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RESEARCH ARTICLE

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

Joseph M. Santin[1,2,*] and Lynn K. Hartzler[1]

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, the need to breathe or the 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 with 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 with 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 to no use of lung breathing in cold-aquatic overwintering habitats.

KEY WORDS: Temperature acclimation, Control of breathing, Bullfrog, Environmental physiology, Hypercarbia, Hypoxia

INTRODUCTION

Anurans 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, 2000; St-Pierre et al., 2000; Tattersall and Boutilier, 1997, 1999a,b; 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 because of their low metabolic rates and high capacity for extrapulmonary gas exchange. Thus, cold-submerged ranid frogs remain aerobic for >5 months in oxygenated water while maintaining temperature-specific acid–base balance (Ultsch et al., 2004). CO_2 and O_2 chemosensory processes that typically stimulate breathing do not function at temperatures ≤10–15°C (Bícego-Nahas and Branco, 1999; Bícego-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, the need for lung breathing or the 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, 1979; 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 h of mechanical ventilation leads to decreased force production in the diaphragm of rats (Powers et al., 2002), and following hibernation, European ground squirrels do not retain spatial and operant memories learned in fall (Millesi et al., 2001). In addition, 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* Shaw 1802, 14–16 h 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

[1]Wright State University, Department of Biological Sciences, 3640 Colonel Glenn. Hwy, Dayton, OH 45435, USA. [2]Wright State University, Biomedical Sciences PhD Program, 3640 Colonel Glenn. Hwy, Dayton, OH 45435, USA.

*Author for correspondence (santin.2@wright.edu)

J.M.S., 0000-0003-1308-623X

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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, and 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, *L. catesbeianus*, were purchased from Rana Ranch (Twin Falls, ID, USA) and maintained in the laboratory in three plastic tanks in ∼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 h:12 h light:dark cycles and fed crickets twice per week. Experiments were performed on control (warm acclimated; 22°C) bullfrogs after at least 2 weeks of acclimation to the 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 h:12 h to 10 h:14 h 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 was 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. After 6 weeks at 2°C with or without access to air, experiments were performed over the next 3 weeks. Experiments in cold-acclimated bullfrogs from tanks with and without access to air were alternated during the 3-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 of the 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 with air access (n=7) and 2°C submerged (n=5) 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, 2016). Briefly, the evening prior to measurements, bullfrogs were lightly anesthetized with ∼0.5% (v/v) isoflurane until they lost toe pinch and eye reflexes. A small, lightweight facemask created from the bulb of a 3 ml transfer pipette (cat. no. 225-15, Samco Scientific Corporation, 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, Goleta, 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^{-1}.

The only methodological difference between this study and our previous study (Santin and Hartzler, 2016) 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; Table S1).

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 (2016). Open-flow respirometry was performed by pushing experimental gas mixtures at 1.1 ml min^{-1} 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^{-1} by a suction pump (AEI Technologies, Pittsburgh, PA, USA). Gases were pulled through a 4100 series mass flow meter (TSI, Shoreview, MN, USA), a CO_2 analyzer (CD-3A; AEI Technologies), a 10 ml syringe filled with desiccant (DM-AR; Perma Pure, Lakewood, NJ, USA) and finally an infrared O_2 analyzer (S-3A/I; AEI Technologies).

Experimental protocols

All experiments were performed during the light cycle. Approximately 16 h after attachment of the mask and pneumotach, resting ventilation and metabolism were determined when pulling room air through the sealed chamber at 215 ml min^{-1}. Each animal was exposed to both hypercarbia and hypoxia for 30 min, in a random order with 2–3 h between each stimulus. 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^{-1} for 5 min to reduce the time required to exchange the gaseous environment. Fractional concentrations of incurrent gases were checked before each gas transition to minimize errors that are due to drift of the 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 at which 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 min after hypercarbia) because breathing frequency in the 'post-hypoxic' period does not increase (Santin and Hartzler, 2016) (J.M.S., personal observation; data not shown). 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.

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Data analysis

Resting ventilation, breathing pattern and metabolism were determined in room air for 30 min before switching to the experimental gases in each animal group. During hypercarbia and hypoxia, ventilation was measured for 5 min, every 10 min, to elucidate the time course of the ventilatory response. Additionally, ventilation in the 1 min period after hypercarbia (post-hypercarbia) was analyzed as this ventilatory response represents the 'stimulatory' ventilatory response to CO_2 independent of olfactory inhibition (Kinkead and Milsom, 1996) and is less variable between animals (Santin and Hartzler, 2016) 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 ($\dot{V}_E$) was determined by multiplying f (breathing frequency) 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 $\dot{V}_E$ by either $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$. Because 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 means±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 (acclimation temperature and gas) and interactions (acclimation temperature×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 nine of 12 total cold-acclimated frogs during hypercarbia (one cold frog with air access and two without access did not contain breaths occurring in succession during the sampling period). Because 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 or figure legend. Because a repeated-measures design could not be used when animals 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–Šidák multiple comparisons test. Statistical significance was accepted when $P<0.05$.

RESULTS

Resting ventilation and metabolism following cold acclimation

Control, cold-acclimated with air access and cold-submerged bullfrogs weighed 109±9, 112±12 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 h after transition from a cold-aquatic to a warm-terrestrial environment. Fig. 1 shows that cold acclimation (with or without air access) does not influence breathing frequency (one-way ANOVA, P=0.1636, $F_{2,17}$=1.084), tidal volume (one-way ANOVA, P=0.2137, $F_{2,17}$=1.692) or minute ventilation (one-way ANOVA, P=0.9185, $F_{2,17}$=0.0854) at rest. Fig. 2 shows metabolic parameters in the three acclimation groups.

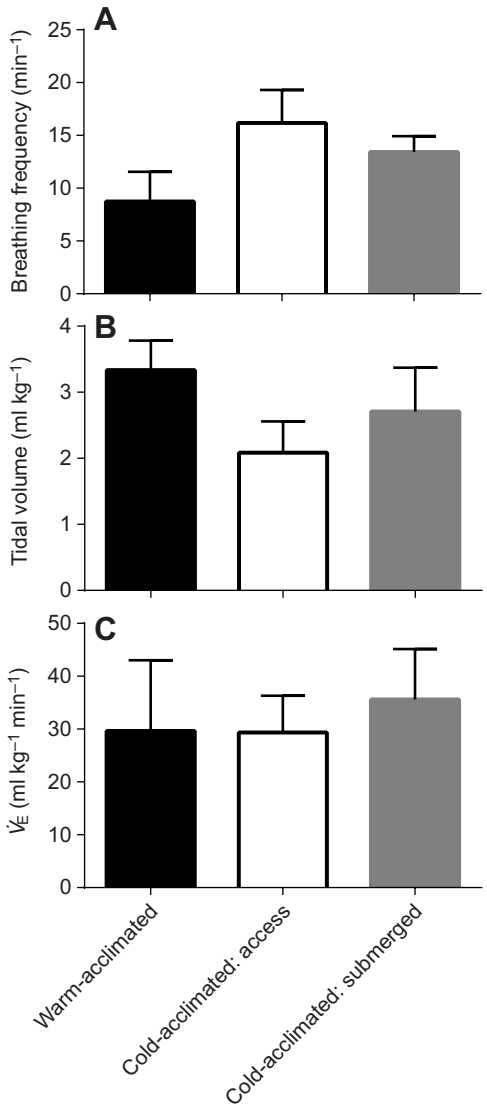

Fig. 1. Cold acclimation with and without access to air does not affect resting ventilatory parameters in the bullfrog *Lithobates catesbeianus*. Breathing frequency (A), tidal volume (B) and minute ventilation ($\dot{V}_E$; C) measured at 24°C were not different (one-way ANOVA, P>0.05 for each parameter) among warm-acclimated 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 (submerged, n=5; gray bars), respectively. Error bars represent ±s.e.m.

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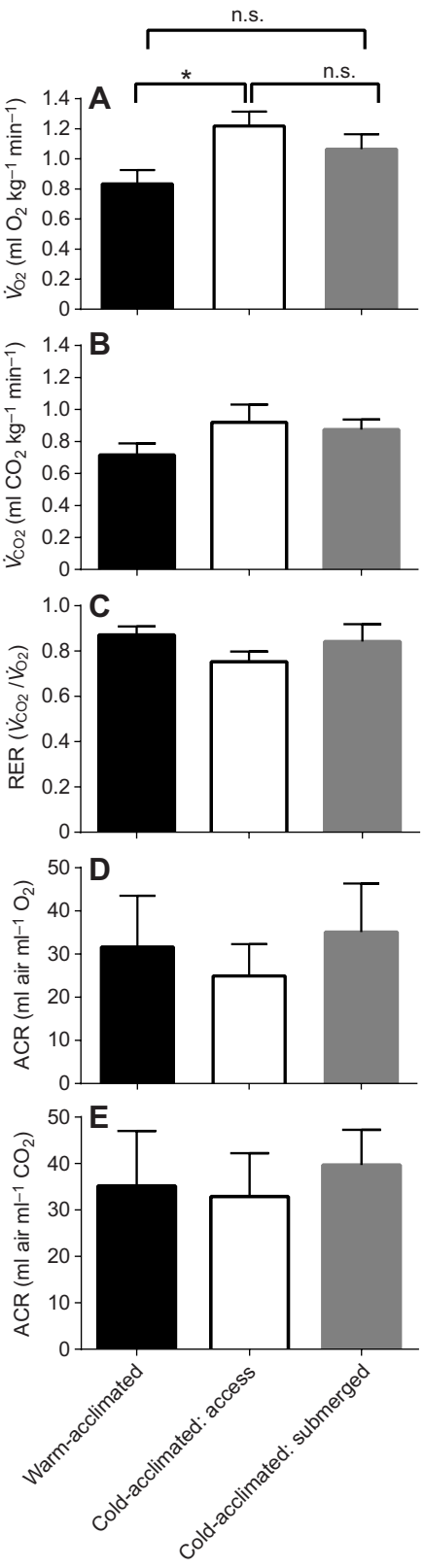

Fig. 2. Bullfrogs ventilate sufficiently to support increased metabolic rate ($\dot{V}_{O_2}$) after overwintering. (A–C) Mean rate of O_2 consumption ($\dot{V}_{O_2}$; A), rate of CO_2 production ($\dot{V}_{CO_2}$; B) and respiratory exchange ratio (RER; C) in warm-acclimated (black bars; n=8), cold-acclimated 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 with controls (Holm–Šidák multiple comparisons test, P<0.05), but not compared with cold-submerged frogs (Holm–Šidák 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–Šidák multiple comparisons test, P>0.05). Although $\dot{V}_{CO_2}$ showed a trend similar to that of $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ (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) were unaffected by temperature acclimation. D and E show air convection requirements (ACR) 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) and those submerged (n=5) breathed sufficiently to match metabolic demands as indicated by statistically indistinguishable ACRs 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 with controls (n=8). Thus frogs removed from conditions that mimic overwintering conditions with and without access to air do not hyper- or hypoventilate compared with control bullfrogs. Error bars represent ±s.e.m. *P<0.05; n.s., not significant.

Resting $\dot{V}_{O_2}$ was elevated in cold-acclimated bullfrogs that had access to air (one-way ANOVA, P=0.0277, $F_{2,18}$=4.404; Holm–Šidák multiple comparisons test, P<0.05 control versus cold-acclimated with air access, P>0.05 for all other comparisons; Fig. 2A). However, $\dot{V}_{CO_2}$ (one-way ANOVA, P=0.2109, $F_{2,18}$=1.699; Fig. 2B) and the RER of each animal ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) (one-way ANOVA, P=0.3526, $F_{2,18}$=1.488; Fig. 2C) were not different among control or either cold-acclimated groups.

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 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 with the controls, we can infer that lung ventilation does not match metabolism after cold acclimation and these animal are hypoventilating. Fig. 2D,E shows that air convection requirements for both O_2 acquisition (one-way ANOVA, P=0.8039, $F_{2,17}$=0.2211) and CO_2 elimination (one-way ANOVA, P=0.9218, $F_{2,17}$=0.0917) 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 RERs comparable to those of 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 the 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. 3 shows $\dot{V}_E$, breathing frequency and V_T in response to hypercarbia. During hypercarbia, none of the three groups showed any change in ventilation at any time point (Holm–Šidák multiple comparisons test, P>0.05 at each time point during hypercarbia; Fig. 3A). We recently showed that there is large variability in ventilatory responses during hypercarbia, presumably as a result of to animal-to-animal differences in CO_2-sensitive olfactory inhibition. The post-hypercarbic hyperpnea (i.e. ventilatory increase in the ∼1 min 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, 2016). Therefore, the post-hypercarbic hyperpnea has

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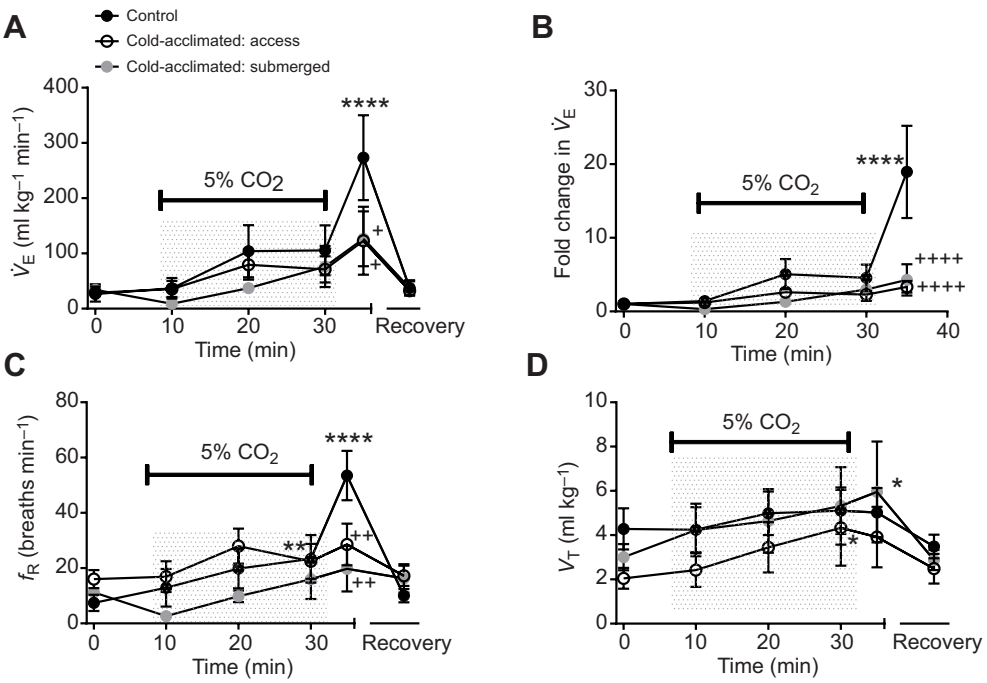

Fig. 3. Cold acclimation with and without air leads to a reduction in the post-hypercarbic hyperpnea, but not differences in ventilation during CO_2 exposure. (A) Absolute $\dot{V}_E$, (B) $\dot{V}_E$ relative to baseline in normocarbia, (C) breathing frequency and (D) tidal volume in warm-acclimated (black circles; n=8), cold-acclimated with air (white circles; n=7) and cold-submerged (gray circles; n=5) bullfrogs. Results shown in A (two-way ANOVA, CO_2 effect, $F_{4,68}$=12.31, P<0.0001) and B (CO_2×temperature acclimation interaction, $F_{8,64}$=3.202, P=0.0038) indicate that hypercarbia does not lead to statistically significant increases in $\dot{V}_E$ in all three groups (Holm–Šidák multiple comparisons test, P>0.05); however, the post-hypercarbic ventilatory response is only significantly greater than normocarbic breathing in control frogs (Holm–Šidák multiple comparisons test, P<0.0001) and is significantly blunted in both cold-acclimated groups compared with control frogs (Holm–Šidák multiple comparisons test, P<0.05). (C) The decreased post-hypercarbic hyperpnea in cold-acclimated frogs (two-way ANOVA; time×acclimation; $F_{8,64}$=4.784; P<0.001) occurs because of 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 compensate for reduced frequency responses to increase $\dot{V}_E$. Error bars represent ±s.e.m. *P<0.05, **P<0.01, ****P<0.0001 compared with normocarbic controls (within-animal comparisons); ^{+}P<0.05, ^{++}P<0.01, ^{++++}P<0.0001 compared with control frogs (between-animal comparisons).

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 (two-way ANOVA, main effect of CO_2, P<0.0001, $F_{4,68}$=12.31; Holm–Šidák multiple comparisons test, baseline versus post-hypercarbia and 30 min in hypercarbia versus post-hypercarbia, P<0.05 for both analyses). However, ventilation did not undergo significant increases during or immediately following hypercarbia compared with normocarbia or hypercarbia in either group of cold-acclimated bullfrogs (Holm–Šidák multiple comparisons test, P>0.05). Additionally, ventilation in the post-hypercarbic period was significantly less in cold-acclimated compared with warm-acclimated frogs (Holm–Šidák multiple comparisons test, P<0.05). When expressing ventilation as a relative change, there was a significant interaction between temperature acclimation and CO_2 (two-way ANOVA, CO_2×temperature acclimation interaction, P=0.0038, $F_{8,68}$=3.202; Fig. 3B), indicating that cold acclimation alters the influence of CO_2 on ventilation through blunting of the post-hypercarbic hyperpnea. The reduced ability to elicit a ventilatory response to hypercarbia stemmed from a smaller increase in breathing frequency during post-hypercarbia (temperature acclimation×CO_2 interaction, P=0.0001, $F_{8,64}$=4.784; Fig. 3B). Unlike warm-acclimated bullfrogs, both groups of cold-acclimated frogs underwent small increases in V_T compared with baseline during or following hypercarbia (two-way ANOVA, main effect of CO_2, P=0.0018, $F_{4,68}$=4.785; Holm–Šidák multiple comparisons test, cold-acclimated air at 30 min, P<0.05; cold-acclimated no air post-hypercarbic hyperpnea, P<0.05; Fig. 3C), but these increases were not large enough to cause statistically significant increases in $\dot{V}_E$. These results imply that ventilatory response to CO_2 challenge is reduced following exposure to overwintering conditions.

To take into account the possibility of different metabolic responses to hypercarbia in warm- and cold-acclimated bullfrogs, we also indirectly assessed metabolism using open-flow respirometry before and during hypercarbia, and then normalized ventilation to metabolism. This allowed us to determine whether CO_2 sensitivity of ventilation per se was reduced in cold-acclimated bullfrogs or whether changes in ventilation during CO_2 were the consequence of a different metabolic response to hypercarbia. Fig. 4A shows that $\dot{V}_{O_2}$ was not influenced by hypercarbia and there were no differences among temperature acclimation groups (two-way ANOVA, P>0.05, no main effects or interactions). The air convection requirement ($\dot{V}_E/\dot{V}_{O_2}$) was influenced by CO_2 treatment (two-way ANOVA, P=0.0056, $F_{2,34}$=6.601). ACR increased significantly during the post-hypercarbic hyperpnea in controls (Holm–Šidák multiple comparisons test, P<0.001), but not in either group of cold-acclimated bullfrogs. ACR was also greater in control bullfrogs compared with both groups of cold-acclimated frogs (Holm–Šidák multiple comparisons test; P<0.05). Thus, cold-acclimated frogs do not undergo hyperventilation during or following hypercarbia. These findings indicate that reduced ventilatory responses to hypercarbic-related

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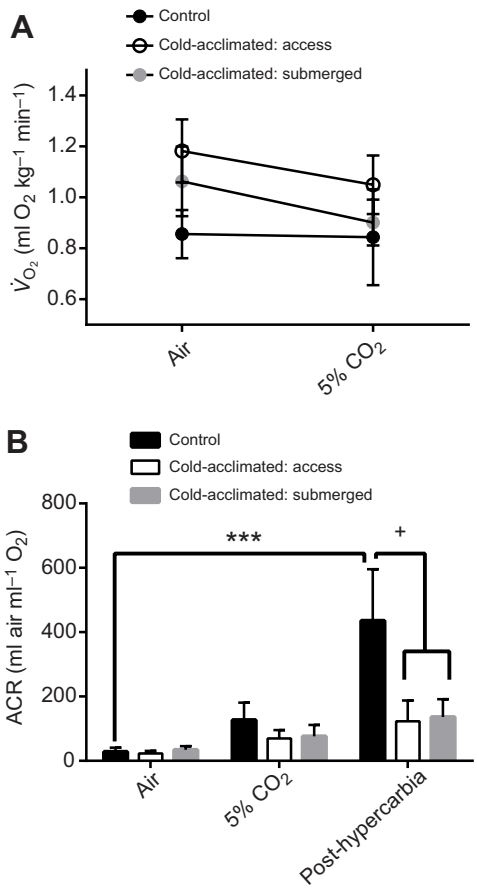

Fig. 4. Cold-acclimated bullfrogs do not increase ventilation relative to metabolism in response to or immediately after hypercarbia. (A) Mean $\dot{V}_{O_2}$ is not affected by environmental hypercarbia in warm-acclimated (black circles; n=8), cold-acclimated with air (white circles; n=7) and cold-submerged (gray circles; n=5) bullfrogs (two-way ANOVA, P>0.05 for main effects and interaction). (B) 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 with rest in normocarbia (Holm–Šidák multiple comparisons test, P<0.001) and was greater during post-hypercarbia compared with both groups of cold-acclimated bullfrogs (Holm–Šidák multiple comparisons test, P<0.05). Error bars represent ±s.e.m. ***P<0.001 compared with control value in normocarbia (within-animal differences); ^{+}P<0.05 compared with control bullfrogs during post-hypercarbic hyperpnea (between-animal differences).

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 anurans. Fig. 5A,B shows that post-hypercarbia resulted in addition of more breaths in each episode of warm-acclimated bullfrogs (Holm–Šidák multiple comparisons test, control versus post-hypercarbic hyperpnea, P<0.05; Fig. 5A) without changing the number of episodes per minute (Holm–Šidák multiple comparisons test, control versus post-hypercarbic hyperpnea, P>0.05; Fig. 5B). Warm-acclimated, control bullfrogs also had shorter non-ventilatory periods in the post-hypercarbic period (Holm–Šidák multiple comparisons test, control versus post-hypercarbic hyperpnea, P<0.05; Fig. 5D). 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 (Holm–Šidák multiple comparisons test, control versus post-hypercarbic hyperpnea, P>0.05; Fig. 5B,D). Additionally, instantaneous breathing frequency (the frequency of successive breaths during episodes of two or more breaths) was decreased in cold-acclimated bullfrogs (two-way ANOVA, main effect of temperature acclimation, P=0.0004, $F_{1,49}$=14.38) and included a statistically significant difference in the post-hypercarbic period compared with controls (Holm–Šidák multiple comparisons test, control versus cold-acclimated, P<0.05; Fig. 5C). 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 versus 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 (two-way ANOVA, main effect of hypoxia, P<0.0001, $F_{4,68}$=18.25; Holm–Šidák multiple comparisons test, P>0.05 for all comparisons between acclimation groups; Fig. 6A). Increases in ventilation were caused by increases in V_T in each acclimation group (two-way ANOVA, main effect of hypoxia, P<0.0001, $F_{4,68}$=16.72; Holm–Šidák multiple comparisons test, P>0.05 for all between-acclimation-group comparisons; Fig. 6B). Although we observed a significant effect of hypoxia on breathing frequency (two-way ANOVA, effect of hypoxia, P=0.0111, $F_{3,51}$=4.099), there were no significant pairwise comparisons between different time points in hypoxia compared with normoxic breathing frequency in any acclimation group (Holm–Šidák multiple comparisons test, P>0.05; Fig. 6B).

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. 7A 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 (two-way ANOVA, main effect of hypoxia, P<0.0001, $F_{1,17}$=68.27; P>0.05 for comparisons between acclimation groups), indicating that 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, 2016), the instantaneous breathing frequency decreased in warm- and cold-acclimated bullfrogs (two-way ANOVA, main effect of hypoxia, P=0.0007, $F_{1,17}$=16.97; Fig. 8C). The number of episodes per minute, breaths per episode and duration of the non-ventilatory period were unaltered by hypoxia and temperature acclimation (two-way ANOVA, P>0.05). Collectively, these results show that bullfrogs that recently emerged from simulated overwintering hibernacula possess ventilatory responses to hypoxia.

DISCUSSION

We performed these experiments to determine whether aspects of the respiratory control system are maintained or suppressed

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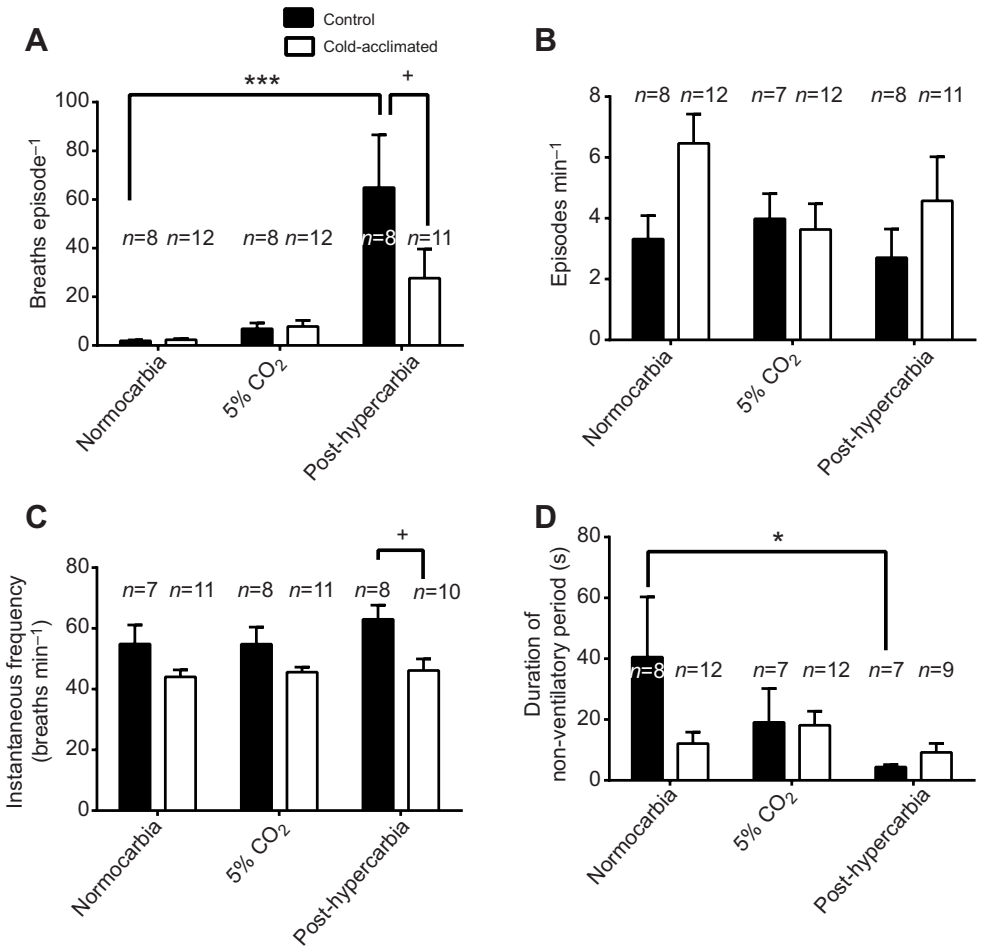

Fig. 5. Cold-acclimated bullfrogs do not undergo changes in breathing pattern that lead to increases in absolute respiratory frequency during post-hypercarbia. (A–D) Breathing pattern parameters including breaths episode^{-1} (A), episodes min^{-1} (B), instantaneous breathing frequency (C) and duration 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 as a result of a reduction in the number of breaths added to each breathing episode (A; Holm–Šidák multiple comparisons test, warm- versus cold-acclimated, $P<0.05$; normocarbia versus post-hypercarbia in warm-acclimated bullfrogs, $P<0.0001$) without changing the total number of episodes (B; Holm–Šidák multiple comparisons test, $P>0.05$ for all comparisons). Instantaneous frequency (i.e. the breathing frequency during breathing episodes) is decreased in cold-acclimated bullfrogs during the post-hypercarbic period compared with warm-acclimated bullfrogs (C; Holm–Šidák multiple comparisons test, warm- versus cold-acclimated during post-hypercarbia, $P<0.05$). Lastly, warm-acclimated bullfrogs decrease the duration of the non-ventilatory period during hypercarbia (D; Holm–Šidák multiple comparisons test, normocarbia versus post-hypercarbia for warm-acclimated bullfrogs, $P<0.05$), but cold-acclimated bullfrogs do not ($P>0.05$ for all comparisons relative to normocarbia). Error bars represent ±s.e.m. $^{*}P<0.05$, $^{***}P<0.001$ compared with normoxia for each acclimation group; $^{+}P<0.05$ compared with warm-acclimated bullfrogs (between-animal differences).

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 with 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 to 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 with 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.

Inactivity of lung ventilation during submergence

Natural (e.g. 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, which burrows for ∼9 months with no muscular atrophy (Hudson and Franklin, 2002). Although submerged northern temperate 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 involuntarily (i.e. trapped under the ice) or voluntarily (i.e. no need

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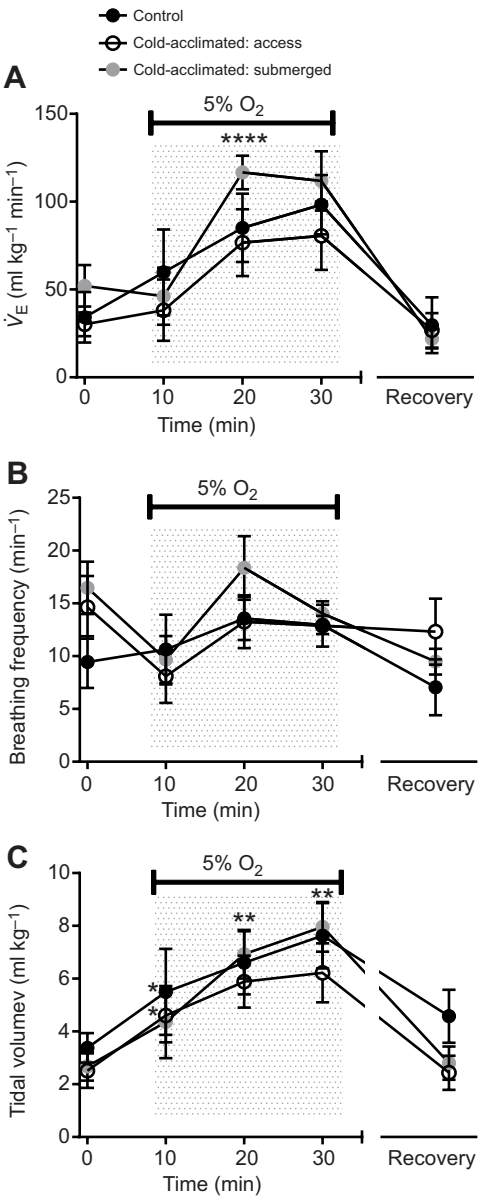

Fig. 6. Cold acclimation with and without access to air does not influence ventilatory response to acute hypoxia. (A) Absolute $\dot{V}_E$, (B) breathing frequency and (C) tidal volume during exposure to hypoxia (5% O_2) in warm-acclimated (black circles; *n*=8), cold-acclimated with air (white circles; *n*=7) and cold-submerged (gray circles; *n*=5) bullfrogs. Asterisks placed above all acclimation 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 with baseline (two-way ANOVA, effect of time in hypoxia, $F_{3,51}$=25.42, P<0.0001) in response to hypoxia because of increases in tidal volume (two-way ANOVA, effect of time in hypoxia, $F_{3,51}$=21.75, P<0.0001). No time in hypoxia×acclimation interactions or significant *post hoc* comparisons existed between acclimation groups at any time point (two-way ANOVA with Holm–Šidák 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 ±s.e.m. *P<0.05, **P<0.01 compared with normoxia for each acclimation group.

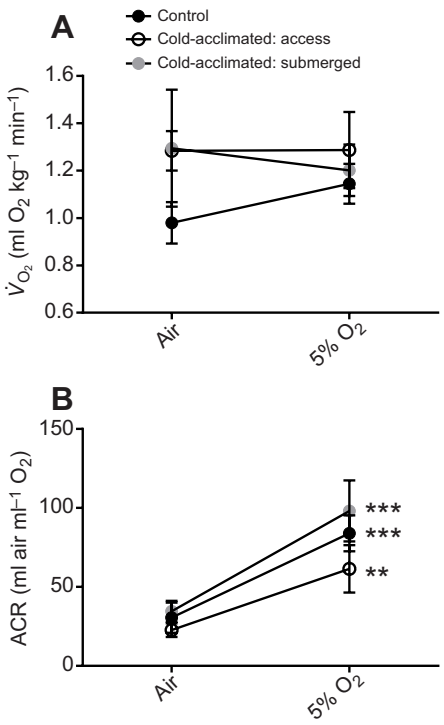

Fig. 7. Metabolic responses and hyperventilation during hypoxia do not differ between acclimation groups. (A) O_2 consumption ($\dot{V}_{O_2}$) before and after 30 min of hypoxia (5% O_2) in control (black circles; *n*=8), cold-acclimated with air access (white circles; *n*=7) and cold-submerged bullfrogs (gray circles; *n*=5). (B) Both groups of cold-acclimated bullfrogs underwent similar magnitude of hyperventilation in response to 30 min of hypoxia as calculated by the ACR for O_2 (two-way ANOVA, main effect of hypoxia, $F_{1,17}$=68.27, P<0.0001; significant *post hoc* analysis for each acclimation group). Error bars represent ±s.e.m. **P<0.01, ***P<0.001 compared with normoxic for each acclimation group.

to surface) (Tattersall and Ultsch, 2008; J.M.S. and L.K.H., personal observations) endure long periods of submergence (≥12 h 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 (Bícego-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 (Tattersall and Ultsch, 2008). 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 (J.M.S. and L.K.H., personal observation). Given that both groups of cold-acclimated bullfrogs had similar levels of resting ventilation and ventilatory responses to gas challenges when assessed

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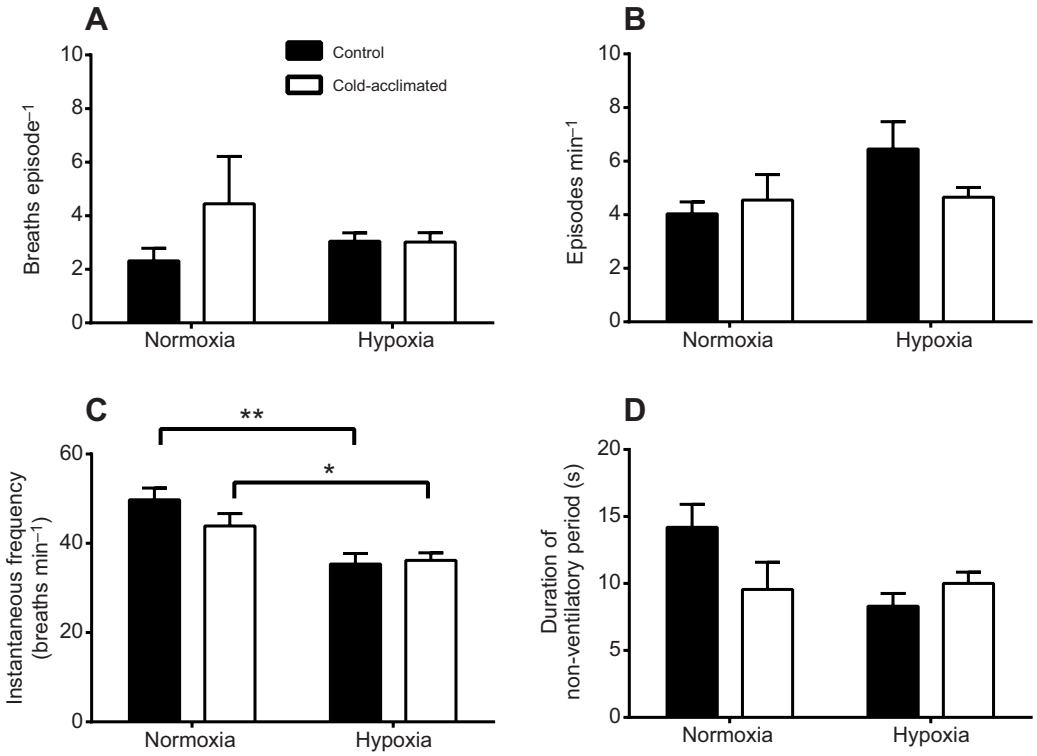

Fig. 8. Breathing pattern does not differ between warm- and cold-acclimated bullfrogs in response to hypoxia. Pooled breathing pattern parameters including (A) breaths episode^{-1} (control: n=8, cold: n=12), (B) episodes min^{-1} (control: n=8, cold: n=12), (C) instantaneous breathing frequency (control: n=8, cold: n=11) and (D) duration of the non-ventilatory period (control: n=8, cold: n=12) 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). Instantaneous breathing frequency (C) decreased during hypoxia in both acclimation groups (two-way ANOVA, main effect of hypoxia, but not acclimation, P=0.0007; Holm–Šidák multiple comparisons test, warm-acclimated, P<0.001, cold-acclimated, P<0.01). Error bars represent ±s.e.m. * P<0.05, ** P<0.01.

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 that affected 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. 1) and match ventilation to metabolism (Fig. 2) ~14–16 h after warming from 2°C to 24°C. Measuring ventilation using facemask pneumotachography required us to 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 visually 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 h 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 with control bullfrogs at 24°C (Fig. 2A), consistent with a classic study of temperature acclimation of metabolism in anurans (Feder, 1982). In contrast, submerged bullfrogs did not have greater $\dot{V}_{O_2}$ at 24°C compared with warm-acclimated frogs, perhaps because of 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 among 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 ACR 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, 1979; Wang et al., 1998), given that RER, $\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$ were the same in each temperature acclimation group (Fig. 2C–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 versus 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

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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, 2016). 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, 2016), 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, 2016) 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, 2016). In cold-acclimated bullfrogs, the post-hypercarbic hyperpnea was reduced by ∼50% compared with control bullfrogs (Fig. 3A). Relative to baseline ventilation in normocarbia, cold-acclimated bullfrogs did not undergo significant increases in ventilation immediately following hypercarbia (Fig. 3A,B). The reduction in the ventilatory response following CO_2 washout did not occur on a background of a different metabolic response to CO_2 (Fig. 4), strongly suggesting that bullfrogs from overwintering environments underwent a reduction in CO_2 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. 3C). 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, increases in breathing frequency 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. 5). 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 CO_2 chemosensitivity arises from the brainstem (near the fourth ventricle) and ∼20% from peripheral chemoreceptors (or chemoreceptors outside the fourth ventricle) (Branco et al., 1993). Interestingly, our results showing blunted ventilatory responses to CO_2 challenge are consistent with preliminary evidence that overwintering submergence leads to a reduction in CO_2 chemosensitivity of locus coeruleus neurons (Santin, 2015; Santin and Hartzler, 2015) that drive ventilation during local brain and arterial acidemia (Noronha-de-Souza et al., 2006). This implies that overwintered bullfrogs have a disrupted CO_2/pH chemosensory system. However, the instantaneous frequency was reduced in cold-acclimated frogs (Fig. 5C), suggesting that issues associated with respiratory rhythmogenesis may, in part, account for lower ventilatory responses to CO_2 challenge independent of modulating chemosensitivity per se. Future work should be directed toward understanding the mechanisms underlying reduced ventilatory responses to elevated inspired CO_2 in cold-acclimated bullfrogs (i.e. central versus peripheral changes in chemosensitivity versus 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_{CO_2} 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 min at 25°C (Lillo, 1978). Furthermore, dive time in turtles inversely correlates with respiratory gas chemosensitivity (Reyes and Milsom, 2009). Because 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 and/or pH to facilitate the occurrence of breaths at warmer temperatures after overwintering may confer the advantage of longer dive time to avoid additional springtime aerial and terrestrial predators. 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_2) at 24°C (Figs 6–8). In this study, an augmentation of tidal volume mediated the ventilatory increase in response to hypoxia that occurred in each acclimation group (Fig. 6). O_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_2-sensitive processes meditating 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 anurans during hypoxia (Gamperl et al., 1999). Although we measured similar ventilated volumes of gas and rates of O_2 consumption before and during hypoxia among acclimation groups (Fig. 7), altered cardiac shunt patterns could lead to drastically different Pa_{O_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_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

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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 and/or 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, 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 the preservation (respiratory motor output and hypoxia sensitivity) and deterioration (CO_2 sensitivity) of sensorimotor function in the same species, individual and neural control system after ecologically relevant inactivity.

Conclusions

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 CO_2. 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.M.S. and L.K.H. conceived and designed the research; J.M.S. performed the experiments; J.M.S. analyzed the data; J.M.S. and L.K.H. interpreted the results; J.M.S. wrote the manuscript; and J.M.S. and L.K.H. revised, edited and approved the final version of the manuscript.

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Supplementary information

Supplementary information available online at
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