Effects of environmental hypoxia and hypercarbia on ventilation and gas exchange in Testudines

Pedro Trevizan-Baú 1, 2, Augusto S Abe 3, Wilfried Klein Corresp. 1

1 Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
2 Programa de Pós-graduação em Biologia Comparada, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
3 Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP, Brazil

Corresponding Author: Wilfried Klein
Email address: wklein@usp.br

Background. Ventilatory parameters have been investigated in several species of Testudines, but few species have had their ventilatory pattern fully characterized by presenting all variables necessary to understand changes in breathing pattern seen under varying environmental conditions.

Methods. We measured ventilation and gas exchange at 25°C in the semi-aquatic turtle Trachemys scripta and the terrestrial tortoise Chelonoidis carbonarius under normoxia, hypoxia, and hypercarbia and furthermore compiled respiratory data of testudine species from the literature to analyze the relative changes in each variable.

Results. During normoxia both species studied showed an episodic breathing pattern with 2-3 breaths per episode, but the non-ventilatory periods ($T_{NVP}$) were 3-4 times longer in T. scripta than in C. carbonarius. Hypoxia and hypercarbia significantly increased ventilation in both species and decreased $T_{NVP}$ and oxygen consumption in T. scripta but not in C. carbonarius.

Discussion. Contrary to expectations, the breathing pattern in C. carbonarius did show considerable non-ventilatory periods with more than one breath per breathing episode, and the breathing pattern in T. scripta was found to diverge significantly from predictions based on mechanical analyses of the respiratory system. A quantitative analysis of the literature showed that relative changes in the ventilatory patterns of chelonians in response to hypoxia and hyperbarbia were qualitatively similar among species, although there were variations in the magnitude of change.
Effects of environmental hypoxia and hypercarbia on ventilation and gas exchange in Testudines

Pedro Trevizan-Baucons*; Augusto S. Abe; Wilfried Klein*

*Programa de Pós-graduação em Biologia Comparada, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

bInstituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP, Brazil

cDepartamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

*Corresponding author: wklein@usp.br

Corresponding author address: Wilfried Klein, Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo. Av. Bandeirantes 3900, Bairro Monte Alegre, CEP: 14040-901 Ribeirão Preto, SP, Brazil. Email: wklein@usp.br; Tel.: +551633150452; FAX: +551633154886
Abstract

Background. Ventilatory parameters have been investigated in several species of Testudines, but few species have had their ventilatory pattern fully characterized by presenting all variables necessary to understand changes in breathing pattern seen under varying environmental conditions.

Methods. We measured ventilation and gas exchange at 25°C in the semi-aquatic turtle *Trachemys scripta* and the terrestrial tortoise *Chelonoidis carbonarius* under normoxia, hypoxia, and hypercarbia and furthermore compiled respiratory data of testudine species from the literature to analyze the relative changes in each variable.

Results. During normoxia both species studied showed an episodic breathing pattern with 2-3 breaths per episode, but the non-ventilatory periods (T_NVP) were 3-4 times longer in *T. scripta* than in *C. carbonarius*. Hypoxia and hypercarbia significantly increased ventilation in both species and decreased T_NVP and oxygen consumption in *T. scripta* but not in *C. carbonarius*.

Discussion. Contrary to expectations, the breathing pattern in *C. carbonarius* did show considerable non-ventilatory periods with more than one breath per breathing episode, and the breathing pattern in *T. scripta* was found to diverge significantly from predictions based on mechanical analyses of the respiratory system. A quantitative analysis of the literature showed that relative changes in the ventilatory patterns of chelonians in response to hypoxia and hyperbarbia were qualitatively similar among species, although there were variations in the magnitude of change.
**Introduction**

The order Testudines differs from the other groups of reptiles by the presence of a rigid shell, impeding lung ventilation through movement of the ribs (Lyson, 2014). To overcome this morphological constraint, Testudines contract abdominal muscles associated with their legs, thereby compressing or expanding the body cavity and resulting in lung ventilation (Gans and Hughes, 1967; Gaunt and Gans, 1969).

Testudines can be divided into two suborders. The 100 species of Pleurodira are characterized by a retraction of the neck in the horizontal plane, whereas the 250 species of Cryptodira retract their neck in the vertical plane (Werneburg et al., 2015; Uetz, Freed & Hošek, 2018). All Pleurodira occur in freshwater habitats, just as the majority of cryptodiran species. However, some Cryptodira live in the marine environment and all representatives of the family Testudinidae and some species of Emydidae are terrestrial.

Gas exchange and ventilation have been studied in several species of turtles, tortoises, and terrapins, but few species were fully characterized regarding their breathing variables. In particular, two species of the family Emydidae (Cryptodira), the semi-aquatic *Trachemys scripta* and *Chrysemys picta* have been used in numerous respiratory studies (see Table S1).

Although oxygen consumption has been determined in many chelonian species (see Ultsch, 2013, for review), data on ventilatory parameters, such as overall breathing frequency, tidal volume, and minute ventilation, are available only for a small number of species, representing a limited range of the taxonomic diversity, especially when considering responses to hypoxic and hypercarbic exposures (Table S1). While the number of studies listed in table S1 seems extensive, few have actually characterized the ventilatory pattern by providing data such as inspiratory time, expiratory time, total duration of a ventilatory cycle, duration of the non-ventilatory period, breathing frequency during breathing
episodes, frequency of breathing episodes, as well as breathing frequency, tidal volume, and minute ventilation (Benchetrit & Dejours, 1980; Cordeiro, Abe & Klein, 2016). Furthermore, most of these data have been obtained for *Chrysemys picta* (e.g. Milsom & Jones, 1980; Milsom & Chan, 1986; Funk & Milsom, 1987; Wasser & Jackson, 1988). The totality of these variables is needed to fully characterize the ventilatory behavior of a species under varying environmental conditions, especially in ectothermic vertebrates where ventilation can show highly episodic burst breathing or regular singlet breathing (for review see Shelton, Jones & Milsom, 1986). Fong, Zimmer & Milsom (2009) suggested that an increasing respiratory drive changes episodic into continuous breathing and Johnson, Krisp & Bartman (2015) used duration of inspiration and expiration as measures for inspiratory and expiratory drive, respectively. Furthermore, Milsom and Wang (2017) argued that the regulation of the ventilatory responses is complex and cannot be totally understood with few variables measured, especially since Testudines possess an undivided heart that allows for intracardiac shunting of blood between the pulmonary and systemic circulations.

Among the Testudines, the terrestrial species belonging to the family Testudinidae are also very poorly characterized regarding their ventilatory response to hypoxia or hypercarbia, and only data on breathing frequency, tidal volume, minute ventilation, and oxygen consumption are available (Altland & Parker, 1955; Benchetrit, Armand & Dejours, 1977; Benchetrit & Dejours, 1980; Burggren, Glass & Johansen, 1977; Glass, Burggren & Johansen, 1978; Ultsch & Anderson, 1988). Burggren, Glass & Johansen (1977) and Glass, Burggren & Johansen (1978) showed that under normoxic conditions, the terrestrial *Testudo pardalis* employs regular single breaths separated by short breath-holds. A regular singlet breathing behavior has also been shown by Burggren (1975) for the tortoise *Testudo*
graeca and by Benchetrit, Armand & Dejours (1977) for Testudo horsfieldi. The semi-aquatic Pelomedusa subrufa, on the other hand, uses breathing episodes containing several ventilations interspaced by longer breath-holds (Burggren, Glass & Johansen, 1977; Glass, Burggren & Johansen, 1978). Such a pattern has been interpreted as adaptation to the aquatic life-style observed in P. subrufa and other aquatic or semi-aquatic species, where the episodic breathing reduces the amount of time spent at the water surface, reducing the risk of predation, as well as reducing the cost of ascending to the surface (Randall et al. 1981).

Depending on the gas concentration, hypoxia, as well as hypercarbia, stimulates breathing in turtles, with moderate concentrations of hypercarbia generally increasing ventilation more than very low oxygen concentrations (Shelton, Jones & Milsom, 1986). Interestingly, Altland & Parker (1955) found a more episodic breathing pattern in Terrapene carolina under normoxia that changed to a more regular singlet breathing pattern under hypoxic conditions. The normal response to either hypoxia or hypercarbia results in reduced non-ventilatory periods, but may or may not increase breathing frequency or tidal volume (Shelton, Jones & Milsom, 1986). In a recent study, Cordeiro, Abe & Klein (2016) demonstrated that two closely related pleurodirans exhibit different ventilatory responses to hypoxia and hypercarbia. While both species reduce significantly the non-ventilatory period and increase breathing frequency during hypoxic and hypercarbic exposures, Podocnemis unifilis significantly increases the breathing frequency during breathing episodes during hypercarbia but significantly decreases the breathing frequency during breathing episodes during hypoxia, whereas Phrynops geoffroanus significantly decreases breathing frequency during breathing episodes under hypoxia, but does not change this variable during hypercarbia.
Given these variations in the breathing pattern during normoxia, hypoxia, and hypercarbia among testudines, and considering the very few ventilatory data available for terrestrial species, the aim of the present study was to analyze the ventilatory response to different gas mixtures in two cryptodirans, the red-eared slider *Trachemys scripta* (Emydidae) and the South American red-footed tortoise *Chelonoidis carbonarius* (Testudinidae). *Trachemys scripta*, the model species for cardiorespiratory studies, was investigated because no previous study reported all ventilatory variables obtained from the same animals and experimental protocols, both under hypoxic and hypercarbic conditions, whereas *C. carbonarius* was chosen because it is a widespread South American tortoise that has not had its respiratory physiology investigated previously. Furthermore, the present data were compiled together with available data from the literature to characterize the general response of testudines to hypoxia and hypercarbia and to verify if terrestrial species show a significantly different ventilatory pattern compared to semi-aquatic species.

Materials and methods

Animals

Adults of both sexes of *T. scripta* (body mass: $M_B = 1.08 \pm 0.10 \text{ kg}; N = 8$) and *C. carbonarius* ($M_B = 3.77 \pm 0.61 \text{ kg}; N = 6$) living under natural conditions were obtained from the Jacarezário, Univeridade Estadual Paulista “Júlio de Mesquita Filho”, Rio Claro, SP, Brazil, transported to the laboratory at the University of São Paulo in Ribeirão Preto, SP, and maintained for at least 3 months before experimentation to acclimate to laboratory conditions. Experiments were performed between November 2014 and February 2015 following approval by the Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO; license number 35221-1) and Comissão de Ética no Uso de Animais (CEUA.
Animals were maintained under a 12h light/dark photoperiod cycle, in a temperature-controlled room at 25 ± 2 °C and received a mixed diet supplemented with amino acids, vitamins and minerals (Aminomix Pet, Vetnil®, Louveira, Brazil) three times a week. *T. scripta* were housed in a box with a water reservoir for diving whereas *C. carbonarius* were housed in boxes whose bottom was covered with wooden chips.

**Setup**

Animals were submitted to open respirometry following Glass, Wood & Johansen (1978), Wang and Warburton (1995) and Silva et al. (2011) to measure ventilation and gas exchange. Individuals of *T. scripta* were placed in an aquarium with a single access to an inverted funnel, and each individual only needed to extend its neck and protrude its nostrils into the chamber for air breathing. *C. carbonarius* were placed in a plastic box and a mask was fitted to the head of each animal for respirometry and a collar was fixed to the neck to prevent head retraction. The dead space of the funnel or the mask was never larger than 40 ml. The exit of the funnel and the frontal tip of the mask were equipped with a pneumotach (Fleisch tube), which was connected to a spirometer (FE141 ADInstrumens). The gas inside the funnel or mask was sampled at 180 ml.min\(^{-1}\), dried, and pulled to a gas analyzer (ML206ADInstrumients). Data were recorded and analyzed using PowerLab 8/35 and LabChart 7.0 (ADInstruments).

Both the funnel and the mask were calibrated by injections of known volumes, using an Inspira ventilator (Harvard Apparatus), and concentrations of gas, supplied by a Pegas 4000MF gas mixer (Columbus Instruments). Air was used for the spirometer calibration with volumes ranging from 1 to 60 ml and different volumes and concentrations of O\(_2\) were
used to calibrate the gas exchange measurements. In all cases, calibrations resulted in linear regressions with $R^2 > 0.95$.

### Experimental protocols

Experimental temperature and photoperiod were the same as during maintenance, and all animals were fasted for three to seven days before experimentation to avoid the confounding effects of digestion on metabolism. The animals were weighed one day before the beginning of each experimental treatment. Before any measurements, animals were placed into the experimental setup or equipped with a mask at least 12 h before initiation of experiments. Experimentation started around 08:00, and ventilation and gas exchange were measured first under normoxic conditions, followed by progressively decreasing hypoxic (9, 7, 5, 3% O$_2$) or progressively increasing hypercarbic (1.5, 3.0, 4.5, 6.0% CO$_2$) exposures, and after that animals were exposed again to normoxia. The exposure times of each gas mixture, as well as normoxic conditions, was 2 h. Animals were first exposed to one randomly chosen progressive gas exposure and the following day to the other one.

### Data analysis

The last hour of each exposure was used to extract the following data: breathing frequency during breathing episodes ($f_{\text{Repi}}$, breaths.episode$^{-1}$), frequency of breathing episodes ($f_E$, episodes.h$^{-1}$), duration of non-ventilatory period ($T_{\text{NVP}}$, s; defined as the time between the end of an inspiration and the beginning of the following expiration), duration of inspiration ($T_{\text{INSPI}}$, s), duration of expiration ($T_{\text{EXP}}$, s), total duration of one ventilatory cycle ($T_{\text{TOT}} = T_{\text{EXP}} + T_{\text{INSPI}}$, s), tidal volume ($V_T$, ml.kg$^{-1}$), breathing frequency ($f_R$, breaths.min$^{-1}$), and
oxygen consumption ($V_{O_2}$, mlO$_2$.kg$^{-1}$) (Fig. 1). During episodic breathing, due to the slow response time of the oxygen analyzer, oxygen consumption was determined by integrating the area above the oxygen trace for the entire episode, and then divided by the number of expirations to obtain mean oxygen consumption per breath. From the extracted data, the instantaneous breathing frequency ($f'$, breaths.min$^{-1}$), the relative duration of expiration ($T_{EXP}$/T$_{TOT}$), the relation between inspiration and expiration ($T_{INSP}$/T$_{EXP}$), the expiratory flow rate ($V_T$/T$_{EXP}$, ml.s$^{-1}$), minute ventilation ($\dot{V}_E$, ml.kg$^{-1}$.min$^{-1}$), oxygen consumption ($\dot{V}_{O_2}$, mlO$_2$.kg$^{-1}$.min$^{-1}$), and air convection requirement ($\dot{V}_E$/\dot{V}_{O_2}, ml.mlO$_2$^{-1}) were calculated.

Data were analyzed using GraphPad Prism 6.0 and applying Repeated Measures ANOVA followed by a Tukey’s multiple comparison test. Values of $P < 0.05$ were considered significant.

To compare the results of the present study with previously published data, we searched for relevant publications using Pubmed, Web of Science, and Google Scholar databases using keywords such as ‘turtle’, ‘Testudines’, ‘hypoxia’, ‘hypercarbia’, ‘hypercapnia’, ‘ventilation’, ‘gas exchange’, etc.. Values of respiratory variables of testudines obtained under exposure to environmental hypoxia or hypercarbia (but not anoxia or hypoxic-hypercarbia) measured at temperatures between 20 and 30°C were included (Table 1). Data from animals that had their trachea cannulated, or that had their respiratory system surgically manipulated, were not included. Values were directly obtained from the text or tables given, or by extracting values from published figures using the free software PlotDigitizer (version 2.6.2). To enable comparison among species, data were expressed as changes relative to normoxic values. Due to the very low number of chelonian species with
a complete set of respiratory variables available and the varying experimental protocols applied at different temperatures, levels of hypoxia or hypercarbia, phylogenetically informed multivariate analysis was not possible.

Results

Ventilation and oxygen consumption in T. scripta and C. carbonarius

During normoxia, both species showed an episodic breathing pattern with 2-3 ventilatory cycles interspersed by non-ventilatory periods (Fig. 2). The $T_{NVP}$ was, on average, 3-4 times longer in T. scripta than in C. carbonarius. In T. scripta, hypoxia significantly increased $f_E$, $V_T$, $\dot{V}_E$ and $\dot{V}_E/\dot{V}O_2$, whereas $f_{Repi}$, $T_{NVP}$ and $\dot{V}O_2$ were significantly reduced (Figs. 3-6).

Under hypoxic exposure, C. carbonarius significantly increased $f_E$, $T_{INSP}$, $V_T$, $f_R$, $\dot{V}_E$, and $\dot{V}_E/\dot{V}O_2$ (Figs. 3-6). Once the hypoxic exposure ended, all variables returned to pre-hypoxic values within one hour, with the exception of $f_R$ in C. carbonarius, which was significantly greater when compared to the pre-hypoxic value. Exposure to CO$_2$ increased $\dot{V}_E$ and $\dot{V}_E/\dot{V}O_2$ significantly and decreased $\dot{V}O_2$ significantly in T. scripta, whereas in C. carbonarius $T_{INSP}$, $T_{TOT}$, $V_T$, $f_R$, $\dot{V}_E$, and $\dot{V}_E/\dot{V}O_2$ significantly increased but $T_{NVP}$ and $f'$ significantly decreased (Fig. 3-6). One hour after the withdrawal of CO$_2$, all variables had returned to pre-hypercarbic values. The relationships between $T_{EXP}$, $T_{INSP}$ and $T_{TOT}$ (i.e. $T_{EXP}/T_{TOT}$, and $T_{INSP}/T_{EXP}$ respectively) were not significantly affected by either hypoxia nor hypercarbia, just as expiratory flow rate ($V_T/T_{EXP}$), but the latter did show a tendency to increase in both species with increasing levels of hypoxia and hypercarbia (Fig. 5).
Relative changes in respiratory variables

Both hypoxia and hypercarbia increased ventilation. This increase was achieved by increasing the number of breathing episodes, caused by decreasing the non-ventilatory period (Fig. 7). $T_{NVP}$ at 3% $O_2$, for example, consistently represented about 20% of the $T_{NVP}$ seen during normoxia in all species investigated, whereas 6% $CO_2$ roughly reduced $T_{NVP}$ by 50%. Interestingly, hypercarbia about doubled $f_E$, with the exception of $P. geoffroanus$, and slightly increased $f_{Repi}$ (exceptions $P. geoffroanus$ and $C. carbonarius$), whereas hypoxia caused a greater increase in $f_E$, but slightly decreased $f_{Repi}$ (exception $C. carbonarius$). Neither hypoxia nor hypercarbia drastically altered $T_{INSP}$, $T_{EXP}$, $T_{TOT}$, and $f'$ (Fig. 8), as well as $T_{EXT}/T_{TOT}$ and $T_{INSP}/T_{EXP}$ (Fig. 9).

$V_T/T_{EXP}$ increased 2 to 5-fold under hypoxic and hypercarbic conditions (Fig. 9) in all species studied, which was mainly caused by an about 2 to 3-fold increase in $V_T$ at severe levels of hypoxia and hypercarbia (Fig. 10). $C. carbonarius$, showing a 12-fold, and $P. geoffroanus$, showing a 6-fold increase in $V_T$, were the only species showing much larger increases in $V_T$. Several species increased $f_R$ during hypercarbia about 6 to 7-fold, but many species only doubled or tripled $f_R$ (Fig. 10). The only species that increased $f_R$ more than 3-fold during hypoxia were $C. picta$ at 30°C (Glass, Boutilier & Heisler, 1983) and $P. geoffroanus$ at 25°C (Cordeiro, Abe & Klein, 2016). The product of $V_T$ and $f_R$, minute ventilation, showed the greatest relative increases, with $P. geoffroanus$ increasing $\dot{V}_E$ 42 times and $C. carbonarius$ about 30 times, both at 6% $CO_2$. The relative increase at 6% $CO_2$ ranged from 4 to 12 times, whereas at 3% $O_2$ the increase in $\dot{V}_E$ ranged between 3 and 6 or between 12 and 17 for $C. picta$, $C. carbonarius$ and $P. geoffroanus$. 
With the exception of *P. geoffroanus*, both under hypoxia and hypercarbia, and of *P. unifilis* under hypercarbia, $\dot{V}O_2$ decreased or remained unaltered during both exposures (Fig. 11). The resulting air convection requirement, however, increased about 10 to 30-fold in *T. scripta* (Jackson (1973) and Lee & Milsom (2016) *versus* this study, respectively), in *C. picta* (3% O$_2$; Glass, Boutilier & Heisler, 1983), and in *C. carbonarius* (4.5 and 6% CO$_2$; this study) (Fig. 11). In the remaining species, $\dot{V}_E/\dot{V}O_2$ increased about 3 to 12 times under both hypoxic and hypercarbic conditions.

**Discussion**

*Ventilation and oxygen consumption in T. scripta and C. carbonarius*

Breathing pattern of both species followed the general reptilian behavior of intermittent lung ventilation. Burggren (1975) and Glass, Burggren & Johansen (1978) observed intermittent ventilation in *Testudo graeca* and *T. pardalis*, respectively, but in both species breathing pattern consisted of just one ventilatory cycle interspersed by short and regular non-ventilatory periods. In the present study, both, *T. scripta* and, unexpectedly, *C. carbonarius*, showed more than one ventilatory cycle per breathing episode, but the mean duration of the non-ventilatory periods was lower in *C. carbonarius* when compared to *T. scripta*. Vitalis & Milsom (1986a) consider episodic breathing an adaptive mechanism that decreases the energetic cost of ventilation in ectotherms, and Randall et al. (1981) consider such a breathing behavior advantageous for aquatic species, since it reduces the energetic cost to surface and also reduces the exposure time at the surface, possibly lessening risks of predation. Since episodic breathing with long non-ventilatory periods leads to a significant change in arterial blood gases, decreasing $P_aO_2$ and pH and increasing $P_aCO_2$ (Glass,
Burggren & Johansen, 1978), as well as decreasing the efficiency of pulmonary CO₂ excretion (Malte, Malte & Wang, 2013), it should be more advantageous for a terrestrial species to ventilate regularly and thereby maintain homeostasis of arterial blood gases. It is therefore interesting to ask why the terrestrial *C. carbonarius* employs episodic breathing under normoxic conditions, thereby possibly increasing variation in arterial blood gases instead of maintaining a regular breathing pattern, such as seen in this species only under severe levels of hypoxia or hypercarbia (Fig. 2). *C. carbonarius* does frequently seek shelter in shallow burrows or other small spaces and remains non-ventilatory for long periods (A. S. Abe, personal observation), possibly explaining the episodic breathing seen in this terrestrial species, but currently a physiological explication for this behavior is lacking. Interestingly, other ectothermic terrestrial species such as varanid (Thompson & Withers, 1997) and agamid lizards (Frappell & Daniels, 1991) also breathe intermittently, however, concomitant blood gas analyses have not been performed in these species to verify accompanying variations in blood gases or pH.

Comparing our data with previous studies on the effect of hypoxia or hypercarbia on ventilation and gas exchange in *Trachemys scripta*, Frankel et al. (1969) found values for $T_{TOT}$ about three times larger during normoxia, hypoxia and hypercarbia when compared to our study, however, animals in their study had their tracheas cannulated which may have influenced the length of the ventilatory cycle, since $T_{TOT}$ values reported by Vitalis & Milsom (1986b) (calculated from their $f'$: 1.7 s during normoxia and 4% O₂ and 1.8 s during 3-5% CO₂) are similar to ours. Reyes & Milsom (2009) report similar values for $f_E$ as in the present study (from 8.4 ± 1.6 in normoxia during winter up to 37.1 ± 2.3 episodes.h⁻¹ in summer), but found considerable variation in $f_{Rept}$ through different seasons, ranging from 3.6 ± 0.4 breaths.episode⁻¹ in normoxia during winter up to 26.1 ± 5.4
breaths.episode\(^{-1}\) in hypoxic-hypercarbia during autumn, thereby demonstrating considerable seasonal variation in breathing pattern in \(T.\ scripta\). Lee & Milsom (2016) report nearly identical values as in the present study for \(f_{\text{Repi}}\) and \(f_E\) during normoxia and hypoxia, and Frankel et al. (1969) report a comparable \(f_{\text{Repi}}\) during normoxia. Johnson & Creighton (2005), on the other hand, report greater values of \(f_{\text{Repi}}\) during both normoxia and hypercarbia, and Frankel et al. (1969) found \(f_{\text{Repi}}\) at 10-12% \(\text{CO}_2\) to be 5.6 ± 1.0 at 28°C. More data are available regarding \(V_T\), \(f_R\), \(V_E\), \(\dot{V}O_2\), and \(V'_E/\dot{V}O_2\) during both, hypoxic and hypercarbic exposures. In general, data obtained in the present study for normoxia are similar to the ones obtained by other authors, such as \(\dot{V}O_2\), which at 25°C varies from 0.82 (Hicks & Wang, 1999; this study) to 1.1 ml\(\text{O}_2\).kg\(^{-1}\).min\(^{-1}\) (24°C; Jackson & Schmidt-Nielsen, 1966), whereas the values given by Vitalis & Milsom (1986b) for \(V_T\) and \(V_E\) are the lowest ones reported for \(T.\ scripta\) exposed to hypoxia or hypercarbia. The overall changes observed in the ventilatory responses of \(T.\ scripta\) to hypoxia and hypercarbia are also comparable between the present study and data from the literature. Only \(V'_E/\dot{V}O_2\) in the present study, both during hypoxia and hypercarbia, was greater when compared to data from the literature. This difference was caused by a much lower \(\dot{V}O_2\) during hypoxic and hypercarbic exposures when compared to data from other authors, since \(V'_E\) was very similar to data obtained by others at similar temperatures (Jackson, Palmer & Meadow, 1974; Lee & Milsom, 2016). The oxygen consumption measured by us during hypercarbia was similar to the one obtained by Jackson, Palmer & Meadow (1974) at 10°C, a 15°C difference, that may represent a variation in chemosensivity seen in this species during different seasons (Reyes & Milsom, 2009), as we found similarly low \(\dot{V}O_2\) values during
hypoxic exposures. Interestingly, our normoxic $\dot{V}O_2$ values were well within the range for

*T. scripta* at 25°C reported in the literature (Hicks & Wang, 1999; Jackson & Schmidt-Nielsen, 1966). A significant drop in oxygen consumption during hypoxia has also been
described before (Jackson & Schmidt-Nielsen, 1966; Jackson, 1973; Lee & Milsom, 2016),
whereas other studies did not find a pronounced fall in metabolism during hypercarbia
(Hicks & Wang, 1999; Jackson, Palmer & Meadow, 1974). One motive for the observed
variations could lie in the significant seasonal variations in metabolism, gas exchange, and,
consequently, ventilation found in *T. scripta* (Reyes & Milsom, 2009), variations that
possibly were not eliminated by maintaining the animals at a constant temperature of 25°C.
Furthermore, exposing animals for two hours to each gas mixture may not have been
sufficient to reach a physiological steady-state, as suggested by Malte, Malte & Wang
(2016). Another reason for this discrepancy could be the species physiological phenotypic
plasticity, since animals used in the previous studies were native to the North American
continent and thereby subject to a more temperate climate than the animals used in the
present study, that have been bred under the subtropical climate of southeastern Brazil.
The values for minute ventilation in *T. scripta* at 8% CO$_2$ found by Hitzig & Nattie (1982)
seem somewhat low, when compared to the values found by Johnson & Creighton (2005) at
the same CO$_2$ concentration at a different temperature (20 versus 27-28°C, respectively),
but are somewhat similar to the values found by Jackson, Palmer & Meadow (1974) at
20°C and 6% CO$_2$ (135.0 versus 215 ml.kg$^{-1}$.min$^{-1}$, respectively). The general response of
*T. scripta* to reducing oxygen concentrations can be described by a moderate, when
compared to the response during hypercarbia, increase in minute ventilation, mainly caused
by increasing $V_T$, and a reduction in oxygen consumption, thereby increasing the air
convection requirement. These changes are generally more pronounced below 5% O_2. The
response to hypercarbia also includes an increase in ventilation due to an increase in V_T and
f_R. In *T. scripta* neither hypoxia nor hypercarbia caused significant changes in T_{INSP}, T_{EXP},
T_{TOT}, f^*, and f_{Repl}, whereas f_E and T_{NVP}, increased and decreased significantly, respectively.
In respect to *C. carbonarius* during hypoxic or hypercarbic exposures, only data on V_T, f_R,
\dot{V}_E, and \dot{V}O_2 are available for other terrestrial Testudines belonging to the Emydidae and
Testudinidae. Despite comparing different species, the ventilatory variables are similar
among the terrestrial species studied, with the exception of the normoxic \dot{V}O_2 value given
by Altland & Parker (1955) for *Terrapene carolina carolina*, possibly indicating that
animals in their study may not have been resting quietly during normoxia. However, their
\dot{V}O_2 value reported for 3-5% O_2 is identical to the values from other studies at similar
oxygen concentrations. V_T in *C. carbonarius* is on the lower end of data available for
terrestrial Testudines, which may have been influenced by the relative large amount of bone
tissue present in adult individuals of this species (A. S. Abe, personal observation).
Breathing frequency and \dot{V}_E, on the other hand, were very similar to the data obtained on
other terrestrial Emydidae and Testudinidae (Altland & Parker, 1955; Benchetrit, Armand
& Dejours, 1977; Burggren, Glass & Johansen, 1977; Glass, Burggren & Johansen, 1978;
Benchetrit & Dejours, 1980).
Ultsch & Anderson (1988), studying *Gopherus polyphemus* and *Terrapene carolina*, found
values of oxygen consumption very similar to those of *C. carbonarius* during both
normoxia and hypoxia. Interestingly, *G. polyphemus* spends a significant amount of time in
burrows that may show hypoxia as well as hypercarbia, and whose critical oxygen level
(percentage of O_2 where \dot{V}O_2 starts decreasing) can be found at approximately 1.5% O_2,
whereas the exclusively terrestrial *T. carolina* shows a somewhat larger critical oxygen tension of 3.5% O$_2$ (Ultsch and Anderson, 1988). Since *C. carbonarius* did not show any significant changes in $\dot{V}O_2$ during hypoxia down to 3% O$_2$, the critical oxygen level of this species seems to be similar to the one seen in the former two species, but $\dot{V}O_2$ was consistently lower at any oxygen concentration when compared to *G. polyphemus* and *T. carolina* and e.g. at 3% O$_2$ (0.08 mLO$_2$.kg$^{-1}$.min$^{-1}$) was similar to the lowest $\dot{V}O_2$ given for *G. polyphemus* (0.05 mLO$_2$.kg$^{-1}$.min$^{-1}$) and *T. carolina* (0.08 mLO$_2$.kg$^{-1}$.min$^{-1}$) at less than 1% O$_2$ (Ultsch & Anderson, 1988). *C. carbonarius* is not known to use burrows and therefore may not show a critical oxygen level as low as *G. polyphemus*, but *Cheloneoidis chilensis* has been reported to use shallow burrows for retreat during cold days (Pritchard, 1979) and therefore other species of the Testudinidae may possess a similarly low oxygen level as the testudinidid *G. polyphemus*.

**Relative changes in respiratory variables**

Analyzing the respiratory variables available in the literature for chelonians exposed to hypoxia and hypercarbia (Figs. 7-11), one notices the discrepancy in data availability between commonly studied parameters such as $V_T$, $f_R$, $V_E$, and $\dot{V}O_2$, and less frequently reported ones such as $T_{EXP}$, $T_{TOT}$, or $f_E$, for example. Furthermore, only very few terrestrial species have been studied, when compared to the wealth of data available for *T. scripta* and *C. picta*. Based on the data analyzed, it seems clear that the breathing pattern of terrestrial chelonians does not significantly differ from aquatic or semi-aquatic species when considering the responses to hypoxia and hypercarbia. With few exceptions, both hypoxia and hypercarbia elicit similar respiratory responses, showing variation mainly in the
magnitude of the species’ responses. The different patterns seen in $f_E$ and $f_{Repi}$ during hypoxia and hypercarbia may suggest varying degrees of chemosensivity between species and towards different gas exposures. Previous experimental manipulations transforming episodic breathing into continuous single ventilations in *T. scripta* were vagotomy (Vitalis & Milsom, 1986b) and dissection of the spinal cord (Johnson & Creighton, 2005). Recently, Johnson, Krisp & Bartman (2015) changed episodic breathing in *T. scripta* from episodic to singlet breathing through pharmacological manipulation of serotonin 5-HT$_3$ receptors. Studying the participation of serotonin in central chemoreception under hypoxia and hypercarbia in phylogenetically distant species, as well as species occupying different habitats, might help elucidating the varying responses to hypoxia and hypercarbia seen in chelonian breathing pattern, since the switch from episodic to singlet breathing under hypoxia has been suggested to be caused by an increased respiratory drive (Fong, Zimmer & Milsom, 2009). However, under hypercarbia nearly all species increase the number of breaths per episode, and do not decrease $f_{Repi}$ as under hypoxia, suggesting that CO$_2$ exposure increases respiratory drive by different regulatory pathways than under hypoxia. Interestingly, Herman & Smatresk (1999) demonstrated that in *T. scripta* hypoxia and hypercarbia cause different changes in pulmonary ventilation and perfusion. During hypoxia, lung ventilation and perfusion increased, whereas under hypercarbia only lung ventilation increased, but not pulmonary perfusion, resulting in a ventilation/perfusion mismatch during exposure to CO$_2$. Burggren, Glass & Johansen (1977) found a similar cardiovascular response in *Pelomedusa subrufa* and in *Testudo pardalis*, suggesting a common Testudine response, but its importance or relation with the differences observed in $f_{Repi}$ remains unclear.
P. geoffroanus and C. carbonarius seem to be more sensitive regarding $T_{\text{INS}}, T_{\text{EXP}}, T_{\text{TOT}},$

$f'$, $T_{\text{EXT}}/T_{\text{TOT}}$, and $T_{\text{INS}}/T_{\text{EXP}}$, with the former species increasing these variables mainly
during hypercarbia, but the latter one increasing all variables with increasing levels of
hypoxia and hypercarbia. Such increases in $T_{\text{INS}}$ and $T_{\text{EXP}}$ have been interpreted by
Johnson, Krisp & Bartman (2015) as a stronger respiratory drive from central respiratory
neurons, whose intensity, however, seems to vary among species. The absolute and relative
decrease in instantaneous breathing frequency seen in C. carbonarius implies that breathing
mechanics may be more variable than previously anticipated for Testudines, since Vitalis &
Milsom (1986b) found $f'$ to be unaffected by either hypoxia or hypercarbia in T. scripta
and suggested (Vitalis & Milsom, 1986a, b) that T. scripta breathes at combinations of
volume and frequency to keep the mechanical work of breathing at a minimum. In the
present study, $f'$ in T. scripta, as well as in C. carbonarius, did show larger variations than
reported for T. scripta in earlier studies (Frankel et al. 1969; Vitalis & Milsom, 1986b).
Vitalis & Milsom (1986a) found, based on mechanical analyses of the respiratory system of
T. scripta, that the mechanical work of breathing is minimal at ventilation frequencies of 35
to 45 cycles.min$^{-1}$ for different levels of minute pump ventilation (100, 200, 300 ml.min$^{-1}$),
meaning that for a minute pump ventilation of 200 ml.min$^{-1}$. Animals should therefore
ventilate at a frequency of 40 breaths.min$^{-1}$ and a tidal volume of 5 ml to ventilate the
respiratory system with the lowest mechanical work, but such a breathing pattern would
result in severe alkalosis due to increased CO$_2$ excretion (Vitalis & Milsom, 1986b). In the
present study, however, T. scripta reached the greatest level of minute ventilation (215.9
ml.min$^{-1}$.kg$^{-1}$) at 6% CO$_2$, using a tidal volume of 57.2 ml.kg$^{-1}$ and an instantaneous
breathing frequency of 18.3 breaths.min$^{-1}$ ($f_R = 3.0$ breaths.min$^{-1}$), values much different
from mechanical predictions. The significance of this variation in breathing pattern versus
the mechanical predictions of work of breathing needs to be investigated to better
understand the mechanical work of breathing of the Testudines respiratory system, since
mechanical work of breathing increases markedly with increasing tidal volume, e.g. from
57 to 272 ml.cmH\textsubscript{2}O.min\textsuperscript{-1}.kg\textsuperscript{-1} at 6.2 ml.kg\textsuperscript{-1} and 3.0 breaths.min\textsuperscript{-1} in undisturbed \textit{T. scripta} versus 34.2 and 0.8 breaths.min\textsuperscript{-1} in vagotomized \textit{T. scripta}, each at 4\% O\textsubscript{2} (Vitalis & Milsom, 1986b).

The relatively large increases seen in $V_T/T_{EXP}$ of \textit{C. carbonarius} and \textit{P. geoffroanus} can be explained by very low values of $V_T$ under normoxic conditions. \textit{P. geoffroanus} (3.1ml.kg\textsuperscript{-1}; Cordeiro, Abe & Klein, 2016) and \textit{C. carbonarius} (3.98 ml.kg\textsuperscript{-1}; this study) show much smaller tidal volumes during normoxia than other chelonians (mostly between 10 and 20 ml.kg\textsuperscript{-1}), resulting in relatively larger increases in $V_T$ during hypoxia and hypercarbia than the other species. Both species also showed relatively larger increases in $\dot{V}_E$, which are again attributable to the low values seen in $f_R$ and $V_T$ under normoxic conditions.

Whereas $\dot{V}_E$ increases largely in all species, $\dot{V}O_2$ remains unaltered or even decreases under both hypoxia and hypercarbia in nearly all species investigated. The relative increase seen in \textit{P. geoffroanus} under both hypoxia and hypercarbia can be explained by the very low oxygen consumption under normoxic conditions, which could be a consequence of significant extra-pulmonary gas exchange or hypometabolism in this species (Cordeiro, Abe & Klein, 2016). Increases in $\dot{V}_E/\dot{V}O_2$ have been linked both under hypoxia (e.g. Glass, Boutilier & Heisler, 1983) and hypercarbia (e.g. Funk & Milsom, 1987) to regulation of arterial PO\textsubscript{2}, PCO\textsubscript{2}, and pH, as all turtles investigated maintain control of these variables under varying environmental conditions.
The chelonian respiratory system shows significant variations in lung structure, as well as in associated structures such as the post-pulmonary septum (PPS; Perry, 1998). The PPS is a membrane that partially or completely separates the lungs from the other viscera (Lambertz, Böhme & Perry, 2010). As a testudinid, *C. carbonarius* possesses a complete post-pulmonary septum (W. Klein, personal observation), when compared to the smaller PPS of the emydid *T. scripta* (Lambertz, Böhme & Perry, 2010). The presence or absence of a PPS may significantly influence the mechanics of the respiratory system, as has been shown for the post-hepatic septum of the lizard *Salvator (Tupinambis) merianae*, whose static breathing mechanics was significantly affected by the removal of their post-hepatic septum (Klein, Abe & Perry, 2003). Similarly, a complete PPS in Testudinidae could alter the mechanics of the respiratory system by reducing the impact of the viscera onto the lungs, when compared to species with an incomplete PPS such as *T. scripta*.

**Conclusion**

This is the first study to present all the different variables necessary to fully characterize the breathing pattern in the terrestrial *C. carbonarius* and the semi-aquatic *T. scripta* during hypoxic and hypercarbic conditions. Contrary to most previous reports on breathing pattern in terrestrial Testudines, *C. carbonarius* did show considerable non-ventilatory periods with more than one breath per episode. While our data confirm previous data on the general response of *T. scripta* to hypoxia and hypercarbia, breathing pattern has been found to diverge significantly from predictions based on mechanical analyses of the respiratory system.

Our meta-analysis demonstrates general trends regarding ventilatory parameters of testudines when exposed to hypoxia or hypercarbia, but a multivariate analysis of the taxons respiratory physiology will need a complete set of ventilatory parameters from a
much larger number of species. To date it is not possible to associate the variations in the magnitude of different respiratory variables to phylogeny, habitat, behavior, and/or lung structure, which could provide important information regarding the evolution of cardiorespiratory physiology in chelonians. Especially cardiovascular data regarding intracardiac shunt, pulmonary and systemic perfusion, and blood gases during hypoxia and hypercarbia are needed from more species to fully understand blood gas homeostasis in such an important group of intermittent breathers.

References

Altland PD, Parker M. 1955. Effects of hypoxia upon the Box Turtle. Am. J. Physiol. 180, 421-427.

Benchetrit G, Armand J, Dejours P. 1977. Ventilatory chemoreflex drive in the tortoise, *Testudo horsfieldi*. Resp. Physiol. 31, 183-191.

Benchetrit G, Dejours P. 1980. Ventilatory CO$_2$ drive in the tortoise *Testudo horsfieldi*. J. Exp. Biol. 87, 229-236.

Boyer DR 1963. Hypoxia: Effects on heart rate and respiration in the snapping turtle. Science 140, 813-814.

Boyer DR. 1966. Comparative effects of hypoxia on respiratory and cardiac function in reptiles. Physiol. Zool. 39, 307-316.

Burggren WW. 1975. A quantitative analysis of ventilation tachycardia and its control in two chelonians, *Pseudemys scripta* and *Testudo graeca*. J. Exp. Biol. 63, 367-380.

Burggren WW, Glass ML, Johansen K. 1977. Pulmonary ventilation: perfusion relationships in terrestrial and aquatic chelonian reptiles. Can. J. Zool. 55, 2024-2034.
Cordeiro TEF, Abe AS, Klein W. 2016. Ventilation and gas exchange in two turtles: *Podocnemis unifilis* and *Phrynops geoffroanus* (Testudines: Pleurodira). Respir. Physiol. Neurobiol. 224, 125-131.

Fong AY, Zimmer MB, Milsom WK. 2009. The conditional nature of the “Central Rhythm Generator” and the production of episodic breathing. Respir. Physiol. Neurobiol. 168, 179–187.

Frankel HM, Spitzer A, Blaine J, Schoener EP. 1969. Respiratory response of turtles (*Pseudemys scripta*) to changes in arterial blood gas composition. Comp. Biochem. Physiol. 31, 535-546.

Frappell PB, Daniels CB. 1991. Ventilation and oxygen consumption in agamid lizards. Physiol. Zool. 64, 985-1001.

Frische S, Fago A, Altimiras J. 2000. Respiratory responses to short term hypoxia in the snapping turtle, *Chelydra serpentina*. Comp. Biochem. Physiol. Part A. 126, 223-231.

Funk GD, Milsom WK. 1987. Changes in ventilation and breathing pattern produced by changing body temperature and inspired CO₂ concentration in turtles. Respir. Physiol. 67, 37-51.

Gans C, Hughes GM. 1967. The mechanism of lung ventilation in the tortoise *Testudo graeca* Linné. J. Exp. Biol. 47, 1-20.

Gaunt AS, Gans C. 1969. Mechanics of respiration in the snapping turtle, *Chelydra serpentina* (Linné). J. Morphol. 128, 195-227.

Glass ML, Burggren WW, Johansen K. 1978. Ventilation in an aquatic and a terrestrial chelonian reptile. J. Exp. Biol. 72, 165-179.

Glass ML, Wood SC, Johansen K. 1978. The application of pneumotachography on small unrestrained animals. Comp. Biochem. Physiol. Part A. 59, 425-427.
Glass ML, Boutilier RG, Heisler N. 1983. Ventilatory control of arterial PO$_2$ in the turtle Chrysemys picta belli: Effects of temperature and hypoxia. J. Comp. Physiol. 151, 145-153.

Herman JK, Smatresk NJ. 1999. Cardiorespiratory response to progressive hypoxia and hypercapnia in the turtle Trachemys scripta. J. Exp. Biol. 202, 3205–3213.

Hicks JW, Wang T. 1999. Hypoxic hypometabolism in the anesthetizes turtle, Trachemys scripta. American Physiological Society. 277, R18-R23.

Hitzig BM, Nattie EE. 1982. Acid-base stress and central chemical control of ventilation in turtles. J. Appl. Physiol. 53, 1365-1370.

Jackson DC. 1973. Ventilatory response to hypoxia in turtles at various temperatures. Respir. Physiol. 18, 178-187.

Jackson DC, Schmidt-Nielsen K. 1966. Heat production during diving in the fresh water turtle, Pseudemys scripta. J. Cellular Physiol. 67, 225-231.

Jackson DC, Palmer SE, Meadow WL. 1974. The effects of temperature and carbon dioxide breathing on ventilation and acid-base status of turtles. Respir. Physiol. 20, 131-146.

Jackson DC, Kraus DR, Prange HD. 1979. Ventilatory response to inspired CO$_2$ in the sea turtle: effects of body size and temperature. Resp. Physiol. 38, 71-81.

Johnson SM, Creighton RJ, 2005. Spinal cord injury-induced changes in breathing are not due to supraspinal plasticity in turtles (Pseudemys scripta). Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R1550–R1561.

Johnson SM, Krisp AR, Bartman ME. 2015. Hypoxia switches episodic breathing to singlet breathing in red-eared slider turtles (Trachemys scripta) via a tropisetron-sensitive mechanism. Resp. Physiol. Neurobiol. 207, 48–57.
Klein W, Abe AS, Perry SF. 2003. Static lung compliance and body pressures in *Tupinambis merianae* with and without post-hepatic septum. Respir. Physiol. Neurobiol. 135, 73-86.

Lambertz M, Böhme W, Perry SF. 2010. The anatomy of the respiratory system in *Platysternon megacephalum* Gray, 1831 (Testudines: Cryptodira) and related species, and its phylogenetic implications. Comp. Biochem. Physiol. A. 156, 330-336.

Lee SY, Milsom WK. 2016. The metabolic cost of breathing in red-eared sliders: An attempt to resolve an old controversy. Respir. Physiol. Neurobiol. 224, 114–124.

Lyson TR, Schachner ER, Botha-Brink J, Scheyer TM, Lambertz M, Bever GS, Rubidge BS, de Queiroz K. 2014. Origin of the unique ventilatory apparatus of turtles. Nat. Comm. 5, 5211.

Malte CL, Malte H, Wang T. 2013. Episodic ventilation lowers the efficiency of pulmonary CO₂ excretion. J. Appl. Physiol. 115, 1506–1518.

Malte CL, Malte H, Wang T. 2016. The long road to steady state in gas exchange: metabolic and ventilatory responses to hypercapnia and hypoxia in Cuvier's dwarf caiman. J. Exp. Biol. 219, 3810–3821.

Milsom WK, Jones DR. 1980. The role of vagal afferent information and hypercapnia in control of the breathing pattern in Chelonia. J. Exp. Biol. 87, 53-63.

Milsom WK, Chan P. 1986. The relationship between lung volume, respiratory drive and breathing pattern in the turtle, *Chrysemys picta*. J. Exp. Biol. 120, 233-247.

Milsom WK, Wang T. 2017. Is the hypoxic ventilatory response driven by blood oxygen concentration? J. Exp. Biol. 220, 956-958.
Perry SF. 1998. Lungs: comparative anatomy, functional morphology, and evolution. In: Biology of the Reptilia, Vol. 19 (ed. Gans C), pp. 1-92. Ithaca: Society for the Study of Amphibians and Reptiles.

Pritchard PCH. 1979. Encyclopedia of turtles. T.F.H. Publications, 895p.

Randall D, Burggren WW, Farrell A, Haswell MS. 1981. The evolution of air breathing in vertebrates. Cambridge University Press, 133p.

Reyes C, Milsom WK. 2009. Daily and seasonal rhythms in the respiratory sensitivity of red-eared sliders (Trachemys scripta elegans). J. Exp. Bio. 212, 3339-3348.

Shelton G, Jones DR, Milsom WK. 1986. Control of breathing in ectothermic vertebrates, in: Fishman, A.P., Chermiak, N.S., Widdicombe, J.G., Geiger, S.R. (Eds.) Handbook of Physiology, section 3, The respiratory system, volume II. Control of Breathing, Part 2. Am. Physiol. Soc. Bethesda, pp 857-909.

Silva GSF, Giusti H, Branco LGS, Glass ML. 2011. Combined ventilatory responses to aerial hypoxic and temperature in the South American lungfish Lepidosiren paradoxa. J. Thermal Biol. 36, 521-526.

Silver RB, Jackson DC. 1985. Ventilatory and acid-base response to long-term hypercapnia in the freshwater turtle, Chrysemys picta bellii. J. Exp. Biol. 144, 661-672.

Thompson GG, Withers PC, 1997. Patterns of gas exchange and extended non-ventilatory periods in small goannas (Squamata: Varanidae). Comp. Biochem. Physiol. 118A, 1411-1417.

Uetz P, Freed P, Hošek J. 2018. The Reptile Database, http://www.reptile-database.org, accessed 10/May/2018.

Ultsch GR, 2013. Metabolic scaling in turtles. Comp. Biochem. Physiol A. 164, 590–597.
Ultsch GR, Anderson JF. 1988. Gas exchange during hypoxia and hypercarbia of terrestrial turtles: A comparison of a fossorial species (*Gopherus polyphemus*) with a sympatric nonfossorial species (*Terrapene carolina*). Physiol. Zool. 61, 142-152.

Vitalis TZ, Milsom WK. 1986a. Pulmonary mechanics and the work of breathing in the semi aquatic turtle, *Pseudemys scripta*. J. Exp. Biol. 125, 137-155.

Vitalis TZ, Milsom WK. 1986b. Mechanical analysis of spontaneous breathing in the semi-aquatic turtle, *Pseudemys scripta*. J. Exp. Biol. 125, 157-171.

Wang T, Warburton SJ. 1995. Breathing pattern and cost of ventilation in the American alligator. Respir. Physiol. 102, 29-37.

Wasser JS, Jackson DC. 1988. Acid-base balance and the control of respiration during anoxic-hypercapnic gas breathing in turtles. Respir. Physiol. 71, 213-226.

Werneburg I, Hinz JK, Gumpenberger M, Volpato V, Natchev N, Joyce, WG. 2015. Modeling neck mobility in fossil turtles. J. Exp. Zool. B 324, 230-243.
Figure captions

Fig 1. Example traces of a single ventilatory cycle (A, B) and episodic ventilation (C, D) in *Chelonoidis carbonarius* during normoxia showing respiratory variables measured. A, C: ventilation; B, D: oxygen consumption. For abbreviations see Material and Methods section. The red parts on figures A and B represent the areas integrated to determine tidal volume and oxygen consumption, respectively.

Fig 2. Example traces of ventilation in *Trachemys scripta* (A-C) and *Chelonoidis carbonarius* (D-F) during normoxia (A, D), 6% CO$_2$ (B, E), and 3% O$_2$ (C, F).

Fig 3. Breathing frequency during breathing episodes (A, D), number of breathing episodes (B, E), and duration of the non-ventilatory period (C, F) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-C) and hypercarbia (D-F) (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (T. scripta) and + (C. carbonarius) indicate values significantly different from initial normoxic values.

Fig. 4. Duration of inspiration (A, E), duration of expiration (B, F), total duration of one ventilatory cycle (C, G), and instantaneous breathing frequency (D, H) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-D) and hypercarbia (E-H) (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (T. scripta) and + (C. carbonarius) indicate values significantly different from initial normoxic values.
Fig. 5. The relation between expiration and total duration of one ventilatory cycle (A, D),
the relation between inspiration and expiration (B, E), and the expiratory flow rate (C, F) in
*Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia,
hypoxia (A-C) and hypercarbia (D-F) (open symbols) and one hour after exposure to the
different gas mixtures (closed symbols).

Fig. 6. Tidal volume (A, F), breathing frequency (B, G), minute ventilation (C, H), oxygen
consumption (D, I), and air convection requirement (E, J) in *Trachemys scripta* (triangle)
and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-E) and hypercarbia (F-J) (open symbols) and one hour after exposure to the different gas mixtures (closed
symbols). * (*T. scripta*) and + (*C. carbonarius*) indicate values significantly different from
initial normoxic values. # denotes a post-hypoxia normoxic value significantly different
from the initial normoxia.

Fig. 7. Relative changes in breathing frequency during breathing episodes (A, D), number
of breathing episodes (B, E), and duration of the non-ventilatory period (C, F) in Testudines
under hypoxic (A-C) and hypercarbic (D-F) exposures. For symbols see Supplementary
Information 2.

Fig. 8. Relative changes in duration of inspiration (A, E), duration of expiration (B, F), total
duration of one ventilatory cycle (C, G), and instantaneous breathing frequency (D, H) in
Testudines under hypoxic (A-D) and hypercarbic (E-H) exposures. For symbols see
Supplementary Information 2.
Fig. 9. Relative changes in the relation between expiration and total duration of one ventilatory cycle (A, D), the relation between inspiration and expiration (B, E), and the expiratory flow rate (C, F) in Testudines under hypoxic (A-C) and hypercarbic (D-F) exposures. For symbols see Supplementary Information 2.

Fig. 10. Relative changes in tidal volume (A, D), breathing frequency (B, E), and minute ventilation (C, F) in Testudines under hypoxic (A-C) and hypercarbic (B-F) exposures. For symbols see Supplementary Information 2.

Fig. 11. Relative changes in oxygen consumption (A, C), and air convection requirement (B, D) in Testudines under hypoxic (A-B) and hypercarbic (C-D) exposures. For symbols see Supplementary Information 2.
Figure 1

Example of a single ventilatory cycle and episodic ventilation in *Chelonoidis carbonarius* during normoxia showing respiratory variables measured

Traces of a single ventilatory cycle (A, B) and episodic ventilation (C, D) in *Chelonoidis carbonarius* during normoxia showing respiratory variables measured A, C: ventilation; B, D: oxygen consumption. For abbreviations see Material and Methods section. The red parts on figures A and B represent the areas integrated to determine tidal volume and oxygen consumption, respectively.
Figure 2

Example traces of ventilation in *Trachemys scripta* and *Chelonoidis carbonarius*

Traces of ventilation in *Trachemys scripta* (A-C) and *Chelonoidis carbonarius* (D-F) during normoxia (A, D), 6% CO$_2$ (B, E), and 3% O$_2$ (C, F).
Figure 3

Breathing frequency during breathing episodes, number of breathing episodes, and duration of the non-ventilatory period in *Trachemys scripta* and *Chelonoidis carbonarius*

Breathing frequency during breathing episodes (A, D), number of breathing episodes (B, E), and duration of the non-ventilatory period (C, F) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-C) and hypercarbia (D-F) (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (*T. scripta*) and + (*C. carbonarius*) indicate values significantly different from initial normoxic values.
Figure 4

Duration of inspiration, duration of expiration, total duration of one ventilatory cycle, and instantaneous breathing frequency in *Trachemys scripta* and *Chelonoidis carbonarius*

Duration of inspiration (A, E), duration of expiration (B, F), total duration of one ventilatory cycle (C, G), and instantaneous breathing frequency (D, H) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-D) and hypercarbia (E-H) (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (T. scripta) and + (C. carbonarius) indicate values significantly different from initial normoxic values.
Figure 5

Relation between expiration and total duration of one ventilatory cycle, the relation between inspiration and expiration, and the expiratory flow rate in *Trachemys scripta* and *Chelonoidis carbonarius*

The relation between expiration and total duration of one ventilatory cycle (A, D), the relation between inspiration and expiration (B, E), and the expiratory flow rate (C, F) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-C) and hypercarbia (D-F) (open symbols) and one hour after exposure to the different gas mixtures (closed symbols).
Manuscript to be reviewed

Graphs showing data for:
- Graph A: T_{EXP}/T_{TOT}
- Graph B: T_{INS}/T_{EXP}
- Graph C: V_{I}/T_{EXP} (ml s^{-1})
- Graph D: T_{EXP}/T_{TOT}
- Graph E: T_{INS}/T_{EXP}
- Graph F: V_{I}/T_{EXP} (ml s^{-1})

Each graph plots different variables against either % oxygen or % carbon dioxide.

% oxygen

% carbon dioxide
Figure 6

Tidal volume, breathing frequency, minute ventilation, oxygen consumption, and air convection requirement in *Trachemys scripta* and *Chelonoidis carbonarius*

Tidal volume (A, F), breathing frequency (B, G), minute ventilation (C, H), oxygen consumption (D, I), and air convection requirement (E, J) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia (A-E) and hypercarbia (F-J) (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (T. scripta) and + (C. carbonarius) indicate values significantly different from initial normoxic values. # denotes a post-hypoxia normoxic value significantly different from the initial normoxia.
Figure 7

Relative changes in breathing frequency during breathing episodes, number of breathing episodes, and duration of the non-ventilatory period in Testudines under hypoxic and hypercarbic exposures

Relative changes in breathing frequency during breathing episodes (A, D), number of breathing episodes (B, E), and duration of the non-ventilatory period (C, F) in Testudines under hypoxic (A-C) and hypercarbic (D-F) exposures. For symbols see Supplementary Information 2.
Figure 8

Relative changes in duration of inspiration and expiration, total duration of one ventilatory cycle, and instantaneous breathing frequency in Testudines under hypoxic and hypercarbic exposures

Relative changes in duration of inspiration (A, E), duration of expiration (B, F), total duration of one ventilatory cycle (C, G), and instantaneous breathing frequency (D, H) in Testudines under hypoxic (A-D) and hypercarbic (E-H) exposures. For symbols see Supplementary Information 2.
Figure 9

Relative changes in the relation between expiration and total duration of one ventilatory cycle, the relation between inspiration and expiration, and expiratory flow rate

Relative changes in the relation between expiration and total duration of one ventilatory cycle (A, D), the relation between inspiration and expiration (B, E), and the expiratory flow rate (C, F) in Testudines under hypoxic (A-C) and hypercarbic (D-F) exposures. For symbols see Supplementary Information 2.
Figure 10

Relative changes in tidal volume, breathing frequency, and minute ventilation in Testudines under hypoxic and hypercarbic exposures

Relative changes in tidal volume (A, D), breathing frequency (B, E), and minute ventilation (C, F) in Testudines under hypoxic (A-C) and hypercarbic (B-F) exposures. For symbols see Supplementary Information 2.
Figure 11

Relative changes in oxygen consumption and air convection requirement in Testudines under hypoxic and hypercarbic exposures

Relative changes in oxygen consumption (A, C), and air convection requirement (B, D) in Testudines under hypoxic (A-B) and hypercarbic (C-D) exposures. For symbols see Supplementary Information 2.
Table 1 (on next page)

Respiratory variables extracted from the literature
Table 1: Respiratory variables extracted from the literature.

| Species                      | $f_{Rep}$ | T      | $T_{NV}$ | $T_{INS}$ | $T_{EX}$ | $T_{TO}$ | f   | V  | f  | $p\_E$ | $p\_O_2$ | $p\_E/\ p\_O_2$ | Reference                      |
|------------------------------|-----------|--------|----------|-----------|----------|---------|-----|----|----|--------|---------|------------------|--------------------------------|
| Hypoxia                      |           |        |          |           |          |         |     |    |    |        |         |                  |                                 |
| Chelonoidis carbonarius      | x         | x      | x        | x         | x        | x       | x   | x  | x  | x      | x       | x                | This study                     |
| Chelydra serpentina          |           |        |          |           |          |         |     |    |    |        |         |                  | Boyer, 1963                   |
| Chelydra serpentina          |           |        |          |           |          |         |     |    |    |        |         |                  | Boyer, 1966                   |
| Chelydra serpentina          |           |        |          |           |          |         |     |    |    |        |         |                  | Frische, Fago & Altimiras, 2000 |
| Chelydra serpentina          |           |        |          |           |          |         |     |    |    |        |         |                  | West, Smits & Burggren, 1989   |
| Chrysemys picta              |           |        |          |           |          |         |     |    |    |        |         |                  | Glass, Boutilier & Heisler, 1983 |
| Chrysemys picta              |           |        |          |           |          |         |     |    |    |        |         |                  | Milsom & Chan, 1986            |
| Gopherus polyphemus          |           |        |          |           |          |         |     |    |    | x      |         |                  | Ultsch & Anderson, 1988       |
| Pelomedusa subrufa           |           |        |          |           |          |         |     |    |    | x      |         |                  | Burggren, Glass & Johansen, 1977 |
| Pelomedusa subrufa           |           |        |          |           |          |         |     |    |    | x      |         |                  | Glass, Burggren & Johansen, 1978 |
| Phrynops geoffroanus         | x         | x      | x        | x         | x        | x       | x   | x  | x  | x      | x       |                  | Cordeiro, Abe & Klein, 2016    |
| Podocenmis unifilis          | x         | x      | x        | x         | x        | x       | x   | x  | x  | x      | x       |                  | Cordeiro, Abe & Klein, 2016    |
| Terrapene carolina           |           |        |          |           |          |         |     |    |    |        |         |                  | Altland & Parker 1954          |
| Terrapene carolina           |           |        |          |           |          |         |     |    |    | x      |         |                  | Ultsch & Anderson, 1988       |
| Testudo horsfieldi           |           |        |          |           |          |         |     |    |    | x      |         |                  | Benchetrit, Armand & Dejours, 1977 |
| Testudo pardalis             |           |        |          |           |          |         |     |    |    | x      |         |                  | Burggren, Glass & Johansen, 1977 |
| Testudo pardalis             |           |        |          |           |          |         |     |    |    | x      |         |                  | Glass, Burggren & Johansen, 1978 |
| Trachemys scripta            |           |        |          |           |          |         |     |    |    | x      |         |                  | Frankel et al., 1969          |
| Trachemys scripta            |           |        |          |           |          |         |     |    |    |        |         |                  | Hicks & Wang, 1999            |
| Trachemys scripta            |           |        |          |           |          |         |     |    |    |        |         |                  | Jackson & Schmidt-Nielsen, 1966 |
| Trachemys scripta            | x         | x      |          |           |          |         |     |    |    |        |         |                  | Lee & Milsom, 2016             |
| Trachemys scripta            | x         | x      | x        | x         | x        | x       | x   | x  | x  | x      | x       |                  | This study                     |
| Trachemys scripta            | x         | x      | x        | x         | x        | x       | x   | x  | x  | x      | x       |                  | Vitalis & Milsom, 1986b        |
| Hypercarbia                  |           |        |          |           |          |         |     |    |    |        |         |                  |                                 |
| Chelonia mydas               |           |        |          |           |          |         |     |    |    | x      |         |                  | Jackson, Kraus & Prange, 1979  |
| Chelonoidis carbonarius      |           | x      | x        | x         | x        | x       | x   | x  | x  | x      | x       |                  | This study                     |
| Species                     | Abbreviations                                                                 |
|-----------------------------|-------------------------------------------------------------------------------|
| **Chelydra serpentina**     |                                                                               |
| **Chrysemys picta**         |                                                                               |
| **Chrysemys picta**         |                                                                               |
| **Chrysemys picta**         |                                                                               |
| **Chrysemys picta**         |                                                                               |
| **Pelomedusa subrufa**      |                                                                               |
| **Pelomedusa subrufa**      |                                                                               |
| **Phrynops geoffroanus**    |                                                                               |
| **Podocnemis unifilis**     |                                                                               |
| **Testudo pardalis**        |                                                                               |
| **Testudo pardalis**        |                                                                               |
| **Testudo horsfieldi**      |                                                                               |
| **Testudo pardalis**        |                                                                               |
| **Trachemys scripta**       |                                                                               |
| **Trachemys scripta**       |                                                                               |
| **Trachemys scripta**       |                                                                               |
| **Trachemys scripta**       |                                                                               |
| **Trachemys scripta**       |                                                                               |
| **Trachemys scripta**       |                                                                               |

**Symbols:**
- $f_{\text{Repi}}$: breathing frequency during breathing episodes,
- $f_E$: number of breathing episodes,
- $T_{\text{NVP}}$: duration of non-ventilatory period,
- $T_{\text{INSP}}$: duration of inspiration,
- $T_{\text{EXP}}$: duration of expiration,
- $T_{\text{TOT}}$: total duration of one ventilatory cycle,
- $f'$: instantaneous breathing frequency,
- $V_T$: tidal volume,
- $f_R$: breathing frequency,
- $\dot{V}_E$: minute ventilation,
- $\dot{V}_O_2$: oxygen consumption,
- $\dot{E}_V$: air convection requirement.

This study

**Abbreviations:**
- $f_{\text{Repi}}$: breathing frequency during breathing episodes,
- $f_E$: number of breathing episodes,
- $T_{\text{NVP}}$: duration of non-ventilatory period,
- $T_{\text{INSP}}$: duration of inspiration,
- $T_{\text{EXP}}$: duration of expiration,
- $T_{\text{TOT}}$: total duration of one ventilatory cycle,
- $f'$: instantaneous breathing frequency,
- $V_T$: tidal volume,
- $f_R$: breathing frequency,
- $\dot{V}_E$: minute ventilation,
- $\dot{V}_O_2$: oxygen consumption,
- $\dot{E}_V$: air convection requirement.