

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

Pedro HT Baú^{1,2}, Augusto S Abe³, 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

Ventilatory parameters have been investigated in several species of Testudines, but few species have its ventilatory pattern fully characterized by presenting all variables necessary to understand changes in breathing pattern seen under varying environmental conditions. Since only the totality of ventilatory variables allows understanding a species ventilatory behavior, we measured ventilation and gas exchange in *Chelonoidis carbonarius* and *Trachemys scripta* under normoxia, hypoxia, and hypercarbia. During normoxia both species showed an episodic breathing pattern with 2-3 breaths per episode but the non-ventilatory periods (T_{NVP}) being 3-4 times longer in *T. scripta* than in *C. carbonarius*. Hypoxia and hypercarbia significantly increased ventilation in both species, decreased significantly T_{NVP} and oxygen consumption in *T. scripta* but not in *C. carbonarius*. Contrary to expectations, breathing pattern in *C. carbonarius* did show considerable non-ventilatory periods with more than one breath per episode and breathing pattern in *T. scripta* was found to diverge significantly from predictions based on mechanical analyses of the respiratory system. Analyzing the data available in the literature, the relative changes of ventilatory parameters in chelonians dealing with hypoxia or hypercarbia were similar, mainly showing variations in the magnitude of change.

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Pedro H. T. Baú^a, Augusto S. Abe^{b, c}, Wilfried Klein^{c, d, *}

^a*Master Program in Comparative Biology, University of São Paulo, Brazil.*

^b*State University of São Paulo, Rio Claro, Brazil.*

^c*National Institute of Science and Technology in Comparative Physiology, Brazil.*

^d*School of Philosophy, Sciences and Literature Ribeirão Preto, University of São Paulo, 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

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Abstract

Ventilatory parameters have been investigated in several species of Testudines, but few species have its ventilatory pattern fully characterized by presenting all variables necessary to understand changes in breathing pattern seen under varying environmental conditions. Since only the totality of ventilatory variables allows understanding a species ventilatory behavior, we measured ventilation and gas exchange in *Chelonoidis carbonarius* and *Trachemys scripta* under normoxia, hypoxia, and hypercarbia. During normoxia both species showed an episodic breathing pattern with 2-3 breaths per episode but the non-ventilatory periods (T_{NVP}) being 3-4 times longer in *T. scripta* than in *C. carbonarius*. Hypoxia and hypercarbia significantly increased ventilation in both species, decreased significantly T_{NVP} and oxygen consumption in *T. scripta* but not in *C. carbonarius*. Contrary to expectations, breathing pattern in *C. carbonarius* did show considerable non-ventilatory periods with more than one breath per episode and breathing pattern in *T. scripta* was found to diverge significantly from predictions based on mechanical analyses of the respiratory system. Analyzing the data available in the literature, the relative changes of ventilatory parameters in chelonians dealing with hypoxia or hypercarbia were similar, mainly showing variations in the magnitude of change.

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42 Introduction

43 Gas exchange and  tilation have been studied in several species of turtles, tortoises
 44 and terrapins.  Especially the semi-aquatic *Trachemys scripta* and *Ch~~e~~mys picta* have
 45 been used  multiple times for respiratory studies (see Table S1).  Whereas oxygen
 46 consumption has been determined in many chelonian species (see Ultsch, 2013, for
 47 review), data on ventilatory parameters such as overall breathing frequency (f_R), tidal
 48 volume (V_T), and minute ventilation ($\dot{V}_E$) are available only for a small number of
 49 species  when compared to the taxons diversity, even more so when the ventilatory
 50 responses during hypoxic or hypercarbic exposures are taken into account (Table S1).
 51 While the number of studies  listed seems extensive,  only few studies have actually
 52 characterized the ventilatory pattern by  giving data such as inspiratory time (T_{INSP}), 
 53 expiratory time (T_{EXP}), total duration of a ventilatory cycle ($T_{TOT} = T_{EXP} + T_{INSP}$),  time
 54 of the non-ventilatory period (T_{NVP}), number of ventilations per episode (f_{Repi}),  number
 55 of breathing episodes (f_E), as  as f_R , V_T , and $\dot{V}_E$ (Benchetrit & Dejours, 1980;
 56 Cordeiro, Abe & Klein, 2016), but most of these data have been obtained for *Chrysemys*
 57 *picta* (e.g. Milsom & Jones, 1980; Milsom & Chan, 1986; Funk & Milsom, 1987;
 58 Wasser & Jackson, 1988).  Only the totality of these variables  may fully characterize the
 59 ventilatory behavior of a species under varying environmental conditions, especially in
 60 ectothermic vertebrates where ventilation can show highly episodic burst breathing or
 61 regular singlet breathing  (for review see Shelton, Jones & Milsom, 1986).
 62  The terrestrial species among the Testudines belonging to the family Testudinidae are
 63 also very poorly characterized regarding their ventilatory response to hypoxia or
 64 hypercarbia  and only data on f_R , V_T , $\dot{V}_E$ and oxygen consumption ($\dot{V}O_2$) are available
 65 (Altland & Parker, 1955; Benchetrit, Armand & Dejours, 1977; Benchetrit & Dejours,
 66 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 semi-aquatic *Pelomedusa subrufa* ventilates in episodes of continuous breathing interspaced with longer breath-holds, whereas the terrestrial *Testudo pardalis* employs single breaths separated by short breath-holds. These different patterns have been interpreted as adaptations to the aquatic life-style observed in *P. subrufa*, 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). A regular singlet breathing behavior has also been shown by Burggren (1975) for the tortoise *Testudo graeca* and Benchetrit, Armand & Dejours (1977) for *Testudo horsfieldi*, whereas Altland & Parker (1955) found a more episodic breathing pattern in *Terrapene carolina carolina* under normoxia that changes to a more regular singlet breathing pattern under hypoxic conditions.

Depending on the gas concentration, hypoxia as well as hypercarbia stimulate breathing in turtles, with moderate concentrations of hypercarbia generally increasing ventilation more than very low oxygen concentrations (Shelton, Jones & Milsom, 1986). It has been shown that the normal response to either of the changes in external gas concentrations reduces T_{NVP} , but may or may not increase f_R or V_T (Shelton, Jones & Milsom, 1986). In a recent study, Cordeiro, Abe & Klein (2016) have demonstrated that two closely related pleurodirans exhibit different responses to hypoxia and hypercarbia.

While both species reduce significantly T_{NVP} and increase f_R during hypoxic and hypercarbic exposures, *Podocnemis unifilis* significantly increases f_{Repi} during hypercarbia but significantly decreases f_{Repi} during hypoxia, whereas *Phrynops geoffroanus* significantly decreases f_{Repi} during 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, it was aim of this study to analyze the ventilatory response to different gas mixtures in two cryptodirans, the South American red-footed tortoise *Chelonoidis carbonarius* (Testudinidae) and the red-eared slider *Trachemys scripta* (Emydidae). *C. carbonarius* was chosen because it is a wide-spread South American tortoise that not had its respiratory physiology investigated previously, whereas *T. scripta*, the model species for cardiorespiratory studies, was investigated since no previous study reported all ventilatory variables obtained from the same animals and experimental protocols, both under hypoxic and hypercarbic conditions. Furthermore, the present data were compiled together with available data from the literature to characterize the general response of testudines to hypoxia and hypercarbia.

Materials and methods

Animals

Adults of both sexes of *T. scripta* ($M_B = 1.077 \pm 0.10$ kg; $N = 8$) and *C. carbonarius* ($M_B = 3.773 \pm 0.61$ 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 USP/Campus de Ribeirão Preto; protocol number 12.1.1541.53.0). 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 et al. (1978b), 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 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 ADInstruments). The gas inside the funnel or mask were sampled, dried and pulled to a gas analyzer (ML206ADInstruments). 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 and concentrations of gas. Air was used for the spirometer calibration and different volumes of O₂ and CO₂ were used to calibrate the gas exchange measurements.

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 weighted 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 8:00 am and ventilation and gas exchange were measured under normoxic conditions, followed by progressively decreasing hypoxic (9, 7, 5, 3% O₂) or progressively increasing hypercarbic (1.5, 3.0, 4.5, 6.0% CO₂) exposures. The exposure times of each gas mixture as well as normoxic condition were 2 h.

Data analysis

The last hour of each exposure was used to extract the following data: breathing frequency (f_R), breathing frequency during breathing episodes (f_{Repi}), number of breathing episodes (f_E), tidal volume (V_T), duration of expiration (T_{EXP}), duration of inspiration (T_{INSP}), total duration of one ventilatory cycle (T_{TOT} = T_{EXP} + T_{INSP}), duration of non-ventilatory period (T_{NVP}; defined as the time between the end of an inspiration and the beginning of the following expiration) and oxygen consumption (V̇O₂). From these data 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}), instantaneous breathing frequency (f'), minute ventilation (V̇_E), oxygen consumption (V̇O₂) and air convection requirement (V̇_E/V̇O₂) were calculated.

Data were analyzed using GraphPad Prism 6.0 applying Repeated Measures ANOVA followed by a Tukey's multiple comparison test. A t-test was used to compare both species normoxic values. Values of P<0.05 were considered significant.

To compare the results of the present study with previously published data, we extracted values of respiratory variables from the literature measured at temperatures between 20 and 30°C of testudines exposed either to environmental hypoxia or hypercarbia (Altland

& Parker, 1955; Boyer, 1963; Boyer, 1966; Jackson & Schmidt-Nielsen, 1966; Frankel et al., 1969; Jackson, Palmer & Meadow, 1974; Benchetrit, Armand & Dejours, 1977; Burggren, Glass & Johansen, 1977; Glass, Burggren & Johansen, 1978; Jackson, Kraus & Prange, 1979; Benchetrit & Dejours, 1980; Milsom & Jones, 1980; Hitzig & Nattie, 1982; Glass, Boutilier & Heisler, 1983; Silver & Jackson, 1985; Milsom & Chan, 1986; Vitalis & Milsom, 1986b; Funk & Milsom, 1987; Ultsch & Anderson, 1988; West, Smits & Burggren, 1989; Herman & Smatresk, 1999; Hicks & Wang, 1999; Frische, Fago & Altimiras, 2000; Johnson & Creighton, 2005; Cordeiro, Abe & Klein, 2016; Lee & Milsom, 2016), but not to anoxia or hypoxic-hypercarbia. 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 better compare the data among species, data have been expressed as relative changes 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 applying different temperatures, levels of hypoxia or hypercarbia, no phylogenetically informed multivariate analysis has been carried out.

Results and Discussion

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

During normoxia *T. scripta* and *C. carbonarius* both showed an episodic breathing pattern with 2-3 ventilatory cycles during a breathing episode and interspersed by non-ventilatory periods (Fig. 1, 2). The T_{NVP} was, on average, 3-4 times longer in *T. scripta* than in *C. carbonarius*. A significant difference between both species' normoxic values was found for T_{INSP} , T_{NVP} , f_R , f_E , V_T , $\dot{V}_E$ and $\dot{V}O_2$. Hypoxia significantly increased T_{INSP} , V_T , f_R , f_E , $\dot{V}_E$, and $\dot{V}_E/\dot{V}O_2$ in *C. carbonarius* and f_E , V_T and $\dot{V}_E$ and $\dot{V}_E/\dot{V}O_2$ in *T.*

scripta, whereas f_{Repi} , T_{NVP} and $\dot{V}O_2$ were significantly reduced in *T. scripta* (Figs. 2-4).

While $\dot{f}$ did show a reduction in *C. carbonarius* at 3% O_2 compared to normoxia, only the $\dot{f}$ values at 9 and 7% O_2 , respectively, where significantly different from the value found at 3% O_2 (Fig. 3). 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 resulted

in significant increases of T_{INSP} , T_{TOT} , V_T , f_R , $\dot{V}_E$, and $\dot{V}_E/\dot{V}O_2$ and significant decreases of $\dot{f}$ and T_{NVP} in *C. carbonarius*, whereas in *T. scripta* $\dot{V}_E$ and $\dot{V}_E/\dot{V}O_2$ increased

significantly and $\dot{V}O_2$ decreased significantly (Fig. 2-4). 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} were not significantly affected by either hypoxia nor hypercarbia,

just as V_T/T_{EXP} , but the expiratory flow rate did show a tendency to increase in both species with increasing levels of hypoxia and hypercarbia (Fig. 5).

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 CO_2 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. 1). *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 explanation for this behavior is lacking. Interestingly, other ectothermic terrestrial species such as varanid (Thompson & Wiersma, 1997) and agamid lizards (Frappel & 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 longer 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% O_2 and 1.8 s during 3-5% CO_2) 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



242 winter up to 37.1 ± 2.3 episodes.h⁻¹ in summer, but found considerable variation in f_{Repi}
 243 through different seasons, ranging from 3.6 ± 0.4 breaths.episode⁻¹ in normoxia during
 244 winter up to 26.1 ± 5.4 breaths.episode⁻¹ in hypoxic-hypercarbia during autumn, thereby
 245 demonstrating considerable seasonal variation in breathing pattern in *T. scripta*. Lee &
 246 Milsom (2016) report nearly identical values as in the present study for f_{Repi} and f_E
 247 during normoxia and hypoxia, and Frankel et al. (1969) report a comparable f_{Repi} during
 248 normoxia. Johnson & Creighton (2005), on the other hand, report greater values of f_{Repi}
 249 during both normoxia and hypercarbia, and Frankel et al. (1969) found f_{Repi} at 10-12%
 250 CO₂ to be 5.6 ± 1.0 at 28°C.

251 More data are available regarding V_T , f_R , $\dot{V}_E$, $\dot{V}O_2$, and $\dot{V}_E/\dot{V}O_2$ during both, hypoxic
 252 and hypercarbic exposures. In general, data obtained in the present study for normoxia
 253 are similar to the ones obtained by other authors, such as $\dot{V}O_2$, which at 25°C varies
 254 from 0.82 (Hicks & Wang, 1999; this study) to 1.1 mlO₂.kg⁻¹.min⁻¹ (24°C; Jackson &
 255 Schmidt-Nielsen, 1966), whereas the values given by Vitalis & Milsom (1986b) for V_T
 256 and $\dot{V}_E$ are the lowest ones reported for *T. scripta* exposed to hypoxia or hypercarbia.
 257 The overall changes observed in the ventilatory responses of *T. scripta* to hypoxia and
 258 hypercarbia are also comparable between the present study and data from the literature.
 259 Only $\dot{V}_E/\dot{V}O_2$ in the present study, both during hypoxia and hypercarbia, was greater
 260 when compared to data from the literature. This difference was caused by a much lower
 261 $\dot{V}O_2$ during hypoxic and hypercarbic exposures when compared to data from other
 262 authors, since $\dot{V}_E$ was very similar to data obtained by others at similar temperatures
 263 (Jackson, Palmer & Meadow, 1974; Lee & Milsom, 2016). The oxygen consumption
 264 measured by us during hypercarbia was similar to the one obtained by Jackson, Palmer
 265 & Meadow (1974) at 10°C, a 15°C difference, that may represent a variation in

chemosensitivity 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. 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% CO₂ found by Hitzig & Nattie (1982) seem somewhat low, when compared to the values found by Johnson & Creighton (2005) at the same CO₂ 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% CO₂ (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% O₂. The response to hypercarbia also includes an increase in

291 ventilation due to an increase in V_T and f_R . In *T. scripta* neither hypoxia nor hypercarbia
 292 caused significant changes in T_{INSP} , T_{EXP} , T_{TOT} , f' , and f_{Repi} , whereas f_E and T_{NVP} ,
 293 increased and decreased, respectively.
 294 In respect to *C. carbonarius* during hypoxic or hypercarbic exposures, only data on V_T ,
 295 f_R , $\dot{V}_E$, and $\dot{V}O_2$ are available for other terrestrial Testudines belonging to the Emydidae
 296 and Testudinidae. Despite comparing different species, the ventilatory variables are
 297 similar among the terrestrial species studied, with the exception of the normoxic $\dot{V}O_2$
 298 value given by Altland & Parker (1955) for *Terrapene carolina carolina*, possibly
 299 indicating that animals in their study may not have been resting quietly during
 300 normoxia. However, their $\dot{V}O_2$ value reported for 3-5% O_2 is identical to the values
 301 from other studies at similar oxygen concentrations. V_T in *C. carbonarius* is on the
 302 lower end of data available for terrestrial Testudines, which may have been influenced
 303 by the relative large amount of bone tissue present in adult individuals of this species
 304 (A. S. Abe, personal observation). Breathing frequency and $\dot{V}_E$, on the other hand, were
 305 very similar to the data obtained on other terrestrial Emydidae and Testudinidae
 306 (Altland & Parker, 1955; Benchetrit, Armand & Dejours, 1977; Burggren, Glass &
 307 Johansen, 1977; Glass, Burggren & Johansen, 1978; Benchetrit & Dejours, 1980).
 308 Ultsch & Anderson (1988), studying *Gopherus polyphemus* and *Terrapene carolina*,
 309 found values of oxygen consumption very similar to the ones observed in *C.*
 310 *carbonarius* during both normoxia and hypoxia. Interestingly, *G. polyphemus* spends a
 311 significant amount of time in burrows that may show hypoxia as well as hypercarbia,
 312 and whose critical oxygen level (percentage of O_2 where $\dot{V}O_2$ starts decreasing) can be
 313 found at approximately 1.5% O_2 , whereas the exclusively terrestrial *T. carolina* shows a
 314 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 ($0.08 \text{ mlO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was similar to the lowest $\dot{V}O_2$ given for *G. polyphemus* ($0.05 \text{ mlO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and *T. carolina* ($0.08 \text{ mlO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) 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 *Chelonoidis 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 testudinid *G. polyphemus*.

Relative changes in respiratory variables

Analyzing the respiratory variables available in the literature for chelonians exposed to hypoxia and hypercarbia (Figs. 6-10), one notices the discrepancy in data availability between commonly studied parameters such as V_T , f_R , $\dot{V}_E$, 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*. However, 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. Both hypoxia and hypercarbia increase ventilation. This increase is achieved by increasing the number of breathing episodes, caused by decreasing the non-ventilatory

period (Fig. 6). T_{NVP} at 3% O_2 , for example, consistently represents about 20% of the T_{NVP} seen during normoxia in all species investigated, whereas 6% CO_2 roughly reduces T_{NVP} by 50%. Interestingly, hypercarbia about doubles f_E , with the exception of *P. geoffroanus*, and slightly increases f_{Rep} (exceptions *P. geoffroanus* and *C. carbonarius*), whereas hypoxia causes a greater increase in f_E , but slightly decreases f_{Rep} (exception *C. carbonarius*). These different patterns may suggest varying regulatory mechanisms of breathing pattern during hypoxia and hypercarbia and between species. 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 drug administration that manipulated serotonin 5-HT₃ receptors. Studying the participation of serotonin in central chemoreception under hypoxia and hypercarbia in different species might elucidate the different responses seen in chelonian breathing pattern to hypoxia and hypercarbia, contributing to the understanding of varying chemosensitivity in the taxon.

Neither hypoxia nor hypercarbia drastically altered T_{INSP} , T_{EXP} , T_{TOT} , and f' (Fig. 7), as well as T_{EXT}/T_{TOT} and T_{INSP}/T_{EXP} (Fig. 8). *P. geoffroanus* and *C. carbonarius* seem, however, be more sensitive regarding these variables, the former species mainly during hypercarbia, but the latter one increasing all variables with increasing levels of hypoxia and hypercarbia. 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_r in *C. carbonarius* as well as in *T. scripta* 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 between ventilation frequencies of 35 to 45 cycles $\cdot \text{min}^{-1}$ for different levels of minute pump ventilation (100, 200, 300 $\text{ml} \cdot \text{min}^{-1}$), meaning that for a minute pump ventilation of 200 $\text{ml} \cdot \text{min}^{-1}$, animals would ventilate at a frequency of 40 breaths $\cdot \text{min}^{-1}$ and a tidal volume of 5 ml to ventilate the respiratory system with the lowest mechanical work. In the present study, however, *T. scripta* reached the greatest level of minute ventilation (215.9 $\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) at 6% CO_2 , using a tidal volume of 57.2 $\text{ml} \cdot \text{kg}^{-1}$ and an instantaneous breathing frequency of 18.3 breaths $\cdot \text{min}^{-1}$ ($f_R = 3.0$ breaths $\cdot \text{min}^{-1}$), 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 for a single breath increases markedly with increasing tidal volume (Vitalis & Milsom, 1986b).

As a testudinid, *C. carbonarius* possesses a complete post-pulmonary septum (PPS; 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 merianae*, whose static breathing mechanics is being significantly affected by the removal of their post-hepatic septum (Klein, Abe & Perry, 2003).

V_T/T_{EXP} increases 2 to 5-fold under hypoxic and hypercarbic conditions (Fig. 8) 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. 9). *C. carbonarius*, showing a 12-fold, and *P. geoffroanus*, showing a 6-fold increase in V_T , are the only species showing much larger increases in V_T . These relatively large increases can be explained by very low values of V_T under normoxic conditions. *P. geoffroanus* (3.1 ml.kg^{-1} ; Cordeiro, Abe & Klein, 2016) and *C. carbonarius* (3.98 ml.kg^{-1} ; this study) show much smaller tidal volumes during normoxia than other chelonians (mostly between 10 and 20 ml.kg^{-1}), resulting in relatively larger increases in V_T during hypoxia and hypercarbia than the other species.

Several species, **on the other hand**, increase f_R during hypercarbia about 6 to 7-fold, but many species only double or triple f_R (Fig. 9). The only species that increase f_R more than 3-fold during hypoxia where *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, did show 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*. The relatively larger increases seen in *P. geoffroanus* and *C. carbonarius* are again attributable to the low values seen in these variables under normoxic conditions in both species.

With the exception of *P. geoffroanus*, **both under** hypoxia and hypercarbia, and of *P. unifilis* under hypercarbia, $\dot{V}\text{O}_2$ decreased or remained unaltered during both exposures (Fig. 10). The resulting air convection requirement, however, increased about 10 to 30-fold in *T. scripta* (Jackson (1974) and Lee & Milsom (2016) **versus** this study,

414 **respectively**), in *C. picta* (3% O₂; Glass, Boutilier & Heisler, 1983), and in *C.*
 415 *carbonarius* (4.5 and 6% CO₂; this study) (Fig. 10). In the remaining species $\dot{V}_E/\dot{V}O_2$
 416 increased about 3 to 12 times under both hypoxic and hypercarbic conditions. Such
 417 increases in $\dot{V}_E/\dot{V}O_2$ have been linked both under hypoxia (e.g. Glass, Boutilier &
 418 Heisler, 1983) and hypercarbia (e.g. Funk & Milsom, 1987) to regulation of arterial
 419 PO₂, PCO₂, and pH, as all turtles investigated maintain control of these variables under
 420 varying environmental conditions.

421

422 Conclusion

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

431 **At the current point**, some general trends regarding ventilatory parameters of testudines
 432 when exposed to hypoxia or hypercarbia **can be recognized**, but a multivariate analysis
 433 of the **taxons** respiratory physiology will need a complete set of ventilatory parameters
 434 from a much larger number of species. To date it is not possible **to associate the**
 435 **variations seen in** the magnitude of different respiratory variables **to phylogeny**, habitat,
 436 behavior, and/or lung structure, **for example.**

437

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Figure captions

Fig 1. Example traces of ventilation in *Trachemys scripta* (a-e) and *Chelonoidis carbonarius* (f-j) during normoxia (a, f), 9% O₂ (b, g), 7% O₂ (c, h), 5% O₂ (d, i), and 3% O₂ (e, j). Units are mV (positive values = Expiration and negative values = inspiration) and scale represents 5 min.

Fig 2. Breathing frequency during breathing episodes (f_{Repi}), number of breathing episodes (f_E), and duration of the non-ventilatory period (T_{NVP}) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia and hypercarbia (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (*T. scripta*) and + (*C. carbonarius*) indicate values significantly different from normoxic values before exposure to the different gas mixtures.

Fig. 3. Duration of inspiration (T_{INSP}), duration of expiration (T_{EXP}), total duration of one ventilatory cycle (T_{TOT}), and instantaneous breathing frequency (f') in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia and hypercarbia (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (*T. scripta*) and + (*C. carbonarius*) indicate values significantly different from normoxic values before exposure to the different gas mixtures.

Fig. 4. Tidal volume (V_T), breathing frequency (f_R), minute ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}O_2$), and air convection requirement ($\dot{V}_E/\dot{V}O_2$) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia and hypercarbia (open symbols) and one hour after exposure to the different gas mixtures (closed symbols). * (*T. scripta*) and + (*C. carbonarius*) indicate values significantly

different from normoxic values before exposure to the different gas mixtures. # denotes a post-hypoxia normoxic value significantly different from the initial normoxia.

Fig. 5. The relation between expiration and total duration of one ventilatory cycle ($T_{\text{EXP}}/T_{\text{TOT}}$), the relation between inspiration and expiration ($T_{\text{INSP}}/T_{\text{EXP}}$), and the expiratory flow rate (V_T/T_{EXP}) in *Trachemys scripta* (triangle) and *Chelonoidis carbonarius* (circle) during normoxia, hypoxia and hypercarbia (open symbols) and one hour after exposure to the different gas mixtures (closed symbols).

Fig. 6. Relative changes in breathing frequency during breathing episodes (f_{Repi}), number of breathing episodes (f_E), and duration of the non-ventilatory period (T_{NVP}) in Testudines under hypoxic and hypercarbic exposures.

Chelonia mydas (◇ 25°C, Jackson, Kraus & Prange, 1979); *Chelonoidis carbonarius* (★ 25°C; this study); *Chelydra serpentina* (◇ 25°C, Boyer, 1966; ◇ 22-24°C, West, Smits & Burggren, 1989; ♣ 20°C, Frische, Fago & Altimiras, 2000); *Chrysemys picta* (● 20-23°C Milsom & Jones, 1980; ● 20°C, Glass, Boutilier & Heisler, 1983; ● (30°C, Glass, Boutilier & Heisler, 1983; ● 20°C, Silver & Jackson, 1985; ⊗ 22-23°C, Milsom & Chan, 1986; ⊙ 20°C, Funk & Milsom, 1987; ○ (30°C, Funk & Milsom, 1987); *Gopherus polyphemus* (< 22°C; Ultsch and Anderson, 1988); *Pelomedusa subrufa* (♠ 25°C, Burggren, Glass & Johansen, 1977; ♥ 25°C, Glass, Burggren & Johansen, 1978); ◆ *Phrynops geoffroanus* (25°C, Cordeiro, Abe & Klein, 2016); □ *Podocnemis unifilis* (25°C, Cordeiro, Abe & Klein, 2016); *Terrapene carolina* (✱ 20-23°C, Altland and Parker, 1955; > 22°C, Ultsch and Anderson, 1988); *Testudo horsfieldi* († 25°C; Benchetrit, Armand & Dejours, 1977; ♀ 30°C; Benchetrit et al., 1977; # 23-25°C; Benchetrit and Dejour, 1980); *Testudo pardalis* (✕ 25°C, Burggren,

596 Glass & Johansen, 1977; + 25°C, Glass, Burggren & Johansen, 1978); *Trachemys*
 597 *scripta* (▲ 24°C, Jackson & Schmidt-Nielsen, 1966; ▽ 28°C, Frankel et al., 1696; ▼
 598 20°C, Jackson, 1973; ▴ 30°C, Jackson, 1973; ▽ 20°C, Jackson, Palmer & Meadow,
 599 1974; ▲ 30°C, Jackson, Palmer & Meadow, 1974; ◁ 20°C, Hitzig & Nattie, 1982; ▼
 600 25°C, Hicks & Wang, 1999; ◀ 27-28°C, Johnson & Creighton, 2005; ▲ 20-23.5°C, Lee
 601 & Milsom, 2016; △ 25°C, this study).

602

603 Fig. 7. **Relative changes** in duration of inspiration (T_{INSP}), duration of expiration (T_{EXP}),
 604 total duration of one ventilatory cycle (T_{TOT}), and instantaneous breathing frequency (f')
 605 in Testudines under hypoxic and hypercarbic exposures. For symbols see Figure 6.

606

607 Fig. 8. **Relative changes in** the relation between expiration and total duration of one
 608 ventilatory cycle ($T_{\text{EXP}}/T_{\text{TOT}}$), the relation between inspiration and expiration
 609 ($T_{\text{INSP}}/T_{\text{EXP}}$), and the expiratory flow rate (V_T/T_{EXP}) in Testudines under hypoxic and
 610 hypercarbic exposures. For symbols see Figure 6.

611

612 Fig. 9. **Relative changes** in tidal volume (V_T), breathing frequency (f_R), and minute
 613 ventilation ($\dot{V}_E$) in Testudines under hypoxic and hypercarbic exposures. For symbols
 614 see Figure 6.

615

616 Fig. 10. **Relative changes** in oxygen consumption ($\dot{V}O_2$), and air convection
 617 requirement ($\dot{V}_E/\dot{V}O_2$) in Testudines under hypoxic and hypercarbic exposures. For
 618 symbols see Figure 6.

Figure 1(on next page)

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

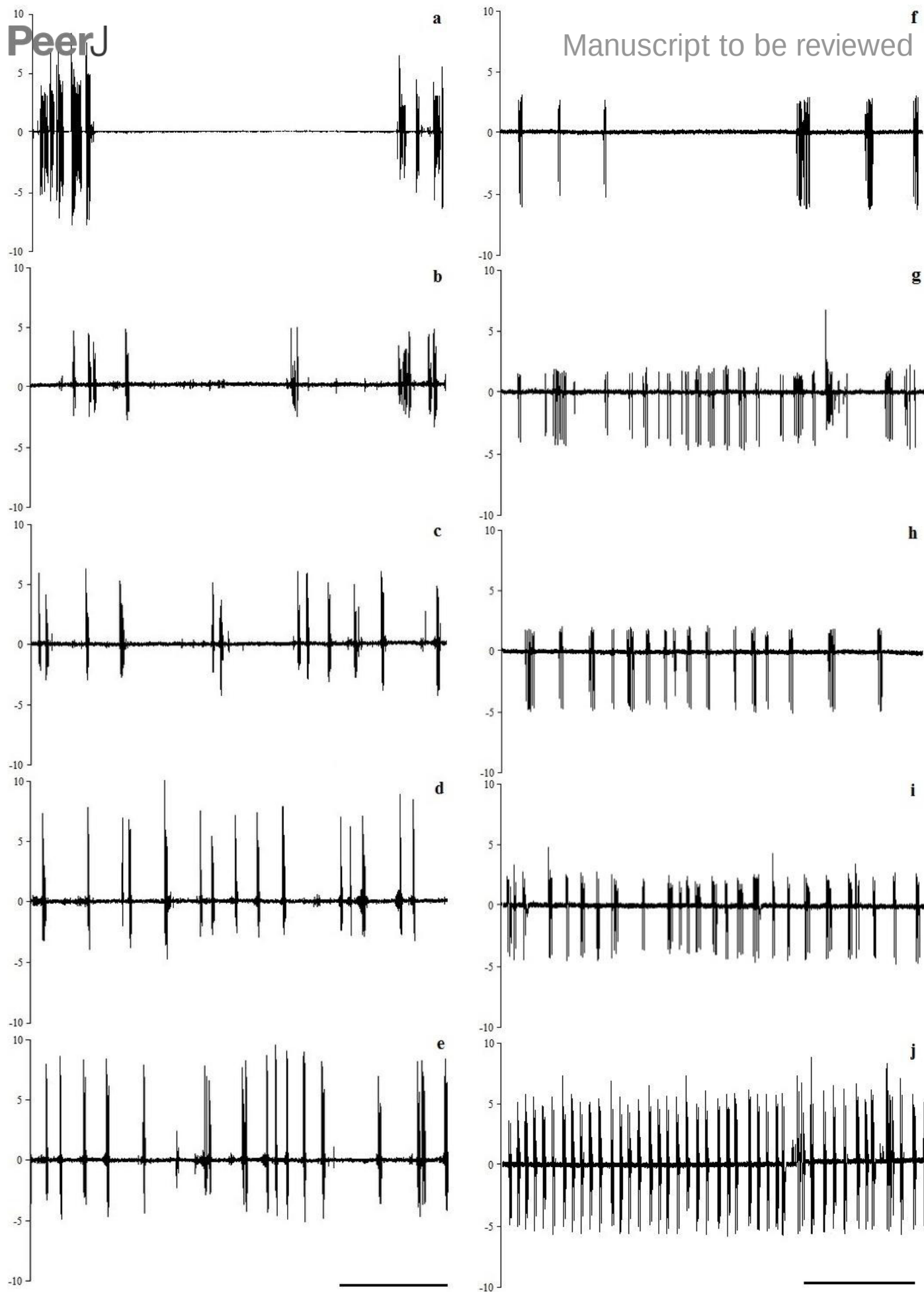


Figure 2

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

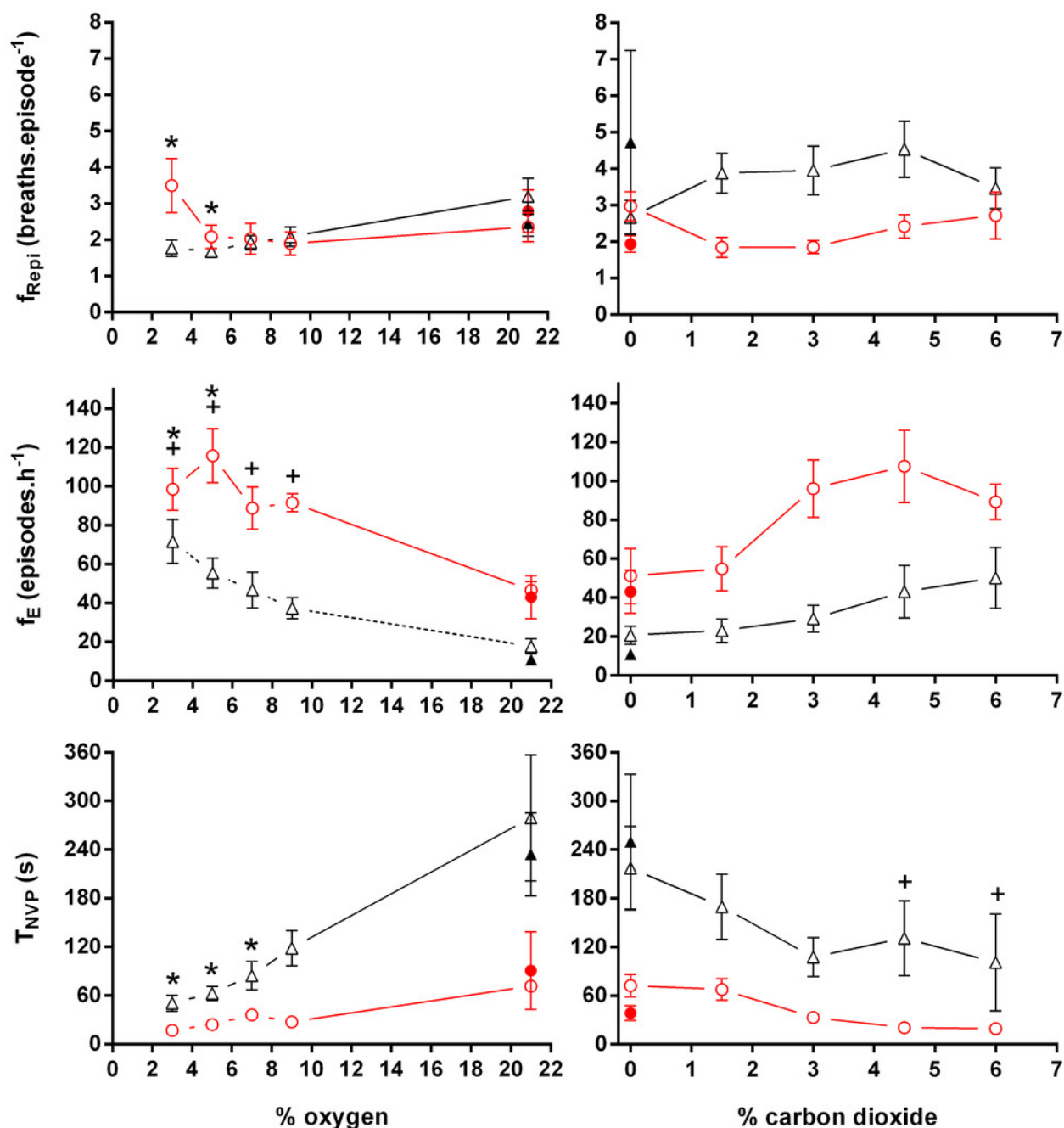


Figure 3

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

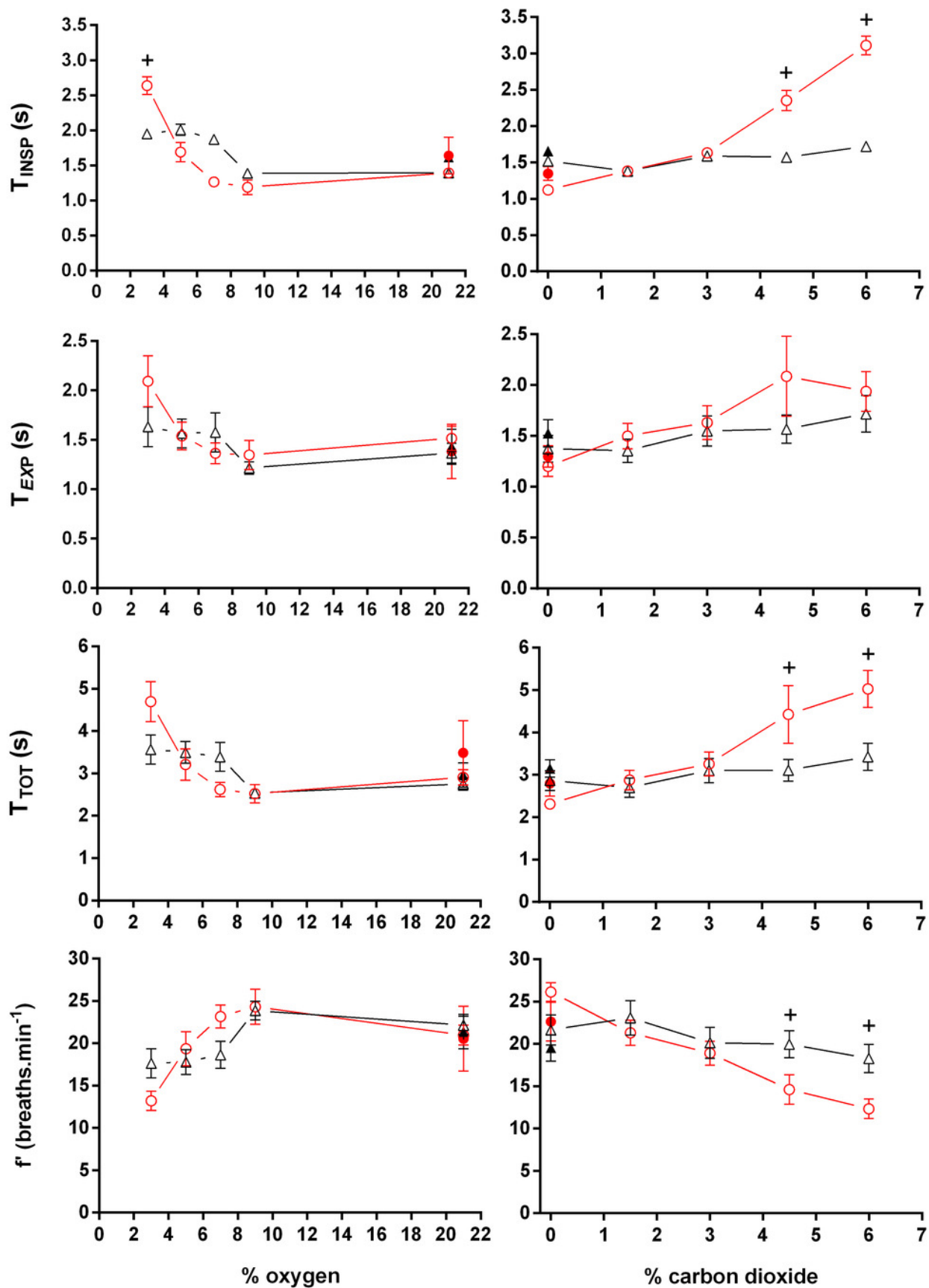


Figure 4

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

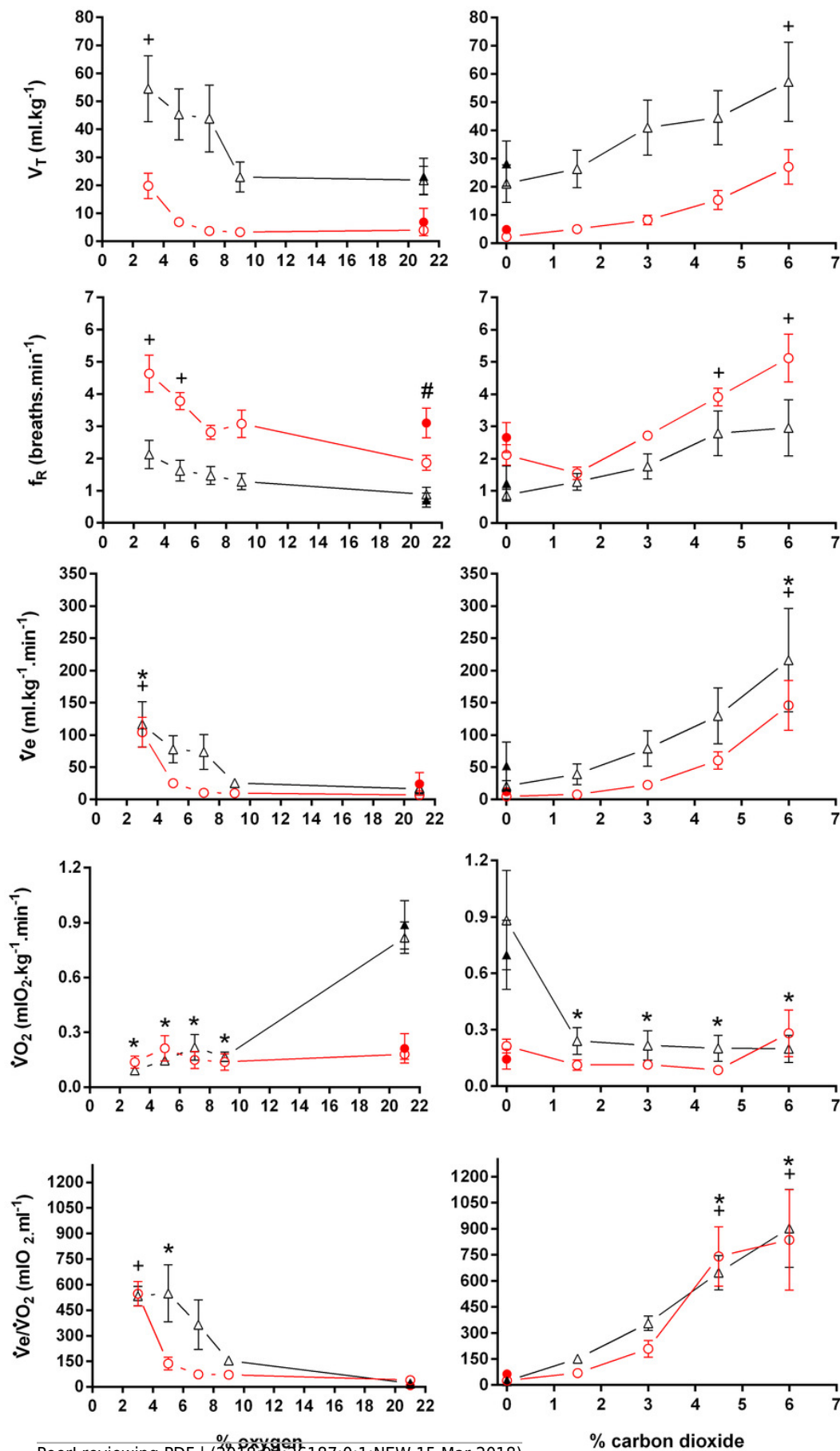


Figure 5

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*

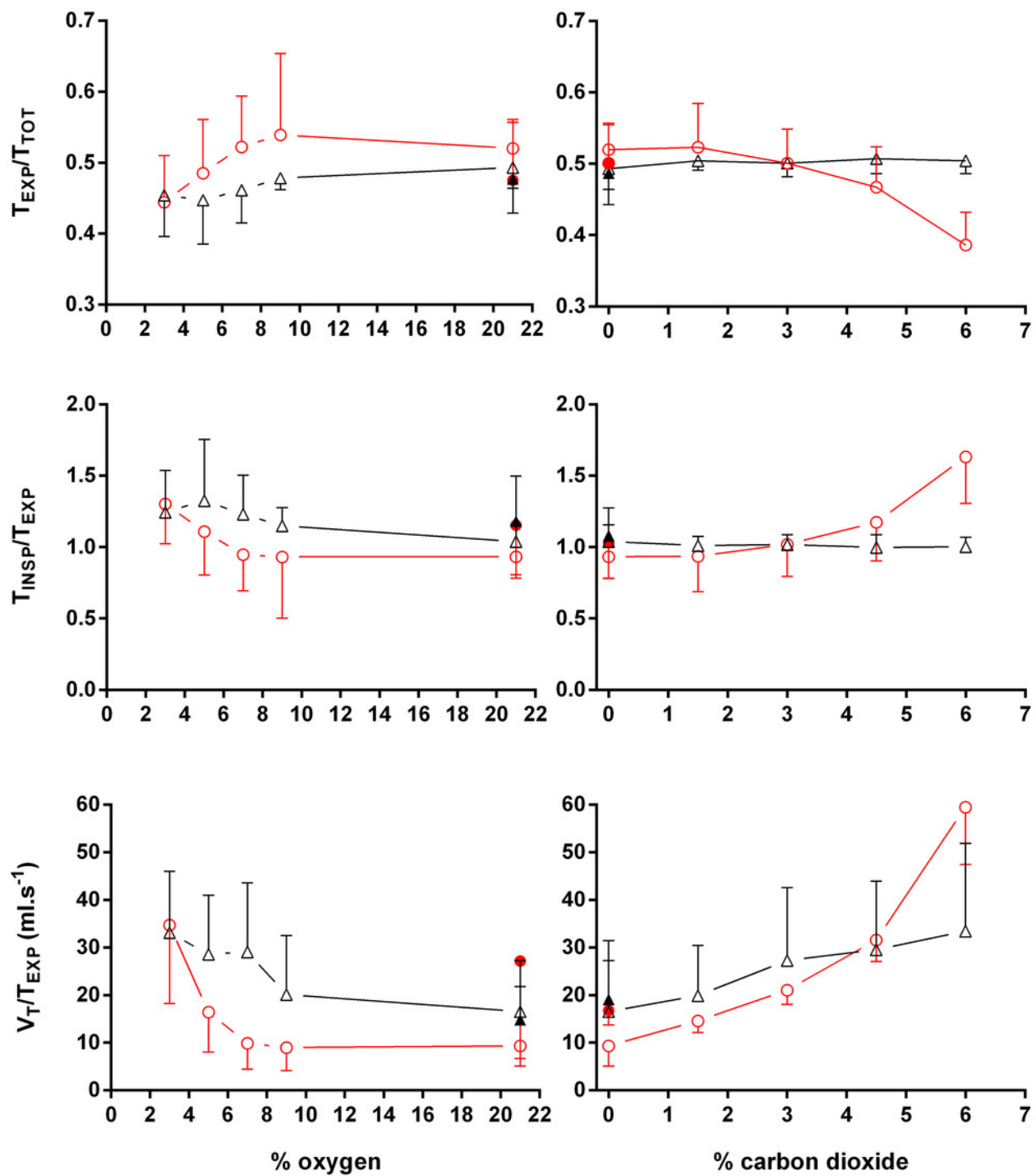


Figure 6

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

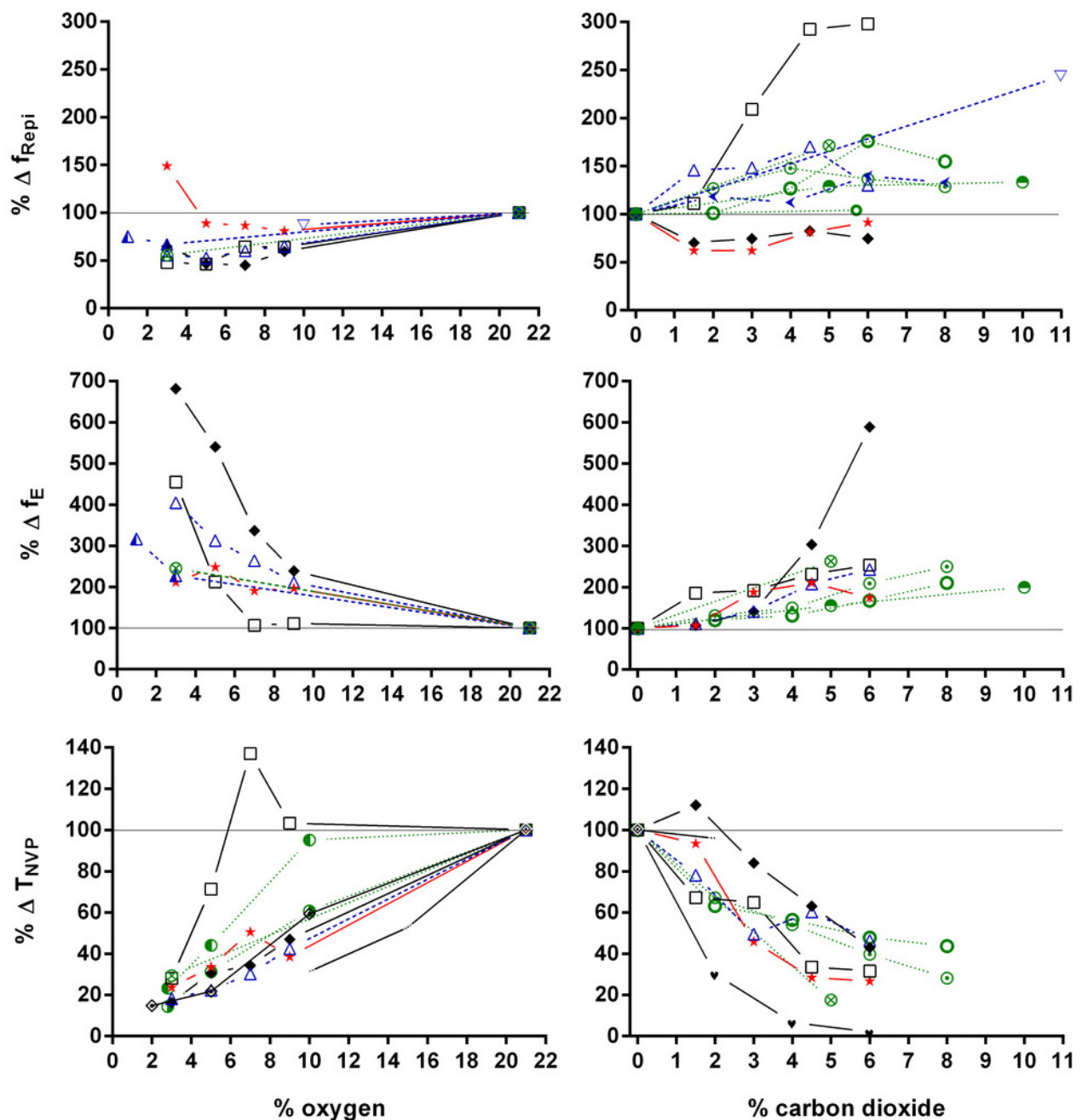


Figure 7

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

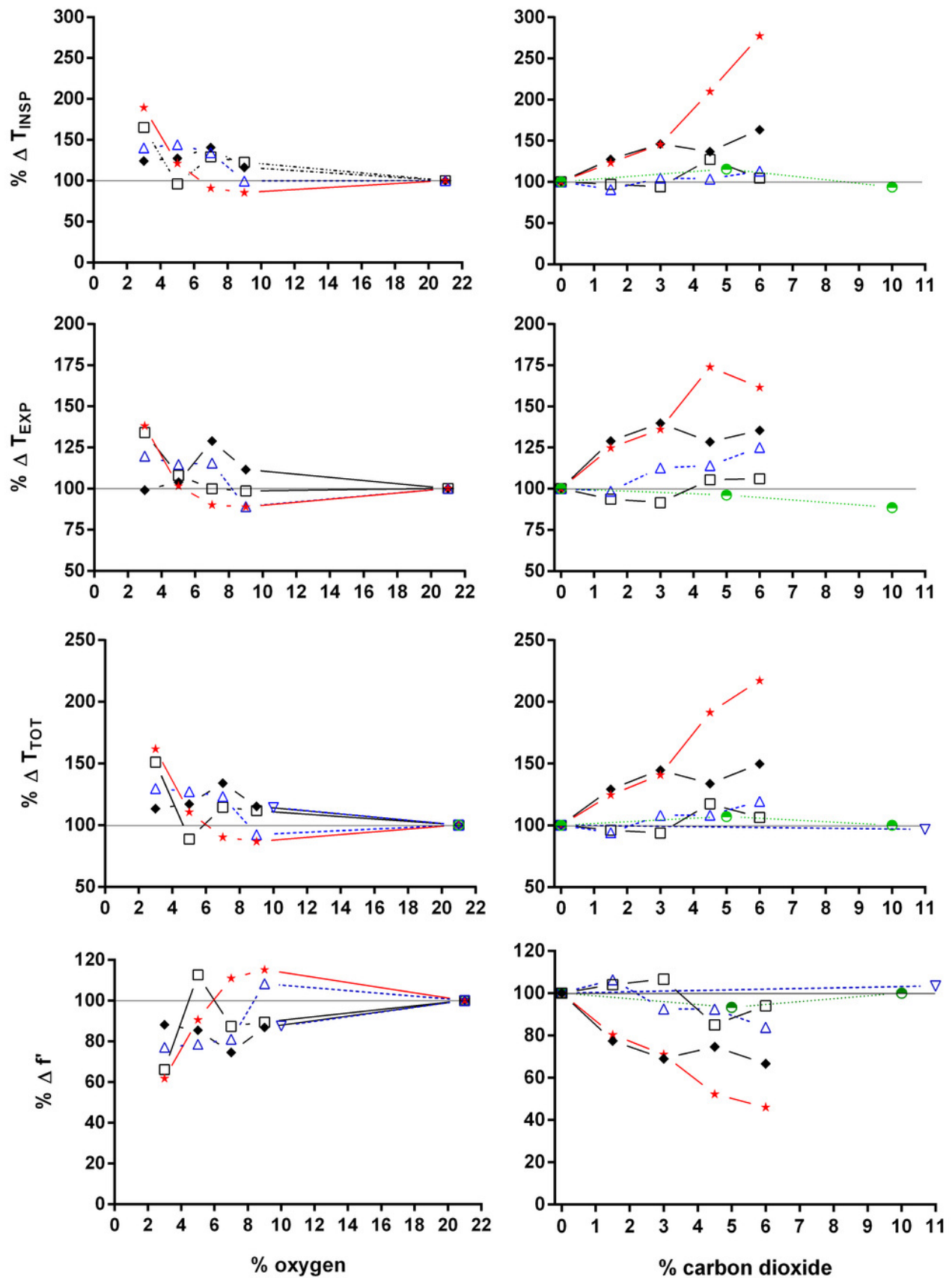


Figure 8

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

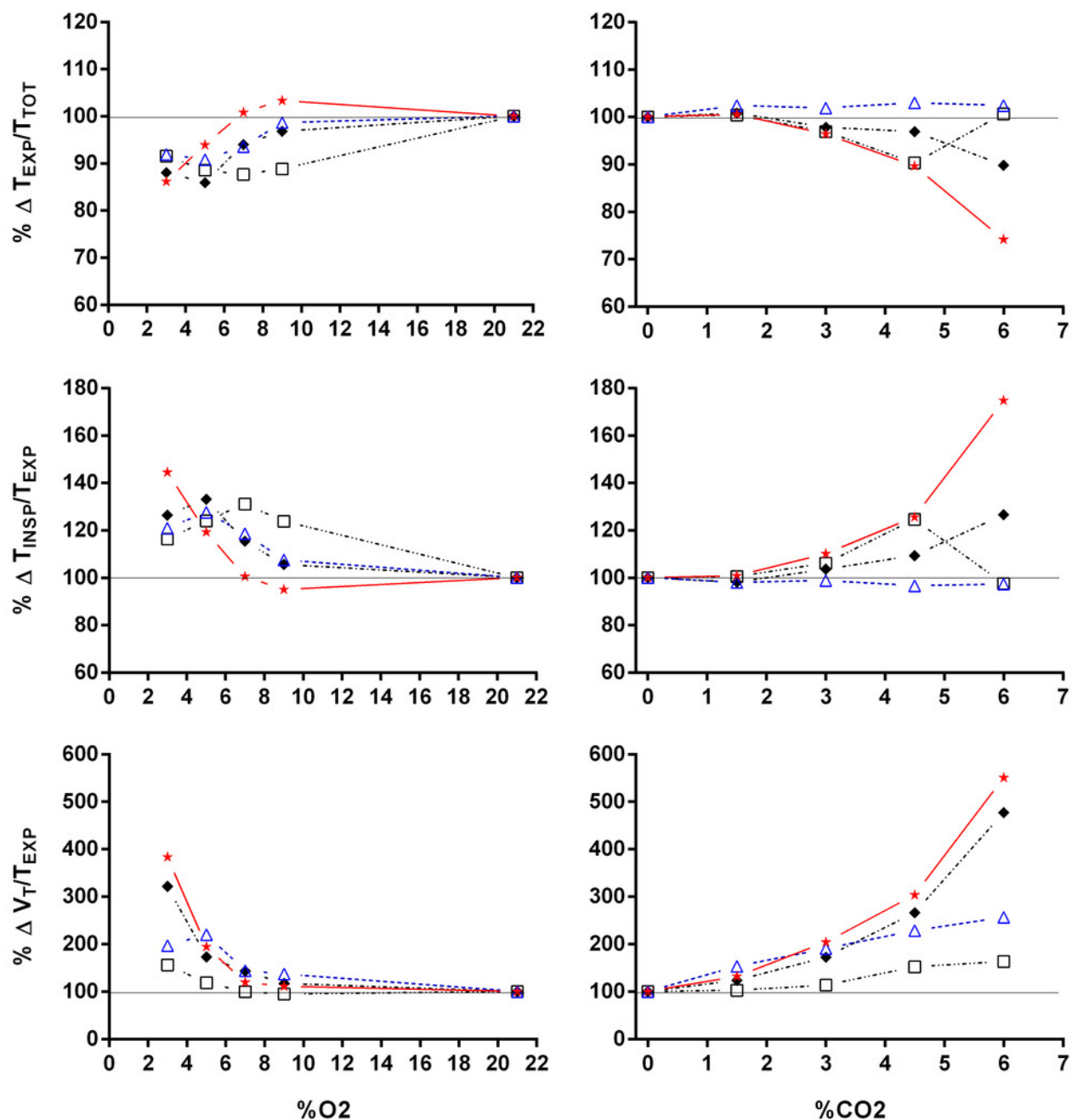


Figure 9

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

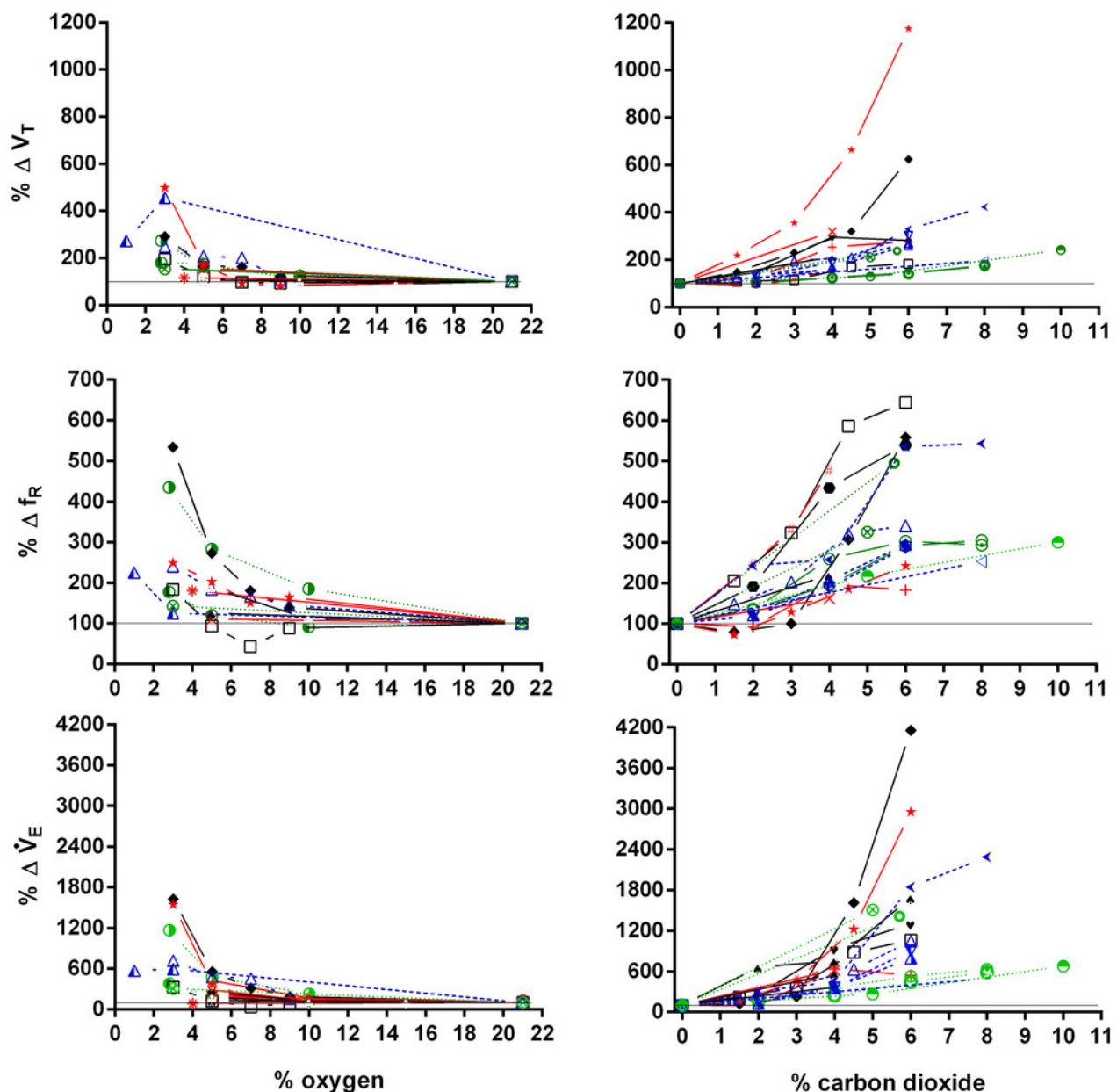


Figure 10

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

