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Daily and seasonal rhythms in the respiratory sensitivity of red-eared sliders (*Trachemys scripta elegans*)

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SUMMARY

The purpose of the present study was to determine whether the daily and seasonal changes in ventilation and breathing pattern previously documented in red-eared sliders resulted solely from daily and seasonal oscillations in metabolism or also from changes in chemoreflex sensitivity. Turtles were exposed to natural environmental conditions over a one year period. In each season, oxygen consumption, ventilation and breathing pattern were measured continuously for 24 h while turtles were breathing air and for 24 h while they were breathing a hypoxic–hypercapnic gas mixture (H–H). We found that oxygen consumption was reduced equally during the day and night under H–H in all seasons except spring. Ventilation was stimulated by H–H but the magnitude of the response was always less at night. On average, it was also less in the winter and greater in the reproductive season. The data indicate that the day–night differences in ventilation and breathing pattern seen previously resulted from daily changes in chemoreflex sensitivity whereas the seasonal changes were strictly due to changes in metabolism. Regardless of mechanism, the changes resulted in longer apneas at night and in the winter at any given level of total ventilation, facilitating longer submergence at times of the day and year when turtles are most vulnerable.

Key words: circadian rhythms, circannual rhythms, respiratory sensitivity, chemoreflexes, turtles.

INTRODUCTION

Circadian and circannual rhythms time biological processes so that specific events take place at appropriate times of the day or year (Underwood, 1992; Tosini et al., 2001; Mortola and Seifert, 2002). Such temporal changes in physiological variables may be particularly crucial for the survival of temperate species, which are subjected to large daily and seasonal variations in their environment (changes in day length and temperature cycles). We previously showed that endogenous circadian and circannual oscillations were present in the metabolism and ventilation of red-eared sliders (*Trachemys scripta elegans* Wied) (Reyes and Milsom, 2009), and were accompanied by changes in breathing pattern that resulted in longer apneas at night and during colder seasons. We speculated that by reducing trips to the surface to breathe, turtles could reduce the cost of locomotion, risk of predation (Cagle, 1950) and, potentially, the cost of breathing (Vitalis and Milsom, 1986) during dormancy.

Little is known about the mechanisms that generate the long apneas that are common to arrhythmic breathing in turtles. Endogenous circadian and circannual oscillations in apnea duration could reflect oscillations in metabolic rate (the need to breathe) or in chemoreflex sensitivity (the drive to breathe). Chemoreflexes are thought to be important in initiating and terminating periods of apnea (Shelton et al., 1986; Milsom, 1990), but the few studies of circadian and circannual variation in chemoreflex responses, performed in mammals and amphibians, have not always observed rhythms in chemoreflex sensitivity (McArthur and Milsom, 1991; Milsom et al., 1993; Peever and Stephenson, 1997; Rocha and Branco, 1998; Bicego-Nahas and Branco, 1999; Mortola and Seifert, 2000; Stephenson et al., 2000; Bicego-Nahas et al., 2001; Mortola and Seifert, 2002; Seifert and Mortola, 2002a; Seifert and Mortola, 2002b; Mortola, 2004).

In the present study, we sought to determine whether daily and seasonal oscillations in chemosensitivity *per se* [defined as the increase in ventilation of turtles breathing a hypoxic–hypercapnic (H–H) gas, after correcting for any changes in metabolism, i.e. the change in air convection requirement; $\Delta ACR=\Delta\dot{V}_E/\Delta\dot{V}_{O_2}$, where $\dot{V}_E$ is ventilation and $\dot{V}_{O_2}$ is oxygen consumption] could be involved in producing the daily and seasonal changes in ventilation and breathing pattern previously reported in red-eared sliders (Reyes and Milsom, 2009). We hypothesized that endogenous rhythms in chemosensitivity contribute to the daily and seasonal oscillations observed in the breathing pattern of red-eared sliders, allowing turtles to remain submerged longer at night and in the winter, which may have implications on their survival.

MATERIALS AND METHODS

Red-eared slider turtles (*Trachemys scripta elegans* Wied) (mean mass=1.09±0.35 kg, N=8) were obtained from commercial suppliers (Lemberger Company, Wisconsin and Sullivan Company, TN, USA) and were housed in a semi-natural pond (2.9×1.9×0.6 m, containing 3.3 m^3 of water), where they experienced the environmental temperature and natural photoperiod of Vancouver (BC, Canada) throughout the year. Two temperature loggers (DS1921 Thermocron iButton, Dallas Semiconductor Corporation, Sunnyvale, CA, USA) were placed on the basking area (1.25×1.9 m) in the shade and submerged in the pond to record ambient and water temperatures, respectively, every hour for one year. Prior to each trial, temperatures recorded by the data loggers were averaged to determine the experimental seasonal temperature. Daily photoperiod and rainfall were obtained from the Meteorological Service of Canada website. The holding and experimental procedures followed Canadian Council on Animal Care guidelines and were approved

Table 1. Temperature and photoperiod used in the experimental series

Seasonal cues		
	Treatment	
Season	Photoperiod	Temperature
Winter	9 h:15 h L:D	9°C
Spring	14 h:10 h L:D	14.6°C
Summer	16 h:8 h L:D	20.8°C
Autumn	10 h:14 h L:D	14.7°C
Constant darkness (outdoor turtles)	0 h:24 h L:D	13.6°C

by the University of British Columbia Animal Care Committee (animal care certificate No. A041006).

Experiments were carried out over a 12 month period (October to October) on unrestrained turtles that were fasted for seven days. In each season turtles were removed from the semi-natural pond and placed in an experimental tank (1.22×0.49×0.64 m) for four days, at the mean seasonal temperature (same day and night water temperatures) and photoperiod prevailing in that season (Table 1). The tank was placed inside a wooden box to insulate the turtle from external stimuli (noise and light). Full spectrum lights were set with a timer to control the light and dark cycle as we have previously described (Reyes and Milsom, 2009), and turtles were acclimated for two days to adjust to the handling stress, the experimental tank and the lack of a thermal cycle. On the first day following acclimation turtles were given humidified air to breathe for 24 h. On the second day after acclimation turtles were given a H–H gas mixture (8% oxygen–3% CO_2) to breathe for 24 h. Water temperature in the experimental tank was recorded every hour for the length of the experimental treatment with a temperature logger (DS1921 Thermocron iButton, Dallas Semiconductor Corporation) to ensure that temperature remained steady during the experimental trial.

To determine the effects of removing all cues that could give any indication of the time of day, the same protocol was repeated in the autumn, except that turtles were maintained under constant darkness for four days. The water temperature used for this experiment was 13.6°C. Day and night differences measured in the autumn under the natural photoperiod were compared with the corresponding day and night values measured under constant darkness.

Measurement of metabolic rate and ventilation

$\dot{V}_{O_2}$ and $\dot{V}_E$ were measured simultaneously using open-flow respirometry (for details, see Reyes and Milsom, 2009). Air or the H–H gas mixture was delivered through a port in the side of a breathing funnel at a rate of 350 ml min^{-1}. The flow rate was regulated with a gas mixing flow meter (Cameron Instrument Company, Port Aransas, TX, USA). The air/gas mixtures entering and leaving the funnel were dried and sampled by a gas analyzer (model 222A version 1.02, Raytech Instruments, Vancouver, BC, Canada). $\dot{V}_{O_2}$ rates were calculated from the flow rate and the difference in fractional concentrations of oxygen between inflow and outflow gas. Rates of $\dot{V}_{O_2}$ are expressed as ml O_2 STPD min^{-1} kg^{-1} (where STPD is standard temperature and pressure, dry).

$\dot{V}_E$ was monitored at the outflow of the breathing funnel with a pneumotachograph (Fleisch, Richmond, VA, USA) attached to a differential pressure transducer (Validyne, Northridge, CA, USA) (Funk et al., 1986). The breathing trace was analyzed for breathing frequency (f_R, breaths min^{-1}) and mean tidal volume (V_T, ml kg^{-1}). Total ventilation ($\dot{V}_E$: $f_R \times V_T$, ml min^{-1} kg^{-1}) was calculated from these values. The components of f_R: number and frequency of breathing episodes, number of breaths in each episode, the instantaneous frequency (the frequency of the breaths within an episode) and the percentage time spent apneic were also calculated. Apnea was defined as a respiratory pause that exceeded the duration of two breaths.

Data analyses

All data were continuously recorded using WINDAQ acquisition software (version 2.19, Dataq Instruments, Akron, OH, USA). Autumn and winter data were analyzed first for every one-hour segment of the 24 h experiment. Based on this, we determined that analyses of data every other hour was sufficient to accurately describe daily rhythms; thus, spring, summer and constant dark runs were analyzed every other hour. Data were averaged over both the daytime and night-time measurements, and over the full 24 h for all turtles (N=6–8) in each season. Segments of breathing traces were removed from the analyses when turtles were active to ensure that breathing and metabolism values used for the analyses were resting rates. Turtle activity produced water movement that induced pressure changes that were sensed by the pneumotacograph (noise). This method was verified by comparing the noise in the breathing trace with 24 h-long video recordings of the turtles during the initial autumn trials.

Calculation of the air convection requirement and chemosensitivity

$\dot{V}_{O_2}$ and $\dot{V}_E$ were used to calculate the ACR (ml air ml^{-1} O_2) for each hour, where ACR=$\dot{V}_E/\dot{V}_{O_2}$.

The H–H ventilatory response was calculated as the absolute change in total ventilation when turtles went from breathing air to breathing the H–H gas mixture ($\Delta\dot{V}_E$). Because our goal was to determine whether ventilatory sensitivity showed endogenous daily and seasonal rhythms (i.e. independent of temperature and metabolism), we eliminated the effects of temperature variation between seasons on our calculations of chemosensitivity using temperature coefficient (Q_{10}) values to correct all data to 14.7°C (the mean annual water temperature). Q_{10} values for $\dot{V}_E$, $\dot{V}_{O_2}$ and ACR were calculated for each turtle under both the air and the H–H treatments. Winter Q_{10} values were calculated by inserting the $\dot{V}_{O_2}$, $\dot{V}_E$ and ACR measured at two different temperatures in this season (19.6°C and 8.8°C, indoor and outdoor temperatures, respectively) into Eqn 1 (Table 2). MR_2 was the variable to be determined (corrected winter $\dot{V}_{O_2}$,

Table 2. Mean ± s.e.m. temperature coefficients (Q_{10}) used to correct values of oxygen consumption, ventilation and air convection requirement measured in the summer and winter to 14.7°C for comparison with other seasons (see Materials and methods for details)

Condition	Season	Q_{10} ($\dot{V}_{O_2}$)	Q_{10}($\dot{V}_E$)	Q_{10} (ACR)
Air	Summer	2.85±0.75	2.13±0.76	0.85±0.18
	Winter	2.11±0.44	1.77±0.38	0.96±0.46
Hypoxia–Hypercapnia	Summer	3.03±0.6	1.06±0.2	0.36±0.11
	Winter	3.54±0.56	2.8±0.61	0.46±0.11

$\dot{V}_{O_2}$, oxygen consumption; $\dot{V}_E$, ventilation and ACR, air convection requirement.

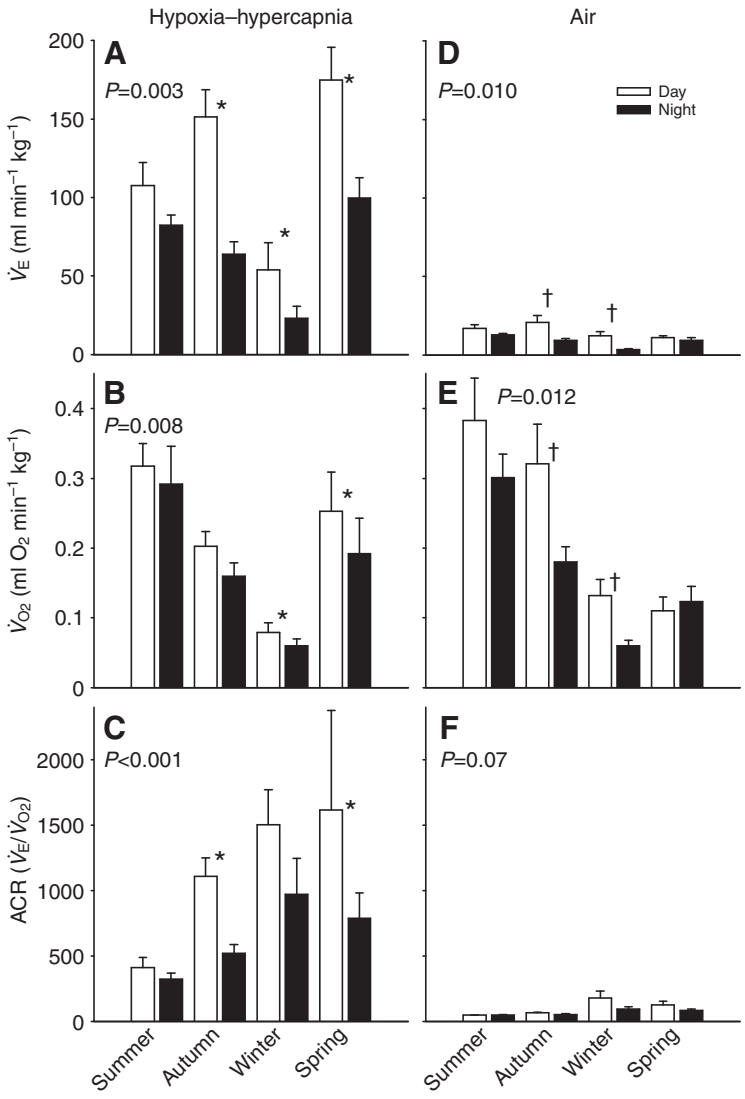

Fig. 1. Day and night values of resting ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}_{O_2}$) and air convection requirement (ACR) ±s.e.m of turtles breathing a hypoxic–hypercapnic (H–H) gas (A–C) and air (D–F) [modified from Reyes and Milsom (Reyes and Milsom, 2009)] in different seasons (N=6 for summer and spring, N=8 for winter and autumn) measured at mean seasonal temperatures and natural photocycle (summer: 20.8°C, 16 h:8 h L:D; autumn: 14.7°C, 10 h:14 h L:D; winter: 9°C, 9 h:15 h L:D; spring: 14.6°C, 14 h:10 h L:D). *Denotes a difference between day and night within seasons under the H–H mixture and † denotes differences between day and night within seasons on air (Holm–Sidak pairwise comparison).

$\dot{V}_E$ and ACR at T_2=14.7°C). MR_1 was the winter value measured at 8.8°C (T_1). As the summer outdoor temperature (20.8°C) was similar to the indoor temperature (19.6°C), we calculated the Q_{10} values by inserting $\dot{V}_{O_2}$, $\dot{V}_E$ and ACR values measured at the summer and autumn outdoor temperatures (20.8°C and 14.71°C, respectively). Autumn values were selected because changes between summer and autumn for all physiological variables were solely the result of changes in temperature (Reyes and Milsom, 2009). Q_{10} values and summer values (MR_2) measured at 20.8°C (T_2) were inserted into Eqn 1 to calculate $\dot{V}_{O_2}$, $\dot{V}_E$ and ACR values (MR_1) at 14.7°C (T_1). Temperature-corrected changes in ventilation ($\Delta\dot{V}_E$), oxygen consumption ($\Delta\dot{V}_{O_2}$) and air convection requirement (ΔACR) were obtained from the temperature-corrected values calculated for $\dot{V}_E$, $\dot{V}_{O_2}$ and ACR under air and the H–H gas:

$$Q_{10} = (MR_2 / MR_1)^{10/(T_2 - T_1)}. \quad (1)$$

We eliminated the effect of changes in metabolism (day–night and seasonal) on chemosensitivity by calculating temperature-corrected changes in ACR when turtles went from breathing air to breathing the H–H gas (ΔACR). Thus, in this study we use changes in ACR calculated from temperature-corrected values of $\Delta\dot{V}_E$ and $\Delta\dot{V}_{O_2}$ as a measure of changes in chemosensitivity.

Statistical analyses

Data are expressed as means ± s.e.m. Differences between day and night, as well as the effects of season on the daily changes were assessed with two-way repeated-measures analysis of variance (RM ANOVA). Differences between seasons (24 h average) were assessed using one-way RM ANOVA. Holm–Sidak multiple comparisons tests were used to determine pairwise differences. Data that did not meet the assumptions of normal distribution or equal variances were natural log (ln) transformed. Sigma Stat (version 3.11, Systat Software, IL, USA) was used for all statistical analyses.

RESULTS

Daily rhythms in the metabolism and breathing of turtles during exposure to H–H

We have previously shown that there are circadian rhythms in $\dot{V}_{O_2}$ and $\dot{V}_E$ in red-eared sliders breathing air (Reyes and Milsom, 2009) (Fig. 1D,E). Here we show for the first time that red-eared sliders also have daily rhythms in $\dot{V}_{O_2}$ and $\dot{V}_E$ while breathing a H–H gas mixture ($\dot{V}_{O_2}$: P=0.008; $\dot{V}_E$: P=0.003, day–night, two-way RM ANOVA) (Fig. 1A,B).

Daytime f_R was higher than night-time f_R in all seasons (P<0.001, day–night, two-way RM ANOVA) (Table 3), while V_T remained

Table 3. Day and night values (±s.e.m.) of tidal volume (ml kg^{-1}), breathing frequency (breaths min^{-1}), frequency of breathing episodes (episodes h^{-1}) and the percentage time spent in apnea for turtles breathing a hypoxic–hypercapnic (H–H) gas and air (Reyes and Milsom, 2009) in different seasons (N=6 for summer and spring, N=8 for winter and fall) and measured at the mean seasonal temperatures and natural photocycle (see Table 1)

		Tidal volume		Breathing frequency		Episodes h^{-1}		% Time in apnea	
Season	Gas/air	Day	Night	Day	Night	Day	Night	Day	Night
Summer	H–H	11.3±0.6	12.7±0.9	9.6±1.2*	6.6±0.6	32.2±3.4	29.9±4.1	74.3±3*	80.9±1.6
	Air	5.5±0.6	6.3±0.7	3.1±0.3	2.1±0.1	22.4±2.9	17.2±1.4	92.5±0.9	94.5±0.4
Autumn	H–H	13.8±0.8	13.1±0.8	10.6±1.3*	4.9±0.6	33.5±5.4*	16.3±3.6	67.7±4.3*	84.4±2
	Air	6.7±0.8	5.7±0.4	2.9±0.3†	1.5±0.2	15.9±1.9†	10.3±1.7	92.4±1.1†	95.9±0.5
Winter	H–H	10.2±2.3	6.8±1.9	3.04±0.7*	1.6±0.4	20.1±4.5*	12.6±4.1	87.6±2.8*	93.5±1.9
	Air	5.4±0.9	3.3±0.7	1.5±0.1†	0.5±0.1	14±2.3†	5±1.5	94.1±0.6†	98±0.6
Spring	H–H	20.3±1.4	19.5±1.3	8.6±1.1*	4.9±0.6	43.1±3.9	28.7±4.5	72.6±3.5*	83.6±2.3
	Air	6.1±0.3	7.99±0.6	1.8±0.2†	1.2±0.2	14.9±1.5†	10.7±2.1	94.9±0.4†	96.6±0.5

*Denotes differences between day and night within seasons under hypoxia–hypercapnia (H–H) and † denotes differences between day and night within seasons on air (Holm–Sidak pairwise comparison).

relatively constant throughout the 24 h in all seasons (P=0.236, day–night, two-way RM ANOVA) (Table 3). Changes in f_R were solely the result of more frequent breathing episodes during the day (P=0.003, day–night, two-way RM ANOVA) (Table 3); the number and frequency of breaths in each episode did not vary with the time of day [P=0.373 and P=0.222, number and frequency of breaths, respectively, two-way RM ANOVA (data not shown)]. Thus, turtles were apneic longer at night than during the day in all seasons (P=0.002, day–night, two-way RM ANOVA) (Table 3).

While day–night differences in the breathing pattern of turtles exposed to the H–H gas were similar to those observed in animals breathing air (Table 3) (Reyes and Milsom, 2009), the daytime increases in breathing under H–H were larger than the increases in $\dot{V}_{O_2}$, giving rise to daily rhythms in ACR (P<0.001, day–night, two-way RM ANOVA) that were not present in animals breathing air (Fig. 1C).

Daily rhythms in chemosensitivity

H–H reduced $\dot{V}_{O_2}$ in the autumn (day: 37%, night: 11%), summer (day: 17%, night: 3%) and winter (day: 40%, night: 0.5%). In spring, however, both daytime and night-time metabolism increased (130% and 56%, respectively) when turtles were breathing the H–H gas. The magnitude of these changes, in absolute terms, was the same during the day and the night (P=0.165, day–night, two-way RM ANOVA) (Fig. 2A).

H–H also caused an increase in $\dot{V}_E$ (summer–day: 536%, summer–night: 538%; autumn–day: 629%, autumn–night: 592%; winter–day: 341%, winter–night: 568%; spring–day: 1496%, spring–night: 990%). This increase was reduced at night in autumn and spring (P<0.05, Holm–Sidak) (Fig. 2B). The ACR increased when animals were breathing the H–H gas (summer–day: 799%, summer–night: 600%; autumn–day: 1627%, autumn–night; 902%; winter–day: 749%, winter–night: 953%; spring–day; 1194%, spring–night: 871%) and this increase in overall ACR (ΔACR: $\Delta\dot{V}_E/\Delta\dot{V}_{O_2}$) varied between day and night across all seasons (P=0.002, day–night, two-way RM ANOVA) (Fig. 2C). Thus, daily changes in $\dot{V}_E$ were not simply due to changes in metabolism but were the result of daily cycles in the sensitivity of chemoreflexes to respiratory stimuli.

Exposure to four days of constant darkness blunted daily oscillations in the chemoreflex response of red-eared sliders. 'Daytime' changes in ventilation ($\Delta\dot{V}_E$) under constant darkness were reduced by 63% from day values under the natural photocycle whereas the decrease in the night ventilatory response was less (21% decrease under constant darkness). As a result no day–night differences in respiratory responses were observed after four days of constant darkness ($\Delta\dot{V}_E$: P=0.118; ΔACR: P=0.480, Holm–Sidak) (Fig. 3).

Seasonal rhythms in the metabolism and breathing of turtles during exposure to H–H

As we have shown for red-eared sliders held in normoxia (Reyes and Milsom, 2009), when turtles were given the H–H gas mixture to breathe they also showed a seasonal cycle in metabolism. We measured the highest rates of $\dot{V}_{O_2}$ in the summer and metabolism decreased seasonally as expected with temperature changes (P<0.001, one-way RM ANOVA) (Fig. 4C). $\dot{V}_E$ showed the same general pattern between seasons as metabolism, except that $\dot{V}_E$ was not as elevated in summer (P=0.013, one-way RM ANOVA) (Fig. 4A). Overall differences in metabolism between seasons seemed to result solely from the effects of temperature on $\dot{V}_{O_2}$, because these differences were not apparent after temperature correction (P=0.266, one-way RM ANOVA, 14.7°C) (Fig. 4D). By contrast, $\dot{V}_E$ remained elevated in the spring and reduced in the winter after correction for temperature (P<0.001, one-way RM ANOVA) (Fig. 4B).

Seasonal changes in the $\dot{V}_{O_2}$ and $\dot{V}_E$ of turtles breathing the H–H gas showed similar trends as those observed in turtles breathing air. Seasonal differences in breathing during H–H were caused by small changes in V_T and large changes in f_R (V_T: P=0.013; f_R: P<0.001, one-way RM ANOVA) (Table 4). f_R in H–H changed due to alterations in the number and frequency of breaths within breathing episodes (P<0.001, one-way RM ANOVA) (Table 4) not due to changes in the frequency of breathing episodes (P=0.076, one-way RM ANOVA) (Table 4). These changes in the breathing pattern resulted in a considerable increase in the time spent in apnea during winter (P<0.001, one-way RM ANOVA) (Table 4).

Because $\dot{V}_E$ and $\dot{V}_{O_2}$ changed largely in parallel, ACR changed very little between seasons. However, ACR was reduced in the summer compared with other seasons (P<0.05, Holm–Sidak) (Fig. 4E). No seasonal differences were found after correcting for temperature (Fig. 4F).

Seasonal rhythms in chemosensitivity

$\dot{V}_{O_2}$ fell when turtles were given the H–H gas instead of air in all seasons (13, 26 and 28% reduced in the summer, autumn and winter,

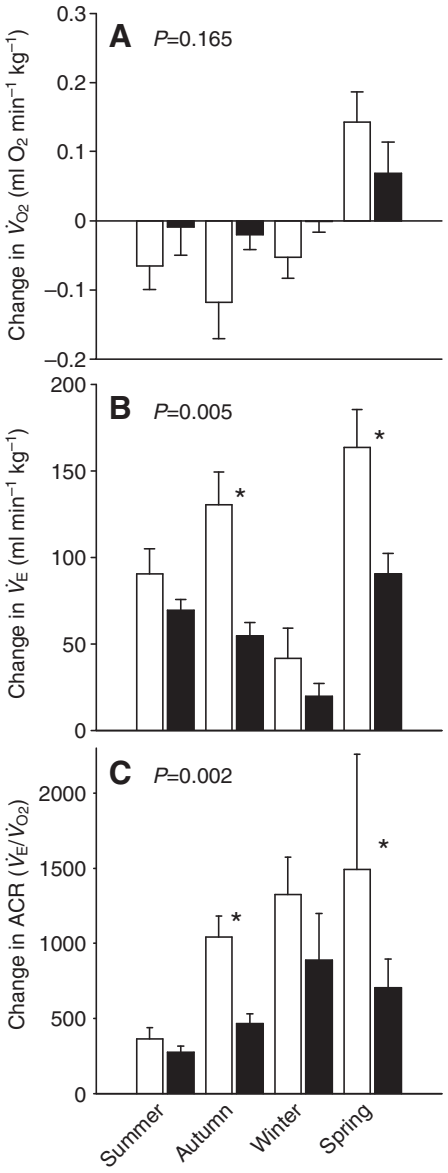

Fig. 2. Mean daytime (open bars) and night-time (closed bars) changes in oxygen consumption ($\Delta\dot{V}_{O_2}$) (A), ventilation ($\Delta\dot{V}_E$) (B) and air convection requirement (ΔACR, the hyperventilatory response, C) as turtles went from breathing air to breathing a hypoxic–hypercapnic (H–H) gas. *P* values reported correspond to overall differences in mean day and night values (two-way RM ANOVA). *Denote day and night differences within seasons (Holm–Sidak pairwise comparison) (*N*=6 for summer and spring, *N*=8 for winter and autumn).

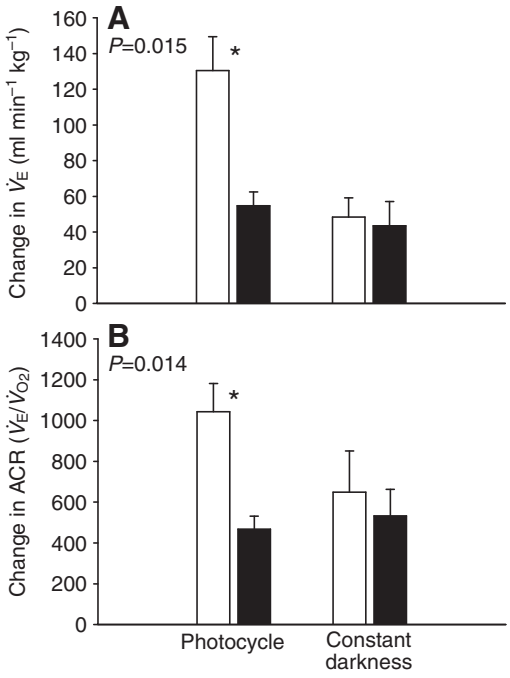

Fig. 3. Comparison of day (open bars) and night (closed bars) changes in ventilation ($\Delta\dot{V}_E$) (A) and air convection requirement (ΔACR) (B) as turtles went from breathing air to breathing a hypoxic–hypercapnic (H–H) gas under the autumn photocycle and constant dark. *P* values reported correspond to overall differences in mean day and night values (two-way RM ANOVA). *Denote day and night differences within the photocycle and constant dark treatments (Holm–Sidak pairwise comparison) (*N*=6 for summer and spring, *N*=8 for winter and autumn).

respectively) (*P*=0.003, one-way RM ANOVA) except spring when metabolism increased by 95% (Fig. 5A). By contrast, $\dot{V}_E$ increased in all seasons during H–H (summer: 539%; autumn: 615%; winter: 416%; spring: 1302%; *P*<0.001) (Fig. 5B) and this increase was greatest in the spring and lowest in the winter. H–H increased the ACR in all seasons (summer: 728.6%; autumn: 1204%; winter: 604%; spring: 1041%; *P*=0.001) but the magnitude of this change was similar in all seasons except summer when it increased the least (Fig. 5C).

To remove the effects of temperature on the metabolism and ventilation of turtles, we then temperature corrected the $\dot{V}_E$ and $\dot{V}_{O_2}$ values measured in all seasons under both H–H and air to 14.7°C using temperature coefficients (Q_{10} values) and used these values to calculate the $\Delta\dot{V}_E$, $\Delta\dot{V}_{O_2}$ and ΔACR. The absolute change in $\dot{V}_{O_2}$ in the spring was significantly different from that in the autumn and summer (*P*<0.05, Holm–Sidak test). Differences between spring and winter were lost, however, after correcting for temperature (*P*>0.05, Holm–Sidak test) (Fig. 6A). Red-eared sliders still showed elevated ventilatory responses to H–H in the spring and reduced ventilatory responses in the winter when temperature effects were removed (*P*<0.001, one-way RM ANOVA) (Fig. 6B). However, after temperature correction, the increase in ventilation relative to metabolism (ΔACR) did not vary between seasons (*P*=0.428, one-way RM ANOVA) (Fig. 6C).

DISCUSSION

The main goal of our present study was to determine whether respiratory chemosensitivity varied daily and/or seasonally in red-eared sliders. Respiratory chemosensitivity is normally defined in mammals as a unit change in $\dot{V}_E$ arising from a unit change in arterial gas partial pressure ($\Delta\dot{V}_E/\Delta Pa_{O_2/CO_2}$). In the present study we did not measure blood gases due to the difficulty of maintaining cannulae open for over a year. As a result we report only the $\Delta\dot{V}_E$ between animals breathing air and the H–H gas (i.e. no units of change in stimulus intensity). In ectotherms, metabolism and temperature can alter the relationship between $\dot{V}_E$ and Pa_{O_2/CO_2}. We therefore corrected for these factors by standardizing $\dot{V}_E$ to $\dot{V}_{O_2}$ (ACR), and also adjusting these values for changes in temperature between seasons. After taking these precautions, we found that the day–night differences in $\dot{V}_E$ resulted from daily changes in chemoreflex sensitivity but that the seasonal changes were directly related to changes in metabolism.

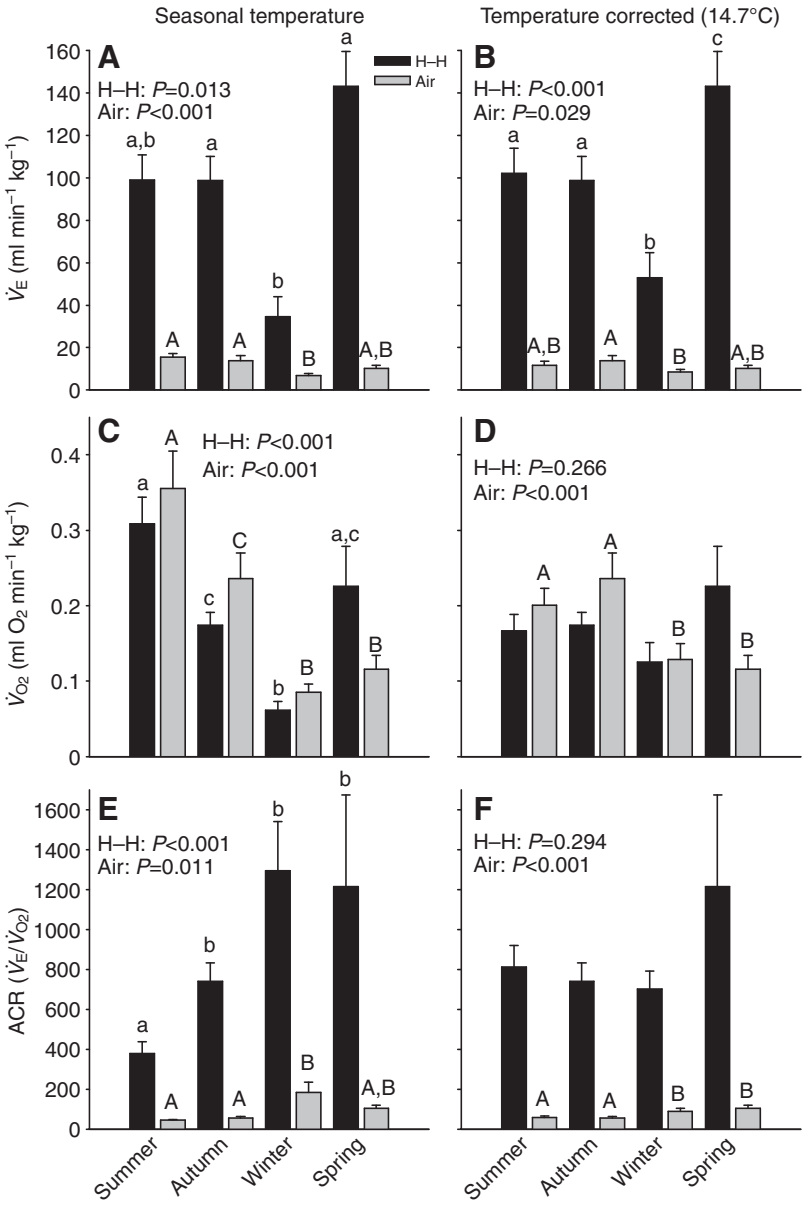

Fig. 4. Ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}_{O_2}$) and air convection requirement (ACR) of turtles breathing a hypoxic–hypercapnic gas (H–H) and air [modified from Reyes and Milsom (Reyes and Milsom, 2009)] in different seasons. Values recorded at seasonal temperatures are given as well as summer and winter values corrected to 14.7°C. Seasonal differences between values measured under H–H are indicated by different lowercase letters. Seasonal differences between values measured in air are indicated by different uppercase letters (N=6 for summer and spring, N=8 for winter and autumn; one-way RM ANOVA).

Daily rhythms in ventilatory sensitivity

Red-eared sliders in our study showed a reduced respiratory response to the H–H stimulus at night compared with the day. Time of the day has been shown to affect the ventilatory response to hypoxia, hypercapnia or both stimuli combined in mammals (Stephenson et al., 2000; Jarsky and Stephenson, 2000; Mortola and Seifert, 2002; Mortola, 2004) and birds (Woodin and Stephenson, 1998), and now we have shown this in reptiles.

The mechanisms underlying day–night changes in ventilatory responses have not yet been elucidated, although interesting species-specific differences have been found. Rats had a higher ventilatory response to hypoxia and hypercapnia at night, when they were active, than during the day. These oscillations in breathing were correlated to daily changes in metabolism and not to changes in chemosensitivity because the hyperventilatory response did not vary between day and night (i.e. there was no %ΔACR between night and day) (Peever and Stephenson, 1997; Mortola and Seifert, 2000; Seifert and Mortola, 2002a; Seifert and Mortola, 2002b). In humans, however, the ventilatory response to hypercapnia was higher in the day and was independent of metabolism or sleep (Stephenson et al., 2000; Mortola and Seifert, 2002; Mortola, 2004), indicative of an endogenous daily cycle in chemosensitivity. In the present study we did not attempt to determine to what extent the day–night differences seen in chemosensitivity were a result of changes in sleep state. State of arousal is known to affect both the hypoxic and hypercapnic ventilatory responses in mammals. Although, the variability between species is large, the general consensus is that the hypercapnic ventilatory response is blunted during sleep and the hypoxic ventilatory response is reduced or unchanged (Phillipson et al., 1978; Bowes et al., 1981; Hunter et al., 1998; Mortola, 2004). Sleep patterns have been described in turtles and lizards (Flanigan, 1973; Flanigan et al., 1974) and periodic reductions in f_R have been reported in pythons and lizards during ‘sleep’ (Donnelly and Woolcock, 1977; Cragg, 1978). Thus, our data support the notion that turtles have a daily rhythm in respiratory sensitivity that is independent of circadian changes in metabolism, activity and temperature but we cannot rule out changes in state. Mortola (Mortola, 2004) suggested that the species difference between rats

Table 4. Mean ± s.e.m. tidal volume (ml kg^{-1}), breathing frequency (breaths min^{-1}), frequency of breathing episodes (episodes h^{-1}), breaths per episode, instantaneous breathing frequency (breaths min^{-1} in an episode) and percentage time spent in apnea of turtles breathing a hypoxic–hypercapnic gas (H–H) and air (Reyes and Milsom, 2009) and exposed to seasonal conditions (see Table 1)

Season	Gas/air	Tidal volume	Breathing frequency	Frequency of episodes	Breaths per episode	Inst. frequency	% Time apnea
Summer	H–H	11.8±0.7	8.6±0.9	31.4±3.3	17.8±2.6	0.60±0.03	76.5±2.4
		a, b	a		a, c	a	a
	Air	5.8±0.6	2.8±0.3	20.6±2.3	8.5±0.8	0.70±0.02	93.2±0.7
		A, B	A	A	A	A	A
Autumn	H–H	13.4±0.7	7.2±0.8	23.3±4.1	26.1±5.4	0.50±0.03	77.8±2.9
		a, b	a		a	a	a
	Air	6.1±0.5	2.1±0.2	12.5±1.6	11.3±2.3	0.60±0.03	94.5±0.7
		A	B	B	A	A	AC
Winter	H–H	8.1±1.8	2.1±0.4	15.4±4.1	5.8±1.4	0.30±0.05	91.3±1.9
		a	b		b	b	b
	Air	4.1±0.6	0.8±0.1	8.4±1.6	3.6±0.4	0.20±0.04	96.6±0.3
		B	C	C	B	B	B
Spring	H–H	19.9±1.2	7.1±0.8	37.1±2.3	13±1.1	0.50±0.03	77.2±2.6
		b	a		bc	a	a
	Air	6.8±0.3	1.5±0.1	13±1.5	7.5±1.1	0.60±0.03	95.6±0.3
		A	B	B	A	A	B, C

Significant differences between seasons (P<0.05, one-way RM ANOVA) are indicated by different lowercase letters (H–H) and uppercase letters (air) (N=6 for summer and spring, N=8 for winter and fall).

and humans could reflect the fact that circadian changes in ventilation are coupled to changes in metabolism in rats where these changes are rapid and sleep state is fragmented but endogenous in humans where the changes in metabolism are small and slow and the sleep cycle is prolonged. Metabolism in turtles also changes slowly, and is further constrained by changes in environmental temperature, perhaps increasing selection pressure on alternate mechanisms to reduce ventilation during periods of inactivity.

Free-running rhythms (constant temperature and D–D or L–L photocycles) in metabolism and ventilation have been shown in the box turtle, garter snake, European green lizard and red-eared slider (Glass et al., 1979; Hicks and Riedesel, 1983; Rismiller and Heldmaier, 1991; Reyes and Milsom, 2009). In the present study we found that four days of constant darkness was sufficient to abolish the day–night difference in chemosensitivity. Interestingly, this was caused by a reduction in the 'daytime' value, while 'night-time' ventilatory sensitivity did not change from the levels seen under the natural photocycle. The same turtles continued to show day–night differences in metabolism and ventilation after three days in constant darkness while breathing air, although the cycles were blunted and the period was slightly shifted (Reyes and Milsom, 2009). The loss in the day–night difference in chemosensitivity after four days of constant darkness does not imply that the daily changes in the physiological variables were not driven by a circadian system. Removal of all environmental cues typically reduces the amplitude of daily rhythms, until the rhythms are lost altogether (Aschoff et al., 1982; Kenagy and Vleck, 1982; Underwood, 1992; Tosini et al., 2001). Tegu lizards have also been shown to lose circadian oscillations in metabolism over time (Milsom et al., 2008). There was large variability in the response of these lizards to constant darkness, with some individuals losing the rhythms after a day in constant darkness and others maintaining the rhythms up to 14 days.

Unfortunately, it is impossible for us to determine where in the reflex arc that underlies the ventilatory response to H–H, changes occurred or even whether the changes were in the hypoxic, hypercapnic or both components of the response. While hypoxia is only sensed by peripheral chemoreceptors, hypercapnia stimulates both central and peripheral chemoreceptors, and the central brainstem sites involved in processing both stimuli are different (Shelton and Boutilier, 1982; Shelton et al., 1986; Milsom, 1990). We used a H–H gas mixture, however, to mimic the respiratory stimuli that turtles experience during submergence (Burggren and Shelton, 1979; Shelton and Boutilier, 1982).

Regardless of the mechanism behind the daily changes in chemosensitivity of turtles, it is clear that night-time reductions in chemosensitivity should reduce sensitivity to a fall in oxygen stores and an accumulation of CO_2 in the blood, and thus allow for longer dives. This appears to be true for other diving species as well. Thus, canvasback ducks showed reduced chemosensitivity to progressive asphyxia during the night-time that was independent of metabolism. These animals forage underwater at night and increasing the level of blood gases at which chemoreflexes are elicited at this time was suggested to allow them to prolong their dives and foraging times (Wooding and Stephenson, 1998). In red-eared sliders, the endogenous circadian rhythms in metabolism (lower at night) (Reyes and Milsom, 2009) along with daily oscillations in chemoreflex responses (independent of metabolism) allow these turtles to reduce the locomotion costs and predation risks associated with surfacing to breathe at night.

Seasonal rhythms in ventilatory sensitivity

Red-eared sliders showed seasonal differences in their ventilatory response to H–H: it was reduced in the winter and elevated in the spring. This may not seem surprising because it is well established that sensitivity to hypoxia or hypercapnic–acidosis increase with temperature (Shelton and Boutilier, 1982; Glass et al., 1983; Glass et al., 1985; Funk and Milsom, 1987). In our study, however, seasonal changes in the H–H response remained after the data were temperature corrected, and appeared to have resulted from the effects of the respiratory stimulus on metabolism and not from changes in chemosensitivity, because the change in ACR (ΔACR) did not vary with season.

The reduced ventilatory response during winter coincides with the dormancy period. Temperate reptiles, including red-eared sliders, often undergo active metabolic suppression, when metabolism

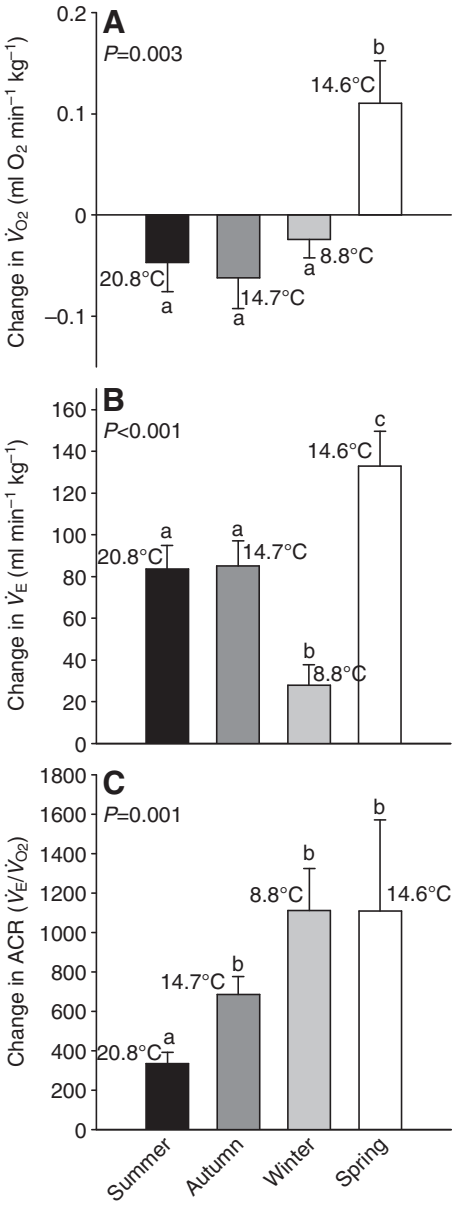

Fig. 5. Changes in oxygen consumption ($\Delta\dot{V}_{O_2}$) (A), ventilation ($\Delta\dot{V}_E$) (B) and air convection requirement (ΔACR) (C) as turtles went from breathing air to breathing a hypoxic–hypercapnic (H–H) gas in different seasons. Significant differences between seasons (P<0.05, one-way RM ANOVA) are indicated by different letters (N=6 for summer and spring, N=8 for winter and autumn).

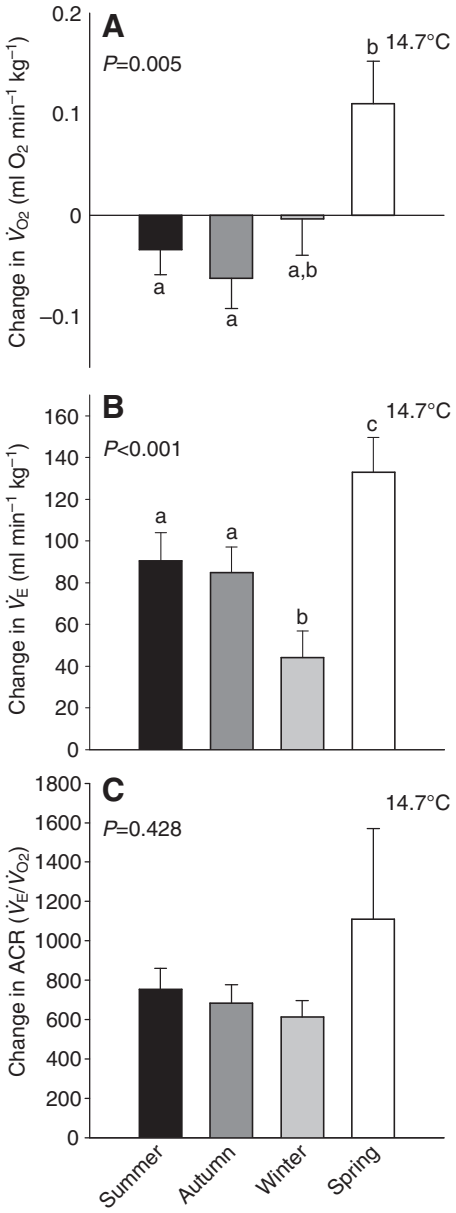

Fig. 6. Temperature-corrected (14.7°C) changes in oxygen consumption ($\Delta\dot{V}_{O_2}$) (A), ventilation ($\Delta\dot{V}_E$) (B) and air convection requirement (ΔACR) (C) as turtles went from breathing air to breathing a hypoxic–hypercapnic (H–H) gas in different seasons. Significant differences between seasons (P<0.05, one-way RM ANOVA) are indicated by different letters (N=6 for summer and spring, N=8 for winter and autumn).

cannot be sustained under winter environmental conditions (Mayhew, 1965; Bennett and Dawson, 1976; Gregory, 1982; Ultsch, 1989; Zari, 1999). Toads and bullfrogs also show reduced metabolism and a lower ventilatory response to hypoxia [toads (Bicego-Nahas et al., 2001)] and hypercapnia [bullfrogs (Bicego-Nahas and Branco, 1999)] in the winter. It would appear that seasonal reductions in both, the temperature-corrected resting ventilation and in the ventilatory response to H–H of red-eared sliders were due to the orchestrated fall in metabolism.

Short photoperiods in the autumn trigger behaviors such as fasting and selection of lower temperatures (Cagle 1950; Gregory, 1982; Rismiller and Heldmaier, 1982), which will slowly reduce metabolism in preparation for dormancy (Bennett and Dawson, 1976). Turtles in our study underwent anorexia in the late autumn and did not eat in the winter. For many reptiles, the highest metabolic rates occur after feeding, with metabolism falling dramatically during fasting. Furthermore, during long periods of fasting the digestive system atrophies removing the daily cost of regeneration (Wang et al., 2001). Although reduced gut mass and lack of feeding may have influenced winter metabolism, the seasonal trends in $\dot{V}_{O_2}$ cannot be solely explained by changes in feeding regime. Turtles in our study stopped eating voluntarily in mid–late autumn and this continued throughout the winter. Thus, if feeding were important, temperature-corrected metabolism in the autumn (in animals

breathing air) should also have been partially reduced and this was not the case (Fig. 4C,D).

$\dot{V}_E$ increased in all seasons with exposure to H–H, which agrees with other studies on reptiles (Jackson, 1973; Jackson et al., 1974; Glass et al., 1985; Shelton et al., 1986). However, the increase in spring $\dot{V}_E$ was larger than that in all other seasons even after temperature correction. Red-eared sliders mate during the spring (Cagle, 1950; Duvall et al., 1982; Ernst and Barbour, 1989; Kuchling, 1999) and hormones involved in reproduction may be the source of the elevated metabolism and the higher ventilatory response during this season (Tatsumi et al., 1997). Higher metabolic rates during oogenesis and mating have also been reported in *Lacerta viridis* (Rismiller and Heldmaier, 1991).

Paradoxically, while the ventilatory response to H–H was greatest in the spring, resting metabolism and ventilation in animals breathing air were very low (Fig. 4) (Reyes and Milsom, 2009) remaining at winter levels. In all cases, however, the changes in ventilation and metabolism occurred in parallel, suggesting that the spring time increases in the H–H ventilatory response were a result of spring time increases in the effects of H–H on metabolism and not the result of seasonal changes in chemosensitivity. The reasons for this are not clear. Hypoxia and/or hypercapnia generally suppress metabolism, if anything, and this was the trend that was seen in all other seasons. It is possible that cause and effect are reversed here and that metabolism increases because of the very large increase in ventilation rather than *vice versa*, but estimates of the oxidative cost of ventilation in turtles remain controversial. Estimates of the relative metabolic cost of breathing based on the elimination of breathing by unidirectional ventilation (Kinney and White, 1977) or based on changes in ventilation and metabolism associated with hypoxic exposure (Wang and Warburton, 1995; Skovgaard and Wang, 2004) suggest that the costs could be as high as 15–20% of resting metabolism. Estimates based on changes in ventilation and metabolism associated with hypercapnic exposure, however, suggest the costs are extremely small and often overshadowed by an acidosis-induced metabolic suppression leading to a paradoxical decrease in metabolism with an increase in breathing (Wang and Warburton, 1995; Skovgaard and Wang, 2004). This issue remains to be resolved, as does the source of the increase in metabolism described here.

Conclusion

What we have measured in this study is the response to a fixed stimulus applied at the lungs. The stimuli for the chemoreceptors involved in ventilatory regulation are the arterial blood gases ($P_{a_{O_2}}$, $P_{a_{CO_2}}$ and pH) at the peripheral receptors and the $P_{a_{CO_2}}$/pH of the cerebral spinal fluid (CSF); however, these were not measured. As a result, the changes in chemosensitivity that we describe may have been due to changes in the sensitivity of the peripheral arterial chemoreceptors *per se* or to changes in the $P_{a_{O_2}}/P_{a_{CO_2}}$/pH at receptor sites. The latter could occur due to daily and/or seasonal changes in the magnitude of the cardiac shunt that is known to be large in turtles. There were seasonal changes in temperature that are also known to give rise to changes in the ACR and hence to resting blood gas tensions. To add to the complexity, similar changes in baseline blood gas tensions initiated from different baseline blood gas levels may give rise to different responses due to the non-linearity of the hypoxic ventilatory response (the same change in blood gases may not amount to the same change in stimulus when starting from different baselines) (Glass, 1992). Finally, even when no changes were seen in chemosensitivity as a function of season, this could have been due to seasonal changes in cardiac shunting acting to maintain constant blood gas tensions (Wang and Hicks, 1996; Wang et al., 1997). Any such changes, however, are part of daily and seasonal rhythms. In this study we are looking at the whole animal output for a given environmental input and determining the mechanistic basis of these changes will be the next challenge.

Mechanism aside, red-eared sliders showed reduced respiratory chemosensitivity at night and in the winter, and enhanced chemosensitivity in the spring. Day and night differences resulted from daily oscillations in the sensitivity of chemoreflexes whereas seasonal differences could be explained by the effects of the H–H stimuli on metabolism. Regardless of the different mechanisms, daily and seasonal changes in the ventilatory response together with circadian and circannual rhythms in metabolism (Reyes and Milsom, 2009) benefit turtles by facilitating longer apneas. These physiological changes will reduce time at the surface and may be important for minimizing the cost of locomotion at times when the cost of surfacing or risk of predation may compromise their survival.

LIST OF ABBREVIATIONS

ACR	air convection requirement
f_R	breathing frequency
H–H	hypoxic–hypercapnic
$P_{a_{CO_2}}$	arterial CO_2 pressure
$P_{a_{O_2}}$	arterial O_2 pressure
Q_{10}	temperature coefficient quotient
RM ANOVA	repeated-measures analysis of variance
$\dot{V}_E$	total ventilation
$\dot{V}_{O_2}$	oxygen consumption
V_T	tidal volume

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