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

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Running Head: Reassessment of breathing control in bullfrogs

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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 .

Highlights

- Preexisting techniques were adapted to measure pulmonary ventilation in conscious bullfrogs.
- Ventilatory responses to hypoxia and hypercarbia have distinct dynamic profiles with tidal volume and frequency differentially regulated.
- Responses to hypercarbia were small; they mainly involved increases in tidal volume (V_T), but a robust post-hypercarbic hyperpnea.

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, 2013), 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., 2012). 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; Gargaglioni et al., 2007; Wilson et al., 2002; Winmill and Hedrick, 2003) and central chemosensing (Fournier and Kinkead, 2008; Santin and Hartzler, 2013; 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 $\dot{V}_E$ 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

47 metabolic changes on breathing control during relevant stimuli (*e.g.*, hypoxia and
48 hypercapnia) cannot be resolved through calculation of the air convection requirement
49 ($\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$) (Wang, 1994). Other methods that do not directly measure tidal
50 volume including body wall impedance measurements and “photoplethysmography” or
51 those that determined tidal volume, but not total gas exchange have also been
52 employed in anurans (Vitalis and Shelton, 1990) (McAneney et al., 2006; Vasilakos et
53 al., 2005). Therefore, the present study was undertaken to reassess chemical control of
54 breathing in bullfrogs by directly measuring V_T , f , and $\dot{V}_E$ with airflow
55 pneumotachography as has been performed in other ectothermic vertebrates including
56 toads (Branco et al., 1993; Gargaglioni and Branco, 2003; Kruhøffer et al., 1987;
57 Noronha-de-Souza et al., 2006) simultaneously with open-flow respirometry. Here, we
58 adapted the methods for airflow pneumotachography in small ectothermic vertebrates
59 developed by Glass et al. (1978) for use in bullfrogs while measuring O_2 consumption
60 ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$) using open flow respirometry in room air, hypercarbia,
61 and severe hypoxia. The use of pneumotachography would enable us to establish
62 whether or not hypercapnia and hypoxia elicited changes in tidal volume and $\dot{V}_E$, in
63 addition to their previously reported effects on breathing frequency (Kinkead et al.,
64 1994; Kinkead and Milsom, 1996; Kinkead and Milsom, 1994; Rocha and Branco, 1997;
65 Taylor et al., 2003a) and improve the understanding of respiratory control in this
66 common comparative model.

2. Materials and Methods

2.1 Animals

Adult bullfrogs, *Lithobates catesbeianus* (n=6; 106±5g) 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 secretions 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 1A), 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 1C; $r^2=0.99$; $n=6$) 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 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 O₂ Consumption and CO₂ Production

O₂ consumption ($\dot{V}_{O_2}$) and CO₂ production ($\dot{V}_{CO_2}$) was measured simultaneously with ventilation using open-flow respirometry. Room air, 5% CO₂/ air balanced, or 5% O₂/ 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 incurrent 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 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).

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% CO₂/ balance air) and hypoxia (5% O₂/ 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 $\dot{V}_{O_2}$ in the post hypercarbic sampling period, ACR for the post hypercarbic hyperpnea was calculated using the $\dot{V}_{O_2}$ measurement made in the hypercarbia immediately before switching to room air.

2.6 Statistics

Data are presented as mean $\pm$ SEM. Statistics were computed using Graphpad Prism (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 1.

Ventilatory and metabolic responses to hypercarbia

Fig. 2 shows the time course of the ventilatory response to elevated inspired CO₂ (5%) in bullfrogs (n=6). Hypercarbia led to slight increases in V_T after 20 and 30 minutes (Figure 2B; 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 2A 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, V_T 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 2.

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 3A, ventilatory responses during hypercarbia separated into two groups; one group that had robust increases in ventilation during hypercarbia (Figure 3A₁) and one group that either decreased or was unaffected (Figure 3A₂). Figure 3B_{1&2} shows that despite differences between frequency and $\dot{V}_E$ during hypercarbia in animals with and without response to hypercarbia, post hypercarbic f and $\dot{V}_E$ does not differ between these two groups (f : $p=0.5204$; $T_4=0.7037$, $\dot{V}_E$: $p=0.190$; $T_4=1.576$; 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 4A; $p=0.8603$; 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.

3.2 Ventilatory and metabolic responses to hypoxia

Exposure to 5% O₂ initially resulted in an increased breathing frequency at 10 minutes (Figure 5. 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

249 minute exposure (Dunnett's multiple comparisons test; $p > 0.05$ for 20 and 30 min). In
250 contrast, V_T increased at 20 and 30 minutes (Figure 5. B; RM-ANOVA; $p = 0.0024$;
251 $F_{(4,20)} = 6.015$; Dunnett's multiple comparisons test; $p < 0.01$ at 20 minutes and $p < 0.001$ at
252 30 minutes), but not during initial exposure to hypoxia (Dunnett's multiple comparisons
253 test; $p > 0.05$ at 10 minutes). As a result of these changes in f and V_T , hypoxia evoked
254 increases in $\dot{V}_E$ throughout the 30 exposure (Figure 5. C; RM-ANOVA; $p = 0.0153$;
255 $F_{(4,20)} = 3.998$; Dunnett's multiple comparisons test; $p < 0.05$ at 10 and 20 minutes and
256 $p < 0.01$ at 30 minutes). $\dot{V}_E$ recovered to near-control values within 2 minutes of replacing
257 hypoxia with room air. Figure 6A shows an example of the ventilatory pattern in
258 normoxia and hypoxia.

259 Figure 6B indicates that hypoxia did not affect $\dot{V}_{O_2}$ (two-tailed paired t test;
260 $p = 0.8140$; $T_3 = 0.2567$). Therefore, hypoxia resulted in an increase in the ACR (two-
261 tailed paired t test; $p = 0.0227$; $T_3 = 3.045$) because of increased minute ventilation.

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 $\dot{V}_E \sim 20 \text{ mL kg}^{-1} \text{ min}^{-1}$ vs. calculated $\dot{V}_A \sim 40 \text{ mL kg}^{-1} \text{ min}^{-1}$) (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: $\sim 20\text{-}40 \text{ mL kg}^{-1} \text{ min}^{-1}$) (Branco et al., 1993; Gargaglioni and Branco, 2003; Gargaglioni et al., 2002; Noronha-de-Souza et al., 2006). Additionally, metabolism (O_2 consumption and 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 CO₂ 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% CO₂ 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

307 hypercarbic hyperpnea between these groups of frogs (Figure 3B). Our results are
308 consistent with previous observations by Kinkead and Milsom (1996) who showed that
309 frogs have nonsignificant increases in breathing during CO₂ and robust increases in
310 breathing immediately following washout of the CO₂ from the airway. CO₂-sensitive
311 olfactory inhibition appears to be responsible for the sub maximal ventilatory response
312 during exposure to hypercarbia in frogs because denervation of this receptor system
313 produces hypercarbic ventilatory responses that are similar to the post hypercarbic
314 hyperpnea (Kinkead and Milsom, 1996). As suggested by Milsom et al. (2004), CO₂-
315 sensitive inhibition probably masks the stereotypical, stimulatory effects of CO₂ on
316 breathing in bullfrogs. Because the animals in our study experienced either strong or
317 weak inhibition of breathing during CO₂, but similar peak stimulatory responses during
318 the post hypercarbic period, our results imply that there is large animal-to-animal
319 variability in the strength of inhibitory influences on breathing control during hypercarbia
320 and not in stimulus strength at central and peripheral chemoreceptors. We cannot
321 confirm this, however, because neither arterial blood nor cerebral spinal fluid gases
322 were measured here. Similar peak responses between these two groups may not be
323 surprising given that CO₂ acutely applied and loaded through the skin elicits increases in
324 $\dot{V}_A$ (Jackson and Braun, 1979) which presumably leads to arterial acidemia in the
325 absence of ventilatory compensation as occurs with an intact olfactory system. Despite
326 differences between large and small ventilatory responses with elevated ambient CO₂,
327 cutaneous CO₂ acquisition probably provides a route to normalize excitatory stimulus
328 strength between these two groups of frogs to account for uniformity in peak, post
329 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 CO₂ among individuals in a population of bullfrogs. Our argument is strengthened by experiments that applied CO₂ exclusively to the airway, but not the lung (thus not activating stimulatory chemoreceptors) of tegu lizards. In this case, elevated airway CO₂ potently inhibited ventilation. Immediate removal of CO₂ 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 CO₂ 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 P_{CO_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 *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 $\dot{V}_E$ 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

376 experimental conditions as ours. While we observed 2-3 fold increases in frequency
377 within the first 10 minutes of hypoxia that drifted toward control values at later time
378 points, Rocha and Branco (1998) showed that hypoxia leads to 2 to 3- fold increases in
379 frequency after 60 minutes of exposure in all seasons except for the summer;

380 unfortunately, $\dot{V}_E$ and V_T (rather $\dot{V}_E$ or V_T “index”) were not determined. It is also worth
381 comparing the hypoxic response *in vivo* to results from bullfrog brainstem preparations
382 *in vitro*. Despite obvious differences between *in vitro* preparations and intact animals
383 (e.g., superfusion with hyperoxic artificial cerebral spinal fluid vs. perfusion, lack of
384 peripheral inputs vs. intact peripheral inputs, etc.), results from each preparation lead to
385 similar conclusions about frequency changes during hypoxia. Specifically, hypoxia
386 initially leads to increases in breathing frequency, but with increased exposure time,
387 frequency decreases (Figure 5; Winwill et al., 2005). Although the magnitudes and
388 manifestation of these responses differ (*in vivo*: slight increase initially, then drift back
389 toward control vs. *in vitro*: increase in episodes/ maintained motor output initially, then
390 silenced output), hypoxic responses of both preparations follow similar time courses.

391 Studies that assessed the hypoxic response of the *in vitro* brainstem preparation of
392 adult bullfrogs (Fournier et al., 2007; Winmill et al., 2005) have compared their results to
393 findings from *Rana pipiens* that shows decreases in breathing frequency upon acute
394 exposure to anoxia (Rose and Drotman, 1967). The results presented here indicate
395 that the mechanisms determined *in vitro* may restrain increases in breathing frequency
396 during less severe bouts of hypoxia (*i.e.*, hypoxic, not anoxic conditions) in intact
397 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 $\sim 100 \text{ ml kg}^{-1} \text{ min}^{-1}$ in bullfrogs (Gamperl et al., 1999; Gargaglioni and Branco, 2001, 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, 2003; Gargaglioni et al., 2002). 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, 2001, 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.

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Figure Legends

Figure 1 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).

Figure 2 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 ($\circ$), after 10, 20, 30 minutes in hypercarbia ($\bullet$),

immediately after the switch back to normocarbia (post-hypercarbia), and the recovery

($\circ$). 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 ($\dot{V}_E$; 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. * $p<0.05$, ** $p<0.01$,

**** $p<0.0001$

Figure 3 Peak ventilatory responses during post hypercarbia do not differ between animals with large or absent hypercarbic ventilatory responses. A_1 (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 CO_2 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. A_2 (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. $B_{1\&2}$ shows summary data of peak, post hypercarbic frequency and $\dot{V}_E$ responses of frogs that did and did not experience increases in ventilation during CO_2 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 4 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 ($\dot{V}_E/\dot{V}_{O_2}$) 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). * $p<0.05$

Figure 5 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 V_E at 10 minutes. Increases in V_T 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 ($\dot{V}_E$; 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 6 Hypoxic 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 hypoxia ($p=0.8410$; two-tailed paired t test). B. summary results for the air convection requirement ($\dot{V}_E/\dot{V}_{O_2}$) before and during exposure to hypoxia indicating a significant hyperventilation during hypoxia ($p=0.0227$; two-tailed unpaired t test). * $p < 0.05$

Table 1 Resting ventilatory and gas exchange parameters in bullfrogs (n=6) at 24°C

Absolute Frequency (fR_{abs} ; br min^{-1})	4.89 ± 0.67
Tidal Volume (V_T ; mL STPD kg^{-1})	4.00 ± 0.48
Minute Ventilation ($\dot{V}_E$; mL STPD $\text{min}^{-1} \text{kg}^{-1}$)	20.42 ± 4.58
Episode Frequency (Minute^{-1})	3.20 ± 0.54
Breaths/Episode	1.57 ± 0.14
Instantaneous Breathing Frequency (fR_{ins})	46.91 ± 3.84
Non-ventilatory period (s)	23.30 ± 3.69
$\dot{V}_{O_2}$ (mL O_2 STPD $\text{min}^{-1} \text{kg}^{-1}$)	0.82 ± 0.04
$\dot{V}_{CO_2}$ (mL CO_2 STPD $\text{min}^{-1} \text{kg}^{-1}$)	0.71 ± 0.04
$\dot{V}_E/\dot{V}_{O_2}$ (mL air mL O_2^{-1})	24.77 ± 5.20
$\dot{V}_E/\dot{V}_{CO_2}$ (mL air mL CO_2^{-1})	28.73 ± 6.06
RER ($\dot{V}_{CO_2}/\dot{V}_{O_2}$)	0.87 ± 0.04

Table 2. The Influence of respiratory gas stimuli on breathing pattern

	Control (n=6)	Hypoxia (n=6)	Hypercarbia (n=5)	Post Hypercarbia (n=6)
Episodes min ⁻¹	3.20 ±0.54	3.63 ±0.36	1.12* ±0.09	2.46 ±0.80
Breaths episode ⁻¹	1.57 ±0.14	2.99 ±0.65	25.51* ±8.79	36.51** ±9.81
fR _{ins} (breaths min ⁻¹)	46.91 ±3.84	33.78* ±1.77	56.06* ±4.22	69.49*** ±1.88
Non-ventilatory period (s)	23.30 ±3.69	17.08 ±5.16	26.44 ±7.61	3.73 ±0.27

Figure 1

Figure 1

Figure 2

A₁

2

3

B₁

2

3

C₁

2

3

Figure 4

A

B

Figure 5

A₁

2

3

B₁

2

3

C₁

2

3

Figure 6