Activation of respiratory muscles does not occur during cold-submergence in bullfrogs, *Lithobates catesbeianus*

Joseph M. Santin* and Lynn K. Hartzler

ABSTRACT

Semiaquatic frogs may not breathe air for several months because they overwinter in ice-covered ponds. In contrast to many vertebrates that experience decreased motor performance after inactivity, bullfrogs, *Lithobates catesbeianus*, retain functional respiratory motor processes following cold-submergence. Unlike mammalian hibernators with unloaded limb muscles and inactive locomotor systems, respiratory mechanics of frogs counterintuitively allow for ventilatory maneuvers when submerged. Thus, we hypothesized that bullfrogs generate respiratory motor patterns during cold-submergence to avoid disuse and preserve motor performance. Accordingly, we measured activity of respiratory muscles (buccal floor compressor and glottal dilator) via electromyography in freely behaving bullfrogs at 20 and 2°C. Although we confirm that ventilation cycles occur underwater at 20°C, bullfrogs did not activate either respiratory muscle when submerged acutely or chronically at 2°C. We conclude that cold-submerged bullfrogs endure respiratory motor inactivity, implying that other mechanisms, excluding underwater muscle activation, maintain a functional respiratory motor system throughout overwintering.

KEY WORDS: Neuromuscular, EMG, Respiratory, Hibernation, Disuse, Inactivity

INTRODUCTION

Vertebrate animals use a diverse set of survival strategies to withstand harsh winter environments in temperate regions. Semi-aquatic ectotherms, including many species of frogs and turtles, overwinter in aquatic habitats without access to air (Milsom and Jackson, 2011; Ultsch, 2006; Ultsch et al., 2004). Because these animals are otherwise considered air-breathers, there has been interest in the processes supporting gas exchange and energetics of overwintering submergence. In ranid frogs, these mechanisms include cutaneous gas exchange, metabolic suppression, behavioral thermoregulation, preservation of activity capacity, and hypoxia tolerance (Boutilier, 2001; Pinder and Feder, 1990; Tattersall and Boutilier, 1997, 1999; Tattersall and Ultsch, 2008). Although underwater survival has received considerable attention, the mechanisms used by overwintering frogs to quickly regulate lung breathing following prolonged submergence in response to metabolic challenges and respiratory requirements associated with early spring (e.g. increased temperature, avoiding terrestrial predators, and calling) has been largely overlooked.

Wright State University, Department of Biological Sciences, 3640 Colonel Glenn Hwy, Dayton, OH 45435, USA.

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

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

Overwintering submergence presents a springtime challenge because disuse of neuromotor processes typically leads to loss-of-function through muscle atrophy and neuroplastic motor defects, especially in humans and laboratory rodents (Bonaldo and Sandri, 2013; Clark et al., 2006; Cormery et al., 2005; Deschenes et al., 2002; Seki et al., 2007). Hibernating, overwintering and estivating vertebrates provide interesting exceptions to this rule as they use integrative mechanisms to curtail muscle atrophy and motor decline during extended inactivity (Hudson and Franklin, 2002). To determine the functionality of the respiratory control system following overwintering conditions in bullfrogs, *Lithobates catesbeianus* Shaw 1802, we assessed breathing control after ~2 months of submergence at 2°C. We demonstrated that bullfrogs maintain resting respiratory motor function despite extended absence of lung breathing (Santin and Hartzler, 2016a,b). Broadly speaking, two plausible processes could maintain motor function throughout winter submergence. First, compensatory mechanisms at the cellular level could offset typical degradative processes caused by reduced activity in muscles and neurons as occurs in heterothermic hibernators and silent neural networks (Lee et al., 2008; Lin et al., 2012; Tessier and Storey, 2014; Turrigiano, 2012; Young et al., 2013). Second, the respiratory motor system may avoid the negative influence of inactivity by maintaining neuromotor activation during overwintering submergence. Although neuromuscular transmission ceases between 0 and 1°C *in vitro* (Jensen, 1972), intact frogs maintain significant motor capacity at temperatures ≥1°C (Licht, 1991; Lotshaw, 1977; Tattersall and Boutilier, 1999). Therefore, it remains possible that preserved respiratory motor function after chronic submergence occurs as a result of persistent neuromotor activation of respiratory muscles during overwintering.

Anuran amphibians use two ventilatory motor patterns – buccal oscillations and lung ventilation (Gargaglioni and Milsom, 2007) – that could counterintuitively persist during submergence in cold water. Buccal oscillations involve compression and dilation of the buccal floor with the lungs sealed-off by the glottis, resulting in tidal air flow within the buccal cavity. Because the lungs remain isolated from the buccal cavity during buccal oscillations, water flow could be generated during overwintering submergence without filling the lungs with water. Buccal floor movements and water flow into and out of the nares have been visualized in submerged bullfrogs at 15°C (Hutchison and Whitford, 1966). During lung ventilation, buccal floor muscles contract more forcefully and drive air into the lungs with the glottis dilated and the nares closed (Vitalis and Shelton, 1990). As the nares are closed during lung ventilation, frogs could produce this motor behavior during overwintering submergence because the lungs are protected from ambient water. Intriguingly, lung and buccal pressure changes have been measured during dives, indicating that lung ventilation cycles occur underwater with the nares sealed (West and Jones, 1976). In addition to phasic activation of respiratory muscles during buccal

oscillations and lung ventilation, water entering the buccal cavity initiates sustained, tonic activity in respiratory muscles (West and Jones, 1976), providing another way for submerged frogs to avoid disuse of respiratory muscles. Collectively, these observations show that respiratory mechanics and neuromotor function can remain active underwater. Respiratory muscle activation during cold-submergence, therefore, may present an inactivity-avoidance strategy that could facilitate motor function of breathing for the spring by circumventing months of inactivity. By measuring muscle activation with chronic electromyography (EMG), we tested the hypothesis that bullfrogs maintain neuromotor activation of respiratory pump muscles during cold-submergence.

MATERIALS AND METHODS
Animals
Adult bullfrogs (n=8 of either sex, 128.8±9.2 g) were kept in plastic tanks in water at 22°C and could access wet and dry areas. Frogs were fed crickets once per week and were exposed to a 12 h:12 h light:dark cycle. Experiments were approved by Wright State University's Institutional Animal Care and Use Committee.

Surgical procedures and implantation of EMG electrodes
Bullfrogs were anesthetized with 0.35% (w/v) MS-222 (Western Chemicals, Ferndale, WA, USA). After loss of the toe pinch and eye reflexes, frogs were placed ventral side up and an incision was made

~0.25 cm from the side of the mouth to the midline in plane with the anterior portion of the laryngeal cartilage. A ~2 cm incision was then made along the midline. The m. intermandibularis and m. dilator laryngis were identified according to Ecker (1889). Two EMG electrodes were constructed for each animal from two 5-0 insulated silver wires (Teflon-coated Ag wire, 0.007 inch coated, 0.005 inch bare, cat no. 786000, A-M Systems, Sequim, WA, USA) with 2 mm bared at the end to expose the silver and implanted into each muscle. The incision was closed using 3-0 non-dissolvable polypropylene suture. After the righting reflex returned, bullfrogs were placed into a 284 l tank that was partitioned into five compartments using plastic mesh containing ~35 cm depth of 20°C water. Electromyograms from four animals were recorded simultaneously. Frogs could swim freely, access the surface and dive. Animals were allowed at least 48 h to recover from surgery before experiments began.

EMG recordings and experimental protocol
The opposite ends of the EMG leads for both muscle groups were connected to bipolar micro-hook electrodes (AD Instruments, Colorado Springs, CO, USA). EMG signals were amplified (BioAmp, AD Instruments), filtered (10–1000 Hz), full-wave rectified and integrated (time constant=60 ms) with the Powerlab 26T system (AD Instruments). EMG recordings of buccal floor compressor and glottal dilator activities were then recorded at water
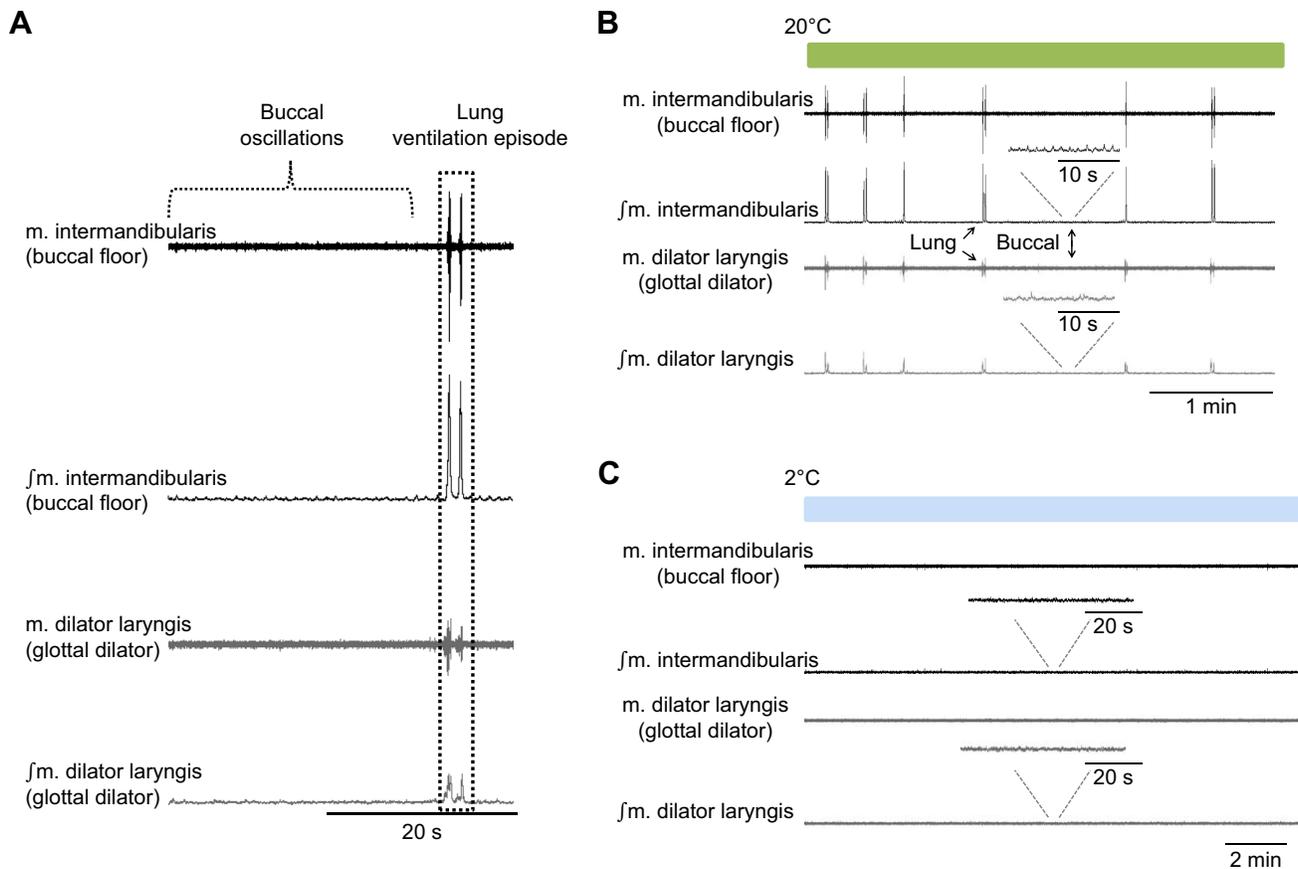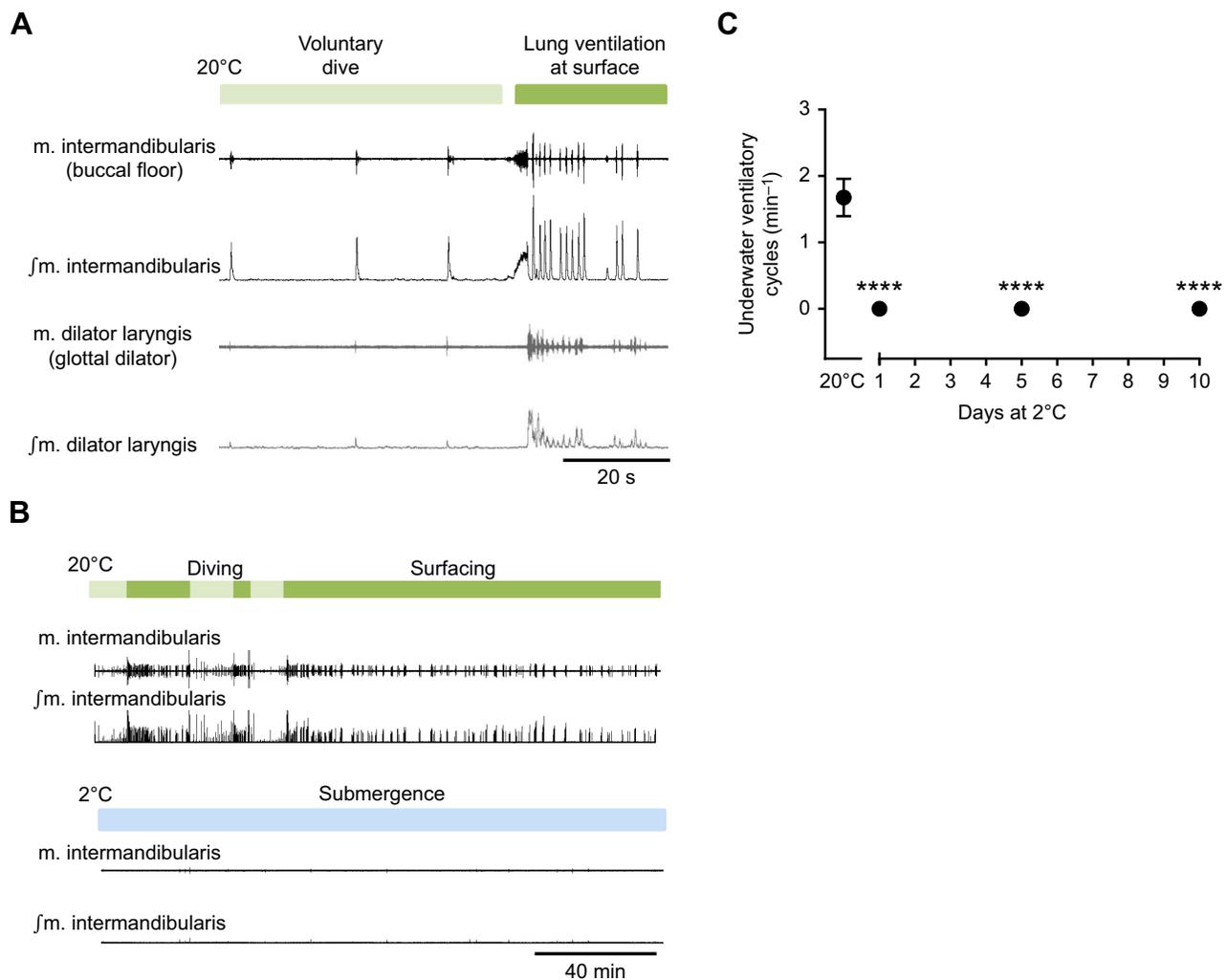

Fig. 1. Cold bullfrogs do not surface to breathe air. (A) Raw (top) and integrated (bottom) electromyography (EMG) recordings of the m. intermandibularis (buccal floor compressors; black) and m. dilator laryngis (glottal dilator; gray) muscles that generate buccal oscillations and lung ventilation. During buccal oscillations, the buccal floor compressors are rhythmically active at small amplitudes. Lung ventilation commences when the glottal dilators activate to open the glottis, which is then followed by high-amplitude activation of the buccal floor compressors. (B) An example of raw (top) and integrated (bottom) EMG recordings of the m. intermandibularis (buccal floor compressors; black) and m. dilator laryngis (glottal dilator; gray) during buccal and lung ventilation at the surface of the tank at 20°C. (C) Surfacing did not occur at 2°C and, therefore, did not produce lung ventilations or buccal oscillations at the surface.

temperatures of 20°C before being stepped to 2°C over 4 days. EMG recordings were made at 2°C for 5 days (n=8) or 10 days (n=4) in a temperature-controlled environmental chamber. Videos of frogs were acquired using Logitec Webcam Software (Logitec, Newark, CA, USA) to determine whether respiratory muscle activation occurred at the surface or underwater. Following experiments, frogs were euthanized and electrodes were checked to ensure they had not moved. All electrodes remained in the muscles as confirmed by dissection.

Data analysis

Ventilatory mechanics of anurans have been described by Vitalis and Shelton (1990). Buccal floor compressor and glottal dilator muscles are activated during the two main ventilatory behaviors: buccal oscillations and lung ventilation. Buccal floor compressors were usually rhythmically active, generating small-amplitude signals (Fig. 1A) that produce tidal airflow within the buccal cavity. Buccal oscillations occur with the glottis closed and,

therefore, the glottal dilator is inactive. During lung ventilation, the glottal dilators activate and open the glottis. Following naril closure, large-amplitude contraction of the buccal floor muscles produces positive pressure that forces air from the previous buccal oscillation into the lung (Fig. 1A). The glottis then closes and stale air is expelled through the nostrils. Therefore, co-activation of glottal dilators and buccal compressors characterizes lung ventilations (Sanders and Milsom, 2001). Lung breaths at 20°C were counted over a 3 h period to determine breathing frequency. As lung breaths and underwater 'ventilatory cycles' both include activation of glottal dilator and buccal floor muscles, these behaviors were distinguished by pairing EMG recordings with video acquired by the webcam. The frequency of underwater ventilatory cycles was determined by counting the events during dives and averaging this value for all dives. Power spectral density analysis (LabChart, AD Instruments) indicated that baseline EMG signals (i.e. when muscles are not phasically active) are predominately electrical noise. We analyzed the integrated baseline EMG signal to determine

Fig. 2. Unlike frogs at warmer temperatures, cold-submerged bullfrogs do not use respiratory muscles to perform 'ventilatory cycles' underwater. (A) Raw (top) and integrated (bottom) EMG recordings of the m. intermandibularis (buccal floor compressors; black) and m. dilator laryngis (glottal dilator; gray) during voluntary diving and surfacing at 20°C. The trace under the light green bar indicates a voluntary dive and shows that lung ventilatory cycles occur underwater (glottal dilation precedes a large-amplitude buccal floor compression). The portion of the trace under the dark green bar shows the frog performing lung ventilations at the surface. (B) Top: raw and integrated m. intermandibularis activity over a 3 h time period, demonstrating that each dive contains underwater activation of the respiratory pump. Bottom: an example recording indicating that submerged bullfrogs at 2°C do not perform underwater ventilatory cycles. (C) Mean data for the frequency of underwater ventilatory cycles at 20°C and at 1, 5 (n=8) and 10 days (n=4) at 2°C. None of the frogs exhibited underwater ventilatory cycles during cold submergence. ****P<0.0001.

whether increases in tonic activity occurred during cold-submergence as is the case when water enters the buccal cavity (West and Jones, 1976). Data were analyzed during the light period at each temperature.

Statistics

Data were analyzed using a one-way ANOVA and Dunnet's multiple comparisons test. Statistical significance was assumed when $P<0.05$. Analyses and graphs were generated using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

We first determined how often bullfrogs breathe when surfacing. Fig. 1B,C shows example recordings of buccal floor compressor and glottal dilator muscles in bullfrogs surfacing at 20°C. The buccal floor muscles were often rhythmically active when surfacing at 20°C, producing buccal oscillations that were interrupted by lung ventilations (mean±1 s.d. of 5.5±4.2 lung breaths per minute; Fig. 1B). At 2°C, bullfrogs remained voluntarily submerged (Fig. 1C). Using video recordings, we confirmed that 7/8 bullfrogs remained submerged for the entire duration of the experiment at 2°C. On the beginning of the first day at 2°C, one frog remained at the surface and had a breathing frequency of 0.14 breaths min^{-1}. This frog dived underwater after a few hours on the first day at 2°C and remained submerged throughout subsequent analysis periods. Therefore, when given the option to breathe air, cold frogs voluntarily submerge and do not surface to take lung breaths.

Next, we analyzed underwater respiratory muscle activation. 'Lung ventilation cycles' counterintuitively occur at warmer temperatures in diving frogs (West and Jones, 1976). This presents the possibility that cold-submerged frogs could maintain respiratory muscle activation without accessing the surface. Fig. 2A shows example raw and integrated EMG recordings of buccal floor and glottal dilator muscles in a freely diving and surfacing bullfrog. As illustrated on the left side of Fig. 2A, glottal dilator and buccal floor muscles were active underwater, consistent with the previous observation that lung ventilation cycles occur in diving frogs (West and Jones, 1976). Fig. 2B (top) shows an example EMG trace of the m. intermandibularis over a 3 h time span, indicating that dives (light green bar over trace) contained underwater lung ventilation cycles. We did not observe buccal oscillations when frogs were underwater at 20°C. In contrast to findings at 20°C, underwater ventilation cycles did not occur in submerged frogs at 2°C (Fig. 2B, bottom). Only movement artifacts, as confirmed by video recording, caused activity on the EMG records (i.e. large-amplitude, long-duration EMG signals over baseline that were only present when the frog was moving on the bottom of the tank and never when the animal remained still) and was thus ignored in the analysis. Of the eight frogs studied, four took voluntary dives at 20°C; therefore, Fig. 2C shows mean data for only these four frogs to avoid biasing means at 20°C. Fig. 2C indicates that underwater ventilatory cycles occurred at a greater frequency at 20°C compared with 1, 5 and 10 days at 2°C ($P<0.001$, $F_{3,10}=28.17$; one-way ANOVA). These findings demonstrate that although the respiratory pump operates
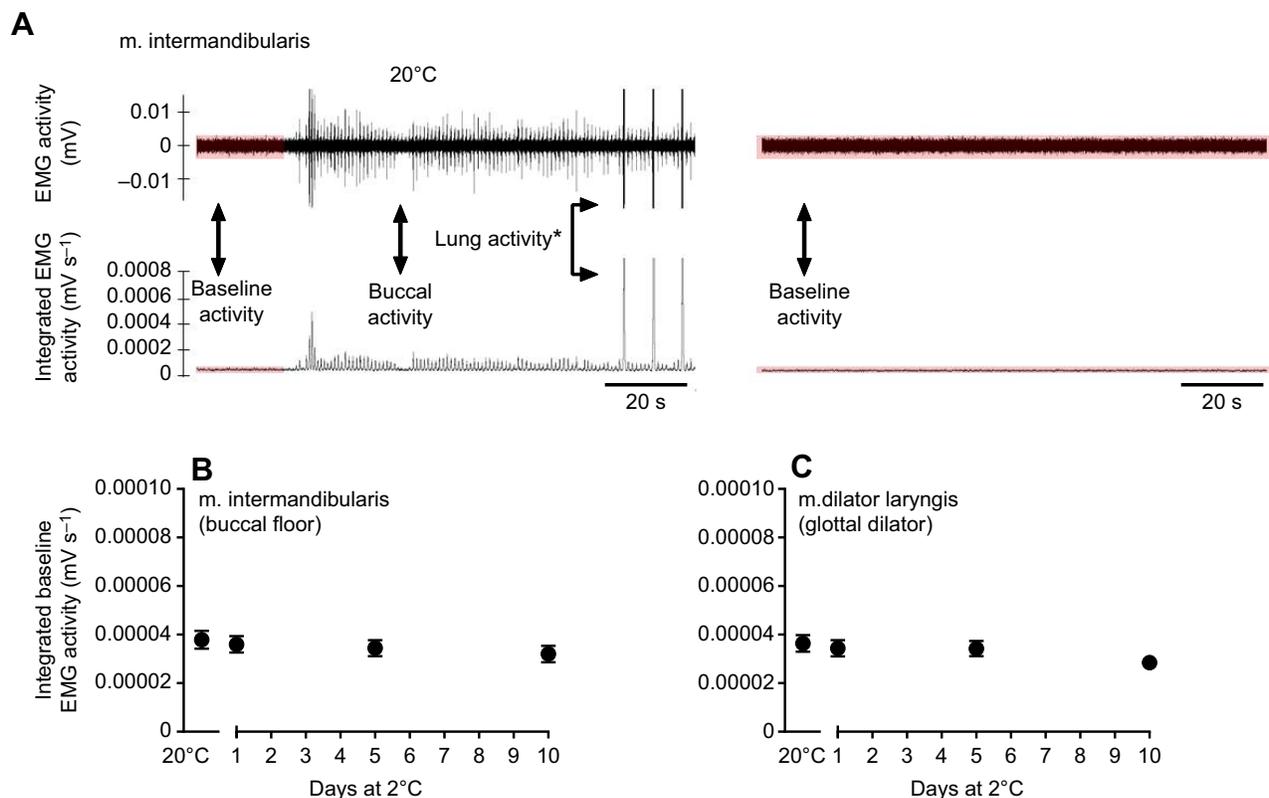
Fig. 3. Baseline EMG activity of respiratory muscles does not change during cold-submergence. (A) Raw (top) and integrated (bottom) EMG activity of the m. intermandibularis at 20°C (left) and after 5 days at 2°C (right). Asterisks indicate truncated lung activity. Baseline EMG activity is shaded in red and can be observed at 20°C when buccal and lung-related EMG activity does not occur and continuously at 2°C. The amount of baseline EMG activity is unchanged at 2°C. Asterisk indicates truncated EMG signals. (B,C) Mean data for integrated tonic EMG activity at 20°C and after 1, 5 ($n=8$) and 10 days ($n=4$) at 2°C in the m. intermandibularis (B) and the m. dilator laryngis (C). Baseline EMG activity does not change during cold-submergence in either muscle group. ****$P<0.0001$.

underwater at warm temperatures, we could not detect phasic respiratory muscle activation in cold-submerged bullfrogs.

Although bullfrogs did not phasically activate respiratory muscles during cold-submergence, sustained, tonic respiratory muscle activity can be triggered when water enters the buccal cavity (West and Jones, 1976). Thus, neuromotor activity in cold-submerged bullfrogs could manifest as tonic activity. The left panel of Fig. 3A illustrates baseline raw and integrated m. intermandibularis EMG signals (shaded in red) at 20°C when small-amplitude buccal and large-amplitude lung activities were not present. The right panel of Fig. 3A shows raw and integrated EMG traces after 5 days at 2°C of similar magnitude to those at 20°C. Fig. 3B,C shows mean data for integrated baseline EMG signal at 20°C and 1, 5 and 10 days at 2°C. In both buccal floor compressor and glottal dilator muscles, the baseline EMG signal did not change with cold-submergence (buccal floor: P=0.7571, $F_{3,24}$=0.3959; one-way ANOVA, glottal dilator: P=0.5533, $F_{3,24}$= 0.7139; one-way ANOVA). No increase in baseline EMG activity suggests that tonic activity is not initiated during cold-submergence. However, it is possible that low levels of activity that we are not able to detect in this study play a role in maintaining neuromotor tone during overwintering.

Diving anurans can activate respiratory muscles underwater (Hutchison and Whitford, 1966; West and Jones, 1976). To reconcile our findings that bullfrogs do not undergo respiratory motor decline following overwintering submergence (Santin and Hartzler, 2016a,b), we hypothesized that bullfrogs would utilize these mechanics during cold-submergence to circumvent the loss of function typically associated with extended disuse in less-active motor systems of non-hibernating mammals (Bonaldo and Sandri, 2013; Cormery et al., 2005; Seki et al., 2007) and certain muscles of heterothermic hibernators (Wickler et al., 1991). We found that underwater lung ventilation cycles, but not buccal oscillations, were present in diving bullfrogs at 20°C, but respiratory muscle activation did not occur during cold-submergence at any time point in this study. Although bullfrogs have respiratory mechanics that function underwater at 20°C, our results indicate that these cycles do not occur in cold-submerged bullfrogs. This indicates that an inactivity-avoidance strategy of the respiratory motor system does not likely explain maintenance of neuromotor function in the spring after overwintering submergence (Santin and Hartzler, 2016a,b).

Our findings demonstrate that cold-submergence associated with overwintering produces neuromotor inactivity; a phenomenon that does not usually occur naturally in highly active motor systems as it incurs detrimental consequences to motor function (Banzrai et al., 2016; Bonaldo and Sandri, 2013; Cormery et al., 2005). Muscle plasticity in natural models of inactivity has received considerable attention (Hudson and Franklin, 2002; James et al., 2013; Nowell et al., 2011; Reid et al., 1995; Wickler et al., 1991). However, mechanisms that support neural stability following months of inactivity remain virtually unexplored. The respiratory control system of bullfrogs provides a natural model that maintains motor function despite chronic and verified inactivity. Understanding how the respiratory motor system of bullfrogs maintains normal function despite lengthy periods of inactivity may generate new insight into mechanisms underlying neuromotor robustness.

Competing interests
The authors declare no competing or financial interests.

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

References
Banzrai, C., Nodera, H., Kawarai, T., Higashi, S., Okada, R., Mori, A., Shimatani, Y., Osaki, Y. and Kaji, R. (2016). Impaired axonal Na⁺ current by hindlimb unloading: implication for disuse neuromuscular atrophy. *Front. Physiol.* **7**, 36.

Bonaldo, P. and Sandri, M. (2013). Cellular and molecular mechanisms of muscle atrophy. *Dis. Model. Mech.* **6**, 25-39.

Boutilier, R. G. (2001). Mechanisms of metabolic defense against hypoxia in hibernating frogs. *Respir. Physiol* **128**, 365-377.

Clark, B. C., Manini, T. M., Bolanowski, S. J. and Ploutz-Snyder, L. L. (2006). Adaptations in human neuromuscular function following prolonged unweighting: II. Neurological properties and motor imagery efficacy. *J. App. Physiol.* **101**, 264-272.

Cormery, B., Beaumont, E., Csukly, K. and Gardiner, P. (2005). Hindlimb unweighting for 2 weeks alters physiological properties of rat hindlimb motoneurones. *J. Physiol.* **568**, 841-850.

Deschenes, M. R., Giles, J. A., McCoy, R. W., Volek, J. S., Gomez, A. L. and Kraemer, W. J. (2002). Neural factors account for strength decrements observed after short-term muscle unloading. *Am. J. Physiol. Reg. Int. Comp. Physiol.* **282**, R578-R583.

Ecker, A. (1889). *The Anatomy of the Frog*, Vol. 2. Oxford: Clarendon Press.

Gargaglioni, L. H. and Milsom, W. K. (2007). Control of breathing in anuran amphibians. *Comp. Biochem. Physiol. A Mol. Int. Physiol.* **147**, 665-684.

Hudson, N. J. and Franklin, C. E. (2002). Maintaining muscle mass during extended disuse: aestivating frogs as a model species. *J. Exp. Biol.* **205**, 2297-2303.

Hutchison, V. H. and Whitford, W. G. (1966). Survival and underwater buccal movements in submerged anurans. *Herpetologica* **22**, 122-127.

James, R. S., Staples, F. J., Brown, J. C. L., Tessier, S. N. and Storey, K. B. (2013). The effects of hibernation on the contractile and biochemical properties of skeletal muscles in the thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*. *J. Exp. Biol.* **216**, 2587-2594.

Jensen, D. W. (1972). The effect of temperature on transmission at the neuromuscular junction of the sartorius muscle of *Rana pipiens*. *Comp. Biochem. Physiol. A Physiol* **41**, 685-695.

Lee, K., Park, J. Y., Yoo, W., Gwag, T., Lee, J.-W., Byun, M.-W. and Choi, I. (2008). Overcoming muscle atrophy in a hibernating mammal despite prolonged disuse in dormancy: proteomic and molecular assessment. *J. Cell. Biochem.* **104**, 642-656.

Licht, L. E. (1991). Habitat selection of *Rana pipiens* and *Rana sylvatica* during exposure to warm and cold temperatures. *Am. Mid. Nat.* **125**, 259-268.

Lin, D. C., Hershey, J. D., Mattoon, J. S. and Robbins, C. T. (2012). Skeletal muscles of hibernating brown bears are unusually resistant to effects of denervation. *J. Exp. Biol.* **215**, 2081-2087.

Lotshaw, D. P. (1977). Temperature adaptation and effects of thermal acclimation in Rana sylvatica and Rana catesbeiana. *Comp. Biochem. Physiol. A Physiol.* **56**, 287-294.

Milsom, W. K. and Jackson, D. C. (2011). Hibernation and gas exchange. *Compr. Physiol.* **1**, 397-420.

Nowell, M. M., Choi, H. and Rourke, B. C. (2011). Muscle plasticity in hibernating ground squirrels (*Spermophilus lateralis*) is induced by seasonal, but not low-temperature, mechanisms. *J. Comp. Physiol. B* **181**, 147-164.

Pinder, A. W. and Feder, M. E. (1990). Effect of boundary layers on cutaneous gas exchange. *J. Exp. Biol.* **154**, 67-80.

Reid, W. D., Ng, A., Wilton, R. K. and Milsom, W. K. (1995). Characteristics of diaphragm muscle fibre types in hibernating squirrels. *Respir. Physiol.* **101**, 301-309.

Sanders, C. E. and Milsom, W. K. (2001). The effects of tonic lung inflation on ventilation in the American bullfrog *Rana catesbeiana* Shaw. *J. Exp. Biol* **204**, 2647-2656.

Santin, J. M. and Hartzler, L. K. (2016a). Control of lung ventilation following overwintering conditions in bullfrogs, *Lithobates catesbeianus*. *J. Exp. Biol.* **219**, 2003-2014.

Santin, J. M. and Hartzler, L. K. (2016b). Environmentally-induced return to juvenile-like chemosensitivity in the respiratory control system of adult bullfrogs, *Lithobates catesbeianus*. *J. Physiol.* **594**, 6349-6367.

Seki, K., Kizuka, T. and Yamada, H. (2007). Reduction in maximal firing rate of motoneurons after 1-week immobilization of finger muscle in human subjects. *J. Electromyogr. Kinesiol.* **17**, 113-120.

Tattersall, G. and Boutilier, R. (1997). Balancing hypoxia and hypothermia in cold-submerged frogs. *J. Exp. Biol.* **200**, 1031-1038.

Tattersall, G. J. and Boutilier, R. G. (1999). Does behavioural hypothermia promote post-exercise recovery in cold-submerged frogs? *J. Exp. Biol.* **202**, 609-622.

Tattersall, G. J. and Ultsch, G. R. (2008). Physiological ecology of aquatic overwintering in ranid frogs. *Biol. Rev.* **83**, 119-140.

Tessier, S. N. and Storey, K. B. (2014). To be or not to be: the regulation of mRNA fate as a survival strategy during mammalian hibernation. *Cell Stress Chaperones* **19**, 763-776.

Turrigiano, G. (2012). Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. *Cold Spring Harb. Perspect. Biol.* **4**, a005736.

Ultsch, G. R. (2006). The ecology of overwintering among turtles: where turtles overwinter and its consequences. *Biol. Rev.* **81**, 339-367.

Ultsch, G. R., Reese, S. A. and Stewart, E. (2004). Physiology of hibernation in *Rana pipiens*: metabolic rate, critical oxygen tension, and the effects of hypoxia on several plasma variables. *J. Exp. Zool. A Comp. Exp. Biol.* **301**, 169-176.

Vitalis, T. Z. and Shelton, G. (1990). Breathing in *Rana pipiens*: the mechanism of ventilation. *J. Exp. Biol.* **154**, 537-556.

West, N. H. and Jones, D. R. (1976). The initiation of diving apnoea in the frog, *Rana pipiens*. *J. Exp. Biol.* **64**, 25-38.

Wickler, S. J., Hoyt, D. F. and van Breukelen, F. (1991). Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis*. *Am. J. Physiol.* **261**, R1214-R1217.

Young, K. M., Cramp, R. L. and Franklin, C. E. (2013). Each to their own: skeletal muscles of different function use different biochemical strategies during aestivation at high temperature. *J. Exp. Biol.* **216**, 1012-1024.