Published in final edited form as:

Respir Physiol Neurobiol. 2015 February 1; 0: 48–57. doi:10.1016/j.resp.2014.12.015.

Hypoxia switches episodic breathing to singlet breathing in red-eared slider turtles (*Trachemys scripta*) via a tropisetron-sensitive mechanism

Stephen M. Johnson, Ashley R. Krisp, and Michelle E. Bartman

Department of Comparative Biosciences, School of Veterinary Medicine University of Wisconsin
Madison, WI 53706 USA

Abstract

Hypoxia-induced changes in the chelonian breathing pattern are poorly understood. Thus, breathing was measured in freely swimming adult red-eared slider turtles breathing air prior to breathing nitrogen for 4 h. Ventilation increased 10-fold within 10 min due to increased breath frequency and tidal volume. Breaths/episode decreased by ~50% within after 1 h of hypoxia while the number of singlet breaths increased from 3.1 ± 1.6 singlets/h to a maximum of 66.1 ± 23.5 singlets/h. Expiratory and inspiratory duration increased during hypoxia. For doublet and triplet breaths, expiratory duration increased during the first breath only, while inspiratory duration increased for all breaths. Tropisetron (5-HT₃ receptor antagonist, 5 mg/kg) administration prior to hypoxia attenuated the hypoxia-induced increase in singlet breath frequency. Along with results from previous *in vitro* studies, this study suggests that 5-HT₃ receptor activation may be required for the hypoxia-induced increase in singlet breathing pattern in red-eared slider turtles.

Keywords

reptile; chelonian; episodic breathing; respiratory motor control; serotonin; 5-HT₃ receptors

1. Introduction

The vertebrate respiratory control system responds to hypoxia depending on the degree and duration of hypoxia, tolerance to hypoxia, and environmental conditions in a species-dependent manner. Under controlled experimental conditions, the acute hypoxic ventilatory response is well characterized with several distinct phases in mammals (Powell et al. 1998) and birds (Mitchell et al. 2001). For ectothermic vertebrates, however, there is less consistency in the length, duration and intensity of the hypoxic exposures, and ambient

© 2014 Elsevier B.V. All rights reserved.

Corresponding Author: Stephen M. Johnson, M.D., Ph.D. Associate Professor Department of Comparative Biosciences School of Veterinary Medicine University of Wisconsin 2015 Linden Drive Madison, Wisconsin 53706 Phone: (608) 261-1104 Fax: (608) 263-3926 johnsons@svm.vetmed.wisc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

temperature (Porteus et al. 2011). Also, hypoxia in ectothermic vertebrates can cause metabolic depression (e.g., Hicks and Wang 1999), decrease arterial PCO₂ (e.g., Davies and Sexton 1987), and increase cardiovascular shunting of blood away from the lungs (Hicks and Wang 1998), all of which confounds interpretation of experimental results. Thus, in ectothermic vertebrates, the hypoxic ventilatory response and its underlying physiological mechanisms are not well characterized.

In chelonians, acute hypoxia increases ventilation, but considerable variation is observed with respect to changes in breath frequency and tidal volume. For example, most studies on semi-aquatic turtles show that hypoxia increases both breath frequency and tidal volume (Glass et al. 1983; Herman and Smatresk 1999; Hitzig and Nattie 1982; Jackson 1973; Wasser and Jackson 1988). However, other studies show that hypoxia increases breath frequency with minimal or no changes in tidal volume in semi-aquatic turtles (Davies and Sexton 1987; Vitalis and Milsom 1986) and aquatic turtles (Burggren et al. 1977). In contrast, snapping turtles exposed to 10% oxygen increase ventilation by decreasing the non-ventilatory period, but there is no correlation between arterial oxygen levels and breath frequency (West et al. 1989). Thus, there is wide variability in hypoxia-induced changes in chelonian breathing.

In addition to altering breath frequency and tidal volume, hypoxia alters breathing pattern by decreasing the number of breaths/episode (defined as “episodicity”) in chelonians. For example, in unidirectionally-ventilated semi-aquatic turtles, the number of breaths/episode decreases by ~50% when oxygen administration is switched from 30% to 0% oxygen (Herman and Smatresk 1999). Likewise, in snapping turtles, exposure to 5% oxygen decreases breathing episodicity from 4.5 to 1.1 breaths/episode (Frische et al. 2000). Hypoxia-induced changes in breathing episodicity have not been systematically characterized, nor has the potential underlying mechanism been tested. Recently, we showed that episodicity in the fictive breathing pattern produced by isolated turtle brainstems can be altered by application of serotonin 5-HT₃ receptor agonist and antagonist drugs (Bartman et al., 2010). Isolated turtle brainstems *in vitro* are advantageous because they produce inspiratory- and expiratory-related motor output that is qualitatively similar to that produced by intact turtles, including episodic breathing (Johnson et al., 1998). Bath-applied 5-HT₃ receptor agonist drugs switch respiratory motor bursts to a singlet pattern, while 5-HT₃ receptor antagonist drugs increase the number of bursts/episode (Bartman et al., 2010). Thus, we postulate that hypoxia may decrease episodicity in intact semi-aquatic turtles by activation of central 5-HT₃ receptors.

Finally, hypoxia transforms the shape and duration of individual breaths. For example, severe hypoxia in mammals transforms phrenic inspiratory-related motor bursts of neural activity from a slowly-incrementing/rapidly-decrementing pattern associated with eupnea to a rapidly-incrementing/slowly-decrementing pattern associated with gasping in intact animals (reviewed in St John 1996) and under *in vitro* conditions (Hill et al. 2011; Lieske et al. 2000). However, very little is known about hypoxia-induced changes in chelonian breathing with respect to breath duration during expiration or inspiration. In semi-aquatic turtles, expiratory and inspiratory duration increases during hypoxia, but this was attributed to the fact that more time is required for expiration and inspiration when tidal volume is

increased (Glass et al. 1983). Thus, the question as to how hypoxia changes the duration of chelonian expiratory and inspiratory phases is not known.

To address these questions, breathing in awake, freely swimming adult red-eared slider turtles (*Trachemys scripta*) was measured by placing turtles in a water-filled tank with a small inverted air-filled chamber for breathing. A pneumotachograph was used to measure ventilation, breathing frequency, tidal volume, breaths/episode, and single breaths (“singlets”). Turtles were allowed to breathe room air before switching to nitrogen gas to induce hypoxia. Specifically, we tested whether: (1) hypoxia augmented breath frequency and tidal volume; (2) hypoxia decreased the number of breaths/episode and increased singlet breath frequency; (3) hypoxia augmented inspiratory duration similar to mammals or altered expiratory duration; and (4) hypoxia-induced decrease in episodicity was altered by serotonin 5-HT₃ receptor antagonist drug administration. Preliminary data were published in abstract form (Johnson, 2010).

2. Methods

2.1 Experimental animals

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine. Adult red-eared slider turtles (*Trachemys scripta*) of either sex ($n = 10$, weight = 790 ± 17 g) were obtained from commercial suppliers and kept in a large open tank where they had access to water for swimming, and heat lamps and dry areas for basking. Room temperature was set to 27-28°C with light 14 h per day. Turtles were fed ReptoMin® floating food sticks (Tetra, Blacksburg, VA) 3-4 times per week. Turtles were adapted to these conditions for at least two weeks before acclimation to the breathing chamber. Experiments were performed throughout the year with no noticeable differences due to seasonal effects (Reyes and Milsom, 2009, 2010).

2.2 Ventilation measurements

Ventilation (V_E ; ml/min/kg) was measured in conscious, freely swimming turtles using established methods (Funk et al., 1986). Individual turtles were placed into respiratory tanks, which were clear plastic containers ($16 \times 42 \times 42$ cm) filled to the top with water at 23°C. A circular breathing chamber (diameter, 8 cm; volume, 250 ml) was sealed into the top, providing the only location within the respiratory tank for the turtle to breathe. Flow meters maintained a constant flow (~ 500 ml/min) of room air through the breathing chamber. Airflow changes were converted to electronic signals with a pneumotachograph (8431 Series, Hans Rudolph Inc., Kansas City, MO) that was connected to an amplifier (Series 1110, Harvard Apparatus, Holliston, MA). Voltage signals were acquired using Clampfit software (Molecular Devices Inc., Sunnyvale, CA). The pneumotachograph was calibrated according to published methods (Funk et al., 1986). One end of plastic tubing (inner diameter, 0.3 mm; length, ~ 40 cm) was inserted into the breathing chamber, while the other end was connected to a motor-driven 25-ml glass syringe. The syringe was set at different volumes (range, 2.5-16.5 ml) and rhythmically moved back and forth at cycle periods of 1.5-4.5 seconds (similar to the duration of one turtle expiratory-inspiratory cycle). For a given syringe volume, expiratory trace areas were averaged at different frequencies and

plotted versus log of the syringe frequency. These plots showed that expiratory area measurements were relatively insensitive to frequency with a maximum of 10% error in V_E and tidal volume (V_T) measurements only at the highest frequencies and V_T in turtles. Thus, the impact of systematic pneumotachograph errors was deemed to be minimal with respect to the major findings.

2.3 Experimental protocols

Turtles were conditioned to the respiratory tank for 2-3 h/day for 1-3 days prior to entering a trial. For each protocol, turtles were placed in the respiratory tank for 3 h to obtain baseline breathing data. Respiratory data after 1 h were discarded to allow for acclimation to the respiratory tank while baseline data were recorded during the 2-3 h period (Johnson and Creighton, 2005). For time control experiments, turtles were allowed to breathe room air for 6 h without being disturbed after the 1 h acclimatization. To test for the effects of severe hypoxia on breathing, turtles breathed room air for 3 h before the gas was switched to 100% nitrogen gas for the next 4 h. To test whether saline injections altered respiratory variables while breathing room air, turtles breathed room air for 3 h before being removed from the tank and injected subcutaneously into the pelvic girdle with saline (1.0 ml). The turtles were returned to the tank and allowed to breathe room air for 4 h. Potential effects of serotonin 5-HT₃ receptor blockade on breathing were determined by allowing turtles to breathe room air for 3 h prior to being removed and injected with tropisetron (3-Tropanyl-indole-3-carboxylate hydrochloride; 5-HT₃ receptor antagonist; Tocris Bioscience, Ellisville, MO) at 1.0, 5.0, or 10 mg/kg (dissolved in 1.0 ml saline) before being returned to the tank to breathe room air for the next 4 h. To test whether hypoxia-induced changes in breathing were altered by serotonin 5-HT₃ receptor drug administration, turtles breathed room air for 3 h before being removed from the tank and injected subcutaneously into the pelvic girdle tropisetron as described above before being returned to the tank to breathe 100% nitrogen gas for the next 4 h. The use of 100% nitrogen gas for inducing hypoxia was based on the finding that turtles respond to very low levels of oxygen, such as 5% oxygen (Frische et al., 2000; Glass et al., 1983). Based on a similar study whereby turtles breathed nitrogen for six hours (Wasser et al., 1991), arterial blood gasses for the turtles in our study were likely approximately $PO_2 = 0-1$ mm Hg, $PCO_2 = 85$ mm Hg, and $pH = 7.4$ after 4 h of breathing nitrogen, consistent with hypoxia, hypercapnia, and acidemia. All experimental protocols were completed on 7/10 turtles.

2.4 Data analysis

Ventilation (V_E , ml/min/kg) was calculated by summing the area under individual expiratory traces within a 60-min period and converting to volume using the pneumotachograph calibration data. Breath frequency was defined as the average number of breaths per minute. Tidal volume (V_T , ml/breath/kg) was calculated by dividing V_E by breath frequency. To determine the breaths/episode, a breath was considered part of an episode if the time interval between two breaths was less than the average duration for an individual breath that includes expiration and inspiration. Data were averaged into 5-min bins (Fig. 5) or 1-h bins (Figs. 2, 6, 8). A two-way repeated measures ANOVA was used to analyze data using Sigma Stat software (Jandel Scientific Software, San Rafael, CA). If the normality assumptions were not satisfied, some data were transformed using the square root

transformation. Results from transformed data were used if normality and equal variance assumptions, or statistical power were improved. Post-hoc comparisons were made using the Student-Newman-Keul's test. All data are expressed as mean $\pm$ SEM. *P*-values < 0.05 were considered significant.

3. Results

3.1 Hypoxia-induced changes in ventilation and breathing pattern

When awake freely swimming turtles ($n=10$) were exposed to air for 6 h, there were no time-dependent changes in ventilation, breath frequency, tidal volume, episodicity, or singlet breath frequency (Figs. 1, 2). During the 6-h exposure to air, ventilation was 9.4-11.8 ml/min/kg, breath frequency was 1.4-1.9 breaths/min, tidal volume was 7.5-7.9 ml/breath/kg, episodicity was 3.5-5.1 breaths/episode, and singlet breath frequency was 2.5-5.9 singlets/h (Fig. 2).

Administration of 100% nitrogen to these same turtles increased ventilation due to increased breathing frequency and tidal volume (Figs. 1, 2). Ventilation increased from a pre-hypoxic baseline of 12.7 ± 1.8 ml/min/kg (2 h timepoint in Fig. 2A) to 67.1 ± 9.4 ml/min/kg after 1 h (designated as the 3 h timepoint in the graph) and a maximum of 126.4 ± 6.6 ml/min/kg after 3 h hypoxia (designated as the 5 h timepoint in the graph) ($p < 0.001$ for hypoxic effect; Fig. 2A). Likewise, breath frequency increased from a pre-hypoxic baseline of 1.9 ± 0.2 breaths/min to 4.7 ± 0.6 breaths/min after 1 h of hypoxia and to a maximum of 7.0 ± 0.6 breaths/min after 3 h of hypoxia ($p < 0.001$ for hypoxic effect; Fig. 2B). Tidal volume increased from a pre-hypoxic baseline of 7.0 ± 1.1 ml/breath/kg to 14.0 ± 1.4 ml/breath/kg after 1 h of hypoxia to a plateau of 19.3 to 19.9 ml/breath/kg after 2-3 h of hypoxia ($p < 0.001$ for hypoxic effect; Fig. 2C). With respect to breathing pattern, there were time- and hypoxia-dependent changes in episodicity and singlet breath frequency (Figs. 1B, 1C). For example, episodicity decreased from a baseline level of 4.8 ± 0.5 breaths/episode in air (hour 2) to 2.6 ± 0.3 breaths/episode (3 h timepoint; $p = 0.006$), returned to near baseline levels of 4.7 ± 1.3 breaths/episode (hour 4; $p > 0.05$), and decreased to 2.5 ± 0.2 and 2.7 ± 0.3 breaths/episode (5-6 h timepoints, $p = 0.001$; $p = 0.004$ for hypoxic effect) (Fig. 2D). Singlet breathing frequency increased from a baseline of 3.1 ± 1.6 singlets/h (2 h timepoint) to a peak of 66.1 ± 23.5 singlets/h during hypoxia (3 h timepoint; $p < 0.001$) and remained elevated at 47-52 singlets/h ($p < 0.002$; $p = 0.016$ for hypoxic effect; Fig. 2E). Hypoxic turtles did not display a breathing pattern of regularly-spaced singlet breaths that would be consistent with a "continuous" breathing pattern. Instead, the singlets occurred interspersed together with doublet, triplet, and larger breathing episodes (Figs. 1, 7B, 7C).

3.2 Hypoxia-induced changes in expiratory and inspiratory duration

To examine hypoxia-induced changes in expiratory and inspiratory duration in turtles ($n=10$), data from an average of the 1-2 h baseline period were compared to each h of the first 2 h of hypoxia (designated as 3 h and 4 h in the bar graphs) (Figs. 3-4). It was necessary to have the first 2 h of baseline used in this analysis because it was important to capture a larger number of specific types of breathing episodes (*e.g.*, singlet breaths, doublet episodes,

triplet episodes) to make adequate statistical comparisons between baseline and hypoxic exposures.

While breathing room air in time control experiments, expiratory and inspiratory durations for all breaths (e.g., singlet breaths, breaths in episodes) were constant at 1.11-1.12 s and 1.33-1.39 s, respectively (data not shown). When turtles were challenged with hypoxia, expiratory duration was 1.00 ± 0.05 s (baseline) and increased to 1.22 ± 0.06 s after 2 h of hypoxia. Inspiratory duration also increased from 1.05 ± 0.08 s (baseline) to 1.51 ± 0.11 s and 1.53 ± 0.10 s after 1-2 h of hypoxia (data not shown). To normalize hypoxia-induced changes in breath durations, as well as present the data more economically, expiratory and inspiratory durations were expressed as percent change from baseline (Figs. 3-4). Accordingly, hypoxia increased expiratory duration of all breaths by $9 \pm 5\%$ and $24 \pm 8\%$ after 1 and 2 h hypoxia (3 h and 4 h timepoints), respectively ($p=0.04$ for hypoxic effect; $p<0.001$ at 4 h timepoint; Fig. 3A₁). Hypoxia increased inspiratory duration of all breaths by $45 \pm 5\%$ and $52 \pm 14\%$ ($p<0.001$ for hypoxic effect; $p<0.001$ at 3 and 4 h timepoints; Fig. 3A₂).

During hypoxia, the increase in inspiratory duration appeared to be mainly due to an increase in the last inspiratory trace within an episode (right panel in Fig. 1C). To test this hypothesis, expiratory and inspiratory duration data from turtles ($n=4$) were graphed separately for singlet breaths only (Figs. 3B₁, 3B₂), an episode of doublet breaths (Figs. 3C₁₋₄), and an episode of triplet breaths (Fig. 4). For all of these data sets, expiratory and inspiratory durations were not altered while breathing room air ($p>0.05$). Thus, we focused on the percent change of expiratory and inspiratory durations caused by hypoxia.

For singlet breaths only, expiratory and inspiratory duration were both increased by hypoxia with somewhat larger increases during the inspiratory phase. Expiratory duration increased by $53 \pm 18\%$ and $64 \pm 19\%$ ($p=0.012$ for hypoxic effect; $p<0.001$ at the 3 h and 4 h timepoints; Fig. 3B₁), and inspiratory duration increased by $80 \pm 14\%$ and $79 \pm 37\%$ ($p=0.047$ for hypoxic effect; $p<0.021$ at the 3 h and 4 h timepoints; Fig. 3B₂).

For episodes of doublet breaths, expiratory and inspiratory duration increased by similar amounts during the first breath of the doublet, but inspiratory duration increased dramatically during the second breath of the doublet. For example, expiratory duration increased by $43 \pm 19\%$ and $55 \pm 19\%$ for the first breath of the doublet during hypoxia ($p=0.053$ for hypoxic effect; $p<0.036$ at the 3 h and 4 h timepoints; Fig. 3C₁). There was, however, no change in expiratory duration for the second breath of the doublet episode ($p=0.095$; Fig. 3C₂). Inspiratory duration increased by $59 \pm 17\%$ and $58 \pm 29\%$ for the first breath of the doublet during of hypoxia ($p=0.037$ for hypoxic effect; $p<0.012$ at the 3 h and 4 h timepoints; Fig. 3C₃) and by $132 \pm 31\%$ and $111 \pm 42\%$ for the second breath of the doublet ($p=0.001$ for hypoxic effect; $p<0.008$ at the 3 h and 4 h timepoints; Fig. 3C₄).

For episodes of triplet breaths, there was an increase in expiratory duration for the first breath of the triplet but not for the second or third breaths. In contrast, inspiratory duration increased for all three breaths of the triplet, especially during the third breath. For example, expiratory duration increased by $58 \pm 8\%$ and $81 \pm 17\%$ for the first breath of the triplet

($p=0.004$ for hypoxic effect; $p<0.008$ at the 3 h and 4 h timepoints; Fig. 4A₁), but there were no changes in expiratory duration for the second ($p=0.74$) or third breath ($p=0.48$). Inspiratory duration increased by $51 \pm 8\%$ and $53 \pm 22\%$, $75 \pm 5\%$ and $75 \pm 23\%$, and $100 \pm 13\%$ and $104 \pm 33\%$ for the first, second, and third breaths, respectively, during hypoxia ($p<0.013$ for hypoxic effect; $p<0.013$ at the 3 h and 4 h timepoints; Figs. 4B₁₋₃).

3.3 Acute changes in breathing pattern during the first hour of hypoxia

To examine the early time-course of hypoxia-induced changes in ventilation and breathing pattern, data from the hypoxic exposures were plotted into 5-min bins. Ventilation increased after 10 min of hypoxia and remained increased compared to controls (breathing air only) for the next 50 min ($p=0.001$ for hypoxic effect; Fig. 5A). Breath frequency increased compared to controls after 10 min ($p<0.001$ for hypoxic effect) and was different from air-breathing controls in about half of the timepoints during 0-60 min after hypoxia onset (Fig. 5B). Tidal volume did not increase above controls until 25 min after the start of the hypoxic exposure ($p=0.048$ for hypoxic effect; Fig. 5C). There was no change in breaths/episode during the first 60 min of the hypoxic exposure ($p=0.62$; Fig. 5D). Singlet breaths did not increase until after 20 min of hypoxia, and then remained increased above controls for the next 45 min ($p=0.011$ for hypoxic effect; Fig. 5E).

3.4 Tropisetron blocks hypoxia-induced changes in breathing pattern

To test whether 5-HT₃ receptor agonist administration attenuated the hypoxia-induced changes in breathing pattern, several control experiments were performed. First, experiments were performed to rule out the possibility that the act of injecting turtles altered their hypoxic ventilatory response. Thus, turtles ($n=11$) were allowed to breathe air for 2 h (baseline), injected with saline, and returned to the tank to breathe air for another 4 h. In general, saline injection did not alter breathing over time, or altered the hypoxic ventilatory response that was shown in Fig. 2 with non-injected turtles. Thus, compared to saline-injected time controls, saline-injected turtles exposed to hypoxia increased ventilation (Fig. 6A), breath frequency (Fig. 6B), and tidal volume (Fig. 6C). For these experiments, there was a decrease in breaths/episode with a significant hypoxic effect ($p=0.01$) and breaths/episode was lower than saline-injected controls after 1-4 h of hypoxia ($P<0.05$; Fig. 6D). Singlet breaths were increased above baseline ($p=0.029$) and different from saline-injected turtles after 1-2 h of hypoxia ($p=0.01$; Fig. 6E).

To test the effects of tropisetron administration on ventilation at two different dosages, ventilatory data from turtles injected with saline (above) were compared with turtles ($n=8$) injected with tropisetron at 1.0 mg/kg and 5.0 mg/kg. Two turtles were injected with 10 mg/kg, but ventilation decreased by $>50\%$ during the 2-3 h period following injection while breathing air. No further experiments were performed at this dosage. For the 1.0 and 5.0 mg/kg tropisetron-injected turtles, there were no time-dependent changes in ventilation ($p=0.19$), breath frequency ($p=0.14$), breaths/episode ($p=0.21$), and singlet number ($p=0.43$; data not shown). There were no hypoxic effects on tidal volume ($p=0.13$), but tidal volume increased from 8.5 ± 1.4 to 12.7 - 15.5 ml/breath/kg during the 4 h of hypoxia ($p<0.001$ compared to baseline). However, these changes were not different from data observed in saline-injected turtles or tropisetron-injected (1.0 mg/kg) turtles. Thus, tropisetron did not

greatly alter ventilation or breathing pattern except for increasing tidal volume at the higher tropisetron dosage.

To test the effects of tropisetron on the hypoxic ventilatory response, turtles ($n=7$) were allowed to breathe nitrogen after being injected with saline or tropisetron at 1.0 mg/kg or 5.0 mg/kg (Fig. 8). Tropisetron (5.0 mg/kg) decreased hypoxia-induced increases in ventilation ($p=0.025$ compared to saline; Fig. 8A) and breath frequency ($p=0.003$ compared to saline; Fig. 8B). However, tropisetron at both dosages did not alter hypoxia-induced changes in tidal volume ($p=0.69$) or episodicity ($p=0.11$; Figs. 8C, 8D). Finally, singlet number increased after 1-2 h of hypoxia for saline-injected turtles ($p=0.034$) and after 1 h of hypoxia in tropisetron-injected (1.0 mg/kg) turtles ($p=0.005$; Fig. 8E). For turtles injected with 5.0 mg/kg tropisetron, singlet number was not increased above baseline ($p=0.085$) and singlet number after 1 h hypoxia was decreased compared to data from saline-injected turtles ($p<0.001$; Fig. 8E).

4. Discussion

Semi-aquatic, red-eared slider turtles are an intriguing species because they are one of the few reptiles that naturally experience hypoxia, and yet these animals are extremely hypoxia-resistant. Some features of their hypoxic ventilatory response are known, but specific details and underlying mechanisms are not well understood. This is the first paper to examine in detail the effects of severe hypoxia on the chelonian breathing pattern with respect to breath duration, episodicity, and singlet breaths. Hypoxia increased ventilation, breath frequency, and tidal volume as expected. However, hypoxia also increased both expiratory and inspiratory duration in singlet breaths, but had variable effects on doublet and triplet breaths. For both doublet and triplet breaths, expiration duration increased only for the first breath while inspiration duration increased for all of the breaths in the episode. Hypoxia increased the occurrence of singlet breaths without any obvious switching to a continuous breathing pattern. The hypoxia-induced increase in singlet breath was nearly abolished with tropisetron administration, suggesting that 5-HT₃ receptor activation is required for this change in breathing pattern. We hypothesize that hypoxia-resistant red-eared slider turtles experience increased “respiratory drive” during hypoxia that switches the breathing pattern temporarily to singlet breathing via a 5-HT₃-dependent mechanism.

4.1 Hypoxia-dependent changes in ventilation, breath frequency, and tidal volume

Past studies on chelonian hypoxic ventilatory responses are variable with respect to species and experimental protocols (Porteus et al., 2011), and are therefore difficult to compare with one another. In addition, the time course of the hypoxic ventilatory response was generally characterized on the scale of hours, rather than minutes, thereby eliminating details with respect to the acute changes in breathing during the onset of hypoxia. Likewise, hypoxia-induced changes in breathing pattern in some studies were presented anecdotally with a single figure or occasional measurement of respiratory variables at one point during the response. Finally, very few studies tested potential peripheral or central mechanisms involved in the response to hypoxia. For example, the hypoxic ventilatory response in chelonians often involves an increase in ventilation due to an increase in breath frequency

and tidal volume (Burggren et al., 1977; Glass et al. 1983; Herman and Smatresk 1999; Jackson 1973; Hitzig and Nattie 1982; Wasser and Jackson 1988). However, other studies in semi-aquatic and aquatic turtles showed relatively little or no change in tidal volume with hypoxia. These studies involved anesthesia (pentobarbital, Vitalis and Milsom, 1987), instrumentation with an endotracheal tube and a stereotaxic frame to measure breathing (Davies and Sexton, 1987), or chronic arterial catheter placement (Burggren et al., 1977). It's possible that turtles in these studies were either not freely behaving during the hypoxic exposures and may have had confounding factors that blunted the tidal volume increase. On the other hand, there was no change in tidal volume with hypoxia (5-15% oxygen) in snapping turtles (Frische et al., 2000), but the respiratory variables in this study were measured after hypoxic exposures lasting 24 h. The acute response to hypoxia was not reported and the turtles may have adapted to the long-term hypoxic exposures. Thus, there are confounding factors and conflicting reports due to the differences in the experiment approach.

In this study with semi-aquatic turtles freely swimming and breathing nitrogen gas to induce acute hypoxia, there was a consistent and robust increase in ventilation, breath frequency, and tidal volume. Overall ventilation increased rapidly within 10 min of the onset of hypoxia while tidal volume increased significantly after 25 min. Without injections that might disturb the turtles, breath frequency appeared to increase within 20 min (Fig. 5B), but the response was more statistically robust when data were pooled into 60-min bins (Fig. 2B). Injections of saline or tropisetron appeared to blunt the frequency increase within 2 h of the onset of hypoxia (Figs. 6B, 8B), consistent with the hypothesis that the aspects of the hypoxic ventilatory response (tidal volume and breath frequency) may be sensitive to instrumentation or manipulation of the turtles during the experiment. Nevertheless, the acute hypoxic ventilatory response in freely behaving red-eared slider turtles involves an increase in both breath frequency and tidal volume.

4.2 Hypoxia-dependent changes in episodic breathing pattern

Episodic breathing is the normal breathing pattern for many ectothermic vertebrates and it can be observed in mammals (Milsom, 1991; Fong et al., 2009). An important unifying hypothesis for all vertebrates is that episodic breathing is related to overall "respiratory drive" (*i.e.*, sum of central or peripheral neural inputs that initiate or augment breathing) such that increasing respiratory drive generally causes episodic breathing pattern to become continuous (Fong et al., 2009). For chelonians, this hypothesis is supported by experimental data (reviewed in Fong et al., 2009), but there may be other factors regulating episodic breathing. For example, it is hypothesized that terrestrial chelonians breathe mostly with a singlet pattern while aquatic chelonians breathe episodically more often (Burggren et al., 1977; Gans and Hughes, 1967; Gaunt and Gans, 1967; McCutcheon, 1943). Unfortunately, there are no studies on terrestrial chelonians in which breathing pattern was analyzed in detail while respiratory drive was systematically altered by hypoxia or hypercapnia. Most data on episodic breathing in chelonians were derived from studies on semi-aquatic red-eared slider turtles.

For freely behaving, red-eared slider turtles, episodic breathing is more readily observed but singlet breathing is not uncommon, especially when the turtles are quiescent in water-filled tanks (Johnson and Creighton, 2005; Johnson et al., 2008; Sladky et al., 2007). The hypoxia-induced decrease in episodic breathing in chelonians was initially reported anecdotally in aquatic turtles and terrestrial tortoises (Benchetrit et al., 1977; Glass et al., 1978). In a later study, the number of breaths per ventilatory period did not change in instrumented and restrained painted turtles breathing 100% nitrogen, but the first hour of exposure to nitrogen was not examined in detail (Wasser and Jackson, 1988). Later studies quantified this response by showing that severe hypoxia (0% oxygen) decreased episodic breathing from 5.3 to 2.5 breaths/episode in red-eared slider turtles (Herman and Smatresk, 1999), while 5% oxygen decreased episodic breathing from 4.5 to 1.1 breaths/episode in snapping turtles (Frische et al. 2000). Thus, hypoxia decreases episodic breathing, but the number of singlet breaths was not quantified.

In this study of freely swimming red-eared slider turtles, breaths/episode as well as singlet frequency was quantified during a 4-h exposure to severe hypoxia. Breathes/episode appeared to be more variable because this variable decreased the first hour of hypoxia, returned to baseline levels, during the second hour of hypoxia, and decreased during the third and fourth hours of hypoxia (Fig. 2D) while, in contrast, singlet frequency increased during the first hour and remained increased for the whole duration of the 4-h hypoxic exposure (Figs. 2E, 6E, 8E). Singlet number increased significantly during the first 15-60 min of the 4-h exposure (Fig. 5E). Thus, severe hypoxia consistently increased the frequency of singlet breaths while causing a general decrease in breaths/episode. Quantifying the number of singlet breaths appeared to be a more robust and sensitive measure of hypoxia-induced changes in breathing pattern.

The mechanisms underlying the decrease in breaths/episode and increased singlet breath frequency in chelonians are not known. Other experimental perturbations that clearly increase singlet breathing include vagotomy (Vitalis and Milsom, 1986) and spinal cord transection (Johnson and Creighton, 2005). Both of these conditions may have increased singlet breathing due to lack of pulmonary stretch receptor feedback (e.g., vagotomy in Vitalis and Milsom, 1986; decreased tidal volume due to spinalization in Johnson and Creighton, 2005). We hypothesized that serotonin 5HT₃ receptors may be involved because *in vitro* studies on isolated turtle brainstems showed that bath-applied serotonin 5-HT₃ agonist drugs caused episodic respiratory motor bursts to become singlet bursts, while 5-HT₃ antagonist drugs caused singlet bursts to become episodic (Bartman et al., 2010). Tropisetron administration at 5.0 mg/kg abolished the hypoxia-induced increase in singlet breathing, suggesting that 5HT₃ receptor activation is required to generate singlet breathing *in vivo* during hypoxia, while 5HT₃ receptor activation is sufficient to induce singlet breathing in isolated *in vitro* brainstems. Since tropisetron was administered systemically, it is not known whether central or peripheral 5HT₃ receptors are involved in the chelonian hypoxic ventilatory response. In fact, one caveat that needs to be mentioned is that tropisetron is not highly selective 5HT₃ receptor agonist, and can activate $\alpha 7$ nicotinic cholinergic receptors (Macor et al., 2001) which are located within and regulate carotid body function in mammals (Joseph and Pequignot, 2009). It is not known whether turtle carotid

bodies are regulated by $\alpha 7$ nicotinic cholinergic receptor activation, but this is one example of a potential peripheral mechanism to explain tropisetron's effects on the hypoxic turtle breathing pattern.

On the other hand, the fact that bath-applied 5HT₃ receptor agonists decrease episodic breathing in isolated turtle brainstems (Bartman et al., 2010) and postmetamorphic frog brainstems (Kinkead *et al.*, 2002) suggests that central mechanisms may be involved. Consistent with this hypothesis, episodic respiratory motor output can be converted to singlet motor bursts in isolated turtle brainstems during hypoxic solution exposure, an effect that is blocked by a 5HT₃ receptor antagonist drug (M.E. Bartman, S.M. Johnson, unpublished observations). The location of these putative central 5HT₃ receptors within the turtle respiratory control system is not known. Also, the mechanisms regulating 5HT release onto these 5HT₃ receptors during hypoxia are also not known.

4.3 Hypoxia-dependent changes in expiratory and inspiratory duration

In mammals, hypoxia-induced changes in breathing pattern are well-characterized in the context of the animal switching from normal breathing to a gasp-like pattern to maintain ventilation during severe hypoxia (Hill et al., 2011; Lieske et al., 2000; St John, 1996). Gaspings is marked by increased inspiratory drive, inhibition of expiration, and a switch to a rapidly incrementing-slowly decrementing phrenic nerve burst (St John, 1996). In semi-aquatic turtles, the hypoxia-induced increase in expiratory and inspiratory duration was attributed to the increase in tidal volume, but changes in breath timing were not quantified (Glass et al. 1983). In anesthetized turtles, breath duration is not changed during exposure to 4% oxygen (Vitalis and Milsom, 1986). For chelonian breathing, very little is known about how hypoxia transforms breathing pattern.

In this study, expiratory and inspiratory duration increased during hypoxia when all breaths were considered, regardless of whether they occurred in singlets, doublets, or triplets (Fig. 3). For singlet breaths, expiratory and inspiratory duration also increased with hypoxia (Fig. 4). Not all breaths increased in duration, however, when individual breaths were measured within a breathing episode. The main findings were that expiratory duration only increased in the first breath of a doublet or triplet. In contrast, inspiratory duration increased for all breaths within a doublet or triplet when graphed as percent change (Figs. 5, 6). Thus, expiratory and inspiratory duration do not appear to increase in duration simply because of increased tidal volume. Instead, the increase in inspiratory duration at the end of a breathing episode may indicate augmented inspiratory drive to respiratory spinal motoneurons. Although hypoxia-resistant red-eared slider turtles may not be capable of gasping, the disproportionate increase in inspiratory drive may have some mechanistic similarities to the augmented inspiratory drive observed during mammalian gasping. Recordings of respiratory-related neurons in the turtle brainstem before and during hypoxia will be required to test this hypothesis further.

Acknowledgments

This work was supported by grants from the National Heart Lung Blood Institute grant (T32 HL07654 to M.B.), Wisconsin Alumni Research Fund (S.J.), and University of Wisconsin Department of Animal Science (Cargill/Benevenga Award to A.K.). The authors gratefully acknowledge the constructive comments on earlier drafts by

Michael S. Hedrick. Also, we acknowledge Claudia Hirsch and the animal care staff at the University of Wisconsin Charny Instructional Facility for exceptional animal care.

References

- Bartman ME, Wilkerson JE, Johnson SM. 5-HT₃ receptor-dependent modulation of respiratory burst frequency, regularity, and episodicity in isolated adult turtle brainstems. *Respiratory Physiology and Neurobiology*. 2010; 172:42–52. [PubMed: 20399913]
- Benchetrit G, Armand J, Dejours P. Ventilatory chemoreflex drive in the tortoise, *Testudo horsfieldi*. *Respiration Physiology*. 1977; 31:183–191. [PubMed: 928996]
- Burggren WW, Glass ML, Johansen K. Pulmonary ventilation: perfusion relationships in terrestrial and aquatic chelonian reptiles. *Canadian Journal of Zoology*. 1977; 55:2024–2034. [PubMed: 606351]
- Davies DG, Sexton JA. Brain ECF pH and central chemical control of ventilation during anoxia in turtles. *American Journal of Physiology*. 1987; 252:R848–R852. [PubMed: 3578552]
- Fong AY, Zimmer MB, Milsom WK. The conditional nature of the “Central Rhythm Generator” and the production of episodic breathing. *Respiratory Physiology and Neurobiology*. 2009; 168:179–187. [PubMed: 19505592]
- Frische S, Fago A, Altimiras J. Respiratory responses to short term hypoxia in the snapping turtle, *Chelydra serpentina*. *Comparative Biochemistry and Physiology A Molecular Integrative Physiology*. 2000; 126:223–231.
- Funk GD, Webb CL, Milsom WK. Non-invasive measurement of respiratory tidal volume in aquatic, air-breathing animals. *Journal of Experimental Biology*. 1986; 126:519–523. [PubMed: 3806001]
- Gans C, Hughes GM. The mechanism of lung ventilation in the tortoise *Testudo graeca* Linné. *Journal of Experimental Biology*. 1967; 47:1–20. [PubMed: 6058978]
- Gaunt AS, Gans C. Mechanics of respiration in the snapping turtle, *Chelydra serpentina* (Linné). *Journal of Morphology*. 1967; 128:195–226.
- Glass ML, Boutilier RG, Heisler N. Ventilatory control of arterial PO₂ in the turtle *Chrysemys picta bellii*: effects of temperature and hypoxia. *Journal of Comparative Physiology*. 1983; 151:145–153.
- Glass M, Burggren WW, Johansen K. Ventilation in an aquatic and a terrestrial chelonian reptile. *Journal of Experimental Biology*. 1978; 72:165–179. [PubMed: 624895]
- Herman JK, Smatresk NJ. Cardiorespiratory response to progressive hypoxia and hypercapnia in the turtle *Trachemys scripta*. *Journal of Experimental Biology*. 1999; 202:3205–3213. [PubMed: 10539969]
- Hicks JW, Wang T. Cardiovascular regulation during anoxia in the turtle: an *in vivo* study. *Physiological Zoology*. 1998; 71:1–14. [PubMed: 9472807]
- Hicks JW, Wang T. Hypoxic hypometabolism in the anesthetized turtle, *Trachemys scripta*. *American Journal of Physiology*. 1999; 277:R18–R23. [PubMed: 10409253]
- Hill AA, Garcia AJ 3rd, Zanella S, Upadhyaya R, Ramirez JM. Graded reductions in oxygenation evoke graded reconfiguration of the isolated respiratory network. *Journal of Neurophysiology*. 2011; 105:625–639. [PubMed: 21084689]
- Hitzig BM, Nattie EE. Acid-base stress and central chemical control of ventilation in turtles. *Journal of Applied Physiology: Respiratory Environmental and Exercise Physiology*. 1982; 53:1365–1370.
- Jackson DC. Ventilatory response to hypoxia in turtles at various temperatures. *Respiration Physiology*. 1973; 18:178–187. [PubMed: 4730749]
- Johnson SM. Hypoxia induces singlet breathing pattern in red-eared slider turtles. *Society for Neuroscience*. 2010 Abstracts 388.5.
- Johnson SM, Creighton RJ. Spinal cord injury-induced changes in breathing are not due to supraspinal plasticity in turtles (*Pseudemys scripta*). *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*. 2005; 289:R1550–R1561.
- Johnson SM, Kinney ME, Wiegel LM. Inhibitory and excitatory effects of micro-, delta-, and kappa-opioid receptor activation on breathing in awake turtles, *Trachemys scripta*. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*. 2008; 295:R1599–R1612.

- Johnson SM, Mitchell GS. N-methyl-D-aspartate-mediated bulbospinal respiratory drive is pH/P(CO₂)-insensitive in turtle brainstem-spinal cord. *Respiration Physiology*. 1998; 113:201–212. [PubMed: 9840329]
- Joseph V, Pequignot JM. Breathing at high altitude. *Cellular and Molecular Life Sciences*. 2009; 66:3565–3573. 2009. [PubMed: 19756384]
- Lieske SP, Thoby-Brisson M, Telgkamp P, Ramirez JM. Reconfiguration of the neural network controlling multiple breathing patterns: eupnea, sighs and gasps. *Nature Neuroscience*. 2000; 3:600–607.
- Macor JE, Gurley D, Lanthorn T, Loch J, Mack RA, Mullen G, Tran O, Wright N, Gordon JC. The 5-HT₃ antagonist tropisetron (ICS 205-930) is a potent and selective alpha7 nicotinic receptor partial agonist. *Bioorganic and Medicinal Chemistry Letters*. 2001; 11:319–321. [PubMed: 11212100]
- McCutcheon FH. The respiratory mechanism in turtles. *Physiological Zoology*. 1943; 3:255–269.
- Milsom WK. Intermittent breathing in vertebrates. *Annual Review of Physiology*. 1991; 53:87–105.
- Mitchell GS, Powell FL, Hopkins SR, Milsom WK. Time domains of the hypoxic ventilatory response in awake ducks: episodic and continuous hypoxia. *Respiration Physiology*. 2001; 124:117–128. [PubMed: 11164203]
- Porteus C, Hedrick MS, Hicks JW, Wang T, Milsom WK. Time domains of the hypoxic ventilatory response in ectothermic vertebrates. *Journal of Comparative Physiology B*. 2011; 181:311–333.
- Powell FL, Milsom WK, Mitchell GS. Time domains of the hypoxic ventilatory response. *Respiration Physiology*. 1998; 112:123–134. [PubMed: 9716296]
- Reyes C, Milsom WK. Daily and seasonal rhythms in the respiratory sensitivity of red-eared sliders (*Trachemys scripta elegans*). *Journal of Experimental Biology*. 2009; 212:3339–3348. [PubMed: 19801438]
- Reyes C, Milsom WK. Circadian and circannual rhythms in the metabolism and ventilation of red-eared sliders (*Trachemys scripta elegans*). *Physiological and Biochemical Zoology*. 2010; 83:283–298.
- Sladky KK, Miletic V, Paul-Murphy J, Kinney ME, Dallwig RK, Johnson SM. Analgesic efficacy and respiratory effects of butorphanol and morphine in turtles. *Journal of the American Veterinary Medical Association*. 2007; 230:1356–1362. [PubMed: 17472564]
- St John WM. Medullary regions for neurogenesis of gasping: noeud vital or noeuds vitals? *Journal of Applied Physiology*. 1996; 81:1865–1877. [PubMed: 8941503]
- Vitalis TZ, Milsom WK. Mechanical analysis of spontaneous breathing in the semi-aquatic turtle, *Pseudemys scripta*. *Journal of Experimental Biology*. 1986; 125:157–171.
- Wasser JS, Jackson DC. Acid-base balance and the control of respiration during anoxic and anoxic-hypercapnic gas breathing in turtles. *Respiration Physiology*. 1988; 71:213–226. [PubMed: 3124237]
- Wasser JS, Warburton SJ, Jackson DC. Extracellular and intracellular acid-base effects of submergence anoxia and nitrogen breathing in turtles. *Respiration Physiology*. 1991; 83:239–252. [PubMed: 2068419]
- West NH, Smits AW, Burggren WW. Factors terminating nonventilatory periods in the turtle, *Chelydra serpentina*. *Respiratory Physiology*. 1989; 77:337–349.

- Hypoxia-induced changes in turtle breathing were examined.
- Hypoxia increased singlet breathing and decreased episodic breathing.
- Hypoxia-induced singlet breathing is abolished by tropisetron administration.

Fig. 1.

Hypoxia-induced changes in turtle breathing. Pneumotachographic traces are shown for a turtle breathing air (A), and 100% nitrogen (to induce hypoxia) at 7 min (B) and 190 min (C) after switching the gases. Time-compressed traces are shown at left while time-expanded traces are shown at right (horizontal black bars in left traces indicate the traces expanded at right). Upward deflections indicate expiratory movements while downward deflections indicate inspiratory movements.

Fig. 2.

Hypoxia-induced changes in respiratory variables. Turtles were allowed to breathe air in the breathing chamber for 2 h before switching to 100% nitrogen for 4 h to induce hypoxia. Data from time control experiments (white circles, air-breathing only) and hypoxia experiments (black circles, nitrogen-breathing) are shown. Hypoxia increased ventilation (A), breath frequency (B), tidal volume (C), and number of singlet breaths/h (E) compared to time controls. (D) The number of breaths/episode remained constant in time control experiments, but decreased at 1, 3, and 4 h of hypoxia (3, 5, 6 h on graph). Symbols: asterisk = $p < 0.05$ compared to baseline (data at second h on graph); pound sign = $p < 0.05$ compared to time control experiment at that hour; dagger = $p < 0.05$ for hypoxic effect.

Fig. 3.

Hypoxia-induced changes in expiratory and inspiratory duration. Percent changes in expiratory (A₁) and inspiratory (A₂) breath durations for all breaths are shown for time control experiments (white vertical bars) and hypoxia experiments (black vertical bars) for baseline (0 h; set to 100%), and 1-2 h of hypoxia. Percent changes in breath durations are shown for expiratory (B₁) and inspiratory (B₂) singlet breaths. Percent changes in breath durations are shown for the first and second breaths of doublet breathing episodes. Expiratory durations for the first (C₁) and second breaths (C₂) are shown along with inspiratory durations for the first (C₃) and second breaths (C₄). Data from the 2-h baseline period were averaged together (designated as “1-2” h) and compared to 1-h data from the first 2 h of hypoxia (designated as “3” and “4” h). Symbols: asterisk = $p < 0.05$ compared to baseline (0 h); pound sign = $p < 0.05$ compared to time control experiment at that hour; dagger = $p < 0.05$ for hypoxic effect.

Triplets breaths

Fig. 4.

Hypoxia-induced changes in expiratory and inspiratory duration in triplet breaths. Percent changes in breath durations are shown for the first, second, and third breaths of triplet breathing episodes. Expiratory durations for the first (A₁), second (A₂), and third breaths (A₃) are shown, along with inspiratory durations for the first (B₁), second (B₂), and third breaths (B₃). Symbols are the same as in Fig. 3.

Fig. 5.

Acute changes in respiratory variables due to hypoxia. To examine the early time course of the turtle hypoxic ventilatory response, the same data from Fig. 2 were graphed into 5-min bins, starting with 20 min prior to hypoxia and including the first 60 min of hypoxia. (A) Ventilation increased after 10 min of hypoxia, and remained increased over the next 50 min. (B) Breath frequency increased (hypoxic effect) with half of the data points increased compared to time controls. (C) Tidal volume increased after 25 min of hypoxia and remained increased for the next 35 min. (D) There were no changes in breaths/episode with hypoxia. (E) The number of singlet breaths increased after 20 min of hypoxia, peaked at 25 min, and remained increased for the next 35 min. Symbols: asterisk = $p < 0.05$ compared to baseline (0-min); pound sign = $p < 0.05$ compared to time control experiment at that time point; dagger = $p < 0.05$ for hypoxic effect.

Fig. 6.

Saline injections did not alter hypoxia-induced changes in breathing. Turtles were injected with saline during the transition from air to nitrogen breathing to control for potential effects of disturbing the turtles during the injections. Saline injections did not alter the hypoxia-induced increase in ventilation (A), breath frequency (B), tidal volume (C), or singlet breaths (E). There was a clear decrease in breaths/episode (D). Symbols are the same as described in Fig. 2.

Fig. 7.

Hypoxia-induced increase in singlet breaths is decreased with 5HT₃ receptor antagonist drug. Pneumotachographic traces are shown for a turtle that breathed air (A) prior to receiving an injection of saline (left traces) or tropisetron (5 mg/kg; right traces), and being switched to breathing 100% nitrogen. (B) Breathing traces are shown after 30 min of hypoxia. (C) Breathing traces are shown at 60 min (left trace) and 100 min (right trace) of hypoxia. Singlet breaths are indicated by asterisks.

Fig. 8.

Hypoxia-induced increase in singlet breaths required 5HT₃ receptor activation. (A, B) Tropisetron at 1.0 mg/kg did not alter hypoxia-induced changes in ventilation and breath frequency, while tropisetron at 5.0 mg/kg decreased the hypoxic response for both variables. (C, D) Tidal volume and breaths/episode were not altered by tropisetron at either dosage. (E) Tropisetron at 5.0 mg/kg decreased the hypoxia-induced increase in singlet breaths within the first 2 h or hypoxia (3 h, 4 h on graph). Symbols are the same as described in Fig. 2. Double-dagger in panel (C) indicates p < 0.05 for time-dependent effects.