensitive} \]

B

\[ \text{r}^2 = 0.6459 \]

\[ p = 0.0051 \]

\[ n = 4 \]

\[ n = 6 \]

\[ 50 \text{ mV} \]

1 Minute

50 mV

1 Minute

Cooling Ramp Applied

B

\[ \text{cAMP Sensitive} \]

\[ \text{cAMP Insensitive} \]

\[ \text{Ih Density (pA/pF)} \]

C

\[ \text{Firing Rate } Q_{10} \]

\[ \text{r}^2 = 0.6459 \]

\[ p = 0.0051 \]
Figure 18 Neurons with cooling responses sensitive to cAMP have higher $I_h$ densities compared to neurons insensitive to cAMP. A$_1$ is a representative action potential recording from a neuron sensitive to cAMP as indicated by reduced firing during cooling. A$_2$ is a representative action potential recording from a neuron dialyzed with cAMP that is insensitive to cAMP as indicated by enhanced firing during cool similar to firing observed in neurons containing control ICF. B. mean $I_h$ density from cAMP-sensitive and cAMP-insensitive neurons indicate that neurons sensitive to cAMP contain higher $I_h$ densities compared to neurons unaffected by cAMP ($p=0.019$; unpaired, two-tailed t test). The enlarged hexagons in B. indicate the $I_h$ density values associated with the representative recordings from A. C. shows that $I_h$ density correlates with firing rate $Q_{10}$ (fold-change over 10°C) in the presence of 100 µM cAMP ($r^2=0.6459$; $p=0.0051$; linear regression). This relationship indicates that neurons with more $I_h$ undergo larger decreases in firing rate upon cooling to 10°C when $I_h$ is initially activated. Gray circles show neurons that are insensitive to cyclic AMP have lower $I_h$ densities and consequently, firing rate $Q_{10}$ values <1 similar to control neurons. In contrast, the black circles indicate neurons that have greater $I_h$ densities exhibit firing rate $Q_{10}$ values >1 when $I_h$ is initially activated. The dotted black line indicates a $Q_{10}$ of 1, or no effect of temperature on firing frequency.

*p<0.05
present and, as a result, had firing rate $Q_{10}s<1$. These findings complement Cs$^+$ antagonist experiments and suggest that when $I_h$ is initially low at physiological $V_m$ [via cAMP+Cs$^+$ (Fig. 17)], cAMP with low $I_h$ density (Figure 18), or closed due to voltage (Fig. 16)] neurons increase excitability when acutely cooled. Conversely, when $I_h$ is activated at rest in neurons that contain a sufficient $I_h$ density, they reduce firing rates upon acute cooling.

**cAMP does not act through $I_h$-independent mechanisms in LC neurons**

In addition to activation of $I_h$, increases in cAMP are linked to activation of protein kinase A (PKA). To assess whether these responses we observed were exclusively due to $I_h$ activation or by additional PKA-dependent mechanisms we performed experiments with the membrane permeable PKA inhibitor, H-89. If a portion of the changes we observed with elevated cAMP occurred as a result of PKA or kinases inhibited by H-89 (Lochner and Moolman, 2006) in addition to $I_h$ activation that we observed, we expected that application of H-89 would result in firing responses that resembled the control condition or cAMP in experiments after applying Cs$^+$. As demonstrated in Fig. 19A (left panel) & 9B, spontaneous firing of neurons at 20°C containing elevated cAMP$_i$ regardless of PKA inhibition was greater compared to control ($p<0.001$; $F_{(2,12)}=12.81$; one-way ANOVA with Tukey’s *post hoc* test). cAMP-dialyzed neurons with and without PKA inhibition did not differ from each other (cAMP: 3.10±0.45 Hz vs. cAMP+H-89: 3.26±0.59 Hz; $p>0.05$). Similar to the results at 20°C, neurons at 10°C containing elevated cAMP$_i$ in the presence and absence of the PKA inhibitor H-89 still resulted in decreased firing rates during cooling (Fig. 19A right panel & 19C) (control:}
Figure 19

A  

**Control**

-54mV

20s

50mV

20°C

100μM cAMP

-54mV

20s

50mV

10°C

100μM cAMP + 10μM H-89

-52mV

20s

50mV

B  

Initial Firing Rate at 20°C (Hz)

Control  cAMP  cAMP + H-89

C  

Firing Rate After Cooling to 10°C (Hz)

Control  cAMP  cAMP + H-89

120
**Figure 19** Protein Kinase A (PKA)-dependent processes are not involved in cAMP sensitivity of LC neurons at 20°C and 10°C. A. 1 minute traces of action potentials representing mean firing frequencies recorded from neurons containing control ICF (first panel), cAMP (second panel), and cAMP in the presence of the PKA inhibitor H-89 (third panel) at 20°C and 10°C. Summary data for 20°C under the three conditions are presented in B (n=5 for all treatments). Increasing cAMP in the presence and absence of PKA inhibition still results in elevated firing frequency compared to control. No differences were observed between neurons containing elevated cAMP with and without inhibition of PKA (n=5; p<0.001; one-way ANOVA with Tukey’s multiple comparisons post hoc test). As indicated by the change in firing rate during cooling, C. shows mean data (n=5 for all treatments) indicating that LC neurons still undergo decreases in firing during cooling in the presence and absence of PKA inhibition with cAMP_i elevated, in contrast to control ICF. Additionally, no differences in firing rate reductions were observed between neurons containing elevated cAMP with and without inhibition of PKA (n=5; p<0.001; one-way ANOVA with Tukey’s multiple comparison post hoc test). **p<0.01; ns=not significant
+1.53±0.50 Hz; cAMP: -2.56±0.62 Hz; cAMP+H-89: -1.95±0.73 Hz; P<0.001; $F_{(2,12)}=12.54$; one-way ANOVA with Tukey’s post hoc test). Therefore, these results indicate that cAMP does not modify excitability at 20°C or 10°C in LC neurons of bullfrogs through PKA-dependent phosphorylation.

Lastly, to identify any further possible cAMP effects independent of $I_h$, we compared firing rate and $V_m$ at 20°C between control and cAMP-containing neurons in the presence of 2mM Cs⁺. Specifically, we used two-tailed unpaired t tests to compare firing rate and $V_m$ data from Cs⁺ exposed neurons in Fig. 15B and 5E. Comparing the firing rates and membrane potentials of these neurons would indicate whether cAMP had other effects independent of $I_h$ (because the analysis was performed in the presence of Cs⁺) and PKA (since we were unable to observe a PKA effect; Fig. 19). Independent of $I_h$, firing rates of neurons dialyzed with cAMP were not different (control + Cs⁺: 0.86±0.4 Hz vs. cAMP+Cs⁺: 0.42±0.21 Hz; p=0.3935; $T_{12}=0.8851$). Similarly, $V_m$ was not different (control + Cs⁺: -59.1±4.4 mV vs. cAMP + Cs⁺: -59.8±1.3 mV; p=0.8972; $T_{12}=0.1320$). These analyses strongly suggest that cAMP does not affect firing and $V_m$ independently of $I_h$ over the time course used in this study. Obtaining cAMP-sensitive responses that persisted during PKA inhibition and were not different from control when $I_h$ was blocked enhances our confidence that cAMP influences firing and cold-sensitivity through its action on $I_h$. 

122
Figure 20
Figure 20  Diagrams illustrating that activation of $I_h$ at 20°C increases firing frequency and offsets cold-activated responses. The top panel shows that increases in $I_h$ at 20°C enhance firing frequency. Pharmacological experiments in current-clamp demonstrated that inhibition of $I_h$ at 20°C was sufficient to reduce firing frequency in the presence of cAMP, but not in control. Therefore, $I_h$ appears to play an excitatory role upon its activation at 20°C. During cooling (bottom-left panel) LC neurons increase firing frequency and depolarize by ~6.5 mV. $I_h$ does not likely contribute to this response because $V_m$ lies positive to the activation $I_h$. Since $I_h$ is closed due to voltage at rest, we include the contribution of $I_h$ as a 0mV to the cooling response. In contrast to control, $I_h$ contributes to the firing frequency and $V_m$ in the presence of cAMP. Pharmacological experiments in current-clamp showed that inhibition of $I_h$ at a greater starting value resulted in ~6 mV membrane hyperpolarization. Because cooling inhibits $I_h$, we estimate that inhibition of $I_h$ by cooling contributes 6 mV of hyperpolarization during the cooling stimulus that is not present under control circumstances. This hyperpolarizing influence emanating from inhibition of a greater starting $I_h$ appears to be sufficient to offset the depolarization associated with cooling and therefore reverse normal elevated firing frequency.
DISCUSSION

We performed these experiments to gain a greater understanding of the ionic basis for regulating firing frequency during cooling in neurons putatively involved in ventilatory control of amphibians. We hypothesized that $I_h$ would contribute to spontaneous firing, but act as a brake to dampen the magnitude of cold-activation typical of LC neurons. Although $I_h$ had marginal basal activity, this hypothesis was supported because activation of $I_h$ by an endogenous agonist, cAMP, elevated firing frequency of neurons (at baseline temperature), but surprisingly, led to decreased firing—contrary to normal increases—during cooling. The interaction between temperature and various neurophysiologic mechanisms including the ionic basis of the action potential (Hodgkin and Katz, 1949), synaptic transmission (Katz and Miledi, 1965), firing frequency (Boulant, 1998), molecular thermosensing (Dhaka et al., 2006), and circuit behavior (Tang et al., 2010) has received attention. This study provides new insight by demonstrating that the direction of temperature-dependent firing can be manipulated by altering a single membrane conductance; specifically, $I_h$.

**Activation of $I_h$ contributes to enhanced excitability at 20°C**

We previously identified electrophysiological evidence of $I_h$ in LC neurons (Santin et al., 2013). This study confirms that $I_h$ in bullfrogs is similar to $I_h$ carried by HCN channels in excitable cells from other animals (Biel et al., 2009). We show that $I_h$ in LC neurons displays inward rectification, has a mean reversal potential ($E_h$) near -40 mV (Fig. 11), exhibits sensitivity to ZD7288 and Cs$^+$ at low
millimolar concentration (Fig. 12), and undergoes a depolarizing shift in voltage-dependent activation with elevated cAMP (Fig. 13). These characteristics indicate that $I_h$ is presumably caused by HCN channel expression. Moreover, because cAMP induced a $\sim +15$ mV depolarizing shift in the $V_{0.5}$, our results suggest that $I_h$ is carried by channels similar to mammalian HCN isoforms 2 or 4, as opposed to the modestly cAMP-sensitive HCN 1 or 3 (Biel et al., 2009; Moroni et al., 2000; Wang et al., 2001).

To elucidate the role of $I_h$ in regulating firing frequency we exposed LC neurons to 2 mM Cs$^+$. Because application of 2mM Cs$^+$ had no net effect on firing rate and $V_m$ in LC neurons containing control pipette solution (Fig. 15A, B, &C), we conclude that $I_h$ plays, at most, a small role in regulating membrane potential and spontaneous firing under control conditions. These results corroborate a study indicating that $I_h$ plays little to no role in determining membrane potential and sensitivity to light in intrinsically photosensitive retinal ganglion cells, most likely because $V_m$ lies positive to the base of the $I_h$ activation curve (Van Hook and Berson, 2010). Furthermore, $I_h$ plays a minimal role in determining membrane potential and thermosensitivity of peripheral thermoreceptive neurons (Orio et al., 2009); however, $I_h$ seems to play a role in determining subthreshold resonance of cold thermoreceptors and may control oscillation frequency of sensory nerve endings (Orio et al., 2012). The control firing responses to Cs$^+$ that we observed were, admittedly, variable (Fig. 15B). Thus interneuron variation in $I_h$ density (Fig. 18), the neuromodulatory environment capable of altering $I_h$ activation at physiological $V_m$ (Peck et al.,
2006), or the strength of an off target effect of Cs⁺ that we were not able to define may determine control firing.

In direct contrast to neurons containing control pipette solution, Iₜₜ clear contributes to spontaneous firing in neurons when activated by elevating cAMP (Fig. 15D, E, & F). As predicted by activating Iₜ (a conductance with a reversal potential positive to Vₘ), mean firing frequency was greater in the presence of cAMP (Fig. 4) and exposure to 2mM Cs⁺ reduced the firing rate and hyperpolarized the membrane in each neuron tested (Figs. 15 E&F). This result implies 1.) that Cs⁺ is reasonably selective antagonist of Iₜ in LC neurons and 2.) similar to other studies (Hawkins et al., 2014; Okamoto et al., 2006), relatively small increases in Iₜ at the base of its activation curve can influence the firing frequency presumably through membrane depolarization (Figs. 15D, E, & F). Therefore, Iₜ plays little to no role in setting firing frequency and Vₘ under normal conditions, but exerts a uniform, excitatory influence when activated due to a depolarizing shift in voltage-dependent activation.

Elevated resting Iₜ induced by cAMP leads to reduced excitability during acute cooling

Under control conditions, nearly all LC neurons increase firing frequency during acute cooling. At present, we do not know the mechanisms that enable cold-activation of LC neurons. Several thermosensitive ion channels or transporters could provide the substrate for cold-activation (Sengupta and Garrity, 2013) and previous work has demonstrated that the conductance
densities of both thermosensitive and non-thermosensitive ion channels can shape firing responses during temperature changes (Madrid et al., 2009; Viana et al., 2002). To achieve greater spontaneous firing frequencies with cooling as observed in majority of LC neurons, cold must enhance depolarizing factors to outweigh inhibitory effects on action potential generation (Hodgkin and Katz, 1949) associated with cold temperatures. This is illustrated in our summary diagram shown in Fig. 10 (left panel) as a ~6.5 mV membrane depolarization (measured in this study; Fig. 16) induced by an unidentified, cold-sensitive inward current ($I_{\text{cold}}$). Previous data indicated that $I_{\text{cold}}$ is intrinsic to LC neurons and preliminary results suggest that $I_{\text{cold}}$ is mediated by a background conductance that sets the membrane potential (J.M. Santin & L.K. Hartzler, preliminary observation).

We hypothesized that $I_h$ would function physiologically to blunt the magnitude of the cold response because this depolarizing current is inhibited by cooling (Santin et al., 2013; Fig. 13); however, a lack of $I_h$ activation at physiological $V_m$ precludes its ability to influence cold activation. In support of this notion, cold sensitivity was unaffected by 2mM Cs$^+$ in neurons containing control solution in the patch pipette. Therefore, $I_h$ does not contribute to a change in membrane potential during cooling and plays no role in regulating the normal cold-activated behavior of LC neurons. This is represented as 0 mV $\Delta V_m$ in the summary diagram (Fig. 20; left panel).

While $I_h$ did not play a role in determining cold-activation under control circumstances, our hypothesis was supported when $I_h$ was initially activated by
dialyzing neurons with elevated concentration of cAMP. Although we predicted a reduction in the magnitude of cold-activation based on the fact that $I_h$ was now making a depolarizing contribution to firing rate and $V_m$ (Figure 15), we unexpectedly found that activation of $I_h$ led to cold-inhibition rather than cold-activation. We arrived at this conclusion because neurons containing elevated cAMP reduced firing frequency upon cooling (Fig. 16) and inhibition of $I_h$ was sufficient to revert cold-inhibited responses back to cold-activated responses that were similar to control neurons (Fig. 17). In support of this pharmacological experiment, neurons with low $I_h$ densities were insensitive to cAMP and exhibited cold-activated responses similar to the control neurons. By contrast, neurons with increasingly greater $I_h$ densities underwent more inhibition with cooling in the presence of cAMP (Figs. 18 B&C), suggesting that cAMP had a more substantial effect in neurons containing greater $I_h$ density. Because of causal (blocking the activated $I_h$ rescued cold-activation) and correlative ($I_h$ density scaled proportionally with sensitivity to cAMP) evidence, our data indicate that augmenting the initial, baseline amount of $I_h$ is sufficient to convert cold-activation to cold-inhibition.

The most parsimonious interpretation of our data is that enhancing the initial amount of $I_h$ active at the physiological membrane potential allows it to be inhibited by cooling, which opposes the $V_m$ depolarization caused by the currently unidentified, cold-depolarizing factors. An estimation of the effects of $I_h$ inhibition on the membrane potential due to cooling was extrapolated from the membrane hyperpolarization caused by Cs$^+$ presented in Fig. 15F and included in the
diagram in Fig. 20 (right panel). We determined that inhibition of \( I_h \) in the presence of cAMP resulted in a ~6mV hyperpolarization (Fig. 15F). We therefore assume that inhibition of \( I_h \) by cooling also contributes ~6 mV of hyperpolarization to the membrane during cooling which was absent from control neurons. Thus greater initial \( I_h \) at 20°C (Fig. 20 left panel) evokes a membrane potential response to cooling that now includes a 6 mV hyperpolarization that offsets the ~6.5 mV depolarization by \( I_{\text{cold}} \), resulting in a net depolarization of <1 mV instead of ~6.5 mV as occurs in control neurons (Fig. 20; right panel). This prediction of the membrane potential response to cooling in neurons dialyzed with cAMP based on findings from Cs\(^+\) experiments that account for added \( I_h \) inhibition (\( \Delta V_m <1 \) mV) is similar to the membrane potential changes measured during cooling in the presence of cAMP (\( \Delta V_m = 1.5 \) mV Fig. 16). Although LC neurons dialyzed with cAMP still underwent modest membrane depolarization (1.5 mV) accompanied by reduced firing frequency during cooling, a similar response involving small membrane depolarization with reduced firing frequency has been observed in *Aplysia* ganglion and rat hypothalamic neurons (Carpenter, 1967; Griffin and Boulant, 1995). Additionally, since \( I_h \) inhibition was sufficient to rescue cold-activation in the presence of cAMP (Fig. 17G) our data do not suggest that cAMP influences thermosensitivity by manipulating \( I_{\text{cold}} \). Stated simply, we conclude that increasing the contribution that \( I_h \) makes to the membrane potential before cooling enables its hyperpolarizing influence upon inhibition by cold temperature to offset normal cold-activated responses.

**Lack of \( I_h \)-Independent Actions of cAMP**
There are currently no selective pharmacological activators of \(I_h/HCN\) channels. To fully address our hypothesis and study baseline firing and cooling responses with \(I_h\) activated, we dialyzed neurons with an endogenous agonist, cAMP. We recognized that cAMP is not a specific agonist of \(I_h\) and that a portion of the changes we observed in the presence of cAMP could have been due to activation of other cAMP dependent process (e.g., activation of PKA). Three lines of evidence indicate that differences in spontaneous firing and cold responses in the presence of cAMP were caused by action on \(I_h\) and not \(I_h\) - independent processes. First, spontaneous firing rates and cold responses did not differ in the presence of the PKA inhibitor, H-89. If a component of the changes we observed in neurons dialyzed with cAMP was caused by PKA phosphorylation, we would have expected inhibition of PKA to cause spontaneous firing and cold responses to resemble the control condition [as occurred during application of Cs\(^+\) (Figs. 15 & 17)]. Second, to rule out unaccountable factors independent of \(I_h\), we compared the firing rates at 20°C in neurons dialyzed with control and cAMP pipette solutions with \(I_h\) blocked using Cs\(^+\). If other factors, outside of \(I_h\), were influencing the firing frequency in neurons dialyzed with cAMP, we would have expected firing rates to differ when comparing these groups of neurons. We found that the firing rates did not differ between neurons containing control and cAMP pipette solutions when \(I_h\) was blocked (\(p \approx 0.4\)). Lastly, we showed that cold-inhibited responses in the presence of cAMP were converted back to cold-activated responses when we blocked \(I_h\) during a second cold exposure. To ensure that cold-activation was
restored due to $I_h$ inhibition by Cs$^+$ application *per se* and not time-sensitive processes that could be activated by cAMP independently of $I_h$, we performed a time control experiment (Fig. 17F). In this series of experiments neurons dialyzed with cAMP were cooled twice in aCSF. If time-dependent processes associated with prolonged dialysis and not inhibition of $I_h$ were responsible for rescuing cold-activation in the presence of cAMP, we would have expected the second exposure of the time control to resemble the cold-activated responses of both control experiments and cAMP experiments performed in the presence of Cs$^+$. As shown in Fig. 17F, the second cold exposure in the time control experiment resulted in reduced firing frequency nearly identical to the first cooling ramp. The time control experiment provides us with confidence that Cs$^+$ application and its probable action on the enhanced initial amount of $I_h$ *per se* is responsible for restoring cold-activated responses in neurons dialyzed with cAMP. Although these control experiments and analyses did not detect any $I_h$-independent effects of cAMP dialysis on baseline firing and cold responses over the time course used in these experiments, cAMP may have had $I_h$ independent effects on cellular processes that did not influence the electrical parameters measured in this study.

**Insights into the amphibian respiratory control system**

LC neurons provide a CO$_2$/pH-chemosensitive drive to breathe in the respiratory network of amphibians (Noronha-de-Souza et al., 2006). Since cold-activation is a property of chemosensitive neurons (Santin et al., 2013), we speculate that LC activity inversely proportional to temperature may stabilize
respiratory parameters during changes in temperature. Our results demonstrating that initial activation of $I_h$ was sufficient to reverse normal cold responses could be important for regulating breathing during changes in temperature. Although the physiological effectors of $I_h$ activation are unidentified in LC neurons from bullfrogs, $I_h$ is activated by several mechanisms that are likely to interact with chemosensitive LC neurons. For example, a soluble form of adenylyl cyclase (sAc) that senses bicarbonate and produces cAMP is expressed (Nunes et al., 2014) and functional (Imber et al., 2014) in chemosensitive LC neurons of rats. Since LC neurons from frogs and rats exhibit similar responses to CO$_2$/pH changes (Santin and Hartzler, 2013a), a sAc-dependent process may elevate cAMP and in turn, activate $I_h$. Additionally, nitric oxide (NO) signaling has been shown to activate $I_h$ (Wilson and Garthwaite, 2010) and also facilitate respiratory discharge from the bullfrog brain stem (Harris et al., 2002; Hedrick and Morales, 1999). We speculate that activation of $I_h$ by NO signaling within the LC may contribute to portion of the excitatory action of NO in the respiratory network of amphibians. The significance of NO signaling on temperature sensitivity of respiratory output has not been addressed, but would be interesting to explore within the context of our findings. These are two physiologically conceivable mechanisms through which $I_h$ could be activated in LC neurons to influence breathing. However, the involvement of cold-activation of LC neurons and its modulation by the battery of factors that interact with $I_h$ (reviewed in Biel et al. 2009) remain unidentified and provide a mechanistic framework for future studies into anuran respiratory control.
General implications for considering tissue temperature in electrophysiological studies

Although the specific mechanisms that physiologically activate $I_h$ in LC neurons of bullfrogs are currently unidentified, we would like to draw attention to the physiological significance associated with considering changes in tissue temperature that are highlighted by this study. These findings provide a unique, perhaps extreme, example demonstrating that the result of $I_h$ activation on firing frequency is non-linear across temperatures (Figs. 15 and 17). Specifically, an increase in firing rate induced by $I_h$ activation that occurred in LC neurons at 20°C transformed firing rate changes in response to cooling from excitatory to inhibitory. Our diagram (Fig. 20), constructed by summing changes in the membrane potential measured under different conditions in this study, suggests that this non-linear effect can be accounted for by enhancing the amount of $I_h$ that contributes to the membrane potential under baseline conditions. Consequently, activation of $I_h$ at baseline results in additional hyperpolarizing pull when cooled due to its inhibition that is not present when $I_h$ is not initially activated under control circumstances. This finding illuminates an important point regarding the effects of $I_h$ activation on activity of single neurons subjected to a poikilothermic neuronal environment; a conclusion drawn at one temperature may not translate to the range of temperatures experienced by those neurons. We assert that accounting for the possibility that $I_h$ activation- or perhaps other membrane conductances- alters firing responses to temperature changes (i.e., blunts, in the
case of this study) in the opposite direction of that which occurs at a stable
temperature (i.e., enhances) is of greater significance than generally appreciated.

Most animals are poikilothermic; that is, they can undergo relatively large
and variable changes in body and brain temperature that depends on season,
environmental condition, microhabitat, time of day, and body size (Stevenson,
1985a; Stevenson, 1985b). Although less obvious, even homeothermic animals
that generally regulate a core body temperature have a physiological range of
brain temperatures (~35°C-39°C) that fluctuates during changes in behavioral
states in vivo and to a greater extent during pathological conditions (Kiyatkin,
2005; Kiyatkin, 2011; Kiyatkin, 2013). Firing frequency of neurons from brain
regions of rodents that express HCN channels including the hippocampus
(increases with cooling), the hypothalamus (increase with warming), and the
substantia nigra pars compacta (increase with warming) exhibit high temperature
sensitivity near this physiological range (de la Peña et al., 2012; Dean and
Boulant, 1989; Guatteo et al., 2005). Thus to a greater or lesser extent, altered
activity of neurons that contain I\textsubscript{h}/HCN channels over a shifting thermal
background is a common phenomenon among many animals, poikilothermic and
homeothermic alike.

The temperature change used in this study (Δ10°C; 20°C to 10°C) is
physiological, and perhaps a mild stimulus, for bullfrogs and many other
poikilothermic animals (Hutchison and Dupre, 1992; Stevenson, 1985a;
Stevenson, 1985b). We have no reason to believe that the modulatory state of I\textsubscript{h}
would affect temperature-dependent firing differently in homeothermic animals
that also experience changes in brain temperature, albeit over a narrower range.

Our results emphasize that considering the possibility of fluctuations in local tissue temperature should be obligatory when extrapolating mechanisms identified in single neurons and networks in vitro to control of behaviors in vivo. Since in vitro experiments from various preparations are often performed under isothermal or even hypothermic conditions (Windels, 2006), the outcomes of experiments intended to probe the effects of ion channel modulation may overlook a physiologically relevant range of firing responses arising from non-linear interactions between activation state of a particular membrane current (e.g., $I_h$) and changes in tissue temperature.

** Perspectives and Significance **

We demonstrated that enhancing $I_h$ under baseline conditions was sufficient to stimulate firing, but led to a reversal of cold-activated firing responses during temperature changes. Given that HCN channels are regulated by various intra and extracellular constituents including cyclic nucleotides, ions, lipids, and interacting proteins (Biel et al., 2009), our results present the possibility that many factors could modulate temperature sensitivity of neuronal firing through actions on HCN channels. This implication is apparent for poikilothermic animals, like bullfrogs used in this study, whose entire nervous system is likely to undergo large changes in temperature, but might also have significance for mammalian nervous systems that also undergo changes in temperature during various physiological and pathological conditions. Results from the present study, therefore, underscore the importance of HCN channel
modulation state as a critical regulator of neuronal activity with effects contingent on changing neuronal temperature.
CHAPTER V
MANUSCRIPT III

Reassessment of chemical control of breathing in undisturbed bullfrogs, Lithobates catesbeianus, using measurements of pulmonary ventilation


Santin, J.M. & Hartzler, L.K.
Abstract

Despite the importance of bullfrogs (*Lithobates catesbeianus*) as models in respiratory control, chemical control of breathing in conscious bullfrogs has never been assessed with methods that measure the tidal volume ($V_T$). This has precluded the calculation of important respiratory variables like minute ventilation ($\dot{V}_E$) and air convection requirement. To address this, we adapted airflow pneumotachography for use in bullfrogs and reassessed chemical control of breathing. We show that $\dot{V}_E$ measured with pneumotachography produces breathing pattern and metabolism values consistent with anurans. Second, we confirm that bullfrogs have small ventilatory responses to hypercarbia that include increases in tidal volume and a post-hypercarbic hyperpnea. We observed that the magnitude of the post-hypercarbic hyperpnea does not depend on ventilatory responses during hypercarbia. Finally, we showed that increases in breathing frequency and $V_T$ during hypoxia are differentially regulated with time. These findings comprise the first complete assessment of hypercarbic and hypoxic $\dot{V}_E$ responses in intact bullfrogs and emphasize the importance of measuring $V_T$.

1. Introduction

Comparative studies serve an important role in the understanding of respiratory control. Determining mechanisms that control breathing in non-model organisms, specifically ectotherms, have provided 1.) insight into phylogenetic trends in the respiratory network (Kinkead, 2009; Milsom, 2010; Wilson et al.,
2006), 2.) robust in vitro preparations that operate under physiological conditions (Hedrick, 2005; Johnson et al., 1998; Santin and Hartzler, 2013a), 3.) tools to elucidate developmental aspects of respiratory control (Hedrick, 2005), and 4.) systems to reveal how environmental factors affect animals during ecological change (da Silva et al., 2013). Although several ectothermic species serve as alternative models, the American bullfrog, *Lithobates catesbeianus*, has provided much of the comparative insight into central mechanisms of respiratory rhythm generation/pattern formation (Galante et al., 1996; Gargagliani et al., 2007; Wilson et al., 2002; Winmill and Hedrick, 2003) and central chemosensing (Fournier and Kinkead, 2008; Santin and Hartzler, 2013a; Taylor et al., 2003b; Winmill et al., 2005). The mechanistic information gained from bullfrogs has been obtained mainly by isolating aspects of the respiratory control system at various degrees of reduction ranging from decerebrated/paralyzed frogs to brain slices. Bullfrog preparations have been essential to uncovering comparative aspects of respiratory control; however, when identifying mechanisms in reduced preparations, it is imperative to interpret identified mechanisms in the context of the conscious, ventilating animal.

Determining minute ventilation ($\dot{V}_E$) requires the measurement of breathing frequency ($f$) and tidal volume ($V_T$) ($\dot{V}_E = f \times V_T$). Whole-body plethysmography can be used as a non-invasive method to estimate $V_T$ and determine $f$ in endotherms, but cannot be used in ectothermic animals such as bullfrogs. As an alternative approach, Glass et al. (1978) adapted airflow pneumotachography for use in
small ectotherms, which became the “gold standard” for measurements of ventilation in unrestrained ectotherms. Airflow pneumotachography enhanced the understanding of control of breathing in a wide-range of terrestrial/semi-terrestrial air-breathing ectotherms including other anurans, specifically; toads (Gargaglioni and Milsom, 2007). However, skin secretions in frogs which are exacerbated by handling stress (Benson and Hadley, 1969) have made it difficult to apply this technique as typically performed in toads (see illustration in Jones (1982), J.M Santin, personal observation)). This has precluded the measurement of $V_T$ and thus pulmonary ventilation in bullfrogs. Therefore, the mechanistic data that have been obtained from reduced preparations in bullfrogs have been interpreted based on $V_E$ measured in toads or by using less direct indices of ventilation in bullfrogs.

Anurans ventilate the lungs through generation of positive pressure which allows breathing in unrestrained bullfrogs to be estimated by measuring changes in buccal pressure that occur during lung inflation/deflation (Jones, 1970; Kinkead and Milsom, 1996; Kinkead and Milsom, 1994). Although measurements of buccal pressure signals provide useful information about the respiratory pattern, the integral or amplitude of this signal does not correlate linearly with tidal volume, limiting the ability to accurately index minute ventilation based on measurements of buccal pressure (Wang, 1994). Given this caveat, Milsom et al. (1999) cautioned about the possibility of misinterpreting results from studies in bullfrogs using buccal pressure as a proxy for $V_T$. Since measurements of buccal pressure do not allow the determination of ventilated
volumes, the influence of metabolic changes on breathing control during relevant stimuli (e.g., hypoxia and hypercapnia) cannot be resolved through calculation of the air convection requirement ($V_e/V_{O_2}$ and $V_e/V_{CO_2}$) (Wang, 1994). Other methods that do not directly measure tidal volume including body wall impedance measurements and “photoplethysmography” or those that determined tidal volume, but not total gas exchange have also been employed in anurans (Vitalis and Shelton, 1990) (McAneney et al., 2006; Vasilakos et al., 2005). Therefore, the present study was undertaken to reassess chemical control of breathing in bullfrogs by directly measuring $V_T$, $f$, and $V_e$ with airflow pneumotachography as has been performed in other ectothermic vertebrates including toads (Branco et al., 1993; Gargaglioni and Branco, 2003; Kruhøffer et al., 1987; Noronha-de-Souza et al., 2006) simultaneously with open-flow respirometry. Here, we adapted the methods for airflow pneumotachography in small ectothermic vertebrates developed by Glass et al. (1978) for use in bullfrogs while measuring $O_2$ consumption ($V_{O_2}$) and $CO_2$ production ($V_{CO_2}$) using open flow respirometry in room air, hypercapria, and severe hypoxia. The use of pneumotachography would enable us to establish whether or not hypercapnia and hypoxia elicited changes in tidal volume and $V_e$, in addition to their previously reported effects on breathing frequency (Kinkead et al., 1994; Kinkead and Milsom, 1996; Kinkead and Milsom, 1994; Rocha and Branco, 1997; Taylor et al., 2003a) and improve the understanding of respiratory control in this common comparative model.
2. Materials and Methods

2.1 Animals

Adult bullfrogs, *Lithobates catesbeianus* (n=6; 106±5 g) were kept in tanks containing ~22 °C water and had access to wet and dry areas. Frogs were exposed to 12:12-h light-dark cycles and fed crickets twice per week. Food was withheld for 3 days before experiments. Experiments were approved by the Wright State University Institutional Animal Care and Use Committee.

2.2 Construction of the Facemask and Measurements of Ventilation

Similar to ventilation measurements from toads and other ectothermic vertebrates, we measured ventilation using air flow pneumotachography as previously described (Glass et al., 1978). Although Glass et al. (1978) show a representative ventilation recording from a bullfrog using their method, we were unable to keep this version of the mask fixed and sealed to the face of the frog for a long enough time period to obtain resting ventilation and perform gas exposure experiments (~24hr total) using this facemask. The two main issues with this facemask were 1.) leaks formed due to skin secretions between the eyes where the pneumotach rests (Jones, 1982) and 2.) a mask of ~2-3g was too heavy to remain stable when the animal moved in the chamber. We observed that the skin secretions around the nostrils were minimal compared to the rest of the head of the animal. Thus, we circumvented mask leaks due to skin
secrections by manufacturing a lighter, smaller facemask/pneumotach that was able to isolate the nostrils and used moderate surgical intervention to keep the mask in place.

To construct the mask (see cartoon in Figure 21A), we made a mold of the head of an anesthetized 100g bullfrog using Mold Gel (ArtMolds, Summit, NJ, USA) and Plaster of Paris (DAP, Baltimore, MD, USA). To create the mask, we found that the bulb of a 3 mL transfer pipette (Samco Scientific Corporation, Cat. No. 225-15, San Fernando, CA, USA) was nearly the correct size and shape to fit over the nostrils. We precisely formed the pipette bulb to the topography of the area around the nostrils using heat to melt the plastic around the mold. We constructed a pneumotach using two small tubes with a nylon screen in the middle which functioned as the resistor. Two 23 gauge needles were glued on either side of the resistor so that the pneumotach could be connected to a differential pressure transducer. The pneumotach was sealed into the top of the mask and calibrated after each experiment by injecting known volumes of air through the mask while connected to the mold of the frog’s head. Flow through the pneumotach produced a linear relationship between the integrated differential pressure signal (used to calculate tidal volume) and injection volume over a range of volumes (0.1 mL-1.2 mL) (Figure 21C; \( r^2 = 0.99 \); n=6) at a flow rate approximating that of the breathing frog.

We used a combination of minor surgical intervention and super glue to attach the mask to the face of the frog. The evening before an experiment, a bullfrog was anaesthetized in ~0.5% (v/v) isoflurane until the loss of a toe pinch
Figure 21

A

To Differential Pressure Transducer

B

1 cm

C

Integrated Differential Pressure Signal (arb. units)

Injection Volume (mL)

$r^2 = 0.99$
**Figure 21** A. Cartoon illustration indicating the position of the facemask/pneumotach covering the snout of the bullfrog. B. Photograph of the facemask containing the embedded pneumotach detached from the snout. C. Calibration showing a linear relationship between the integrated differential pressure signals in response to various volumes of air injected through the pneumotach (n=6; $r^2=0.99$; linear regression).
and eye reflex. Three sutures were used to secure the mask to the face of the animal; two sutures around the lateral maxilla bone through the mouth and one through the skin on the caudal portion of the snout. This procedure usually took ~5 minutes. The mask and mouth were then sealed with cyanoacrylate glue (Loctite Super Glue Gel; Westlake, OH, USA) to ensure that all nasal airflow moved through the pneumotach. After the glue dried, the pneumotach was connected to a differential pressure transducer (TSD 160A differential pressure transducer Biopac Systems Inc., CA, USA) by PE50 tubing and data were collected using a Biopac acquisition system (Biopac MP 150 and Acknowledge software; Biopac Systems Inc., CA, USA).

Because leaks between the mask and the frog were a problem in previous versions of the mask, we took special care to ensure no leaks were present by puffing air around the edges of the mask. If a pressure signal was observed when puffing air on the outside of the mask, the leak was filled by adding a small amount of glue. We had some concern that the mask would not remain sealed throughout the duration of the experiment due to movement of the animal within the experimental chamber. Occasionally, large amounts of noise could be observed on the differential pressure recording, indicating that the animal had moved in the chamber. When this occurred, the frog was removed from the chamber and the mask was, again, checked for leaks. Under circumstances of large and vigorous movement by the animal, the mask always remained sealed, indicating that our mask was robust against movement in the chamber. The 875 mL experimental chamber was flushed with fully humidified room air (1.1 L/min;
24 °C) was covered by a cloth for ~15-18 hours until the start of the experiment the next day. We did not include a water phase in our experimental chamber; however, bullfrogs did not show any evidence of dehydration since they were moist and expelled large volumes of urine during handling following the experiment.

2.3 Measurements of $O_2$ Consumption and $CO_2$ Production

$O_2$ consumption ($\dot{V}o_2$) and $CO_2$ production ($\dot{V}co_2$) was measured simultaneously with ventilation using open-flow respirometry. Room air, 5% $CO_2$/air balanced, or 5% $O_2$/nitrogen balanced (depending on the experiment) were pushed at 1.1 ml/min through a flask half-filled with water to fully humidify the gas. Subsamples of humidified incident gases were then pulled through the sealed acrylic chamber containing the animal at 200ml/min by a suction pump (AEI Technologies Inc., PA, USA). Excurrent gases were pulled, in series, through a 4100 series mass flow meter (TSI Inc., MN, USA), a $CO_2$ analyzer (CD-3A; AEI Technologies Inc., PA, USA), a 10 mL syringe filled with desiccant (DM-AR; Perma pure LLC, NJ, USA), and finally an infrared $O_2$ analyzer (S-3A/I; AEI Technologies Inc., PA, USA).

2.4 Experimental Protocol

Experiments were performed between the hours of 09:00-15:00. The day after attaching the mask (~15-18 hr later), room air was pulled through the respirometry chamber containing the animal at 200 mL/min to establish resting ventilation and metabolism. To minimize the number of animals used, we
performed 30 minute hypercarbia (5% CO$_2$/ balance air) and hypoxia (5% O$_2$/ balance nitrogen) exposures on the same animals in random order approximately 2-3 hours apart. This time period was suitable for recovery of ventilation and metabolism from subsequent gas exposures (see RESULTS). To reduce the time required to replace the gaseous environment in the chamber, we elected to push the humidified gas at 1.1 L/min for the first 5 minutes each gas treatment (hypercarbia or hypoxia). We had a concern that abruptly altering the flow when exchanging the gaseous environment would induce changes in ventilation independent of the gas mixture; however, when switching between 1.1 L/min and the experimental flow 200 mL/min prior to beginning the experiment did not affect breathing frequency (personal observation). Given that sampling periods were after the frog had reached steady state, the only time abrupt changes in flow could affect interpretation of the results was in the post hypercarbic period where we observed large increases in ventilation. To control for this we also analyzed ventilation in the "post hypoxic" period and observed a rapid trend toward recovery; therefore, we are confident that the flow changes in our experiment required to reduce the time constant of the chamber did not affect breathing in a way that alters the interpretation of our results.

2.5 Data Analysis

Resting ventilation and metabolism (n=6) were determined for 15 minutes before switching to the experimental gases. In each experimental gas, ventilation was measured in 5 minutes sampling periods, every 10 minutes, to establish time courses of ventilatory responses to each gas mixture. Additionally,
ventilation in the first 2 minutes post-hypercarbia and post-hypoxia periods were analyzed. $\dot{V}O_2$ was measured in the last 5 minute sampling period of hypoxia and hypercapnia (n=4).

Lung breaths were identified by the peak analysis function on LabChart (AD Instruments Inc. CO, USA) as previously shown and described for anurans (Jones, 1982; Vitalis and Shelton, 1990). Specifically, lung breaths were typically large and contained a biphasic expiratory flow signal, while ventilations of the buccal cavity were always smaller than lung breaths (although variable in amplitude) and monophasic. We integrated the expiratory flow signal associated with lung breaths and interpolated these values into the calibration curve produced for each animal (Figure 1C) to determine tidal volume ($V_T$) and then converted each volume to standard temperature pressure dry (STDP).

Ventilation ($\dot{V}_E$) was determined by multiplying $f$ by $V_T$. Respiratory pattern parameters including the episodes frequency, breaths per episode, instantaneous frequency, and duration of the non-ventilatory period were determined using criteria summarized previously (Noronha-de-Souza et al., 2006).

$\dot{V}O_2$ and $\dot{V}CO_2$ were obtained by determining fractional differences between inspired and expired $O_2$ and expired and inspired $CO_2$, respectively, multiplied by the flow rate, and expressed as STPD. The air convection requirement (ACR) was calculated by dividing $\dot{V}_E$ by either $\dot{V}O_2$ or $\dot{V}CO_2$. Because we did not measure
Vo$_2$ in the post hypercarbic sampling period, ACR for the post hypercarbic hyperpnea was calculated using the Vo$_2$ measurement made in the hypercarbia immediately before switching to room air.

2.6 Statistics

Data are presented as mean ±SEM. Statistics were computed using Graphpad Prism Version 6.01 (GraphPad Software, San Diego CA). Differences between 3 or more means at multiple time points were analyzed using a repeated measures (RM) ANOVA with Dunnett’s multiple comparisons test. Differences between two means were analyzed using a two-tailed paired t test and two-tailed unpaired t test as appropriate. Statistical significance was accepted when p<0.05.

Pertinent statistical information is included in the results section.

3. Results

3.1 Resting ventilation and metabolic parameters

The first goal of this study was to establish pneumotachography as a method for use in bullfrogs. Resting values for ventilatory and metabolic parameters (n=6) are presented in Table 3.
**Table 3** Resting ventilatory and gas exchange parameters in bullfrogs (n=6) at 24°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Frequency ($f_{R_{abs}}$; br min$^{-1}$)</td>
<td>4.89 ± 0.67</td>
</tr>
<tr>
<td>Tidal Volume ($V_t$; mL STPD kg$^{-1}$)</td>
<td>4.00 ± 0.48</td>
</tr>
<tr>
<td>Minute Ventilation ($V_E$; mL STPD min$^{-1}$ *kg$^{-1}$)</td>
<td>20.42 ± 4.58</td>
</tr>
<tr>
<td>Episode Frequency (Minute$^{-1}$)</td>
<td>3.20 ± 0.54</td>
</tr>
<tr>
<td>Breaths/Episode</td>
<td>1.57 ± 0.14</td>
</tr>
<tr>
<td>Instantaneous Breathing Frequency ($f_{R_{ins}}$)</td>
<td>46.91 ± 3.84</td>
</tr>
<tr>
<td>Non-ventilatory period (s)</td>
<td>23.30 ± 3.69</td>
</tr>
<tr>
<td>$V_{O_2}$ (mL $O_2$ STPD min$^{-1}$ *kg$^{-1}$)</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>$V_{CO_2}$ (mL $CO_2$ STPD min$^{-1}$ *kg$^{-1}$)</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>$\dot{V}<em>E/\dot{V}</em>{O_2}$ (mL air mL $O_2$)</td>
<td>24.77</td>
</tr>
<tr>
<td></td>
<td>±5.20</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>$\dot{V}_E/\dot{V}co_2$ (mL air mL CO$_2$⁻¹)</td>
<td>28.73</td>
</tr>
<tr>
<td></td>
<td>±6.06</td>
</tr>
<tr>
<td>RER ($\dot{V}co_2/\dot{V}o_2$)</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>±0.04</td>
</tr>
</tbody>
</table>
Ventilatory and metabolic responses to hypercarbia

Fig. 22 shows the time course of the ventilatory response to elevated inspired CO₂ (5%) in bullfrogs (n=6). Hypercarbia led to slight increases in Vₜ after 20 and 30 minutes (Figure 22B; RM-ANOVA, p=0.0224 F_(4,20)=3.624; 20 min.- p<0.05, Dunnett’s multiple comparisons test; 30 min.- p<0.01, Dunnett’s multiple comparisons test); however, mean frequency and \( \dot{V}_E \) were not significantly changed (Figure 22A and C; p>0.05; Dunnett’s multiple comparisons test). As described previously (Kinkead and Milsom, 1996), bullfrogs in our study experienced a large increase in frequency (p<0.0001; RM-ANOVA; \( F_{4,20}= 15.94; \) p<0.0001; Dunnett’s multiple comparisons test) and \( V_E \) (p=0.0001; RM-ANOVA; \( F_{4,20}= 11.27; \) p<0.0001 for Dunnett’s multiple comparisons test) in the 2 minutes immediately following removal of the hypercarbic gas mixture. In the post hypercarbic period, Vₜ started decreasing to near-control values (p>0.05; Dunnett’s multiple comparisons test). The effect of CO₂ and post hypercarbic exposure on breathing pattern is included in Table 4.

The post hypercarbic hyperpnea resulting from increases in f is consistent with responses for bullfrogs established by Kinkead and Milsom (1996), but we report here that ventilatory responses to elevated inspired CO₂ show high animal-to-animal variability. As illustrated by the representative differential pressure traces shown in Figure 23A, ventilatory responses during hypercarbia separated into two groups; one group that had robust increases in ventilation
<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>Hypoxia (n=6)</th>
<th>Hypercarbia (n=5)</th>
<th>Post Hypercarbia (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Episodes min⁻¹</td>
<td>3.20 ±0.54</td>
<td>3.63 ±0.36</td>
<td>1.12* ±0.09</td>
<td>2.46 ±0.80</td>
</tr>
<tr>
<td>Breaths episode⁻¹</td>
<td>1.57 ±0.14</td>
<td>2.99 ±0.65</td>
<td>25.51* ±8.79</td>
<td>36.51** ±9.81</td>
</tr>
<tr>
<td>fR_{ins} (breaths min⁻¹)</td>
<td>46.91 ±3.84</td>
<td>33.78* ±1.77</td>
<td>56.06* ±4.22</td>
<td>69.49*** ±1.88</td>
</tr>
<tr>
<td>Non-ventilatory period (s)</td>
<td>23.30 ±3.69</td>
<td>17.08 ±5.16</td>
<td>26.44 ±7.61</td>
<td>3.73 ±0.27</td>
</tr>
</tbody>
</table>
**Figure 22** Hypercarbic and post-hypercarbic ventilatory response in *Lithobates catesbeianus* (n=6). The effects of hypercarbia on breathing frequency (1), tidal volume (2), and minute ventilation (3) are expressed absolutely (A), as a change from baseline (B), and as a percentage change from baseline (C). A figure for each parameter shows the control value during normocarbia (○), after 10, 20, 30 minutes in hypercarbia (●), immediately after the switch back to normocarbia (post-hypercarbia), and the recovery (○). Although $V_T$ increased during hypercarbia (RM-ANOVA, Dunnett’s multiple comparisons test; p<0.05; 20 and 30 min.), frequency responses were variable, resulting in lack of statistical significance in minute ventilation (RM-ANOVA, p>0.05). However, immediately following removal of CO$_2$, there was a robust post-hypercarbic hyperpnea ($V_E$; Dunnett’s multiple comparisons test; p<0.05) mediated through increases in breathing frequency (Dunnett’s multiple comparisons test; p<0.05). To highlight the variability, responses from individual animals that experienced increases in ventilation during hypercarbia are shown as broken gray lines and ventilatory response from individuals that did not are shown as solid gray lines. *p<0.05, **p<0.01, ****p<0.0001
during hypercarbia (Figure 23A₁) and one group that either decreased or was unaffected (Figure 23A₂). Figure 3B₁&₂ shows that despite differences between frequency and \( V_E \) during hypercarbia in animals with and without response to hypercarbia, post hypercarbic \( f \) and \( V_E \) does not differ between these two groups (\( f: p=0.5204; T_4=0.7037, V_E: p=0.190; T_4=1.576; \) unpaired two-tailed t test). \( V_T \) also did not differ between low and high CO₂ responding animals in the post hypercarbic period (\( p=0.3263; T_4=1.118; \) two-tailed unpaired t test). This suggests that factors responsible for controlling breathing during the inspiration of high CO₂ differ between these two groups, but elements responsible for controlling breathing immediately following the removal of hypercarbia may be similar.

Airflow pneumotachography enabled us to determine ventilated volumes of air. This allowed us to account for the possibility of changes in metabolism on breathing induced by hypercarbia through calculation of an air convection requirement (ACR). Exposing bullfrogs to 5% CO₂ did not alter metabolism (Figure 24A; \( p=0.8603; \) two-tailed paired t test; \( T_3=0.1916 \)); therefore, the ACR tended to increase during hypercarbia and increased in the post hypercarbic period (RM-ANOVA; \( p=0.0314; F_{(1,5,4,6)}=8.562; p<0.05; \) Dunnett’s multiple comparisons test) because of increases in minute ventilation.
Figure 23

A1
Normocarbia
Hypercarbia (5% CO₂)
Post-hypercarbia

A2
Normocarbia
Hypercarbia (5% CO₂)
Post-hypercarbia

B1
Breathing Frequency (breaths min⁻¹)
Post-hypercarbia
Breathing Frequency during Hypercarbia (breaths min⁻¹)

B2
Ve during hypercarbia (mL kg⁻¹ min⁻¹)
Post-hypercarbia
Ve during hypercarbia (mL kg⁻¹ min⁻¹)
Figure 23 Peak ventilatory responses during post hypercarbia do not differ between animals with large or absent hypercarbic ventilatory responses. A₁ (left) shows an example differential pressure recording from a bullfrog that experienced increases in ventilation during hypercarbia and further increases in ventilation in the post hypercarbic period (2 minute after removing the CO₂ stimulus). Large pressure deflections represent lung ventilations and the smaller deflections indicate buccal ventilations. The arrows point to lung (large deflections) and buccal (small deflections) ventilatory behaviors. A₂ (right) shows an example recording from a bullfrog that did not undergo increases in ventilation during hypercarbia, but experienced increases in breathing during the post hypercarbic period. B₁&₂ shows summary data of peak, post hypercarbic frequency and $\dot{V}_E$ responses of frogs that did and did not experience increases ventilation during CO₂ exposure. Post-hypercarbic breathing frequency and $\dot{V}_E$ responses between these two groups of bullfrogs did not differ (f; $p=0.5204$, $\dot{V}_E$: $p=0.1901$; unpaired two-tailed t test). ns= not significant
Figure 24

A

\[ V_{O_2} (\text{mL O}_2 \text{ kg}^{-1} \text{ min}^{-1}) \]

Control
Hypercarbia

B

\[ ACR (\text{mL air/mL O}_2) \]

Control
Hypercarbia
Post-Hypercarbia

\( n = 4 \)
**Figure 24** Hypercarbic exposure for 30 minutes does not influence oxygen consumption in adult bullfrogs (n=4). A. shows summary data for $\dot{V}O_2$ of bullfrogs in room air and hypercarbia (p=0.8603; two-tailed paired t test). B. summary results for the air convection requirement ($V_E/Vo_2$) before, during, and immediately after exposure to hypercarbia, indicating that significant hyperventilation only occurs in the post hypercarbic period (p=0.0314; RM-ANOVA; p<0.05 control vs. post hypercarbia, p>0.05 control vs. hypercarbia-Dunnett’s multiple comparisons test). *p<0.05
3.2 Ventilatory and metabolic responses to hypoxia

Exposure to 5% O₂ initially resulted in an increased breathing frequency at 10 minutes (Figure 25. A; RM-ANOVA; p=0.0239; F\(_{4,20}\)=3.558; Dunnett’s multiple comparisons test; p<0.05), but this increase was not sustained throughout the 30 minute exposure (Dunnett’s multiple comparisons test; p>0.05 for 20 and 30 min). In contrast, V\(_T\) increased at 20 and 30 minutes (Figure 25. B; RM-ANOVA; p=0.0024; F\(_{4,20}\)=6.015; Dunnett’s multiple comparisons test; p<0.01 at 20 minutes and p<0.001 at 30 minutes), but not during initial exposure to hypoxia (Dunnett’s multiple comparisons test; p>0.05 at 10 minutes). As a result of these changes in f and V\(_T\), hypoxia evoked increases in V\(_E\) throughout the 30 exposure (Figure 25. C; RM-ANOVA; p=0.0153; F\(_{4,20}\)=3.998; Dunnett’s multiple comparisons test; p<0.05 at 10 and 20 minutes and p<0.01 at 30 minutes). V\(_E\) recovered to near-control values within 2 minutes of replacing hypoxia with room air. Figure 26A shows an example of the ventilatory pattern in normoxia and hypoxia. Figure 26B indicates that hypoxia did not affect V\(_b\)\(_2\) (two-tailed paired t test; p=0.8140; T\(_3\)=0.2567). Therefore, hypoxia resulted in an increase in the ACR (two-tailed paired t test; p=0.0227; T\(_3\)=3.045) because of increased minute ventilation.
Figure 25
Figure 25 Hypoxia elicits increases in ventilation initially through increases in breathing frequency and then through increases in tidal volume. The effects of hypoxia (5% O₂) on breathing frequency (1), tidal volume (2), and minute ventilation (3) are expressed absolutely (A), as a change from baseline (B), and as a percentage change from baseline (C). A figure for each parameter shows the control value during normocapnia (○), after 10, 20, 30 minutes in hypercarbia (●), immediately after the switch back to normoxia (post-hypoxia), and the recovery (○). Breathing frequency initially increased during hypoxia (RM-ANOVA, Dunnett’s multiple comparisons test; p<0.05; 10 min.), eliciting increases in Vₑ at 10 minutes. Increases in Vₜ produced increases in ventilation at later time points (p<0.05, RM-ANOVA; 20 and 30 minutes). Unlike washout of hypercapnic gas mixture, removal of hypoxic stimulus did not produce an increase in ventilation (Vₑ; Dunnett’s multiple comparisons test; p>0.05 compared to control). The immediate recovery of breathing after hypoxia indicates that post hypercarbic hyperpnea is specific to washout of hypercapnic gas and not a general effect of changing gas mixture in the experimental chamber. Ventilatory responses of individuals are shown as solid gray lines. *p<0.05, **p<0.01, ***p<0.001
Figure 26

A

Normoxia

Hypoxia (5% O₂)

expiration

20 s

B

\( V̇O_2 (mL O_2 kg^{-1} min^{-1}) \)

Control
Hypoxia

n = 4

C

ACR (mL air/mL O₂)

Control
Hypoxia

*
**Figure 26** Hypoxic exposure for 30 minutes does not influence oxygen consumption in adult bullfrogs (n=4). A. shows summary data for $\bar{V}O_2$ of bullfrogs in room air and hypoxia (p=0.8410; two-tailed paired t test). B. summary results for the air convection requirement ($V_E/V_O_2$) before and during exposure to hypoxia indicating a significant hyperventilation during hypoxia (p=0.0227; two-tailed unpaired t test). *p<0.05
4. Discussion

Despite the long-standing use of bullfrogs as comparative models in the study of respiratory rhythm generation and central chemosensing, this is the first study to assess the chemical control breathing by directly quantifying ventilated volumes in undisturbed, conscious, adult bullfrogs. To our knowledge, we report the first comprehensive assessment of the acute hypercarbic and hypoxic ventilatory ($\dot{V}_E$) response in bullfrogs and highlight similarities and differences compared to responses obtained by measuring buccal pressure in bullfrogs to $\dot{V}_E$ in toads.

4.1 Resting Ventilation and Metabolism in Bullfrogs

Resting ventilation and metabolism in bullfrogs presented in Table 1 are similar to those reported previously for anuran amphibians. Respiratory pattern parameters (absolute frequency, episode frequency, breaths per episode, instantaneous frequency) are comparable to values reported for bullfrogs assessed using measurements of buccal pressure (Bicego-Nahas and Branco, 1999; Kinkead and Milsom, 1996; Rocha and Branco, 1997; Rocha and Branco, 1998). Resting $\dot{V}_E$ that we measure is similar, albeit slightly lower, compared to alveolar ventilation ($\dot{V}_A$) calculated from lung CO$_2$ excretion and arterial Pco$_2$ in bullfrogs (measured $\dot{V}_E \sim$20 mL kg$^{-1}$ min$^{-1}$ vs. calculated $\dot{V}_A \sim$40 mL kg$^{-1}$ min$^{-1}$) (Jackson & Braun, 1979), likely reflecting methodological differences or less
stressed animals in our study. \( \dot{V}_E \) from intact toads is also similar to our value in bullfrogs (toad: ~20-40 mL kg\(^{-1}\) min\(^{-1}\)) (Branco et al., 1993; Gargaglioni and Branco, 2003; Gargaglioni et al., 2002; Noronha-de-Souza et al., 2006).

Additionally, metabolism (\( \text{O}_2 \) consumption and \( \text{CO}_2 \) production) and respiratory exchange ratio (RER) are in general agreement with resting values found in anurans at comparable experimental temperatures (~0.8-0.85) measured using both open-flow and closed-system respirometry (Bicego-Nahas and Branco, 1999; Busk et al., 2000; Gottlieb and Jackson, 1976; Overgaard et al., 2012; Rogers et al., 2007; Wang et al., 1998). Given that ventilation, breathing pattern, metabolic rate, and RER that we measured are in agreement with other studies of undisturbed, resting anurans; our masking procedure did not appear to alter normal ventilation and metabolism.

**4.2 Reassessment of the Hypercarbic Ventilatory Response in Adult Bullfrogs**

Despite methodological differences (*i.e.*, ventilation vs. buccal pressure), our findings corroborate a previous study investigating ventilatory responses to hypercarbia in bullfrogs. Specifically, the changes in frequency, tidal volume, and minute ventilation induced by hypercarbia and post hypercarbia were in the same direction and of similar magnitude as determined by changes in buccal pressure (Kinkead and Milsom, 1996). Ventilatory responses to increased ambient \( \text{CO}_2 \) in bullfrogs and toads differ in the presence of the post hypercarbic hyperpnea, which complicate direct comparisons. However, peak \( \dot{V}_E \) responses— that is, hypercarbic hyperpnea in toads and post hypercarbic hyperpnea in frogs-
were similar in magnitude (~350ml STPD min$^{-1}$ kg$^{-1}$) and primarily driven by increases in breathing frequency (Noronha-de-Souza et al., 2006).

By interpreting the hypercarbic ventilatory responses of individuals, we also made the previously unreported observation that 5% CO$_2$ elicited robust (but sub maximal) increases in breathing in half of the frogs and minimally affected breathing in the other half; however we did not observe differences in the magnitude of the post hypercarbic hyperpnea between these groups of frogs (Figure 3B). Our results are consistent with previous observations by Kinkead and Milsom (1996) who showed that frogs have nonsignificant increases in breathing during CO$_2$ and robust increases in breathing immediately following washout of the CO$_2$ from the airway. CO$_2$-sensitive olfactory inhibition appears to be responsible for the sub maximal ventilatory response during exposure to hypercarbia in frogs because denervation of this receptor system produces hypercarbic ventilatory responses that are similar to the post hypercarbic hyperpnea (Kinkead and Milsom, 1996). As suggested by Milsom et al. (2004), CO$_2$-sensitive inhibition probably masks the stereotypical, stimulatory effects of CO$_2$ on breathing in bullfrogs. Because the animals in our study experienced either strong or weak inhibition of breathing during CO$_2$, but similar peak stimulatory responses during the post hypercarbic period, our results imply that there is large animal-to-animal variability in the strength of inhibitory influences on breathing control during hypercarbia and not in stimulus strength at central and peripheral chemoreceptors. We cannot confirm this, however, because neither arterial blood nor cerebral spinal fluid gases were measured here.
Similar peak responses between these two groups may not be surprising given that CO₂ acutely applied and loaded through the skin elicits increases in $\dot{V}_A$ (Jackson and Braun, 1979b) which presumably leads to arterial acidemia in the absence of ventilatory compensation as occurs with an intact olfactory system. Despite differences between large and small ventilatory responses with elevated ambient CO₂, cutaneous CO₂ acquisition probably provides a route to normalize excitatory stimulus strength between these two groups of frogs to account for uniformity in peak, post hypercarbic breathing.

Since the ventilatory response to hypercarbia involves both stimulation and inhibition from CO₂-sensitive chemoreceptors, and this balance shows variability in a population (Figure 3), previous studies investigating hypercarbic ventilatory responses in intact bullfrogs should be interpreted with caution. For example, ventilatory responses during hypercarbia in adult bullfrogs are reduced in the winter and by cold temperatures independently of the season implying stimulatory CO₂ sensitivity was reduced (Bicego-Nahas and Branco, 1999). Although it may certainly be true that ventilatory responses to hypercarbia are reduced under these conditions, our results offer an alternative explanation that reduced ventilatory responses to hypercarbia could result from strengthened inhibition (i.e., enhanced CO₂ sensitivity of inhibitory systems) from the olfactory chemoreceptors in the cold and during the winter. This complicates the use of hypercarbia for elucidating “stimulatory” CO₂ chemosensitivity that would be involved in the homeostatic regulation of arterial PCO₂. Our observation of a consistent, large increase in minute ventilation immediately following CO₂,
regardless of ventilation during hypercarbia, provides evidence that the post hypercarbic hyperpnea may more consistently approximate the stimulatory effects of CO$_2$ among individuals in a population of bullfrogs. Our argument is strengthened by experiments that applied CO$_2$ exclusively to the airway, but not the lung (thus not activating stimulatory chemoreceptors) of tegu lizards. In this case, elevated airway CO$_2$ potently inhibited ventilation. Immediate removal of CO$_2$ from the airway caused breathing to return to control values and did not elicit a post hypercarbic hyperpnea (Coates et al., 1991). These results suggest that the concomitant acid-base disturbance occurring when CO$_2$ is applied at the lung/skin of bullfrogs produces the ventilatory overshoot immediately after removal of olfactory inhibition in the post hypercarbic period when arterial (and presumably central) pH and Pco$_2$ are still low and high, respectively (Kinkead and Milsom, 1996). Thus we propose that ventilation during and immediately following CO$_2$ should be routinely assessed in bullfrogs to better estimate the relative strength of inhibition and stimulation by CO$_2$ in bullfrogs.

4.3 Reassessment of the Hypoxic Ventilatory Response in Adult Bullfrogs

Although the hypoxic ventilatory response has been assessed in other adult anurans [see Gargaglioni and Milsom (2007) and Porteus et al. (2011) for review], a thorough literature search revealed few studies measuring ventilatory responses to hypoxia (even as assessed by buccal pressure) in intact bullfrogs. We found this surprising given the attention to mechanisms (determined by $\textit{in vitro}$ studies) mediating or potentially playing a role in the central O$_2$ chemoreflex in this species (Belzile et al., 2007; Fournier et al., 2007; Fournier and Kinkead,
2008; Harris et al., 2002; Winmill et al., 2005). As anticipated, we show here that bullfrogs increase minute ventilation in response to acute hypoxia. From the parameters we measured here, our results imply increased ventilation is the dominant compensatory strategy during acute hypoxia since we observed a large increase in the air convection requirement and did not measure a change in O$_2$ consumption. Initially, this increase in $V_e$ was caused by increasing breathing frequency. At later time points, frequency fell to values statistically indistinguishable from normoxia (although the frequency response to hypoxia at later time points showed animal-to-animal variability), while $V_T$ increased.

Here we constrain comparisons of our data to the few studies that used bullfrogs (buccal pressure measurements) or toads (pulmonary ventilation) under similar experimental conditions as ours. While we observed 2-3 fold increases in frequency within the first 10 minutes of hypoxia that drifted toward control values at later time points, Rocha and Branco (1998) showed that hypoxia leads to 2 to 3-fold increases in frequency after 60 minutes of exposure in all seasons except for the summer; unfortunately, $V_e$ and $V_T$ (rather $V_e$ or $V_T$ “index”) were not determined. It is also worth comparing the hypoxic response *in vivo* to results from bullfrog brainstem preparations *in vitro*. Despite obvious differences between *in vitro* preparations and intact animals (*e.g.*, superfusion with hyperoxic artificial cerebral spinal fluid vs. perfusion, lack of peripheral inputs vs. intact peripheral inputs, *etc.*), results from each preparation lead to similar conclusions about frequency changes during hypoxia. Specifically, hypoxia initially leads to
increases in breathing frequency, but with increased exposure time, frequency decreases (Figure 25; Winwill et al., 2005). Although the magnitudes and manifestation of these responses differ (in vivo: slight increase initially, then drift back toward control vs. in vitro: increase in episodes/ maintained motor output initially, then silenced output), hypoxic responses of both preparations follow similar time courses. Studies that assessed the hypoxic response of the in vitro brainstem preparation of adult bullfrogs (Fournier et al., 2007; Winmill et al., 2005) have compared their results to findings from Rana pipiens that shows decreases in breathing frequency upon acute exposure to anoxia (Rose and Drotman, 1967). The results presented here indicate that the mechanisms determined in vitro may restrain increases in breathing frequency during less severe bouts of hypoxia (i.e., hypoxic, not anoxic conditions) in intact bullfrogs. We exercise caution in this interpretation, however, because breathing frequency response to acute hypoxia in anurans shows variability from study to study (Porteus et al., 2011).

In comparison to toads, we report a similar mean peak minute ventilation of ~100ml kg\(^{-1}\) min\(^{-1}\) in bullfrogs (Gamperl et al., 1999; Gargaglioni and Branco, 2001; Gargaglioni and Branco, 2003; Gargaglioni et al., 2002; Kruhøffer et al., 1987; Wang, 1994). Although changes in frequency and tidal volume with time are not presented in these studies, our results are consistent with findings in toads that exposure to 5% O\(_2\) for ~30 minutes leads to increases in \(V_E\) by increasing \(V_T\) with little influence on breathing frequency (Fonseca et al., 2014; Gargaglioni and Branco, 2001; Gargaglioni and Branco, 2003; Gargaglioni et al.,
Although hypoxia leading to increases in tidal volume appears to be a consistent trend in anurans, other aspects of the breathing pattern tend to show variable responses among studies (Gargaglioni and Branco, 2000; Gargaglioni and Branco, 2001; Gargaglioni and Branco, 2003; Gargaglioni et al., 2002).

Whether or not the influence of hypoxia on breathing pattern characteristics (e.g., the instantaneous frequency) determined in this report on bullfrogs will remain a consistent finding awaits further study.

4.4 Conclusions and Perspectives

To alleviate the concern that understanding respiratory control through measurements of buccal pressure may lead to misinterpretation of breathing control in bullfrogs (Milsom et al., 1999), we measured pulmonary ventilation in bullfrogs using airflow pneumotachography as is typically done in toads. This enabled us, for the first time, to determine minute ventilation and air convection requirements at rest and during hypercarbia/hypoxia in bullfrogs. Respiratory responses to hypercarbia corroborated previous studies that indexed breathing through changes in buccal pressure. We also made the new finding that inhibition of breathing during hypercarbia shows variability in a population, but leads to consistent increases in post hypercarbic ventilation. Additionally, despite the interest in central mechanisms of breathing control during hypoxia in bullfrogs, we provide the first comprehensive characterization of the acute hypoxic ventilatory response in the unrestrained bullfrog. Based on our findings, a general trend among anurans seems to be an increase in tidal volume, but relatively constrained frequency during hypoxia. Although mechanisms that
suppress motor output during hypoxia in bullfrogs have received considerable attention, perhaps the methods described here for measuring ventilation in conscience bullfrogs could ease the transition between *in vitro* and *in vivo* studies to determine how important mechanisms regulating both frequency and $V_T$ during hypoxia are integrated in the intact animal.
CHAPTER VII

MANUSCRIPT IV

Control of lung ventilation following overwintering conditions in bullfrogs, *Lithobates catesbeianus*


Santin, J.M. & Hartzler, L.K.
Abstract:

Ranid frogs in northern latitudes survive winter at cold temperatures in aquatic habitats often completely covered by ice. Cold-submerged frogs survive aerobically for several months relying exclusively on cutaneous gas exchange while maintaining temperature-specific acid-base balance. Depending on the overwintering hibernaculum, frogs in northern latitudes could spend several months without access to air, need to breathe, or chemosensory drive to use neuromuscular processes that regulate and enable pulmonary ventilation. Therefore, we performed experiments to determine whether aspects of the respiratory control system of bullfrogs, *Lithobates catesbeianus*, are maintained or suppressed following minimal use of air breathing in overwintering environments. Based on the necessity for control of lung ventilation in early spring, we hypothesized that critical components of the respiratory control system of bullfrogs would be functional following simulated overwintering. We found that bullfrogs recently removed from simulated overwintering environments exhibited similar resting ventilation when assessed at 24°C compared to warm-acclimated control bullfrogs. Additionally, ventilation met resting metabolic and, presumably, acid-base regulation requirements, indicating preservation of basal respiratory function despite prolonged disuse in the cold. Recently emerged bullfrogs underwent similar increases in ventilation during acute oxygen lack (aerial hypoxia) compared to warm-acclimated frogs; however, CO$_2$-related hyperventilation was significantly blunted following overwintering. Overcoming challenges to gas exchange during overwintering have garnered attention in
ectothermic vertebrates, but this study uncovers robust and labile aspects of the respiratory control system at a time point correlating with early spring following minimal/no use of lung breathing in cold-aquatic overwintering habitats.
Introduction:

Anuran amphibians and other ectothermic vertebrates operate over a wide range of body temperatures that depend on time of day, microhabitat, local weather, and season. Ranid frogs living in northern latitudes (e.g., bullfrogs, common frogs, leopard frogs) survive cold winters in aquatic habitats that may be completely covered by ice (Emery et al., 1972; Willis et al., 1956). The respiratory and energetic challenges associated with overwintering submergence in frogs including complete reliance on cutaneous gas exchange, exercise capacity, acid-base balance, hypoxia tolerance, and induction of metabolic suppression are well understood (Donohoe et al., 1998; Donohoe et al., 2000; St-Pierre et al., 2000; Tattersall and Boutilier, 1997; Tattersall and Boutilier, 1999a; Tattersall and Boutilier, 1999b; Tattersall and Ultsch, 2008; West et al., 2006).

Unlike most other vertebrates, overwintering frogs and some turtles do not require lung ventilation for gas exchange due to their low metabolic rates and high capacity for extrapulmonary gas exchange (reviewed by Milsom and Jackson, 2011). Thus cold-submerged ranid frogs remain aerobic for >5 months in oxygenated water while maintaining temperature-specific acid-base balance (Ultsch et al., 2004). CO₂ and O₂ chemosensory processes that typically stimulate breathing do not function at temperatures ≤10-15°C (Bicego-Nahas and Branco, 1999; Bicego-Nahas et al., 2001; Morales and Hedrick, 2002; Santin et al., 2013). Therefore, depending on the overwintering hibernaculum, frogs from northern latitudes could potentially spend several months without access to air,
need for lung breathing, and chemosensory drive to use pulmonary ventilation. Rising ambient temperature typically triggers emergence from overwintering hibernacula (Willis et al., 1956). Warming of body temperature and accomplishing energetically costly behaviors (e.g., calling, foraging, mating) in early spring (Tattersall and Ultsch, 2008; Willis et al., 1956) undoubtedly increase rates of $O_2$ consumption and $CO_2$ production above that provided by cutaneous gas exchange (Gottlieb and Jackson, 1976; Jackson and Braun, 1979a; Mackenzie and Jackson, 1978). Despite minimal use of lung breathing during aquatic overwintering, frogs emerging in the spring should quickly require lung ventilation to sustain adequate gas exchange.

Air breathing vertebrates, including frogs, regulate ventilation through a complicated sensorimotor system (Feldman et al., 2013; Kinkead, 2009). Although extreme exceptions exist, prolonged disuse of and reduced sensory input to other neural-muscular systems may contribute to decreased function. For example, 24 hours of mechanical ventilation leads to decreased force production in the diaphragm of rats (Powers et al., 2002). Following hibernation, European ground squirrels do not retain spatial and operant memories learned in fall (Millesi et al., 2001). Pharmacological silencing of neurons in culture leads to a reduction in functional synaptic connections (Mitra et al., 2012). Moreover, simulated overwintering in ranid frogs decreases jump performance when assessed at warm, spring-like temperatures (Renaud and Stevens, 1983). It remains unknown how well frogs match ventilation to metabolism and combat disturbances to blood gas homeostasis upon acute transition from a cold-
submerged hibernaculum to a warm-terrestrial habitat as occurs after emergence from overwintering without lung breathing and chemosensory control of ventilation.

Here we determined the consequences of cold-acclimation on respiratory control of intact bullfrogs, *Lithobates catesbeianus*, 14-16 hr following forced emergence and warming from simulated overwintering hibernacula. This allowed us to establish how well the breathing control system works after prolonged absence of (cold-acclimated at 2°C without air access) or negligible (cold-acclimated at 2°C with air access) pulmonary ventilation. Soon after spring emergence from underwater habitats, frogs perform costly behaviors at higher temperatures that require control of pulmonary ventilation to sustain metabolic rates despite months of disuse. Thus, we hypothesized that bullfrogs can breathe to 1.) enable resting metabolic processes and 2.) combat challenges to blood gas homeostasis upon transition from a cold-submerged to a warm-terrestrial environment as occurs following emergence in early spring. To test these hypotheses we measured ventilation, metabolism, breathing pattern, hypercarbic, and hypoxic chemoreflexes at 24°C, after forced emergence from simulated overwintering environments in adult bullfrogs. This approach provided insights into aspects of the respiratory control system that diminish and remain intact following overwintering submergence with no reliance on air breathing.
Materials and Methods

Experimental Animals

Adult female bullfrogs, *Lithobates catesbeianus*, were purchased from Rana Ranch (Twin Falls, ID, USA) and maintained in the lab in three plastic tanks at ~22°C aerated water with access to wet and dry areas (n=8 animals per tank). The depth of the water was 17 cm in each tank. Frogs were exposed to 12:12-h light-dark cycles and fed crickets twice per week. Experiments were performed on control (warm-acclimated; 22°C) bullfrogs after at least two weeks of acclimation to conditions of the animal facility. Food was withheld for 3 days before experiments. To produce the cold-acclimated groups, after ~2 weeks of laboratory acclimation, two of the tanks were cooled in a walk-in environmental chamber at ~3-4°C per week over 6 weeks until a final temperature of ~2°C was reached. During the cooling period, the light:dark cycle was gradually transitioned from 12:12 to 10:14 to shorten the day length. During the cooling ramp, bullfrogs were fed twice per week; however, feeding stopped and frogs tended to voluntarily submerge when water temperature reached ~7°C. At this time food was withheld and access to dry areas were removed. At 6 weeks, a plastic screen containing small holes was placed near the surface of one of the tanks undergoing cold-acclimation to deny air access. Bullfrogs were then maintained for the next 6 weeks with or without access to air at ~2 °C. Following 6 weeks at 2°C with or without access to air, experiments were performed over the next three weeks. Experiments in cold-acclimated bullfrogs from tanks with and without access to air were alternated during the three-week experimental
period. Experiments were approved by the Wright State University Institutional Animal Care and Use Committee.

We occasionally monitored the surfacing behavior of cold-acclimated bullfrogs that had access to air. Similar to previous reports (Lillo, 1980; Tattersall and Ultsch, 2008) bullfrogs spent most time voluntarily submerged and were only occasionally observed at the surface. Based on the lack of a requirement for pulmonary ventilation during cold-overwintering, we are confident that surfacing behavior and reliance on pulmonary ventilation were minimal in the group provided air access, but we did not quantify these parameters.

**Measurement of Ventilation**

We measured ventilation in bullfrogs acclimated to room temperature (22°C; n=8), 2°C (air access; n=7), and 2°C (submerged; n=5) air groups at 24°C (a temperature requiring lung ventilation as would occur in spring) using air-flow pneumotachography (Glass et al., 1978) closely following methods that we recently adapted for bullfrogs (Santin and Hartzler, 2016c). Briefly, the evening prior to measurements, bullfrogs were lightly anesthetized with ~0.5% (v/v) isoflurane until losing toe pinch and eye reflexes. A small, lightweight facemask created from the bulb of a 3 mL transfer pipette (Samco Scientific Corporation, Cat. No. 225-15, San Fernando, CA, USA) containing a small pneumotach was fitted to the snout of the frog using gel super glue (Loctite Super Glue Gel; Westlake, OH, USA). After the glue dried, the pneumotach was connected to a
differential pressure transducer (TSD 160A differential pressure transducer Biopac Systems Inc., CA, USA) by PE50 tubing and the mask was carefully checked for leaks to ensure all air-flow produced by the animal moved across the pneumotach. Bullfrogs fitted with the mask and pneumotach were then placed in a dark 875 mL experimental chamber overnight at 24 °C and exposed to airflow of 1.1 L/min.

The only methodological difference between this study and Santin and Hartzler (2016c) is that we omitted the use of sutures to secure the mask before applying super glue. This step was deemed unnecessary as the super glue alone held the mask in place and remained sealed in most animals. A comparison between 22°C acclimated animals with and without sutures to secure the mask revealed that all ventilation and metabolic parameters (frequency, tidal volume, minute ventilation, breathing pattern, $\dot{V}o_2$, $\dot{V}co_2$, and respiratory exchange ratio) were the same (p>0.05 for all parameters; two-tailed unpaired t test; Supplementary Table 1).

Measurement of Metabolic Rate

The rates of O$_2$ consumption ($\dot{V}o_2$) and CO$_2$ production ($\dot{V}co_2$) were determined in concert with ventilation as described in Santin and Hartzler (2016c). Open-flow respirometry was performed by pushing experimental gas mixtures at 1.1 ml/min through a flask half-filled with water to fully humidify the gas. Subsamples of humidified gases were then pulled through the sealed
acrylic chamber (875 mL) containing the animal at 215 mL/min by a suction pump (AEI Technologies Inc., PA, USA). Gases were pulled through a 4100 series mass flow meter (TSI Inc., MN, USA), a CO₂ analyzer (CD-3A; AEI Technologies Inc., PA, USA), a 10 mL syringe filled with desiccant (DM-AR; Perma pure LLC, NJ, USA), and finally an infrared O₂ analyzer (S-3A/I; AEI Technologies Inc., PA, USA).

**Experimental Protocols**

All experiments were performed during the light cycle. After ~16 hrs following attachment of the mask and pneumotach, resting ventilation and metabolism was determined when pulling room air through the sealed chamber at 215 mL/min. Each animal was exposed to both hypercarbia and hypoxia for 30 min, in a random order with 2-3 hours between each stimulus. This was a long enough time period for recovery from the previous stimulus (Santin and Hartzler, 2016c) and we did not observe an order effect (i.e., exposure to hypercarbia did not influence the hypoxic ventilatory response and *vice versa*; personal observation).

When switching from room air to either experimental gas mixture or experimental gas mixture back to room air, we pushed the humidified gas through the chamber at 1.1 mL/min for 5 minutes to reduce the time required to exchange the gaseous environment. Fractional concentrations of incurrent gases were checked before each gas transition to minimize errors due to drift of gas analyzers. We observed that flow changes in the chamber do not influence ventilation and given that most
measurements were taken at steady-state this does not likely present a problem. The only time point where ventilation measurements were made immediately after altering the flow was the post-hypercarbic period. It is unlikely that changes in flow influence the increase in breathing observed in the post hypercarbic period (1-2 minutes after hypercarbia) since breathing frequency in the “post-hypoxic” period does not increase (Santin and Hartzler, 2016c) (personal observation; data not included). At the end of each experiment, the facemask was removed and 0.1-1.2 mL air injections were pushed through the facemask to calibrate the pneumotach.

Data Analysis

Resting ventilation, breathing pattern, and metabolism were determined in room air for 30 minutes before switching to the experimental gases in each animal group. During hypercarbia and hypoxia, ventilation was measured for 5 minutes, every 10 minutes, to elucidate the time course of the ventilatory response. Additionally, ventilation in the 1 minute period after hypercarbia (post-hypercarbia) was analyzed as this ventilatory response represents the “stimulatory” ventilatory response to CO₂ independent of olfactory inhibition (Kinkead and Milsom, 1996) and is less variable between animals (Santin and Hartzler, 2016c) presumably because of variable activation of inhibitory olfactory responses. \( \dot{V}_{O_2} \) and breathing pattern were measured in the last 5 min. of hypoxia and hypercarbia.
Lung breaths were identified by their large amplitude relative to buccal ventilations and biphasic expiratory flow pattern (Jones, 1982; Vitalis and Shelton, 1990). We integrated the expiratory flow signal caused by lung breaths and interpolated differential pressure integrals into the calibration curve for each animal to establish tidal volume ($V_T$) and then converted $V_T$ to standard temperature pressure dry (STPD). Ventilation ($V_E$) was determined by multiplying $f$ by $V_T$. Breathing pattern characteristics including episode frequency, breaths per episode, instantaneous frequency, and duration of the non-ventilatory period were analyzed according to previously established criteria (Kinkead and Milsom, 1996).

$\dot{V}O_2$ and $\dot{V}CO_2$ were determined by measuring the fractional differences between inspired and expired $O_2$ and expired and inspired $CO_2$, respectively, multiplied by the flow rate, and expressed as STPD. The air convection requirement (ACR) was calculated by dividing $V_E$ by either $\dot{V}O_2$ or $\dot{V}CO_2$. Since our experimental setup did not allow us to measure $\dot{V}O_2$ during the post-hypercarbic period, ACR for the post hypercarbic hyperpnea was estimated using the $\dot{V}O_2$ measurement taken during hypercarbia before switching to room air.

**Statistics**

Data are presented as mean ± S.E.M. Resting ventilation and metabolic parameters between experimental groups were analyzed using a one-way ANOVA. When assessing the influence of hypoxia or hypercarbia on ventilation,
breathing pattern, and metabolism, main effects (temperature acclimation and gas) and interactions (temperature acclimation x gas) on ventilation and metabolic parameters were analyzed using a repeated measures two-way ANOVA with time point in gas as the repeated measure. Some animals did not contain certain breathing pattern characteristics; therefore these could not be analyzed. For example, instantaneous breathing frequency could only be measured in 9/12 total cold-acclimated frogs during hypercarbia (1 cold frog with air access and 2 without air access did not produce breaths occurring in succession during the sampling period). Since there were no statistical differences in absolute breathing frequency between acclimation groups and to ensure adequate sample sizes for pattern analysis, we pooled data from cold-acclimated tanks for these analyses and indicate the sample size for each analysis in the figure and legend. Because a repeated measures design could not be used when records contained aspects of the breathing pattern in room air, but not hypercarbia/post hypercarbia, data were analyzed using an ordinary two-way ANOVA. Pairwise comparisons were performed using the Holm-Sidak multiple comparisons test. Statistical significance was accepted when p<0.05. Pertinent statistical information is included in the results section. Statistics were performed using GraphPad Prism Version 6.01 (GraphPad Software, San Diego CA).
Results

Resting Ventilation and Metabolism following Cold-Acclimation

Control, cold-acclimated with air access and cold-submerged bullfrogs weighed 109±9 g, 112±12 g, and 102±6 g, respectively immediately following experiments. To understand the capacity for lung ventilation to match metabolism after emerging from an overwintering habitat, we measured ventilation and metabolism in cold-acclimated bullfrogs ~16 hours after transition from a cold-aquatic to a warm-terrestrial environment. Fig. 27 shows that cold-acclimation (with or without air access) does not influence breathing frequency (p=0.1636; F\( (2,17) =1.084 \); one-way ANOVA), tidal volume (p=0.2137; F\( (2,17) =1.692 \); one-way ANOVA) and minute ventilation (p=0.9185; ; F\( (2,17) =0.0854 \); one-way ANOVA) at rest. Figure 28 shows metabolic parameters in the three acclimation groups. Resting \( \dot{V}o_2 \) was elevated in cold-acclimated bullfrogs that had access to air (Fig. 2A; p=0.0277; F\( (2,18) =4.404 \); one-way ANOVA; p<0.05 control vs. CA (cold-acclimated): Air; p>0.05 for all other comparisons; Holm-Sidak’s multiple comparisons test). However \( \dot{V}co_2 \) (Fig. 2B; p=0.2109; F\( (2,18) =1.699 \); one-way ANOVA); and the respiratory exchange ratio of each animal (RER; \( \dot{V}co_2/\dot{V}o_2 \)) (Fig. 2C; p=0.3526; F\( (2,18) =1.488 \); one-way ANOVA) were not different among control and both cold-acclimated groups.
Figure 27

A

Breathing Frequency (min⁻¹)

Warm Acclimated
Cold Acclimated: Access
Cold Acclimated: Submerged

B

Tidal Volume (mL·kg⁻¹)

Warm Acclimated
Cold Acclimated: Access
Cold Acclimated: Submerged

C

VE (mL·kg⁻¹·min⁻¹)

Warm Acclimated
Cold Acclimated: Access
Cold Acclimated: Submerged
Figure 27 Cold-acclimation with and without access to air does not affect resting ventilatory parameters. Breathing frequency (A), tidal volume (B), and minute ventilation ($V'_E$; C) measured at 24°C were not different (one-way ANOVA; $p>0.05$ for each parameter) among warm and groups of cold-acclimated bullfrogs. Black, white, and gray bars represent mean control ($n=8$; black bars), cold-acclimated with air ($n=7$; white bars), and cold-acclimated with no air ($n=5$; gray bars), respectively. Error bars represent ± SEM.
The air convection requirement (ACR; $\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$) normalizes ventilation to metabolic rate, and ACR can therefore be used to infer hyper- or hypo- ventilation between animal groups. For example, if one treatment (e.g., cold-acclimation) has a reduced ACR compared to control, we can infer that lung ventilation does not match metabolism after cold-acclimation and these animal are hypoventilating. Fig. 2D & 2E show that both air convection requirements for $O_2$ acquisition ($p=0.8039; F_{(2,17)}=0.2211$; one-way ANOVA) and $CO_2$ elimination ($p=0.9218; F_{(2,17)}=0.0917$; one-way ANOVA) are not different among warm-acclimated and both groups of cold-acclimated bullfrogs. Taken together, these results show that cold-acclimated bullfrogs match breathing to metabolism similarly to controls and have respiratory exchange ratios comparable to control bullfrogs at rest (i.e., they use similar metabolic fuel and/or have a similar acid-base status). This suggests that baseline ventilation is well buffered against cold and disuse and is capable of supporting resting organismal metabolism after emergence despite near-complete to complete disuse of lung breathing.

Cold-acclimation reduces the post-hypercarbic hyperpnea

Aside from regulating gas exchange to sustain resting metabolism, the respiratory control system must respond to acid-base disturbances and elicit ventilatory compensation. To test responsiveness of the respiratory control system to acid-base perturbations, we exposed control and recently emerged bullfrogs to environmental hypercarbia (5% $CO_2$ in air). Fig. 29 shows $\dot{V}_E$,
Figure 28 Bullfrogs ventilate sufficiently to support increased metabolic rate ($\dot{V}O_2$) after overwintering. A-C shows mean rate of $O_2$ consumption ($\dot{V}O_2$; A), rate of $CO_2$ production ($\dot{V}CO_2$; B), and respiratory exchange ratio (RER; C) in warm-acclimated (black bars; n=8), cold-acclimated with air (white bars; n=7), and cold-submerged (gray bars; n=6) bullfrogs. As shown in A, $\dot{V}O_2$ was influenced by temperature acclimation (one-way ANOVA; p=0.0274; $F_{(2,18)}$= 4.404). Cold-acclimation with access to air resulted in elevated $\dot{V}O_2$ compared to control (Holm-Sidak multiple comparisons test; p<0.05), but not compared to cold-submerged frogs (Holm-Sidak multiple comparisons test; p>0.05) at 24°C. In contrast, cold-submerged frogs did not have $O_2$ consumption greater than control or cold-acclimated frogs with air access (Holm-Sidak multiple comparisons test; p>0.05). Although $\dot{V}CO_2$ showed a similar trend as $\dot{V}O_2$, $\dot{V}CO_2$ (B; one-way ANOVA; p>0.05; $F_{(2,18)}$=1.6099) and RER (C; one-way ANOVA; p=0.26; $F_{(2,18)}$= 1.448) was unaffected by temperature acclimation. D and E show air convection requirements for $O_2$ ($\dot{V}_E/\dot{V}O_2$) and $CO_2$ ($\dot{V}_E/\dot{V}CO_2$), respectively. On an animal to animal basis, cold-acclimated bullfrogs with access to air (n=7) or submerged (n=5) breathed sufficiently to match metabolic demands as indicated by statistically indistinguishable air convection requirements for $O_2$ (D; one-way ANOVA; p=0.8039; $F_{(2,18)}$= 0.2211) and $CO_2$ (E; one-way ANOVA; p=0.9128; $F_{(2,18)}$= 0.0917 ) compared to control (n=8). Thus frogs removed from conditions that mimic overwintering conditions with and without access to air do not hyper- or hypoventilate compared to control bullfrogs. Error bars represent ± SEM. *p<0.05, ns= not significant
Figure 29 Cold-acclimation with and without air leads to a reduction in the post-hypercarbic hyperpnea, but not differences in ventilation during CO$_2$ exposure. A-D shows absolute $\dot{V}_E$, $\dot{V}_E$ relative to baseline in normocarbia, breathing frequency, and tidal volume, respectively, in warm-acclimated (black circles; n=8), cold-acclimated with air (white circles; n=7), and cold-submerged (gray circles; n=5) bullfrogs. A. (two-way ANOVA; CO$_2$ effect; $F_{(4,68)}= 12.31$; p<0.0001) and B (CO$_2$ x temperature acclimation interaction; $F_{(8,64)}$=3.202; p=0.0038) shows that hypercarbia does not lead to statistically significant increases in $\dot{V}_E$ in all three groups (Holm-Sidak multiple comparisons test; p>0.05); however, the post-hypercarbic ventilatory response is only significantly greater than normocarbic breathing in control frogs (Holm-Sidak multiple comparisons test; p<0.0001) and significantly blunted in both cold-acclimated groups compared to control frogs (Holm-Sidak multiple comparisons test; p<0.05). C. (two-way ANOVA; time x acclimation; $F_{(8,64)}$=4.784; p<0.001) shows that the decreased post-hypercarbic hyperpnea in cold-acclimated frogs occurs due to smaller increases in breathing frequency (Holm-Sidak’s multiple comparisons test; p<0.0.01). Tidal volume (D) only increased in response to hypercarbia or post hypercarbic stimulation in the cold-acclimated groups; however, this was not enough to significantly alter $V_E$ due to the reduced frequency responses. Error bars represent ± SEM. *p<0.05, **p<0.01, ****p<0.0001 compared to normocarbic control (within animal comparisons). +p<0.05, ++p<0.01, ++++p<0.0001 compared to control frog (between animal comparisons).
breathing frequency, and $V_T$ in response to hypercarbia. During hypercarbia, all three groups did not change ventilation at any time point (Fig. 29A; $p>0.05$ at each time point during hypercarbia; Holm-Sidak’s multiple comparisons test).

We recently showed that there is large variability in ventilatory responses during hypercarbia presumably due to animal-to-animal differences in $CO_2$-sensitive olfactory inhibition (Santin & Hartzler, 2016). The post-hypercarbic hyperpnea (i.e., ventilatory increase in the ~1 minute after removal of $CO_2$ from the airway when arterial $CO_2$ and pH remain elevated and reduced, respectively (Kinkead and Milsom, 1996)) was consistently large regardless of breathing during hypercarbia (Santin and Hartzler, 2016c). Therefore, the post hypercarbic hyperpnea has been suggested to represent a uniform index of stimulatory $CO_2$ chemosensitivity in vertebrates with large inhibition of breathing by olfactory chemoreceptors (Milsom et al., 2004). We observed that control bullfrogs had a robust post-hypercarbic hyperpnea (main effect of $CO_2$; $p<0.0001$; $F_{(4,68)}=12.31$; two-way ANOVA; baseline vs. post hypercarbia and 30 minutes in hypercarbia vs. post hypercarbia; $p<0.05$ for both analyses; Holm-Sidak’s multiple comparisons test). However, ventilation did not undergo significant increases during or immediately following hypercarbia compared to normocarbia or hypercarbia in both groups of cold-acclimated bullfrogs ($p>0.05$; Holm-Sidak’s multiple comparisons test). Additionally, ventilation in the post hypercarbic period was significantly less in cold-acclimated compared to warm-acclimated frogs ($p<0.05$; Holm-Sidak’s multiple comparisons test). When expressing ventilation as a relative change, there was a significant interaction between
temperature acclimation and CO₂ (Fig. 29B; CO₂ x temperature acclimation interaction; p=0.0038; F(8,68)=3.202; two-way ANOVA), indicating that cold-acclimation alters the influence of CO₂ on ventilation through blunting the post-hypercarbic hyperpnea. The reduced ability to elicit ventilatory response to hypercarbia stemmed from a smaller increase in breathing frequency during post-hypercarbia (Fig. 29B; temperature acclimation x CO₂ interaction; p=0.0001; F(8,64)=4.784; ). Unlike warm-acclimated bullfrogs, both groups of cold-acclimated frogs underwent small increases in Vₜ compared to baseline during or following hypercarbia (Fig. 29C; main effect of CO₂; p=0.0018; F(4,68)=4.785; cold-acclimated air at 30 min. p<0.05; Holm-Sidak’s multiple comparisons test; cold-acclimated no air post hypercarbic hyperpnea; p<0.05; Holm-Sidak’s multiple comparisons test), but these increases were not large enough to cause statistically significant increases in ˙Vₑ. These results imply that ventilatory response to CO₂ challenge is reduced following overwintering conditions.

To take into account the possibility of different metabolic responses to hypercarbia compared to warm and cold-acclimated bullfrogs, we also indirectly assessed metabolism using open-flow respirometry before and during hypercarbia then normalized ventilation to metabolism. This allowed us to determine whether CO₂ sensitivity of ventilation per se was reduced in cold-acclimated bullfrogs or changes in ventilation during CO₂ were the consequence of a different metabolic response to hypercarbia. Fig. 30A shows that ˙Vₒ₂ was not influenced by hypercarbia and there were no differences among temperature acclimation groups (p>0.05; two-way ANOVA; no main effects or interactions).
Figure 30

A

- Control
- Cold-Acclimated: Access (n=7)
- Cold-Acclimated: Submerged (n=5)

B

- Control (n=8)
- Cold-Acclimated: Access (n=7)
- Cold-Acclimated: Submerged (n=5)

CO₂: p=0.0056
Acclimation: p=0.1180
CO₂ × Acclimation: p=0.1384
Figure 30 Cold-acclimated bullfrogs do not increase ventilation relative to metabolism in response to hypercarbia or immediately after return to normoxia.

A. shows that mean \(\dot{V}O_2\) is not affected by environmental hypercarbia in warm-acclimated (black circles; n=8), cold-acclimated with air (white circles; n=7), and cold-submerged (gray circles; n=5) bullfrogs (two-way ANOVA; p>0.05 for main effects and interaction). B. presents mean air convection requirements (ACR; \(\dot{V}_E/\dot{V}O_2\)) before, during and after hypercarbia. ACR of warm-acclimated bullfrogs increased in the post-hypercarbic period compared to rest in normocarbia (p<0.001; Holm-Sidak’s multiple comparisons test) and was greater during post hypercarbia compared to both groups of cold-acclimated bullfrogs (p<0.05; Holm-Sidak’s multiple comparisons test). Error bars represent ± SEM. ***p<0.001 compared to control value in normocarbia (within animal differences). +p<0.05 compared to control bullfrogs during post-hypercarbic hypervnea (between animal differences).
The air convection requirement ($\dot{V}_E/\dot{V}_{O_2}$) was influenced by CO$_2$ treatment ($p=0.0056; F_{(2,34)}=6.601$; two-way ANOVA). ACR increased significantly during the post-hypercarbic hyperpnea in controls ($p<0.001$; Holm-Sidak’s multiple comparisons test), but not in either group of cold-acclimated bullfrogs. ACR was also greater in control bullfrogs compared to both groups of cold-acclimated frogs ($p<0.05$; Holm-Sidak’s multiple comparisons test). Thus, cold-acclimated frogs do not undergo hyperventilation during or following hypercarbia. These findings indicate that reduced ventilatory responses to hypercarbic-related stimulation results from reductions in ventilatory sensitivity to CO$_2$ challenge and not from changes in metabolic responsiveness to hypercarbia.

To assess possible causes of the reduction in CO$_2$ sensitivity of the breathing frequency, we analyzed aspects of the episodic breathing pattern that is characteristic of anuran amphibians. Fig. 31A & B show that post-hypercarbia resulted in addition of more breaths in each episode of warm-acclimated bullfrogs (Fig. 31A; $p<0.05$ control vs. post-hypercarbic hyperpnea; Holm-Sidak’s multiple comparisons test) without changing the number of episodes per minute (Fig. 31B; $p>0.05$ control vs. post-hypercarbic hyperpnea; Holm-Sidak’s multiple comparisons test). Warm-acclimated, control bullfrogs also had shorter non-ventilatory periods in the post-hypercarbic period (Fig. 31D; $p<0.05$ control vs. post-hypercarbic hyperpnea; Holm-Sidak’s multiple comparisons test). In contrast, cold-acclimated bullfrogs did not undergo significant increases in the number of breaths per episode and did not shorten the duration of the non-ventilatory period (Fig. 31B &D; $p>0.05$ control vs. post-hypercarbic hyperpnea;
Figure 31

- **Control**
- **Cold-acclimated**

A

B

C

D
Figure 31 Cold-acclimated bullfrogs do not undergo changes in breathing pattern that lead to increases in absolute respiratory frequency during post-hypercarbia. A-D shows breathing pattern parameters including breaths episode\(^{-1}\) (A), episodes min\(^{-1}\) (B), instantaneous breathing frequency (C), and duration of the non-ventilatory period (D) in warm (black bars) and cold-acclimated (white bars) bullfrogs. Sample sizes are included in the figure.

Reduced breathing frequency responses during post hypercarbia of cold-acclimated bullfrogs manifests due to a reduction in the number of breaths in each breathing episode (A; p<0.05; warm vs. cold-acclimated; Holm-Sidak’s multiple comparisons test, p<0.0001; normocarbia vs. post-hypercarbia in warm-acclimated bullfrogs; Holm-Sidak’s multiple comparisons test) without changing the total number of episodes (B., p>0.05 for all comparisons; Holm-Sidak’s multiple comparisons test). C. shows that instantaneous frequency (i.e., the breathing frequency during breathing episodes) is decreased in cold-acclimated bullfrogs during the post-hypercarbic period compared to warm acclimated bullfrogs (p<0.05; warm vs. cold-acclimated during post-hypercarbia; Holm-Sidak’s multiple comparisons test). Lastly, D. indicates that warm-acclimated bullfrogs decrease the duration of the non-ventilatory period during hypercarbia (p<0.05; normocarbia vs. post-hypercarbia for warm-acclimated bullfrogs; Holm-Sidak’s multiple comparisons test), but cold-acclimated bullfrogs do not (p>0.05 for all comparisons relative to normocarbia). Error bars represent ± SEM.

*p<0.05, ***p<0.001 compared to normoxia for each acclimation group and +p<0.05 compared to warm-acclimated bullfrogs (between animal differences).
Holm-Sidak’s multiple comparisons test). Additionally, instantaneous breathing frequency (the frequency of successive breaths during episodes of two or more breaths) was decreased in cold acclimated bullfrogs (main effect of temperature acclimation; p=0.0004; F_{(1,49)}=14.38; two-way ANOVA) and included a statistically significant difference in the post hypercarbic period compared to controls (Fig. 31C; p<0.05 control vs. cold-acclimated; Holm-Sidak’s multiple comparisons test). Processes responsible for these changes are therefore insensitive to CO\(_2\) in cold-acclimated frogs.

*Cold-acclimation does not alter the ventilatory response to hypoxia*

To determine whether there was a global vs. CO\(_2\)-specific reduction in respiratory gas sensitivity, we also compared the ventilatory response to hypoxia (5% O\(_2\)) in cold and warm-acclimated bullfrogs. In contrast to reductions in ventilatory responses to CO\(_2\) challenge, ventilatory responses to hypoxia were similar among control and both cold-acclimated groups. Warm and both cold-acclimated groups increased \(\dot{V}_e\) during hypoxia (Figure 32A; main effect of hypoxia; p<0.0001; F_{(4,68)}=18.25; two-way ANOVA; p>0.05 for all comparisons between acclimation groups; Holm-Sidak’s multiple comparisons test). Increases in ventilation were caused by increases in V\(_T\) in each acclimation group (Figure 32B; main effect of hypoxia; p<0.0001; F_{(4,68)}=16.72; two-way ANOVA; p>0.05 for all between acclimation group comparisons; Holm-Sidak’s multiple comparisons test). Although we observed a significant effect of hypoxia on breathing frequency (two-way ANOVA; effect of hypoxia; F_{(3,51)}= 4.099; p=0.0111), there were no significant pairwise comparisons between different time points in
Figure 32

- Control (n=8)
- Cold-Acclimated: Access (n=7)
- Cold-Acclimated: Submerged (n=5)

A

5% O₂

Hypoxia: p < 0.0001
Acclimation: p = 0.5643
Hypoxia x Acclimation: p = 0.5641

B

5% O₂

Hypoxia: p = 0.0111
Acclimation: 0.4976
Hypoxia x Acclimation: p = 0.3440

C

5% O₂

Hypoxia: p < 0.0001
Acclimation: p = 0.7355
Hypoxia x Acclimation: p = 0.8695
Figure 32 Cold-acclimation with and without access to air does not influence ventilatory response to acute hypoxia. A-C shows absolute $\dot{V}_E$, breathing frequency, and tidal volume, respectively, during exposure to hypoxia (5% $O_2$) in warm-acclimated (black circles; n=8), cold-acclimated with air (white circles; n=7), and cold-submerged (gray circles; n=5) bullfrogs. Asterisks placed above all acclimation demonstrate groups at specific time points in hypoxia indicates the same significance level achieved for each group. All temperature acclimation groups increased $\dot{V}_E$ compared to baseline (two-way ANOVA; effect of time in hypoxia; $F_{(3,51)} = 25.42; p<0.0001$) in response to hypoxia due to increases in tidal volume (two-way ANOVA; effect of time in hypoxia; $F_{(3,51)} = 21.75; p<0.0001$). There was no time in hypoxia x acclimation interactions or significant post hoc comparisons between acclimation groups at any time point (two-way ANOVA with Holm-Sidak multiple comparisons test; $p>0.05$ for all). Although there was an effect of hypoxia on breathing frequency (two-way ANOVA; effect of time in hypoxia; $F_{(3,51)} = 4.099; p=0.0111$), there were no statistically significant pairwise comparisons between different time points in hypoxia. Error bars represent ± SEM. *$p<0.05$, **$p<0.01$ compared to normoxia for each acclimation group.
hypoxia compared to normoxic breathing frequency in any acclimation group (Figure 32B; p>0.05; Holm-Sidak’s multiple comparisons test).

We also measured $\dot{V}_{O_2}$ during hypoxia so that we could calculate changes in the air convection requirements among acclimation groups in response to hypoxia. This would allow us to determine whether similar ventilatory responses to hypoxia were occurring amid different metabolic responses to hypoxia among acclimation groups. Fig. 33A shows that acute hypoxia did not alter $\dot{V}_{O_2}$ in any acclimation group (no effect of temperature acclimation or hypoxia; p>0.05; two-way ANOVA). Given that ventilation increased in warm and both cold-acclimated groups, $\dot{V}_E/\dot{V}_{O_2}$ increased significantly in response to hypoxia (Fig. 33B, main effect of hypoxia; p<0.0001; $F_{(1,17)}= 68.27$; two-way ANOVA; p>0.05 for comparisons between acclimation groups), indicating ventilatory sensitivity to hypoxia is intact following cold-acclimation with or without access to the surface.

Lastly, we measured aspects of the breathing pattern in normoxia and hypoxia. Similar to previous reports in warm-acclimated bullfrogs (Santin and Hartzler, 2016c), the instantaneous breathing frequency decreased in warm and cold-acclimated bullfrogs during hypoxia (Fig. 34C; main effect of hypoxia; p=0.0007; $F_{(1,17)}=16.97$; two-way ANOVA). The number of episodes per minute, breaths per episode, and the duration of the non-ventilatory period were unaltered by hypoxia and temperature acclimation (Fig. 34A,B,D; p>0.05; two-way ANOVA). Collectively, these results show that bullfrogs that recently emerged from simulated overwintering hibernacula possess ventilatory responses to hypoxia that are similar to controls.
Figure 33

A

- **Control (n=8)**
- Cold-Acclimated: Access (n=7)
- Cold-Acclimated: Submerged (n=5)

B

- Hypoxia: p=0.8255
- Acclimation: 0.139
- Hypoxia \times Acclimation: p=0.6384

- Hypoxia: p<0.0001
- Acclimation: 0.3039
- Hypoxia \times Acclimation: p=0.3159
Figure 33 Metabolic responses and hyperventilation during hypoxia do not differ between acclimation groups. A. shows $O_2$ consumption ($\dot{V}o_2$; A) before and after 30 minutes of hypoxia (5% $O_2$) in control (black circles; n=8), cold-acclimated with air access (white circles; n=7), and cold-submerged bullfrogs (gray circles; n=5). B. indicates that both groups of cold-acclimated bullfrogs underwent similar magnitude of hyperventilation in response to 30 minutes of hypoxia as calculated by the air convection requirement for $O_2$ (main effect of hypoxia; two-way ANOVA; $F_{(1,17)}=68.27$; p<0.0001; significant post hoc analysis for each acclimation group). Error bars represent ± SEM. **p<0.01, ***p<0.001 compared to normoxic for each acclimation group.
Figure 34

- **Control**
- **Cold-acclimated**

**A**

Breaths Episode$^{-1}$

**B**

Episodes min$^{-1}$

**C**

Instantaneous frequency (breaths min$^{-1}$)

**D**

Duration of non-ventilatory period (s)
Figure 34 Breathing pattern does not differ between warm and cold-acclimated bullfrogs in response to hypoxia. A-D shows pooled breathing pattern parameters including breaths episode$^{-1}$ (control: n=8, cold: n=12) (A), episodes min$^{-1}$ (control: n=8, cold: n=12) (B), instantaneous breathing frequency (control: n=8, cold: n=11) (C), and duration of the non-ventilatory period (control: n=8, cold: n=12) (D) before and after hypoxia (5% O$_2$) in warm (black bars) and pooled, cold-acclimated (white bars) bullfrogs. Breaths episode$^{-1}$ (A), episodes min$^{-1}$ (B), and duration of the non-ventilatory period (D) were affected by hypoxia in both acclimation groups (two-way ANOVA; p>0.05). The instantaneous breathing frequency (C) decreased during hypoxia in both acclimation groups (main effect of hypoxia, but not acclimation; two-way ANOVA; p=0.0007, warm-acclimated; p<0.001; Holm-Sidak’s multiple comparisons test, cold-acclimated; p<0.01; Holm-Sidak’s multiple comparisons test). Error bars represent ± SEM. * p<0.05, **p<0.01
Discussion

We performed these experiments to determine whether aspects of the respiratory control system are maintained or suppressed following aquatic hibernation. Maintenance of the respiratory control system throughout extended disuse would ensure adequate gas exchange and blood gas homeostasis at higher temperatures following emergence from overwintering hibernacula. We found that bullfrogs recently removed from simulated overwintering environments exhibited similar levels of resting ventilation when assessed at 24°C compared to warm-acclimated bullfrogs. Additionally, ventilation matches resting metabolism and, presumably contributed to acid-base regulation requirements under control conditions, indicating preservation of basal respiratory function despite 6-9 weeks of minimal/no activity in the cold. Recently emerged bullfrogs underwent similar increases in ventilation during acute hypoxia, but not in response to hypercarbia-related stimulation, compared to warm-acclimated frogs. Altogether, this study reveals new insights into the function of the respiratory control system following extended disuse of lung breathing as inevitably occurs in cold-aquatic overwintering habitats of bullfrogs and other northern temperate frogs.

Respiratory Inactivity during Submergence

Natural (hibernation, microgravity, bedrest) or experimental (limb immobilization) scenarios associated with neuromotor disuse in vertebrates generally lead to some degree of functional degradation through muscle atrophy
or maladaptive neuroplastic changes (Clark et al., 2006; Hudson and Franklin, 2002; Langlet et al., 2012; Renaud and Stevens, 1983; Wickler et al., 1991). A notable and extreme exception, however, includes the green-striped burrowing frog who burrows for ~9 months with no muscular atrophy (Hudson and Franklin, 2002). Although submerged northern temperature frogs such *L. catesbeianus* and *R. temperaria* move (Stinner et al., 1994; Tattersall and Boutilier, 1999b) and perform cardiovascular function (Lillo, 1980), lung breathing is suspended. Several lines of evidence suggest that the entire sensorimotor system controlling lung ventilation is inactive during the overwintering period. First, air access may be eliminated in overwintering hibernacula. Northern temperate frogs involuntary (*i.e.*, trapped under the ice) or voluntarily (*i.e.*, no need to surface) (Tattersal and Ultsch, 2008; personal observations) endure long periods of submergence (≥12 hours to months). Second, low metabolic rates associated with cold temperatures can be satisfied exclusively through cutaneous gas exchange without negative consequences to temperature-specific blood gas homeostasis (Donohoe et al., 1998; Ultsch et al., 2004), eliminating a “need” to breathe. In fact, the isolated central respiratory control system of adult bullfrogs does not produce respiratory-related nerve activity below 10°C (Morales and Hedrick, 2002) suggesting that mechanism(s) may be in place to inhibit breathing under metabolic conditions that do not require lung ventilation. Third, anuran amphibians do not have chemical respiratory drive at low temperatures. Evidence across scales of organization indicate that CO$_2$ and O$_2$ sensory systems that alter ventilation for respiratory gas homeostasis do not detect
chemosensory stimuli or drive breathing at cold temperatures (Bicego-Nahas and Branco, 1999; Morales and Hedrick, 2002; Rocha and Branco, 1998; Santin et al., 2013). Therefore, the system that generates, drives, and regulates lung ventilation, does not appear to function for several months during the winter.

We have made the argument here that bullfrogs contain a minimally active respiratory control system during overwintering conditions in deep, well-oxygenated water. However, contradictory evidence exists suggesting that bullfrogs at 1°C will surface in shallow water (5 cm) when provided air access (Tattersal and Ultsch, 2008; unpublished observation). Although we did not quantify surfacing and lung ventilation in the group provided air access during simulated overwintering, we rarely observed frogs at the surface of the tank (personal observation). Given that both groups of cold-acclimated bullfrogs had similar levels of resting ventilation and ventilatory responses to gas challenges when assessed following forced emergence, we have no reason to believe that minimal surfacing that may have occurred in the group provided air access influenced breathing control compared with the submerged group. Therefore, we assume that bullfrogs with air access were “functionally submerged” with respect to changes that occurred to affect lung breathing after emergence.

**Maintenance of Resting Ventilation**

We found that cold-acclimation without or with (but minimally used) access to the surface did not affect the ability to generate lung ventilation (Fig. 27) and match ventilation to metabolism (Fig. 28) ~14-16 hours after warming from 2°C to 24°C.
Measuring ventilation using facemask pneumotachography required us disturb the bullfrog to attach a mask to its face, precluding the use of an earlier time point in analysis. However, after removing bullfrogs from the cold environmental chamber before attaching the mask, we observed buccal pumping, naril opening and closing, and axial contraction associated with lung ventilation (Vitalis and Shelton, 1990) within a few minutes. Although we measured ventilation ~14-16 hours after emergence, this observation implies that basic neuromotor properties associated with breathing remain intact and do not require a recovery period. The strategies used by bullfrogs that result in resilience of the respiratory output despite overwintering disuse are currently unknown. Cold-acclimated bullfrogs with air access had a slightly larger $\dot{V}O_2$ compared to control bullfrogs at 24°C (Fig. 2A), consistent with a well known temperature acclimation of metabolism of anurans (Feder, 1982). In contrast, submerged bullfrogs did not have greater $\dot{V}O_2$ at 24 °C compared to warm-acclimated frogs, perhaps due to metabolic suppression induced by forced submergence (i.e., lower O$_2$ consumption during submergence than predicted by cold temperature alone) during overwintering conditions (Donohoe et al., 1998). $\dot{V}CO_2$ paralleled $\dot{V}O_2$ in both groups of cold-acclimated bullfrogs, but did not reach the same statistical significance. Regardless of these slight differences in metabolic backgrounds, ventilation evidently satisfied metabolism because average $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ for individuals in each group (air convection requirement; ACR) were similar for each temperature acclimation group (Fig. 2D & E). We did not measure blood gases in this study; however, interpreting the respiratory exchange ratio (RER;
\( \dot{V}_{CO_2}/\dot{V}_{O_2} \) in concert with the air convection requirement provides an indication of organismal acid-base balance. Although we cannot account for gas exchange partitioning between lungs and the skin, rates of total \( O_2 \) consumption, total \( CO_2 \) production, and ventilation influence acid-base balance (Jackson and Braun, 1979a; Wang et al., 1998). Given that RER, \( \dot{V}_E/\dot{V}_{O_2} \), and \( \dot{V}_E/\dot{V}_{CO_2} \) did not vary across each temperature-acclimation group (Fig.28 C, D, & E), the most parsimonious explanation for the combination of results we obtained is that ventilation matched metabolism and, presumably, contributed to maintenance of similar acid-base balance at rest. If we had found a lower ACR with similar RER or a similar ACR with a lower/ higher RER in cold-acclimated vs. control bullfrogs, we would have interpreted our results differently to include the potential for changes in resting cutaneous gas exchange, intracardiac shunt, or acid-base balance, but this did not occur. Thus we conclude that breathing matches metabolism and probably contributes to maintenance of acid-base balance at rest in bullfrogs after 6-9 weeks of disuse in the aquatic overwintering environment shortly after forced transition to land and elevated temperature.

**Sensitivity to Environmental Hypercarbia**

We reasoned that ventilation may sufficiently satisfy resting metabolism after simulated overwintering, but the respiratory control system may have a limited capacity to respond with increases in ventilation during perturbations to blood gas homeostasis. Hypercarbia and hypoxia increase ventilation via distinct mechanisms by predominately elevating breathing frequency and tidal volume, respectively, in adult bullfrogs (Santin and Hartzler, 2016c). Therefore, we
assessed the ventilatory responses to hypercarbia and hypoxia after the transition from cold-aquatic to warm-terrestrial environments.

Consistent with previous reports (Kinkead and Milsom, 1996; Santin and Hartzler, 2016c), we showed here that ventilation did not increase significantly in any acclimation group at any time point during hypercarbia. Warm-acclimated bullfrogs in our study, however, underwent a large “post-hypercarbic hyperpnea” immediately after the exchange of room air for hypercarbia. Because the ventilatory response during exposure to environmental hypercarbia is under strong (Kinkead and Milsom, 1996) and presumably variable (Santin and Hartzler, 2016c) inhibition by olfactory chemoreceptors, the post-hypercarbic hyperpnea has been suggested to better reflect stimulatory chemosensitivity associated with activation of central and peripheral chemoreceptors (Milsom et al., 2004; Santin and Hartzler, 2016c). In cold-acclimated bullfrogs, the post-hypercarbic hyperpnea was reduced by ~50% compared to control bullfrogs (Fig. 29A). Relative to baseline ventilation in normocarbia, cold-acclimated bullfrogs did not undergo significant increases in ventilation immediately following hypercarbia (Fig. 29A&B). The reduction in the ventilatory response following CO₂ washout did not occur on a background of a different metabolic response to CO₂ (Fig. 30), strongly suggesting that bullfrogs from overwintering environments underwent a reduction in CO₂ chemosensitivity per se. As hypercarbia/post-hypercarbia minimally influences the tidal volume in bullfrogs, a limited breathing frequency response underlies reduction in post-hypercarbic ventilation (Fig. 29C). Bullfrogs and other anurans contain an inherently episodic breathing
pattern; specifically, breaths occur in clustered episodes, rather than a regular pattern (Kinkead and Milsom, 1994; Smatresk and Smits, 1991). Under the experimental conditions of the present study, breathing frequency increases during the post-hypercarbic period of control bullfrogs resulted from adding breaths to each episode and shortening the duration of the non-ventilatory period (Fig. 31). Our findings suggest that overwintering conditions lead to an inability to add breaths to episodes and shorten the duration of the non-ventilatory period. In anurans, ~80% of the stimulatory CO₂ chemosensitivity arises from the brainstem (near the 4th ventricle) and ~20% from peripheral chemoreceptors (or chemoreceptors outside the 4th ventricle) (Branco et al., 1993). Interestingly, our results showing blunted ventilatory responses to CO₂ challenge are consistent with preliminary evidence that overwintering submergence leads to a reduction in CO₂ chemosensitivity of locus coeruleus neurons (Santin, 2015; Santin and Hartzler, 2015b) that drive ventilation during local brain and arterial acidemia (Noronha-de-Souza et al., 2006). This implies that overwintered bullfrogs have a disrupted CO₂/pH chemosensory system. However, the instantaneous frequency was reduced in cold-acclimated frogs (Fig. 31C) suggesting that issues associated with respiratory rhythmogenesis may, in part, account for lower ventilatory responses to CO₂ challenge independent of modulating chemosensitivity per se. Future work should be directed toward understanding the mechanisms underlying reduced ventilatory responses to elevated inspired CO₂ in cold-acclimated bullfrogs (i.e., central vs. peripheral changes in chemosensitivity vs. rhythmogenic dysfunction).
Although cutaneous gas exchange eliminates a large proportion of metabolically produced CO$_2$ in anurans, acid-base regulation requires lung ventilation in bullfrogs (Gottlieb and Jackson, 1976). The CO$_2$ chemosensory system controls ventilation to regulate arterial P$_{CO2}$ and pH in response to blood gas disturbances in air-breathing vertebrates (da Silva et al., 2013; Guyenet and Bayliss, 2015). Our results showing that overwintering conditions lead to a reduction in ventilatory responses to CO$_2$ challenge when assessed at warmer temperatures soon after emergence imply that there may be reduced capacity for compensation of acid-base disturbances by chemosensory-driven lung ventilation. During forced diving, bullfrogs also accumulate CO$_2$ (11 to 18 mm Hg), leading to acidemia (pH 7.9 to 7.6), after ~10 minutes at 25°C (Lillo, 1978). Furthermore, dive time in turtles inversely correlates with respiratory gas chemosensitivity (Reyes and Milsom, 2009). Since prolonged dives represent a behavioral mechanism for predator avoidance in ranid frogs (Gregory, 1979; Heatwole, 1961; McIntyre and McCollum, 2000), a reduced ability for CO$_2$/pH to facilitate the occurrence of breaths at warmer temperatures after overwintering may confer the advantage of longer dive time to avoid additional aerial and terrestrial predators of the spring. However, the consequences of reduced ventilatory sensitivity to CO$_2$ in the early-spring environment suggested here are speculative and require further study to place our results in an ecological context.

Sensitivity to Hypoxia

In contrast to reduced ventilatory responses to hypercarbia, warm and cold-acclimated bullfrogs had similar ventilatory responses, air convection
requirements, and breathing patterns in response to hypoxia (5% O\textsubscript{2}) at 24°C (Fig. 32, 33, 34). In this study, an augmentation of tidal volume mediated the ventilatory increase in response to hypoxia that occurred in each acclimation group (Fig. 32). O\textsubscript{2}-sensitive processes that drive ventilation located in the carotid labyrinth and the aortic arch effect ventilatory increases during hypoxia in anurans (Van Vliet and West, 1992). Additionally, a centrally-driven, hypoxic ventilatory depression occurs in adult bullfrogs, presumably to dampen breathing frequency during hypoxia for energy conservation (Fournier et al., 2007; Winmill et al., 2005). Similar to resting breathing, our findings suggest that overwintering conditions would not affect “early spring” function of O\textsubscript{2} sensitive processes mediating ventilatory responses to hypoxia. However, it is important to acknowledge that our measurements do not allow for speculation about changes in ventilation-perfusion matching and regulation of blood gases by alterations in right-to-left cardiac shunting that occur in anuran amphibians during hypoxia (Gamperl et al., 1999). Although we measured similar ventilated volumes of gas and rates of O\textsubscript{2} consumption before and during hypoxia among acclimation groups (Fig. 33), altered cardiac shunt patterns could lead to drastically different Pao\textsubscript{2} values even with identical ventilation and metabolism (Wang and Hicks, 1996; Wang et al., 1997). Therefore, similar hypoxic ventilatory responses could potentially be a result of varied O\textsubscript{2} chemosensitivities if differences in cardiac shunts produced different changes in blood gases among temperature acclimation groups during hypoxia. Regardless of the mechanistic causes and effects emanating from differences in sensory processing or system integration,
our findings demonstrate that recently emerged frogs possess ventilatory sensitivity to hypoxia, but blunted sensitivity to CO$_2$ in response to constant stimuli supplied in the ambient environment following overwintering conditions.

**Perspectives and Significance**

Following long-term depression of lung breathing and chemosensory respiratory drive during cold submergence, bullfrogs breathed normally at rest and possessed normal ventilatory sensitivity to oxygen shortage, but had reduced ventilatory responses to CO$_2$ challenge after transition to land at a warmer temperature. This is an intriguing scenario because prolonged inactivity generally results in loss of function through muscle atrophy or neuroplastic deficits (Clark et al., 2006; Phillips and McGlory, 2014), unless evaded through physiological/behavioral compensatory mechanisms. For example, hibernators employ a suite of unique cellular mechanisms to avoid muscle atrophy (Lee et al., 2008; Lin et al., 2012; Nowell et al., 2011; Tessier and Storey, 2014; Van Breukelen and Martin, 2002; Young et al., 2013) and can even experience muscle hypertrophy (Reid et al., 1995). However, some rodent hibernators may also experience skeletal muscle atrophy that should hinder performance (Wickler et al., 1987; Wickler et al., 1991). From the neural perspective, a wealth of information exists regarding how neurons and circuits maintain target levels of activity despite removing or enhancing activity inputs (termed homeostatic plasticity). However, models to study these processes use pharmacological or pathological paradigms to manipulate neuronal activity *in vivo* and *in vitro* primarily at early developmental stages (Hengen et al., 2013; Knogler et al.,
2010; Ngodup et al., 2015; Schacher and Hu, 2014; Turrigiano, 2012; Wilhelm and Wenner, 2008). Thus, the bullfrog respiratory control system following overwintering offers a powerful ability to uncover mechanisms leading to and resulting in preservation of (respiratory motor output and hypoxia sensitivity) and deterioration (CO₂ sensitivity) of sensorimotor function in the same species, individual, and neural control system after ecologically relevant inactivity.

**Conclusion**

We performed these experiments to determine whether aspects of the respiratory control system were functional following inactivity in the overwintering period. We found that bullfrogs breathed sufficiently to satisfy a greater metabolic rate associated with warmer temperatures soon after forced emergence. We also showed that cold-acclimated bullfrogs had preserved ventilatory responses to oxygen lack, but not elevated CO₂. The ability to dissect mechanisms underlying preservation and loss of sensorimotor function following prolonged disuse in this natural context may provide a powerful model to improve the basic understanding of consequences following inactivity in other normal or pathological states.
CHAPTER VIII

MANUSCRIPT V

Environmentally induced return to juvenile-like chemosensitivity in the respiratory control system of adult bullfrog, *Lithobates catesbeianus*


Santin, J.M. & Hartzler, L.K.
Abstract:
An unanswered question in developmental physiology is to what extent does the environment vs. a genetic program produce phenotypes. Developing animals inhabit different environments and switch from one to another. Thus a developmental time course overlapping with environmental change confounds interpretations as to whether development (i.e., permanent processes) or environmental plasticity (i.e., reversible processes) generates phenotypes. Tadpoles of the American bullfrog, *Lithobates catesbeianus*, breathe water at early life-stages and minimally use lungs for gas exchange. As adults, bullfrogs rely on lungs for gas exchange, but spend months per year in ice-covered ponds without lung breathing. Aquatic-submergence, therefore, removes environmental pressures requiring lung breathing and enables separation of adulthood from environmental factors associated with adulthood that necessitate control of lung ventilation. To test the hypothesis that postmetamorphic respiratory control phenotypes arise through permanent developmental changes vs. reversible environmental signals, we measured respiratory-related nerve discharge in isolated brainstem preparations and action potential firing from CO₂-sensitive neurons in bullfrogs acclimatized to semi-terrestrial (air-breathing) and aquatic-overwintering (no air-breathing) habitats. We found that aquatic-overwintering significantly reduced neuroventilatory responses to CO₂ and O₂ involved in lung breathing. Strikingly, this gas sensitivity profile reflects that of water-breathing tadpoles. We further demonstrated that aquatic-overwintering reduced CO₂-induced firing responses of chemosensitive neurons. In contrast, respiratory rhythm generating processes remained adult-like after submergence. Our results
establish that phenotypes associated with life-stage can arise from environmental plasticity \textit{per se}. This provides evidence that developmental time courses coinciding with environmental changes obscure interpretations regarding origins of stage-dependent physiological phenotypes by masking plasticity.
Introduction:

A major question in developmental physiology is to what extent does the environment vs. a genetic program produce phenotypes (Burggren and Warburton, 2005). Anatomical processes that determine body plan may be expected to follow strict genetic programming (Mallo et al., 2010), but plastic physiological functions could exhibit high sensitivity to environmental factors. Animals across vertebrate taxa inhabit drastically different environments (e.g., uterus, eggs, water, land) and abruptly switch from one to another at key developmental stages (e.g., birth, metamorphosis, hatching), during which time, maturation of physiological processes from cells to whole systems occurs (Hedrick, 2005; Henning, 1981; Imber and Putnam, 2012; Monyer et al., 1991). Thus a developmental time course overlapping with irreversible shifts in the environment confounds interpretations as to whether particular phenotypes arise through genetically determined developmental processes (i.e., permanent changes during life-history) or extrinsic factors associated with environmental plasticity (i.e., potentially reversible changes). Despite a well-appreciated interaction between environmental influences on development (Burggren and Warburton, 2005; Gilbert, 2012), the possibility remains that environmental plasticity *per se* may generate phenotypes attributed to irreversible developmental processes (Ho et al., 2011). As potential treatments from certain human diseases assume genetically “hardwired,” developmental underpinnings (Köhler et al., 2014), deciphering whether development (irreversible) or environmental plasticity (potentially reversible) dictates certain phenotypes may
have important ramifications for human health. Unfortunately, use of traditional mammalian model systems hinders disambiguating whether phenotypes observed at particular life stages occur due to development or to the environments in which life-stages occur because these two factors often change simultaneously and irreversibly.

Anuran amphibians (frogs and toads) experience profound environmental changes during development and throughout adult life. Therefore, anurans make unparalleled models to determine whether phenotypes observed at different developmental stages occur due to developmental trajectories or the environments in which developmental stages occur. Larval anurans have a respiratory system adapted for water breathing, but they transition to air breathing during metamorphosis (Burggren and Infantino, 1994; Burggren and West, 1982). Along with maturation of lungs and loss of gills, a sensorimotor circuit in the brainstem controlling gill and lung ventilation changes to accommodate the switch from water to air. Neurobiological modifications to central control of ventilation during development from tadpole to frog involve 1.) increased expression of a lung respiratory rhythm (Torgerson et al., 1997) 2.) larger absolute lung breathing frequency responses to changes in CO$_2$/pH (Taylor et al., 2003a; Torgerson et al., 1997) and 3.) enhanced inhibition of lung breathing frequency by hypoxia (Fournier et al., 2007; Winmill et al., 2005). As with other air-breathing vertebrates such as mammals, these neural changes facilitate air breathing and blood gas homeostasis on land; however, many adult frog species from northern latitudes overwinter for several months in completely
aquatic habitats without breathing air (Bradford, 1983; Stinner et al., 1994; Ultsch et al., 2004; Willis et al., 1956). Processes including cutaneous gas exchange and hypoxia tolerance promote survival of frogs in submerged overwintering environments (Tattersall and Ultsch, 2008). Intriguingly, overwintering in adult frogs recapitulates salient features of the juvenile physiological environment, namely, gas exchange in water without lung ventilation. Overwintering submergence, therefore, provides a unique opportunity to uncouple developmental stage (adulthood) from environmental factors associated with adulthood (terrestrial life) in the respiratory control system. Understanding the influence of overwintering submergence on respiratory control could provide insight into whether permanent developmental shifts or changes in the environment associated with terrestrially generate mature respiratory phenotypes typically attributed to development of the breathing control system (Gargaglioni and Milsom, 2007; Gdovin et al., 1999; Milsom, 2010; Torgerson et al., 1997; Winmill et al., 2005).

After bullfrogs emerge from submerged overwintering conditions, breathing operates normally at rest, indicating functional respiratory rhythmogenic processes, but has diminished CO$_2$ sensitivity (Santin and Hartzler, 2016a). In response to increased brain and arterial CO$_2$, air breathing vertebrates undergo hyperventilation primarily mediated by brainstem chemoreceptors. (Milsom, 2010; Sundin et al., 2007; Taylor et al., 2003a; Taylor et al., 2003c; Torgerson et al., 1997). Although early-stage tadpoles appear to have central CO$_2$ chemoreceptors (Rousseau et al., 2016; Taylor and Brundage,
2013; Taylor et al., 2003b), the bulk of the evidence indicates that the ability for CO₂/acidification to increase lung breathing frequency increases absolutely in magnitude with maturity (Davis et al., 2006; Taylor et al., 2003a; Taylor et al., 2003c; Torgerson et al., 1997) in accordance with requirements for chemoreceptive control of lung ventilation (Gargaglioni and Milsom, 2007; Milsom, 2010). This trend may be conserved among air-breathing vertebrates because increased ventilatory CO₂ responsiveness also occurs during postnatal development in rats (Davis et al., 2006). Diminished ventilatory chemosensitivity after aquatic submergence, therefore, presents an intriguing case where an adult air-breathing vertebrate has an environmentally-induced loss of ventilatory CO₂ responsiveness under conditions that otherwise result in large increases in ventilation. This suggests that stereotypical respiratory control phenotypes may be driven by environments associated with adulthood and immaturity (i.e., terrestrial and aquatic life) rather than maturation state per se. Using an in vitro neurophysiological approach, we tested the hypothesis that submerged overwintering in adult bullfrogs would reduce brainstem “fictive” breathing frequency responses to respiratory gases CO₂ and O₂, similar in magnitude to immature, water-breathing tadpoles. In contrast, since we previously found that aquatic submergence does not affect resting ventilation, we hypothesized that respiratory rhythmogenic function would remain adult-like.
Methods

Ethical Approval

Experiments were approved by the Wright State University Institutional Animal Care and Use Committee.

Experimental Animals

Three groups of bullfrogs were used in this study: 1.) semi-terrestrial controls at 22°C, 2.) overwintered at 2°C with access to air, 3.) overwintered at 2°C without access to air. The generation of the experimental animal groups has been described in detail (Santin and Hartzler, 2016a). Briefly, semi-terrestrial bullfrogs were maintained in 22°C aerated water, fed crickets twice per week, and had access to wet and dry areas. Both aquatic overwintered groups underwent an initial cooling phase from ~22°C to 2 °C over 6 weeks in aerated water. A plastic screen containing holes was placed at the surface of one tank containing aquatic overwintered bullfrogs and both tanks (one with air access and the other without) were maintained at 2°C for another six weeks. After six-nine weeks of acclimation, experiments were performed on aquatic overwintered bullfrogs, alternating between the two experimental tanks.

Brainstem-spinal cord preparation and brainstem slices:

To address whether aquatic overwintering alters the central control of breathing and cellular CO₂ sensitivity in bullfrogs we used the in vitro brainstem-spinal cord (Galante et al., 1996; Harris et al., 2002; Kinkead et al., 1994) and locus
coeruleus brain slice preparations (Santin and Hartzler, 2013a), respectively. For both preparations, the brainstem-spinal cord was dissected as previously described (Santin et al., 2013). Bullfrogs were euthanized by rapid decapitation. To generate the *in vitro* brainstem-spinal cord preparation, the dissected brainstem-spinal cord was then transected rostral to the optic tectum and caudal to spinal nerve II and then pinned ventral side up in the recording chamber (6 mL) constructed from a petri dish coated with Sylgard (Dow Corning, Midland, MI; U.S.A). To produce brain slices containing the locus coeruleus, the whole-brain was glued to an agar block and cut into ~400 μm-thick slices using a Vibratome tissue slicer (Leica Microsystems, Buffalo Grove, IL). Brain slices containing the locus coeruleus were identified anatomically (González and Smeets, 1993) and transferred to a 1 mL recording chamber, and stabilized with a nylon grid prior to cellular electrophysiology experiments. The brainstem-spinal cord preparation was superfused with control bullfrog artificial cerebral spinal fluid (aCSF) containing (in mM) 104 NaCl, 4 KCl, 1.4 MgCl₂, 7.5 glucose, 40 NaHCO₃, 2.5 CaCl₂, and 1 NaH₂PO₄, and gassed with 90% O₂, 1.3% CO₂, and balance N₂ at 6 mL/min, and circulated using a peristaltic pump (Rainin Instrument Co., Oakland, CA; U.S.A.). Brain slice preparations were superfused with the same aCSF, but bubbled with 80% O₂, 1.3% CO₂, and balance N₂ at 2 mL/min using gravity-fed, stainless steel drip lines, respectively. Preparations were allowed to recover in their experimental chambers at 22°C for ~1 hr following dissection.

*Whole-nerve recordings from brainstem-spinal cord preparations*
Cranial nerves V (trigeminal) and X (vagus) contain branches that innervate the muscles that operate the respiratory muscles of amphibians; therefore, spontaneous rootlet activity correlates with respiratory-related central nervous system activity that drives breathing in intact frogs (Sakakibara, 1983). CN V and CN X were drawn into borosilicate glass bipolar suction electrodes. Each glass electrode was pulled using a two-stage micropipette puller (PC-10; Narishige; East Meadow, NY; U.S.A.), broken to size to fit snugly around each nerve rootlet, and then fire polished. Activity of each nerve was amplified (X1000) using Warner Instruments differential amplifiers (DP-311; Warner Instruments, Hamden, CT, U.S.A), filtered (100 Hz-1000 Hz), full-wave rectified, integrated (time constant = 60ms), and recorded using the Powerlab 8/35 data acquisition system (AD Instruments Inc. CO, USA) onto a personal computer.

**Whole-cell patch clamp electrophysiology**

Whole-cell current clamp recordings of spontaneous changes in membrane voltage (i.e., action potentials) in LC neurons were performed as previously described (Santin et al., 2013). Briefly, ~5 MΩ borosilicate glass pipettes were back-filled with artificial intracellular solution containing (in mM) 110 potassium gluconate, 2 MgCl₂, 10 HEPES, 1 Na₂-ATP, 0.1 Na₂-GTP, 2.5 EGTA, pH 7.2, with KOH, and positioned over an AgCl₂-coated Ag wire. The chamber was located under a fix-stage microscope (Nikon, Elgin, IL, U.S.A.) where the slice was visualized at 4X magnification to identify the LC. Individual neurons located in the area identified anatomically as the locus coeruleus (González et al., 1994) were observed at 60X magnification. The electrode was positioned near the
neuron of interest using a Burleigh micromanipulator while applying positive pressure through the glass pipette (PCS 5000; Thorlabs, Newton, NJ, U.S.A.). When the pipette touched the neuron, positive pressure was removed and slight negative pressure was applied by mouth until the formation of a GΩ seal. Rapid, but gentle, negative pressure was applied to break the membrane and gain whole-cell electrochemical access. Membrane potential was determined in current-clamp mode using Axopatch 200 B integrating patch clamp amplifier (Molecular Devices; Sunnyvale, CA, U.S.A) and collected using P10 Clampex software (Molecular Devices; Sunnyvale, CA, U.S.A). Current-clamp recordings were analyzed off-line using pCLAMP software (Molecular Devices).

Experimental Procedures

Brain-Stem Spinal Cord Experiments: In semi-terrestrial (n=9), aquatic overwintered air access (n=7), and aquatic overwintered submerged (n=5) frogs, baseline burst activity from CN V and CN X was recorded from brainstem-spinal cord preparations. After acquiring stable baseline recordings (90% O₂/ 1.3% CO₂, balanced N₂; pH=7.9), we applied hypercapnia (90% O₂, 5% CO₂ balanced N₂; pH=7.5) for 30 minutes or hypoxia (1.3% CO₂, balanced N₂; pH=7.9) for 15 minutes sequentially, but in random order (i.e., the first application, either hypercapnia or hypoxia, was alternated). Two limitations must be acknowledged. One limitation to this repeated exposures design is the possibility that previous exposure to hypercapnia influences the hypoxia responses and vice versa; however, by alternating the order of stimulus application this effect would be the same in each animal group. Within groups responses were consistent and
preparations always recovered to near-initial baseline activity when returned to control aCSF (Fig. 3 and 6). Secondly, we are aware that transitioning from 90% O₂ (hyperoxic control) to anoxic aCSF as a hypoxic stimulus is not physiological. Similar methods to ours (same flow rate and chamber volume) determined tissue Po₂ to be 0mmHg within 5 minutes (at any depth) using this aCSF anoxia as the stimulus (Winmill et al., 2005). Since intact frogs survive temporary anoxia (Rose and Drotman, 1967; Tattersall and Ultsch, 2008), interpretation errors from this experimental paradigm may stem from high control Po₂ values and not the anoxic stimulus (Fournier and Kinkead, 2008).

**CO₂ Chemosensitivity of LC Neurons:** Chemosensitive responses were identified in LC neurons from control (n=8) and aquatic overwintered frogs (n=13). After entry into the whole-cell configuration, stable baseline firing in control aCSF (80% O₂, 1.3% CO₂, balanced N₂; pH= 7.9) was recorded for 5 minutes. Neurons were then exposed to hypercapnia (80% O₂, 5% CO₂, balanced N₂; pH= 7.5) for 5 minutes to elicit increases in firing frequency. We have shown previously that exposing neurons to 5% CO₂ is sufficient to observe increases in firing frequency in ~90% of LC neurons from bullfrogs (Santin et al., 2013). Neurons then were returned to control aCSF and firing rates recovered to near control values. We have previously reported that chemosensitive LC neurons from bullfrogs are paradoxically activated by cooling (Santin and Hartzler, 2015a; Santin et al., 2013). We used cold-activation to 1.) determine if cold-acclimation influences neuronal properties besides chemosensitivity, and 2.) to provide a positive control for increases in firing frequency. Because the primary goal of this study
was to understand if cold-acclimation alters cellular chemosensitivity, cooling was always applied after the 5% CO\textsubscript{2} exposure.

\textit{Data Analysis and Statistics:}

\textit{Classification of nerve activities:} Fictive lung (large amplitude-low frequency) and buccal (small amplitude- high frequency) activities were analyzed according to previously determined criteria (Taylor et al., 2003c; Winmill et al., 2005). Since CN V and CN X must burst in phase for air to move in and out of the lungs of an intact frog (Sanders and Milsom, 2001), large amplitude bursts were classified as fictive lung activity only when CN V and CN X nerves fired in phase (Fournier et al., 2007; Fournier and Kinkead, 2008). In our hands, large amplitude CN V bursts almost always occurred simultaneously with CN X bursts. Similar to recent reports, CN V usually contained both fictive buccal and lung activities; however, as previously demonstrated (Baghdadwala et al., 2015), some post metamorphic preparations exhibited inconsistent or “waxing and waning” buccal activity (Baghdadwala et al., 2015; Taylor et al., 2003c). Thus analysis of buccal motor activity only from preparations exhibiting a discernable “buccal” rhythm during the control and hypercapnic sampling periods were used in analysis. Buccal activities from both aquatic overwintered groups were therefore pooled. CN X only contained large amplitude bursts that occurred in phase with large amplitude bursts of CN V.

\textit{Brain-spinal cord preparation analysis:} 5 minutes of baseline properties (fictive lung burst frequency CN X duration, and normalized CN X rise time) were
compared among control, cold-acclimation air access, and aquatic overwintered submerged preparations before switching to hypercapnia or hypoxia. For hypercapnia experiments, burst properties (fictive lung burst frequency, fictive buccal frequency, burst duration, normalized rise time, burst amplitude, and burst integral) were analyzed 5 minutes before treatment and in the last 5 minutes of each treatment. Results for burst frequency are expressed both absolutely and as a percent of baseline. For hypoxia experiments, burst frequency was analyzed in the 5 minutes before transitioning to hypoxia and in the last 5 minutes of hypoxia. Data were analyzed using Labchart peak analysis (AD Instruments Inc. CO, USA). Burst frequencies obtained in hypercapnia and hypoxia are expressed absolutely and as percentages of baseline.

*Chemosensitivity of LC neurons analysis:* Firing frequency of LC neurons was integrated into 10s bins using Clampfit (Molecular Devices; Sunnyvale, CA, U.S.A). Integrated firing rate of LC neurons from semi-terrestrial and aquatic overwintered bullfrogs was analyzed in the two minutes preceding the switch to hypercapnia and in the last 2 minutes of the hypercapnic treatment. Recovery firing rates were recorded ~5 minutes after the return to control aCSF. The recovery firing rate then was used as the control value for acute cooling experiments. Neurons (n=7 for semi-terrestrial and n=6 aquatic overwintered) were then cooled from 20°C to 10°C over ~4 minutes. One minute of steady-state firing was measured at 10°C. Changes in firing rate during hypercapnia and cooling are expressed absolutely and as a percent increase from baseline. Interspike membrane potential was determined by measuring membrane
potential in between action potentials, following the after hyperpolarization and before the next action potential threshold ramp. Voltages are corrected for a +12 mV liquid junction potential between the pipette filling solution and the aCSF calculated by the Henderson Equation using the “junction potential calculator” function in P10 Clampex (Molecular Devices; Sunnyvale, CA, U.S.A).

Statistics

Whole-nerve recordings: Each baseline characteristic was analyzed using a one-way ANOVA. For gas exposure experiments, absolute and normalized data were analyzed using a two-way ANOVA. Since we randomized the order of hypercapnia and hypoxia, each parameter was treated as independent. When a main effect (temperature acclimation or gas treatment) or interaction (temperature acclimation x gas treatment) was detected, Holm-Sidak’s multiple comparisons test was used to determine differences between individual means.

Chemosensitivity of LC neurons: Absolute firing frequencies of LC neurons in normo- and hypercapnia were compared between semi-terrestrial and aquatic overwintered bullfrogs using a two-way ANOVA. When a main effect (temperature acclimation or gas treatment) or interaction (temperature acclimation x gas treatment) was detected, Holm-Sidak’s multiple comparisons test was used to determine differences between means. Differences in normalized increases in firing increases in firing rates of LC neurons between semi-terrestrial and aquatic overwintered bullfrogs due hypercapnia or cold were analyzed during a two-tailed unpaired t-test. If standard deviations were different
between groups as determined by the F test, a Welch’s correction was applied to
the t-test to correct for unequal variances. Statistics were performed using
Graphpad Prism Version 6.01 (GraphPad Software, San Diego CA).
Results

In vitro brainstem preparation

To assess central processes controlling breathing we employed in vitro brainstem preparations (Baghdawala et al., 2015). In vitro brainstem preparations from bullfrogs produce motor patterns similar to the ventilatory pattern observed in intact bullfrogs (Santin and Hartzler, 2016c). This preparation generates two distinct motor patterns: 1.) low frequency, large amplitude, near-synchronous bursts on cranial nerve (CN) V and X and 2.) high frequency, small amplitude bursts that occur mainly on CN V (Fig.35A). Near-synchronous, large amplitude bursts from CN V and X correspond with neural activity driving ventilation of the lungs, while small amplitude bursts correspond with motor activity that ventilates the oropharyngeal (buccal) cavity (Kogo et al., 1994; Sanders and Milsom, 2001).

Burst characteristics of the brainstem-spinal cord preparation under control conditions

We used two aquatic overwintered groups: one that had access to air and one that was submerged. Overwintered aquatic bullfrogs with air access rarely surfaced, but rather remained voluntarily submerged in their tank (Santin and Hartzler, 2016a). Since these animals were housed in separate tanks, we analyzed these groups separately. To determine if disuse of the respiratory control system affects respiratory rhythm generation and pattern formation, we
Figure 35 Aquatic overwintering increases lung burst frequency, but does not fundamentally change adult motor behavior. A. shows representative raw and integrated neurograms of cranial nerve (CN) V and X, illustrating the typical respiratory motor pattern of adult bullfrogs. Small amplitude, high frequency bursts observed on CN V represent neural correlates of buccal ventilation (i.e. pumping of air in and out of the oropharyngeal cavity that does not contribute to breathing). Near-synchronous, large amplitude bursts on CN V and X represent neural correlate of lung ventilation, as this motor output would result in air flow in or out of the lung. B. shows representative integrated CN X neurograms from semi-terrestrial (top) and aquatic overwintered (bottom) bullfrogs. Sample sizes are presented in the figure and apply to C and D. The left panel shows CN X bursts at a condensed time scale, illustrating a faster frequency in aquatic overwintered bullfrogs. The right panel shows individual bursts at an expanded time scale to demonstrate that duration (time from first to third gray bar) and normalized rise time (time from the first to the second grey bar divided by the burst duration) do not differ between semi-terrestrial and overwintered aquatic bullfrogs. C. mean lung burst frequency data from semi-terrestrial and both groups of aquatic overwintered bullfrogs demonstrating that lung burst frequency increases following aquatic overwintering. D. & E. mean data for lung burst duration and normalized rise time for semi-terrestrial and overwintered aquatic bullfrogs from CN X (top) and V (bottom). Lung burst morphology did not change after aquatic overwintering. p+<0.05 and ++p<0.01. Error bars represent ± standard error of the mean (SEM).
compared burst frequency, duration, and rise time among semi-terrestrial and both aquatic groups of bullfrogs under baseline conditions. Figure 35B shows example CN X neurograms at compressed (left panel) and expanded (right panel) time scales from semi-terrestrial and aquatic overwintered bullfrogs. Preparations from aquatic frogs with and without air access had burst frequencies greater than semi-terrestrial frogs (Fig.1C; p=0.0021; F(2,18)= 8.860; one-way ANOVA; both aquatic overwintered groups greater than control, but not different from each other; p<0.05 and p>0.05, respectively; Holm-Sidak’s multiple comparisons test). Although the burst frequency was elevated, buccal frequency did not differ between groups (semi-terrestrial: 41.1±5.2 bursts per min. vs. 40.1±4.2 bursts per min.; p=0.9767; T16=0.0296; two-tailed unpaired t-test).

To gain an understanding of whether or not aquatic overwintering influences pattern formation of the network and/or motor function, we assessed total burst duration (Shao and Feldman, 2005) and burst rise time normalized to the total duration of each burst. In contrast to the frequency, duration and rise time of individual fictive breaths recorded from cranial nerves V and X were similar among all semi-terrestrial and overwintered, aquatic frogs (Fig.35D & E; p>0.05 for both nerves; one-way ANOVA). In summary rhythmogenic processes producing lung breaths persist, albeit at a greater frequency, and maintains the normal burst morphology of the motor output after aquatic overwintering.
Aquatic overwintering reduces sensitivity to hypercapnia of brain-stem spinal cord preparations

We next took advantage of the brainstem-spinal cord preparation to understand whether the reduction in CO\textsubscript{2} sensitivity that we previously observed in the intact animal (Santin and Hartzler, 2016a) is reflected in the hypercapnic sensitivity of the brainstem. The in vitro brainstem preparation from adult bullfrogs undergoes increased fictive lung breathing during hypercapnia (Harris et al., 2002; Morales and Hedrick, 2002), while the brain of premetamorphic tadpoles undergoes little-to-no change (Taylor et al., 2003c; Torgerson et al., 1997). Fig.36 illustrates examples of raw and integrated nerve discharge from CN V and X from semi-terrestrial and aquatic overwintered bullfrogs. The left panel shows resting burst frequency under control (1.3% CO\textsubscript{2}) and the right panel shows burst frequency during hypercapnia (5% CO\textsubscript{2}). Aquatic overwintering reduced the sensitivity of the brainstem-spinal cord preparation to hypercapnia (Figures 36 and 37; temperature acclimation x CO\textsubscript{2} interaction; p=0.0024; F\textsubscript{4,36}=5.056; two-way ANOVA). Fig.37 A-C shows the individual and mean neuroventilatory response to hypercarbia in semi-terrestrial and aquatic overwintered bullfrogs. Preparations from semi-terrestrial bullfrogs underwent significant increases in fictive lung-related discharge (p<0.0001; Holm-Sidak’s multiple comparisons test), but preparations from both groups of aquatic overwintered bullfrogs did not (p>0.05; Holm-Sidak’s multiple comparisons test). Fig.37D shows mean data expressed as a percentage increase from baseline (temperature acclimation x CO\textsubscript{2} interaction; p<0.0001; F\textsubscript{4,36}=8.299; two-way
Figure 36

A. Semi-terrestrial

1.3% CO₂

5% CO₂

B. Aquatic Overwintering

1.3% CO₂

5% CO₂
**Figure 36** Example raw and integrated CN V and X neurograms from semi-terrestrial and aquatic overwintered bullfrogs before and after 30 minute exposure to hypercapnia (5% CO$_2$). Notice that lung-related nerve discharge from the semi-terrestrial bullfrog (A) increases after exposure to hypercapnia (right panel) compared to baseline (left panel). In contrast, the lung discharge from the aquatic overwintered bullfrog maintains a similar frequency in baseline conditions (left) and hypercapnia (right).
Figure 37

A

Semi-terrestrial: n=9

Baseline 5% CO₂

B

Aquatic Overwintering - access: n=7

Baseline 5% CO₂

C

Aquatic Overwintering - submerged: n=5

Baseline 5% CO₂

D

Semi-terrestrial
Aquatic: Access
Aquatic: Submerged

Baseline 5% CO₂ Recovery
Figure 37 Lung burst frequency lose sensitivity to hypercapnia after aquatic overwinterring. A.-C. mean data and individual responses to hypercapnia in semi-terrestrial and aquatic overwintered bullfrogs. In each figure, the gray lines connect baseline burst frequency to the response of that individual in hypercapnia. Sample sizes for each group are included in the figure. A. demonstrates that hypercapnia induces significant increases in lung burst frequency (p<0.0001; repeated measures two-way ANOVA with Holm-Sidak’s multiple comparisons test), but B. & C. show that both groups of aquatic overwintering bullfrogs do not undergo increases in lung burst frequency after exposure to hypercapnia (p>0.05; repeated measures two-way ANOVA followed by Holm-Sidak’s multiple comparisons test). D. summarizes mean responses from each normalized as a percent of baseline burst frequency. The same sample sizes for groups in A.-C. apply to Fig. D. Only semi-terrestrial controls increased lung burst frequency with exposure to hypercapnia compared to baseline (p<0.0001; repeated measures two-way ANOVA with Holm-Sidak’s multiple comparisons test). Relative changes in lung burst frequency of semi-terrestrial bullfrogs during hypercapnia were also greater compared to both groups following aquatic overwintering (p<0.0001; repeated measures two-way ANOVA with Holm-Sidak’s multiple comparisons test **p<0.001 and ****p<0.0001 for within group comparisons. +++p<0.0001 for between group comparisons. Error bars represent ± standard error of the mean (SEM).
ANOVA). This highlights that preparations from semi-terrestrial bullfrogs undergo ~3.5-fold increase in fictive lung-burst frequency during exposure to hypercapnia (p<0.0001; Holm-Sidak’s multiple comparisons test), but preparations from aquatic overwintered bullfrogs are not influenced by hypercapnia (p>0.05; Holm-Sidak’s multiple comparisons test).

Additionally, lack of increases in lung burst frequency in overwintered aquatic bullfrogs do not appear to occur because of a “ceiling effect” (i.e., no change due to a higher starting frequency) since initial burst frequency does not correlate with the change in burst frequency induced by hypercapnia (Fig.38). If lack of responsiveness to hypercapnia occurred due to a ceiling effect, we would have expected to observe an inverse relationship between initial burst frequency and change induced by CO₂, but this did not occur in either group of bullfrogs.

In contrast to altered frequency responses to hypercarbia, burst properties including duration, rise time, and integral have been shown to be unaffected by hypercarbia in in vitro preparations from adult bullfrogs (Morales and Hedrick, 2002). We reasoned that these properties could be altered as a compensatory response to reduced hypercapnic frequency stimulation in overwintered bullfrogs. Similar to a previous report (Morales and Hedrick, 2002), hypercapnia did not alter burst shape properties in semi-terrestrial and overwintered aquatic groups (Fig.39 A-C; no effect of temperature acclimation, CO₂, or interaction; p>0.05; two-way ANOVA). These results indicate that aquatic overwintering conditions reduces aspects of the central respiratory control network that dictate the
Figure 38

In initial Lung Burst Frequency (min⁻¹) vs. Increase in Lung Frequency (% of Control) for Control and Aquatic Overwintering conditions:

- Control: $r^2 = 0.07, p = 0.49$
- Aquatic Overwintering: $r^2 = 0.02, p = 0.60$
Figure 38 Change in burst frequency by hypercapnia does not correlate with initial burst frequency. A possible explanation for lack of CO₂ sensitivity of lung-related nerve discharge in aquatic overwintered bullfrogs is that the higher initial burst frequency creates a “ceiling effect,” preventing further increases in burst discharge independent of reduced CO₂ chemosensitivity. If higher burst frequencies accounted for lack of CO₂ chemosensitivity, we would expect preparations with low starting burst frequencies to have large changes in lung bursting during hypercapnia and preparations with high starting frequencies to have small changes in lung bursting during hypercapnia. In both semi-terrestrial and aquatic overwintered (pooled) preparations, there was no relationship initial burst frequency and change in burst frequency. If a “ceiling effect” caused elimination of CO₂ sensitivity, we would have expected an inverse linear correlation between initial burst frequency and change in burst frequency in aquatic overwintered bullfrogs. P values and R² values are presented in the figure.
Figure 39

A

**Semi-terrestrial**

**Aquatic Overwintering: Access**

**Aquatic Overwintering: Submerged**

Duration (ms)

Baseline

5% CO₂

Recovery

0

500

1000

1500

B

Normalized Rise Time

(Rise time duration⁻¹)

Control

5% CO₂

Recovery

0

20

40

60

C

Burst Integral

(% of baseline)

Control

5% CO₂

Recovery

0

50

100

150
Figure 39 Aquatic overwintering does not alter burst morphology during hypercapnia. Hypercapnia did not affect lung burst duration (A), normalized rise time (B), and integral (C) in any acclimation group (no main effects of interactions). Given that there was no frequency response to hypercapnia after aquatic overwintering, there could have been a compensatory increase in burst characteristics; however, this did not occur further confirming that CO$_2$ chemosensitivity of the respiratory control system is eliminated after overwintering.
frequency responses to CO₂, but does not induce compensatory changes that lead to changes in burst properties during hypercapnia.

Aquatic overwintering reduces firing responses to CO₂/pH challenge in putative respiratory chemoreceptive neurons

The locus coeruleus (LC) is an important CO₂ chemoreceptive brain region that regulates lung ventilation in amphibians (Noronha-de-Souza et al., 2006). This area also contains neurons that are stimulated by high CO₂/acidification in in vitro brain slice preparations (Santin and Hartzler, 2013a). Since the LC is an important chemosensory brain region involved in increasing breathing, we performed whole-cell patch clamp electrophysiology experiments in brain slices to further determine whether aquatic overwintering leads to alterations in cellular processes determining CO₂/pH sensitivity of lung breathing. Figures 40A&B show example integrated firing rate traces and whole-cell action potential recordings before, during, and after exposure to hypercapnia (5% CO₂) in LC neurons from semi-terrestrial and aquatic overwintered bullfrogs, respectively. LC neurons from aquatic overwintered frogs had reduced firing responses to hypercapnia (Fig.40C; temperature acclimation x CO₂ interaction; p= 0.0062; F(2,38)=5.830; two-way ANOVA), but both groups underwent statistically significant increases in firing frequency during hypercapnia (semi-terrestrial; p<0.0001; Holm-Sidak’s multiple comparisons test, aquatic overwintered; p<0.05; Holm-Sidak’s multiple comparisons test). The reduced firing responses to hypercapnia of LC neurons after aquatic overwintering is also apparent when
Figure 40

**Change in Firing Rate (Hz)**

- **Semi-terrestrial**: n = 8
- **Aquatic Overwintering**: n = 13

*co2 x acclimation interaction: p = 0.006*

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**Specific Figure Details**

- **Figure A** and **Figure B** show firing rate histograms for 5% CO2 in semi-terrestrial and aquatic overwintering conditions, respectively.
- **Figure C** displays a bar graph contrasting firing rates under different CO2 levels and acclimation conditions, with statistically significant interactions indicated.
- **Figure D** illustrates change in firing rate (%) from baseline for both conditions, with individual data points and mean values.
Figure 40 CO₂/pH sensitive neurons from a chemoreceptive nucleus, the locus coeruleus (LC), have reduced firing responses to hypercapnia after submerged overwintering. A. and B. show representative integrated firing rate traces (top) and whole cell current-clamp recordings of action potentials (bottom) in LC neurons from semi-terrestrial and aquatic overwintered (air access only) bullfrogs. The dashed gray line indicates the interspike $V_m$ (i.e., $V_m$ between action potentials). A. demonstrates that upon transition from normocapnia (1.3% CO₂) to hypercapnia (5% CO₂) LC neurons from semi-terrestrial bullfrogs increase action potential firing frequency and undergo slight membrane depolarization. This increase is reversed upon returning to normocapnia. In contrast, B. shows that transition to hypercapnia in an LC neuron from an aquatic overwintered bullfrog results in a less robust increase in firing frequency with minimal membrane depolarization. C. mean firing frequencies before, during, and after hypercapnia. Samples sizes are included in the figure. There is a significant interaction between CO₂ and acclimation group indicating that aquatic overwintered bullfrogs have reduced firing responses to hypercapnia (temperature acclimation x CO₂ interaction; p= 0.0062, two-way ANOVA), even though LC neurons from both groups of bullfrogs undergo slight increases in firing frequency during hypercapnia (p<0.05; Holm-Sidak’s multiple comparisons test). D. expresses firing rate in LC neurons from semi-terrestrial bullfrogs as percentage increase from baseline firing rate. Firing rate increases by ~215% in semi-terrestrial bullfrogs, while aquatic overwintered bullfrogs increase firing frequency by ~90% (p=0.01; two-tailed unpaired t-test). *p<0.05, ****p<0.0001 for
within group comparisons. \( p < 0.05 \) for between group comparisons. Error bars represent \( \pm \) standard error of the mean (SEM).
expressed as an increase relative to baseline (Fig.6D; ~215% increase vs. ~90% increase; p=0.0175; T_{19}=2.603; two-tailed unpaired t-test) and also when examining changes in membrane potential induced by 5% CO\textsubscript{2} (semi-terrestrial: +4.3±0.7 mV vs. +1.5±0.4 mV; p=0.0012; T_{19}=3.810). Normocapnic, interspike membrane potential did not differ in LC neurons from semi-terrestrial and cold-aquatic bullfrogs (semi-terrestrial: -54.1±1.6 mV vs. aquatic overwintered: -51.8±1.1 mV; p=0.2472; T_{19}=1. Given that a large fraction of CO\textsubscript{2}/pH sensitivity of the respiratory control system of amphibians emanates from the LC (Noronha-de-Souza et al., 2006), these results indicate that reduced central sensitivity to hypercapnia is, in part, mediated by reduced firing responses of individual LC neurons to CO\textsubscript{2} challenge.

We sought to gain a clearer understanding of whether aquatic overwintering altered to the ability for CO\textsubscript{2} to stimulate firing or resulted in a change in LC neuron excitability which might account for reduced firing responses to hypercapnia. We previously showed that LC neurons from bullfrogs are paradoxically activated by acute exposure to cold temperatures (Santin and Hartzler, 2015a; Santin et al., 2013). Thus, the next series of experiments was performed to determine whether firing responses to stimulation by another modality are disrupted following aquatic overwintering. Figures 41 A&B show example whole-cell action potentials recorded from LC neurons before and after a 4 minute cooling ramp from 20°C to 10°C in semi-terrestrial and aquatic overwintered bullfrogs. As expected, acute cooling increased firing frequencies of LC neurons, but unlike hypercapnic stimulation, there was no
Figure 41

A  Semi-terrestrial

B  Aquatic Overwintering

C  

D  

-55 mV - 50 mV

-54 mV - 50 mV

Firing Rate (Hz)

Temperature x acclimation interaction: p = 0.3237

Change in Firing Rate (% change from baseline)

Semi-terrestrial: n = 7
Aquatic Overwintering: n = 6
Figure 41 Aquatic overwintering does not disrupt firing responses to another modality of stimulation, acute cooling. Paradoxically, LC neurons are stimulated by acute decreases in temperature. This provides a convenient way to assess whether reduced firing responses to CO$_2$ reflect a general decrease in responsiveness to stimulation vs. decreases in CO$_2$/pH-sensitivity. A. and B. show representative whole cell current-clamp recordings of action potentials in LC neurons from semi-terrestrial and aquatic overwintered (air access only) bullfrogs before (left panel) and after (right panel) acute transition from 20°C to 10°C. The dashed gray line indicates the interspike $V_m$ (i.e., $V_m$ between action potentials). Both A. and B. illustrate that firing rate stimulation by acute cooling is similar between semi-terrestrial and aquatic overwintered bullfrogs. C. shows mean data by indicating that both semi-terrestrial and aquatic overwintered bullfrogs increase firing frequency during acute cooling ($p=0.3237$ for temperature x acclimation group interaction; $p=0.0003$ for main effect of temperature). Sample sizes are included in the figure. D. expresses firing rate increases induced by acute cooling as a percentage of baseline firing frequency. Relative firing rate increases by acute cooling do not differ between semi-terrestrial and aquatic overwintered bullfrogs indicating that reduced firing responses to CO$_2$ result from decreases CO$_2$/pH sensitivity per se. **$p<0.01$ and ***$p<0.001$ for within group comparisons. Error bars represent ± standard error of the mean (SEM).
interaction between acclimation group and response to acute cooling (Fig.41C; p>0.05; two-way ANOVA) indicating that cold sensitivity of LC neurons does not depend on temperature acclimation. Additionally, unlike hypercapnic stimulation, acute cooling results in relative increases in firing frequency (Fig.41D; p=0.6608; $T_{11}=0.4409$; two-tailed unpaired t test) and membrane depolarization (semiterrestrial: +5.5±0.4 mV vs. aquatic overwintered: 5.7±0.9 mV; p=0.8419; $T_{11}=0.2042$) that do not differ between LC neurons from semiterrestrial and aquatic overwintered bullfrogs. Lastly, when comparing the percentage increase in firing rate induced by CO$_2$ and cooling in aquatic overwintered bullfrogs, cooling induces a significantly larger increase in firing frequency (92.3±29.2% increase by CO$_2$ vs. 704.6±217.8% increase by cooling; p=0.0371; $T_{5.18}=2.787$; two-tailed unpaired t test with Welch's correction). Collectively, these findings imply that reduced firing responses to hypercapnia occur by altering mechanisms underlying the ability of CO$_2$/pH to manipulate firing (be they intrinsic or extrinsic to the neurons) and not a consequence of generally decreasing cellular excitability or extra-CO$_2$ sensory processing. This further confirms that changes in respiratory gas sensitivity in the absence of air breathing are induced by environmental plasticity acting on chemosensory mechanisms and not general dysfunction of the respiratory network.

Aquatic overwintering reduces central sensitivity to hypoxia

In addition to understanding how aquatic overwintering influences aspects of the CO$_2$ sensitivity of the respiratory network, we also assessed the sensitivity of the central respiratory control system to acute hypoxia (aCSF bubbled with
control CO₂ and balanced N₂). Acute hypoxia suppresses respiratory discharge in the central respiratory control system of adults, while premetamorphic tadpoles maintain bursting during hypoxia (Fournier et al., 2007; Winmill et al., 2005).

Fig.42A shows examples of integrated CN V and X discharge before during and after 15 minute exposure to hypoxia in semi-terrestrial and aquatic overwintered bullfrogs. As illustrated in Fig. 42A (left panel), semi-terrestrial bullfrogs underwent stereotypical reductions in nerve discharge. In 7/9 preparations, bursting was completely and reversibly silenced by acute hypoxia in semi-terrestrial frogs. As represented in Fig. 42A (right panel), hypoxia did not silence rhythmic bursting in preparations from aquatic overwintered bullfrogs (access: 0/6; submerged: 0/5). The proportion of preparations silenced by hypoxia did not occur by chance (i.e., preparations from aquatic overwintered bullfrogs were more likely to continue bursting during hypoxia) (p=0.0014; χ² test). Mean data are shown in Fig.42B. There was a significant effect of hypoxia on lung burst frequency (hypoxia effect; p=0.0031; F₁,₁₇= 11.85; two-way ANOVA) because of decreases in burst frequency in semi-terrestrial preparations during hypoxia (p<0.05 for semi-terrestrial bullfrogs; Holm-Sidak multiple comparisons test). Both groups of aquatic overwintered bullfrogs did not undergo significant decreases in lung bursting during hypoxia (Fig. 42C&D; p>0.05; Holm-Sidak’s multiple comparisons test). When expressed a percentage change from baseline, it was apparent that aquatic overwintering reduced the hypoxia sensitivity of the fictive-lung rhythm (Fig.42E; temperature acclimation x hypoxia interaction; p=0.0037; F₄,₃₄= 4.749; two-way ANOVA). Aquatic overwintering,
therefore, decreases hypoxia sensitivity of the respiratory rhythm in adult bullfrogs.
Figure 42

A  

Control (95% O₂)

Semi-terrestrial

Hydroxyl (0% O₂; 15 min)

Aquatic Overwintering

B  

Normalized Frequency (% of baseline)

Semi-terrestrial: n=9

Aquatic Overwintering: Access

Aquatic Overwintering: Submerged

C  

Lung Burst Frequency (min⁻¹)

Baseline 0% O₂

Aquatic Overwintering: access: n=6

D  

Lung Burst Frequency (min⁻¹)

Baseline 0% O₂

Aquatic Overwintering: submerged: n=5

E  

Normalized Frequency (% of baseline)

Baseline 0% O₂ Recovery

Semi-terrestrial

Aquatic Overwintering: Access

Aquatic Overwintering: Submerged
Figure 42 Aquatic overwintering eliminates hypoxic ventilatory depression characteristic of the mature central breathing control system. A. illustrates integrated CN V neurograms from semi-terrestrial (top) and aquatic overwintered (bottom) bullfrogs. Upon transition to hypoxic aCSF (0% O$_2$) (and presumable tissue anoxia) burst activity recorded through CN V underwent complete inhibition in semi-terrestrial bullfrogs. In contrast the normal adult response to hypoxia, brainstems from aquatic overwintered bullfrogs did not stop bursting during hypoxia. B.-D. show mean data and individual lung burst frequency responses to hypoxia. Sample sizes are included in each figure. In each figure, the gray lines connect baseline burst frequency to the response of that individual after 15 minutes of hypoxia. There was a main effect of hypoxia (p=0.0031; Two-way ANOVA) because of decreases in burst frequency in semi-terrestrial bullfrogs (B; p<0.05; Holm Sidak’s multiple comparisons test). Both groups of aquatic overwintered bullfrogs did not change burst frequency during hypoxia (C & D; p>0.05 Holm Sidak’s multiple comparisons test). E. expresses the response to hypoxia as a percent of baseline. There was a significant interaction between acclimation group and hypoxia (p=blank; Two-way ANOVA). Hypoxia depressed lung bursting in semi-terrestrial bullfrogs (p<0.001; Holm Sidak’s multiple comparisons test), but not in both group of aquatic overwintered bullfrogs. Bursting during hypoxia decreased more in semi-terrestrial bullfrogs compared to both groups of overwintered groups. *p<0.05, ***p<0.001 for within group comparisons. +p<0.05, ++p<0.01 for between group comparisons. Error bars represent ± standard error of the mean (SEM).
Discussion:

Our aim was to gain insight into whether permanent developmental changes or environmental factors associated with terrestrial life produce the respiratory control system adapted for breathing air. Therefore, we examined function of central control of lung breathing in adult bullfrogs following an absence of air breathing in conditions that mimic aquatic overwintering. Lack of air breathing is a common respiratory scenario in both larval and overwintering-adult bullfrogs as lungs are minimally used for gas exchange, leading to little need for chemical control of lung breathing. After aquatic overwintering, motor output driving lung breathing was similar to semi-terrestrial controls suggesting that permanent neural changes convert gill control to lung control during development. However, we identified that central control of lung breathing lost sensitivity to respiratory gases CO₂ and O₂. This is intriguing because until now, simultaneous absence (or small absolute levels) of centrally-mediated CO₂ and O₂-induced breathing responses has mainly been restricted to immature vertebrates and water breathing fish. Therefore, our findings suggest that a developmental time course overlapping with major environmental changes can convolute interpretations regarding developmental origins of certain phenotypes by masking environmental plasticity.

Aquatic overwintering eliminates hypercapnic and hypoxia sensitivity characteristic of adult, air breathing vertebrates.
Larval bullfrogs have little necessity for chemical control of lung ventilation because the majority of metabolically produced CO$_2$ is lost across the gills and skin. Although extrapulmonary gas exchange dominates in premetamorphic tadpoles, there is controversy as to whether the central respiratory control system of premetamorphic tadpoles stimulates lung breathing during hypercapnia. Specifically, some reports demonstrate relative increases during hypercapnia (Rousseau et al., 2016; Taylor et al., 2003a; Taylor et al., 2003c), while others do not (Infantino, 1992; Torgerson et al., 1997; Walker et al., 1990). Despite these discrepancies, hypercapnia always results in small absolute (Rousseau et al., 2016; Taylor and Brundage, 2013; Taylor et al., 2003c) or no significant (Infantino, 1992; Taylor et al., 2003a; Torgerson et al., 1997; Walker et al., 1990) increases in lung breathing compared with consistent significant and larger absolute increases in post-metamorphic tadpoles and adult bullfrogs. On these grounds, it has been interpreted that CO$_2$ chemoreceptors, although present in the brainstem at early stages of development, increase importance in ventilatory control of acid-base balance in accordance with the transition to air-breathing (Gargaglioni and Milsom, 2007; Milsom, 2010). In addition to more uniform and robust CO$_2$ responsiveness, post-metamorphic tadpoles exhibit a central hypoxic ventilatory depression (HVD) that is absent in early-stage tadpoles (Fournier et al., 2007; Winmill et al., 2005). Similar trends seem to exist in rats and mice since ventilatory responses to CO$_2$ challenge and central HVD increase throughout pre and postnatal development (Davis et al., 2006;
Appearance of ventilatory control through central chemosensing during development seems to have adaptive advantages for controlling breathing during acid-base disturbances, providing a drive to breathe, and maintaining energy homeostasis in air-breathing vertebrates (Guyenet and Bayliss, 2015; Milsom, 2002; Milsom, 2010; Winmill et al., 2005). This is presumably the case because after central CO$_2$ (Milsom, 2010) and O$_2$ (Ramirez et al., 1997; Winmill et al., 2005) chemoreceptors appear during development, they generally produce predictable and reproducible effects on ventilation. Therefore we found it intriguing that an air-breather, the adult bullfrog, exposed to fully aquatic overwintering conditions lacks stereotypical increases and decreases in respiratory output during hypercapnia and hypoxia, respectively.

Several lines of evidence suggest that mechanisms underlying CO$_2$ chemosensitivity of lung breathing are reduced following overwintering. First, ventilatory responses to CO$_2$ challenge are reduced following aquatic overwintering in vivo through inability to increase breathing frequency (Santin and Hartzler, 2016a). Second, although we showed that aquatic overwintered bullfrogs had a higher baseline lung frequency, reduced brainstem chemosensitivity did not likely occur as a result of a “ceiling effect.” CO$_2$/pH sensitivity did not inversely correlate with burst frequency under control conditions (Fig. 38) as would be expected if a ceiling effect prevented further increases in burst frequency during hypercapnia. Third, altered burst properties...
did not compensate for decreases in frequency sensitivity to hypercapnia (Fig.5).

Fourth, we show that individual CO$_2$/pH sensitive neurons in the locus coeruleus, a brain region involved in increasing lung ventilation during hypercapnia (Noronha-de-Souza et al., 2006), have reduced firing responses to CO$_2$ in aquatic overwintered bullfrogs (Fig.40). Finally, we demonstrate that reduced ability to increase firing during CO$_2$/pH challenge occurs specifically through altering CO$_2$/pH-dependent processes, since acute cooling (a paradoxical known stimulus of LC neurons) elicits large increases in firing after aquatic overwintering (Fig.41). This strongly implies that environmental plasticity decreases CO$_2$/pH sensitivity in response to aquatic overwintering by acting on chemosensory mechanisms (be they intrinsic or extrinsic to the neuron) and not general dysfunction of neurons in the respiratory control network. Collectively, these data indicate that aquatic overwintering conditions lead to an (at least temporary) reduction of ventilatory (Santin and Hartzler, 2016a) and central (Fig.36-38) CO$_2$ chemosensitivity, at least in part, by decreasing CO$_2$-induced firing responses of presumed respiratory control neurons (Fig.40 &41).

We also provide evidence that aquatic overwintering reduces O$_2$ sensitivity of the respiratory control system. In hypoxia intolerant vertebrates including frogs, rats, and mice, a central hypoxic ventilatory depression (HVD) appears during development and dampens breathing frequency during sustained hypoxia. Unlike the typical HVD of hypoxia intolerant adult vertebrates, we observed that bullfrogs emerging from conditions mimicking aquatic overwintering did not express a central HVD (Fig.42). Specifically, hypoxia (tissue anoxia) completely
and reversibly suppressed respiratory-related discharge in 7/9 brainstem preparations from semi-terrestrial adult bullfrogs, while lung bursting persisted in aquatic overwintered bullfrogs. Continued bursting during hypoxia in adult bullfrogs after overwintering resembles that of immature vertebrates and water breathing fish (Côté et al., 2014; Hedrick et al., 1991; Neubauer and Sunderram, 2004; Ramirez et al., 1997; Viemari et al., 2003; Winmill et al., 2005). Based on evidence that pharmacological inhibition of anaerobic metabolism during hypoxia did not decrease respiratory motor output faster than hypoxia alone, Winmill et al. (2005) suggested that a brainstem oxygen/energy sensor mediates the central HVD in post metamorphic bullfrogs. Although we did not determine mechanisms here, our results imply that persistent respiratory motor output during hypoxia in overwintered bullfrogs may result from decreasing hypoxia sensitivity of the noradrenergic mechanism(s) responsible for depressing respiratory motor output (Fournier et al., 2007; Fournier and Kinkead, 2008). Alternatively or additionally, aquatic overwintering may improve hypoxia tolerance of the central respiratory control system of adult bullfrogs through enhancement of anaerobically generated ATP as occurs in premetamorphic tadpoles (Winmill et al., 2005) and anoxia tolerant turtles (Johnson et al., 1998). Regardless of mechanism, our data demonstrate that unlike most other hypoxia intolerant adult vertebrates, bullfrogs that recently emerge from aquatic overwintering conditions do not contain central HVD and therefore resemble juveniles.

*Development of Respiratory Rhythm Generation in American Bullfrogs*
The neurobiological basis for a developmental transition from water to air breathing involves a switch from control of gill to lung ventilation. In premetamorphic bullfrog tadpoles, gill ventilation dominates respiratory motor output, with occasional lung-related nerve activity (Hedrick, 2005). Activity of the gill/buccal rhythm generator phasically inhibits the lung rhythm generator to suppress the occurrence of lung breaths through GABA$_B$-dependent mechanisms (Straus et al., 2000). A greater occurrence of lung breaths during metamorphosis corresponds with decreased inhibition of the lung rhythm generator by the gill/buccal rhythm generator and also a switch from a pacemaker-driven to a network-driven rhythm generator (Broch et al., 2002; Duchcherer et al., 2013; Winmill and Hedrick, 2003).

Unlike respiratory gas sensitivity of the breathing control system, our results do not suggest that aquatic overwintering results in neuroplasticity that drastically alters mature rhythmogenic processes for lung breathing. Specifically, semi-terrestrial and aquatic overwintered frogs had a relatively high number of fictive lung breaths and relatively long burst durations as is typical of motor output from mature bullfrogs. Premetamorphic tadpoles typically produce lungs bursts at a rate of $\leq 2$ per min, while adult preparations typically produce $\sim 5$-15 lung bursts per min (Broch et al., 2002; Winmill et al., 2005) (Fig.35A). In fact, brainstem preparations from aquatic overwintered bullfrogs produced lung bursts at a greater frequency compared to controls (Fig.35C), although these values fall within the range determined by other studies (Broch et al., 2002; Winmill et al., 2005). Possible mechanisms that could underlie the elevated lung frequency in
overwintered frogs may include further disinhibition from the buccal generator on the lung generator (Duchcherer et al., 2013), less tonic inhibition by hypoxia through reductions in $O_2$ sensitivity (Fig.42), increased excitability of the lung rhythm generator through changes in intrinsic and neuromodulatory processes, or increased excitability of motor neurons involved in lung bursts. In addition to adult-like burst frequency, burst duration and relative rise time of both cranial nerves remained unchanged (Fig. 35 D& E). Burst duration of respiratory-related nerve activity tends to increase throughout vertebrate development (Hedrick, 2005; Viemari et al., 2003), presumably, since lung breathing requires increased neural drive to produce more forceful contractions of respiratory muscles. How the respiratory motor system of bullfrogs defends function despite probable inactivity during aquatic overwintering remains unclear; nonetheless, these results suggest that the mature motor pattern dominates throughout adulthood after environmental challenges that diminish air breathing.

*Developmental change or environmental plasticity coinciding with a developmental time course?*

Although the mechanisms by which the environment manipulates development are well appreciated (Gilbert, 2012), a key question remains: is a physiological phenotype observed at a particular developmental stage driven by a genetically determined processes (that may or may not be regulated by the environment) or by environmental factors specific to that stage independent of a developmental
trajectory? Our results demonstrate that removal of air breathing in adults through aquatic overwintering, surprisingly, results in specific neuroventilatory responses to CO$_2$ and hypoxia combinatorially observed typically in immature air-breathers and adult water-breathing teleost fishes (Côté et al., 2014; Milsom, 2010). Our intriguing findings imply that a developmental trajectory coinciding with major environmental shifts may lead to incorrect conclusions about developmental causes underlying phenotypes by masking what is truly environmental plasticity. We acknowledge that a contradictory argument could be made because the proportion of CO$_2$-stimulated neurons in the respiratory control system from rats has been shown to increase over a developmental time course in cell culture (i.e., independent of native environmental factors) which correlates with similar developmental changes observed in acute brain slices (i.e., native environmental factors present during development) (Wang and Richerson, 1999). Unfortunately, the magnitude of excitatory and inhibitory firing responses to CO$_2$ in brain slices over development was not reported, making comparisons to results obtained from cell culture difficult to draw. Furthermore, differences in the proportion of CO$_2$-inhibited neurons between cell culture and brain slices at early time points were observed suggesting that the native developmental environment influences normal cellular chemosensitivity in rats.

Our findings have important implications for identifying proximate causes of phenotypes observed at different stages of development. First, although we performed these experiments in an adult amphibian, our speculation that developmental time course masks environmental plasticity likely applies to
developing organisms in general. All animals experience internal (e.g., developing organ systems with interacting hormones) (Mueller et al., 2014) and external (e.g., transition from inside to outside of the uterus or egg) environmental changes during development. Therefore, phenotypes at different developmental stages have the potential to be driven by plasticity resulting from the developmental environment (Ho et al., 2011) in parallel with permanent changes associated with a genetic developmental program. Second, it would be difficult, if not impossible, to determine whether phenotypes at specific stages of development result from environmentally-induced plasticity (as central chemoreception appears to be) or developmentally-directed change (as lung rhythm generation appears to be) with exclusive use of gene manipulation approaches. Although genetic tools are common for deducing generation of phenotypes (Amsterdam and Hopkins, 2006; Guo et al., 2011; Sauvageau et al., 2013), our results imply that exclusive use these approaches has the potential to dramatically underestimate the role of environmental plasticity, per se, in producing specific phenotypes. In the framework of our study, if a particular genetic manipulation resulted in disruption of the “development” of respiratory chemosensitivity in bullfrogs, then the function of this gene would likely be involved in transducing an environmental signal required for modulating chemosensitivity, but not regulating its development. In contrast, if a genetic manipulation inhibited maturation of the lung rhythm generator, then the interpretation would be that the gene in question is involved in development of lung breathing. Without a comparative-environmental physiology approach, this
non-trivial distinction would not have been obvious because conventional laboratory animals develop while irreversibly shifting to novel environments.

In conclusion, when we exposed adult bullfrogs to entirely aquatic environments without lung breathing, the respiratory gas sensitivity of the breathing control system was reduced in magnitude to appear more similar to that of predominately water breathing tadpoles. However, baseline “fictive” breathing frequency remained similar to that of adults. Further studies should determine how mechanisms resulting in the reduced ability for respiratory gases to modulate breathing frequency and maintained basal respiratory motor output after overwintering compare to those of immature tadpoles and semi-terrestrial adult bullfrogs. Given that our findings suggest that respiratory responses to gas stimuli may be determined by environmental plasticity independent of development, future work must acknowledge that overlap of developmental time course with co-occurring environmental changes may result in an inability to differentiate genetically programmed phenotypes from those generated by environmental plasticity per se.
CHAPTER IX

CONCLUSIONS AND FUTURE DIRECTIONS
Although it is satisfying to conclude a dissertation with a visual model summarizing the major findings, I have chosen rather to emphasize the importance of context dependence on interpretation of physiology data and describe how my research has led me into the following questions that I am now or will be pursuing in my own research lab.

1. **How does a motor network operate after months after inactivity?**

   Neuromotor inactivity in mammals can lead to decreased function through reductions in motor neuron excitability (Cormery et al., 2005; Seki et al., 2007) that occurs independent of muscle atrophy (Cormery et al., 2005; Langlet et al., 2012; Lundbye-Jensen and Nielsen, 2008). Since ventilation was unchanged and retains scope for increases (through ventilatory responses to hypoxia) following two months of disuse in bullfrogs, respiratory motor neurons have the ability to maintain functionality (Santin and Hartzler, 2016a; Santin and Hartzler, 2016b) throughout prolonged inactivity (Santin and Hartzler, 2017). Interestingly, locomotor performance decreases after the winter in ranid frogs (Renaud and Stevens, 1983), indicating that preservation of respiratory motor function is not simply a result of suspended animation in the cold. How do bullfrogs preserve motor function of the respiratory system throughout disuse?

   Compensatory plasticity of neurons may hold some clues. Neurons compensatory changes in excitability and synaptic strength as a function of their own activity to preserve overall network output (Turrigiano, 2011). Decreasing neuronal activity through pathological and pharmacological approaches can
induce compensatory increases in cell excitability and synaptic transmission that stabilizes network output (Turrigiano, 2011; Watt and Desai, 2010; Wilhelm et al., 2009). For example, motoneurons from chicks increase excitability after pharmacological suppression of activity which stabilizes spontaneous network activity (Wilhelm et al., 2009). My results that respiratory network stability is maintained in the face of prolonged inactivity suggest that homeostatic plasticity could act to support breathing after months of overwintering inactivity. Therefore, I hypothesize respiratory inactivity during simulated overwintering in bullfrogs leads to enhanced intrinsic excitability and/or excitatory synaptic transmission onto respiratory motor neurons. These changes would stabilize motor output for proper respiratory function following overwintering inactivity. Interestingly, faithful compensation of inactivity does not appear to exist in chronic motor disuse and spinal cord injury (Cormery et al., 2005; Gazula et al., 2004), making respiratory motoneurons in adult bullfrogs amenable to understanding how to access such plasticity in adult vertebrate motoneurons. These studies are currently underway.
2. Is CO₂ chemosensitivity required for normal ventilation and acid-base balance?

Over 100 years ago, Haldane and Priestley (1905) discovered that pulmonary ventilation is exquisitely sensitive to inspired CO₂. This discovery spurred nearly a century’s worth of work that has led to the identification of many brain structures and molecules involved in generating the hypercapnic ventilatory response (HCVR). Mechanisms involved in generating the HCVR have been presumed to play a major role in homeostatic control of arterial Pco₂/pH by effecting subtle changes in ventilation; however, there are two issues with this broad interpretation. First, there are no detectable Pₐco₂/pHₐ error signals under physiological conditions (e.g., at rest and low-moderate intensity exercise) to alter ventilation in most mammals (Forster et al., 2012). Second, along with my data in bullfrogs (Santin and Hartzler, 2016a; Santin and Hartzler, 2016b), rodents with severely blunted or absent hypercapnic chemoreflexes have relatively normal ventilation, gas exchange, acid-base status, and exercise hyperpnoea (Hodges et al., 2002; Kumar et al., 2015; Puissant et al., 2015) indicating that negative feedback mechanisms through the HCVR may play a smaller role in controlling ventilation and acid-base balance under physiological conditions than previously interpreted. Therefore, the question remains: why is the CO₂ chemosensory system seemingly on the “wrong side” of the circulation for its participation in acid-base regulation of mammals? In a review I am writing, I am taking a comparative approach to this problem by reviewing environmental scenarios and physiological characteristics common among ectothermic, air-
breathing vertebrates that produce an acute respiratory acidosis requiring ventilatory compensation (i.e., activation of the HCVR) for acid-base regulation. Chemoreflexes likely contribute to normal ventilatory adjustments for acid-base balance as these animals regularly experience respiratory acidosis. In contrast, mammals use – as of yet unidentified - mechanisms that match ventilation to CO$_2$ production for maintenance of normal acid-base balance because ventilation-metabolism matching does not require the presence of a HCVR (Forster et al., 2012; Shea et al., 1993). Central chemoreceptors, however, may detect local changes in brain CO$_2$/pH that do not reflect arterial blood, act as a relay sites for peripheral metabolic information independent of P$_a$CO$_2$/pH$_a$, or fine-tune ventilation during activity. Furthermore, plasticity (i.e., changes evoke by a stimulus) and flexibility (i.e., built in malleability of responsiveness) of the CO$_2$ detection system appears to play an adaptive role in ectothermic vertebrates as they inhabit environments with variable gas exchange demands (Santin and Hartzler, 2016a; Santin et al., 2013); yet, plasticity of this system in mammals, including humans, appears to manifest as or with respiratory disease. The HCVR and its plasticity may, therefore, serve a prominent homeostatic role in ectothermic vertebrates facing demands that routinely require compensation of a respiratory acidosis. In addition, I argue the CO$_2$ chemoreflex may have played a major role in ventilatory regulation in early tetrapods, but decreased importance in mammals with the appearance of more efficient gas exchange and improved ventilation-metabolism matching. Understanding the evolution of the elusive mechanisms that match ventilation to CO$_2$ excretion, resulting prevention of
arterial acid-base disturbances in healthy mammals, presents a major challenge to both medically-oriented respiratory physiologists and comparative respiratory physiologists alike.
3. Can development be uncoupled from the developmental environment?

A surprising finding from Santin and Hartzler (2016b) was that overwintered bullfrogs that have not been breathing exhibit central respiratory gas chemosensitivity similar to predominately water-breathing tadpoles. Low levels of CO$_2$ and hypoxia sensitivity coexisting have typically characterized immaturity of the central respiratory control system. My data represent the first example of an air-breathing vertebrate that express plasticity to, apparently recapitulate, a previous stage of maturation. This plasticity suggests that central respiratory gas chemosensitivity is not necessarily a developmental phenotype, but may rather be a plastic response to environmental demands associated with (or without) air breathing. This hypothesis remains to be tested. In future studies I will manipulate the aquatic and/or metabolic environment of tadpoles and the overwintering environment of adult frogs to determine whether environmental factors regulate mechanisms of central chemosensitivity independent of developmental stage. This has potential to serve as a powerful model in developmental physiology because, as far as I know, there are no other organisms for which developmental “program” and developmental environment can be easily uncoupled across the life span of the animal.
4. How does the respiratory network maintain stability during temperatures changes?

Brains from healthy individuals have neural circuits with high robustness and therefore are able to maintain function even when disturbances occur. It therefore follows that failure to maintain circuit function might contribute to neuronal dysfunction and consequences of brain disorders like epilepsy, Parkinson’s, and stroke (Carmichael, 2012; Turrigiano, 2011). Mechanisms that maintain neural stability in vertebrate brains during chronic disturbances have been suggested to involve intrinsic and synaptic homeostatic mechanisms, but the inherent mechanisms that provide the brain with stability during acute disturbances are largely unexplored.

Temperature changes represent a major environmental disturbance that can potentially disrupt brain function. Some animals, such as mammals and birds, are able to generate body heat to minimize fluctuations in brain temperature to ~3-4°C, while most others conform to the temperature of their surrounding environments. Animals that cannot maintain body temperature, like bullfrogs, can undergo large temperature changes throughout the course of a day depending on microhabitat. Given the high temperature-sensitivities of many ion channels, drastic and fast temperature disturbances ought to destabilize neural circuits. Intriguingly, the circuit that controls breathing in bullfrogs maintains stable function over a large range of temperatures disturbances in vivo and in vitro (Bicego-Nahas and Branco, 1999; Morales and Hedrick, 2002) in the range of ~15-25°C. Most circuits increase their activity proportionally with temperature
(Robertson and Money, 2012; Tang et al., 2010), so the frog respiratory circuit is an ideal system with which to determine mechanisms that underlie stability of circuits in the vertebrate brain during large, acute disturbances.

The results described here in my dissertation suggest that neurmodulation may play a role in generating this stability. The locus coeruleus (LC) is a brainstem region involved in breathing control. This nucleus has been identified as the main supplier of norepinephrine in the brain. Chapters I and II described that, in bullfrogs, ≥85% of LC neurons involved in regulation of breathing (i.e., chemosensitive neurons) are counterintuitively activated by decreases in temperature within the physiological range. This cold-activated response is interesting because 1. the rates of most physiological processes slow as temperature decreases and 2. it may oppose rate influences typically associated changing temperature.

I have begun a preliminary examination to test this hypothesis that cold-activation (i.e., firing responses inversely proportion to temperature) of LC neurons contribute to frequency stability of the respiratory network. My preliminary data demonstrate that when the LC is removed by transection, the stability of the whole network is altered during temperatures changes (Figure 43). These preliminary data together with the cold-activated phenomenon presented in my dissertation suggest that the LC plays a key role in maintaining neural network stability. Norepinephrine, presumably from LC neurons, has been shown to act on post synaptic neurons involved in breathing control of amphibians. Although the brainstem targets of LC neurons are not yet
determined, specific receptors, $\alpha_1$ and $\alpha_2$ receptors, bind norepinephrine to elicit respiratory modulation in bullfrogs. $\alpha_1$ receptors have been shown to play a major role in the noradrenergic modulation of breathing, while $\alpha_2$ have a relatively low importance (Fournier et al., 2007). Future work will, therefore, test the hypothesis that cold-activation, through noradrenergic and potentially other neuromodulatory mechanisms, maintains respiratory network stability during acute temperature changes. This project is currently being performed by a master’s student, Maurico Vallejo, with whom I will continue collaborating.
Figure 43

Burst Frequency
(% of baseline at 20°C)

- LC Present (n=2)
- LC removed (n=1)

Temperature (°C)
Figure 43. Preliminary evidence suggests that respiratory motor stability across temperatures requires intact LC. Burst frequency in brainstem (n=2) is kept constant as temperature changes from 15°C to 25°C (Solid line). Burst frequency in brainstem with LC removed (n=1) increases as temperature changes from 15°C to 25°C (dotted line).
CHAPTER X

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