Measuring the oxidative cost of breathing:
A comparison of methods using red-eared sliders
(Trachemys scripta elegans)

by

Stella Yim Jung Lee

B. Sc. University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

January 2011

© Stella Yim Jung Lee, 2011
Abstract

There is a metabolic cost associated with the work required to overcome the elastic and resistive forces associated with breathing. These forces vary widely as a function of the anatomy of the lungs and body wall in different species. In turtles, the lungs are relatively compliant but the body wall with the carapace is very stiff. Studies designed to measure the cost of ventilation in turtles have proven to be difficult. Two different methods have been used to estimate costs and each method has produced a different result. In an attempt to resolve this controversy, the present study obtained data using three different methods in a single group of turtles; a data regression method, a unidirectional ventilation method (UDV), and a hybrid method combining data regression and unidirectional ventilation. All three methods produced highly variable results (individual variability, differences between use of hypoxia and hypercapnia, evidence of hypoxia and hypercapnia-induced metabolic suppression). Based on data plotted for individual animals versus groups of animals and using all data points versus mean values for different levels of inspired gases, oxidative costs were obtained ranging from -0.0005 to 0.022 ml O$_2$/ml air ventilated using the regression method, 0.014 ml O$_2$/ml air for the UDV method and 0.025 ml O$_2$/ml air for the hybrid method. Most values were high compared to a theoretical estimate of 0.0003 ml O$_2$/ml air obtained based on measurements of mechanical work of breathing and an assumed respiratory muscle efficiency of 10%. The experimental results from the two methods appeared to be different as a result of CO$_2$-induced metabolic rate suppression or the non-linearity of the oxygen uptake-ventilation relationship. However, assuming the theoretical estimate best represents the oxygen uptake-ventilation relationship and the true oxidative costs of
ventilation, neither theory would fit. Instead, attributing the discrepancy in results to the
differences in the breathing patterns and their irregularity provided the most logical
explanation and resulted in the conclusion that the oxidative cost of ventilation in turtles
to be similar to that of mammals.
Table of contents

Abstract .............................................................................................................................. ii
Table of contents .............................................................................................................. iv
List of tables .................................................................................................................... vi
List of figures ................................................................................................................... vii
Acknowledgements ........................................................................................................ viii
Dedication ....................................................................................................................... x

1. General introduction .................................................................................................... 1
   1.1. Mechanism of ventilation .................................................................................... 1
   1.2. Mechanics of ventilation ..................................................................................... 5
   1.3. Mechanical cost of ventilation ............................................................................. 7
   1.4. Patterns of ventilation ....................................................................................... 8
   1.5. Oxidative cost of ventilation .............................................................................. 9
       1.5.1. Turtles ....................................................................................................... 10
       1.5.2. Other reptilian species ............................................................................. 11
   1.6. Aim of the thesis .............................................................................................. 11

2. Determining ventilatory cost: comparison of three methods .................................. 16
   2.1. Introduction .................................................................................................... 16
   2.2. Materials and methods .................................................................................... 19
       2.2.1. Animals ................................................................................................... 19
       2.2.2. Series 1 (regression method) .................................................................... 19
       2.2.3. Series 2 (unidirectional ventilation (UDV) method) .................................. 21
       2.2.4. Series 3 (the “hybrid” method) ................................................................. 23
       2.2.5. Theoretical estimation ............................................................................. 23
       2.2.6. Data analysis ........................................................................................... 24
   2.3. Results ............................................................................................................. 31
       2.3.1. Overall response to respiratory stimuli ..................................................... 31
       2.3.2. Sample length variation .......................................................................... 32
       2.3.3. Mean data ............................................................................................... 32
       2.3.4. Individual data ......................................................................................... 33
       2.3.5. Theoretical calculations ......................................................................... 35
   2.4. Discussion ....................................................................................................... 46
       2.4.1. Overall response to respiratory stimuli ..................................................... 46
       2.4.2. Relative versus absolute cost .................................................................. 46
       2.4.3. Mean data ............................................................................................... 47
       2.4.4. Individual data ......................................................................................... 50
       2.4.5. Theoretical estimate .............................................................................. 52
       2.4.6. Applications of the research findings ...................................................... 54

3. Conclusion .............................................................................................................. 63
   3.1. Summary ......................................................................................................... 63
   3.2. Shortcomings and future directions ................................................................. 64
3.3. Overall conclusion ................................................................................................. 65

Bibliography .................................................................................................................... 66
List of tables

Table 2.1. Comparison of respiratory variables for semi-aquatic turtles taken from the literature ............................................................................................................................ 58
Table 2.2. Comparison of calculated oxidative cost values between different methods .. 60
Table 2.3. Comparison of calculated oxidative cost values using the regression method taken from the literature .................................................................................................... 61
Table 2.4. Comparison of theoretically estimated relative oxidative cost of breathing for semi-aquatic turtles from the literature ................................................................. 62
List of figures

Figure 1.1. An illustration of the steps in ventilation ....................................................... 13
Figure 1.2. Minute work at various levels of ventilation.................................................. 14
Figure 1.3. Work at constant minute ventilation across breathing frequency range....... 15

Figure 2.1. Experimental set-up: regression method ........................................................ 28
Figure 2.2. Experimental set-up: unidirectional ventilation method ............................... 29
Figure 2.3. Experimental set-up: hybrid method ............................................................. 30
Figure 2.4. Mean values of number of breaths per episode and episode frequency ...... 37
Figure 2.5. Changes in overall breathing pattern in response to respiratory stimuli ...... 38
Figure 2.6. Re-evaluation of ventilation and O₂ consumption from normoxic traces .... 38
Figure 2.7. Control values of resting, spontaneously ventilating turtles (normoxia) ...... 39
Figure 2.8. Three different groups of mean data (UDV trial, hypoxia and hypercapnia-regression trials) ................................................................................................................ 40
Figure 2.9. Mean data of UDV and regression trials (a single plot)................................. 41
Figure 2.10. O₂ consumption vs. ventilation plots for individual data ......................... 42
Figure 2.11. All individual data (a single plot)................................................................ 43
Figure 2.12. Three-dimensional mesh plots of work vs. instantaneous frequency vs. tidal volume ....................................................................................................................... 44
Figure 2.13. A theoretical O₂ consumption vs. ventilation plot derived from the three-dimensional mesh plots................................................................. 45
Figure 2.14. An example of the lack of correlation between apnea and breathing episode length .................................................................................................................. 56
Figure 2.15. O₂ consumption vs. instantaneous frequency at three different levels of ventilation ...................................................................................................................... 57
Acknowledgements

First and foremost, I’d like to thank my supervisor, Dr. William Milsom. He gave me a chance to experience the field of research and opened the eyes of an ignorant undergraduate student to a whole new world. His knowledge and expertise provided a learning atmosphere with new challenges that was always followed by a sense of accomplishment. I would also like to thank my committee members, Dr. Patricia Schulte and Dr. Robert Shadwick for their ideas and input into this project.

Along with my supervisor, the rest of the Milsom lab deserves a big “thank you” (Catalina Reyes, Cosima Ciuhandu, Jessica Meir, Dee Brink and Yvonne Dzal) for their enthusiasm and willingness to lend a hand whenever needed. Special thanks goes out to Catalina Reyes for always reminding me that I can rely on her like a big sister and helping me through the good times and bad.

To former lab members, Dr. Angelina Fong and Dr. Graham Scott: I am forever grateful for what they’ve done. Angelina kept me standing through very tough times, both in and out of the lab, and will always be the best lab mom a graduate student can ask for. Graham, who in my eyes is still the incredibly smart PhD student that took me on as his volunteer, first showed me the ways of academia and continues to be a source of motivation.

I have many friends and fellow students to thank (Christie Anderson, Jessica Shirley and her family, Jane Lee, Georgie Cox, the Richards lab and Micha Ben-Zvi) but a special thanks goes out to Gigi Lau. She has been a true friend for patiently listening to my endless rants and sharing my enthusiasm for food after a bad day.
I would like to thank the Natural Sciences and Engineering Research Council of Canada for their financial support.

Last but certainly not least, I would like to thank my mom and sister for their endless love and support. Because of them, I can stay strong and inspired. And a thank you to my dad, who made me the person I am today and continues to be my mentor and rock in spirit.
To my dad, for all that he’s done.
1. General introduction

All turtles and tortoises (order Chelonia) have a rigid shell. While this shell confers such advantages as armour-like protection, it also creates some disadvantages. For instance, during ventilation, the stiffness of the shell greatly restricts the necessary expansion and contraction of the body wall (Gans and Hughes, 1967), impeding both the volume and pressure changes required to draw air in and out of the lungs. How chelonians overcome this impediment during breathing has been a topic of interest for decades.

1.1. Mechanism of ventilation

In all air-breathing vertebrates, inspiration is an active process during which respiratory muscles actively inflate the lungs. Many mammals expire passively under normal circumstances, a result of the difference in pressure between the lungs and the atmosphere, and only recruit muscles to actively exhale when the respiratory drive is high (Milsom, 1991). Reptiles, however, always exhibit an active expiration (Gans and Hughes, 1967; Milsom, 1991) that also requires the work of respiratory muscles.

Lung ventilation in reptiles begins with an expiration followed by an inspiration (Milsom, 1991; Druzisky and Brainerd, 2001). For episodic breathers, such as the turtle, a breathing episode always begins with expiration and ends on an inspiration with a varying number of breaths in between. The last breath ends with an end-inspiratory pause, a passive process during which the glottis closes and blocks the airways to hold the air in the lungs (Milsom, 1991).
Early speculations on how turtles overcome the rigid shell to actively alter volume and pressure within the body cavity to ventilate their lungs gave rise to several different theories. Some suggested that they use a buccal pumping mechanism similar to that of frogs to push or force air into the lungs from the buccal cavity (Morgagni, 1719, Hansen, 1941). Although this theory originally appeared convincing, an investigation of the terrestrial tortoise *Testudo graeca* indicated that buccal movements did not correlate with intrapulmonary pressure changes and that buccal pumping did not play a significant role in ventilating the lungs (Mitchell and Morehouse, 1863; McCutcheon, 1943). Further evidence against the buccal pump theory emerged in a study conducted on the semi-aquatic turtle *Platysternon megacephalum* (Druzisky and Brainerd, 2001) where X-ray videos indicated very little to no buccal oscillation during lung ventilation. Moreover, airflow measurements pointed out that air drawn in and out of the buccal cavity never travelled down to the lungs. Instead, when the buccal cavity compressed and expelled air, air travelled back out through the nares into the atmosphere. This is similar to observations in other aquatic turtle species including *Chelydra serpentina* (Bagatto and Henry, 1999), *Sternotherus minor* (Belkin, 1968) and *Trachemys scripta* (Belkin, 1968). Today, three functions for buccal oscillation in reptiles are proposed: buccopharyngeal respiration (Dunson, 1966), thermoregulation (Heatwole et al., 1973) and olfaction (McCutcheon, 1943; Root, 1949).

A second theory proposed by Wolf (1933) suggested that the pumping movements of the limbs provide the primary power behind respiration. The musculature responsible for moving the pectoral girdle and limbs, the pectoralis and the serratus magnus muscles (Walker, 1963; Gans and Hughes, 1967), clearly function as locomotory
muscles. However, they are also active while girdles move synchronously with the ventilatory movements, implying that they also play a role in respiration (Gans and Hughes, 1967).

The pectoralis muscle induces an inward rotation of the shoulder girdle, bringing the limbs into the shell whereas the serratus magnus produces an outward rotation and consequently brings the limbs out. The fan-shaped pectoralis originates from the plastron and inserts via a tendon on the humerus. It becomes active at the beginning of the inward movement of the limbs and stops when the limbs are fully retracted. Immediately following this, as the forelimbs begin to move outward and the pressure within the lungs equalizes to atmospheric pressure, the serratus magnus becomes active. The serratus magnus (also known as testocoracoideus) originates on the anterior end of the carapace dorsal to the limbs and inserts on the antero-dorsal section of the coracoid (a part of the assembly of shoulder bones). This muscle appears to stay active until the limbs are fully extended to the anterior limit of the visceral cavity (Gans and Hughes, 1967). The pelvic girdle itself is immobile but the large flank membranes with each hindlimb consist only of skin, a thin sheet of muscle and connective tissue, providing more flexibility. This acts as the posterior limiting membrane of the visceral cavity that moves in and out with the hindlimbs. Here lie the two abdominal muscles, the transverse and oblique abdominus muscles, which will be discussed later in greater detail.

Locomotion presumably has a mechanical effect on ventilation as any retraction or protraction of the limbs will inevitably alter the body cavity volume (Landberg et al. 2009). Although it has been established that the girdle and limb movements can play a role in producing volume and pressure changes during breathing (McCutcheon, 1943;
Gans and Hughes, 1967; Gaunt and Gans, 1969), they are believed to play an accessory
role rather than a primary one. This is supported by observations made of turtles’
tendencies to rest for long periods of time in tight gaps that restrict limb movements
(McCutcheon, 1943). This was also clearly demonstrated in the terrestrial box turtle
_Terrapene carolina_ (Landberg et al. 2003) and the red-eared slider _Trachemys scripta_
(Landberg et al. 2009), two species which sustained breathing in the presence of
locomotion, suggesting that locomotion has no direct effect on ventilation. It should be
pointed out, however, that the degree of interference varied between species; breathing
completely ceased in the green sea turtle _Chelonia mydas_ as walking interfered with the
movements of breathing (Jackson and Prange, 1979). Regardless, establishing a fuller
understanding of the link between locomotion and ventilation still appears to be a work in
progress.

Currently, the contraction of two muscles of the posterior flank cavities, the
transverse (expiratory) and oblique (inspiratory) abdominus, are considered to be
primarily responsible for altering lung volume and consequently ventilating the lungs
(Gans and Hughes, 1967; Gaunt and Gans, 1969; Landberg et al., 2003). The transverse
abdominus originates from the carapace and inserts on a sheet of connective tissue that
forms the posterior wall of the visceral cavity. When it contracts and pulls in the flank
membranes, the posterior limiting membrane that normally forms a concave cup and
encloses the viscera will flatten and reduce the volume of the body cavity. This increases
the intrapulmonary pressure, resulting in an exhalation as air is pushed out. Following
this, the exhalation alternates with the inhalation induced by the contraction of the
oblique abdominus. The oblique abdominus originates from the postero-dorsal portion of
the flank membrane and inserts immediately postero-ventral to where the transverse abdominus inserts. The activation of the oblique abdominus brings out the flank membranes to increase the concavity of the posterior limiting membrane and increase the volume of the lungs along with the volume of the body cavity (Fig. 1.1). Consequently the pressure drops within the lungs and pulls air in, resulting in an inhalation. The inspiratory oblique abdominus is truly antagonistic to the expiratory transverse abdominus as the activation of the first inhibits the second.

1.2. Mechanics of ventilation

The mechanical costs associated with turtle ventilation have attracted much interest (Duncker, 1978; Perry, 1983; Vitalis and Milsom, 1986a, b; Milsom, 1989) and one of the factors determining the mechanical cost of breathing is lung structure. Both the broncho-alveolar lung of mammals and the para-bronchial lung of birds (Duncker, 1978) are highly subdivided. The lungs of reptiles fall within one of three categories: unicameral, paucicameral and multicameral. The simplest type, the unicameral lung, can be found in most families of lizards (Perry, 1983) and consists of a single, undivided chamber with the elaborations of the lung wall acting as the respiratory surface. The paucicameral lung consists of a chamber within which a small number of large septa divide the central lumen and is found among chameleons (Klaver, 1973), agamids and iguanids (Perry, 1983). Lastly, in the multicameral lung, which can be seen in crocodilians, varanids and chelonians, a cartilage-reinforced intrapulmonary bronchus connects multiple, separate chambers of the lung (Duncker, 1978).

Lung volumes of reptiles vary in size from approximately 1.0 to over 100 ml/100 g (Milsom, 1989). The two forces that must be overcome to produce the movements of
breathing, flow resistive and elastic forces, also vary with the size of the lungs and the respiratory system. The flow resistive force is a function of the overall flow resistance, rate of tissue movement and the rate at which gas travels through the system (Milsom, 1989). Subsequently, this is a function of the dimensions of the lungs and the airways. The flow resistance within a respiratory system will decrease as lung volume increases, evidently as a result of larger airway dimensions (Crossfill and Widdicombe, 1961; Tenney and Remmers, 1963; Rodarte and Rehder, 1986) and reduced tissue viscous resistance. Tissue viscous resistance is proportional to the velocity of the linear movements of the tissue, which in turn decrease with increasing volume (Grimby et al., 1968). In addition, flow resistance is expected to be less in heterogeneous lungs than homogenous ones as the former provides large expansible chambers. In general, reptilian lungs are larger in proportion to the body than those of birds which in turn, are larger than those of mammals. With the large size and the heterogeneous chambers, the multicameral lung provides less flow resistance than the relatively smaller, homogeneous lungs of birds and mammals.

Flow resistance of the body wall is also dictated by volume and tissue viscous resistance. Similar to flow resistance in the lungs, increasing volume decreases the velocity of tissue movement and the tissue viscous resistance. Since tissue resistance is proportional to tissue velocity, there is less flow resistance at greater volume.

The elastic forces are a function of the compliance of the respiratory system. Reptiles possess simpler, more compliant lungs than mammals which translate into less elastic forces opposing breathing; instead, the majority of the elastic forces to be overcome originate from the body wall (Perry and Duncker, 1978; Milsom and Vitalis,
1984; Vitalis and Milsom, 1986a). The body wall of most reptiles is also considered more compliant than that of mammals (Milsom, 1989), particularly when normalized to body weight. When normalized to respiratory system volume, however, body wall compliance values are remarkably similar across most vertebrate groups with the exception of turtles (Milsom, 1989). Turtles, with their rigid shell, have stiffer body walls and lower body wall compliance than most other vertebrates; approximately double the stiffness (Milsom, 1989).

1.3. Mechanical cost of ventilation

The mechanical work of ventilation can be defined as the product of volume and pressure; therefore, if one measures the volume and pressure changes associated with breathing in a turtle, the work required for ventilation can be determined. At any given level of ventilation, there is an optimal combination of tidal volume ($V_T$) and breathing frequency ($f_R$) at which work and cost of breathing are minimal (Otis et al., 1950; Crossfill and Widdicombe, 1961; Milsom and Vitalis, 1984; Vitalis and Milsom, 1986a; Milsom, 1989; Wang and Warburton, 1995). Turtles naturally breathe at this optimal combination at various levels of ventilation (Vitalis and Milsom, 1986a,b) and altering either variable inevitably increases work (Fig. 1.2). If $V_T$ decreases, $f_R$ must increase as a result if the same level of total ventilation is to be maintained; this is equivalent to shallower, more frequent breaths. Although the elastic forces will decrease with decreasing $V_T$, greater $f_R$ means more air traveling in and out of the respiratory system at a faster rate, rapidly increasing the flow resistive forces. Ultimately, the resultant overall resistance will be greater and subsequently increase the work of breathing (see Fig. 1.3). Conversely, if $f_R$ decreases, this reduces the flow resistive forces; however, $V_T$ must
increase in order to maintain the same level of ventilation. With deeper breaths, expansions and contractions happen to greater degrees, increasing the elastic forces involved with each breath. This, again, increases the work of breathing. The relationship between $V_T$, $f_R$, ventilatory resistance and mechanical work demonstrates how tightly breathing pattern is associated with mechanical work of breathing.

1.4. Patterns of ventilation

Turtles are episodic breathers; they exhibit a breathing pattern in which episodes of continuous breathing are separated by longer non-ventilatory periods ($T_{NVP}$), or apneas. Within each episode, breathing appears to take place at the optimal combination of $V_T$ and $f_{R_{inst}}$ (instantaneous frequency, the frequency within an episode) that minimizes the work of breathing. As the drive to breathe increases, increasing total ventilation by simply increasing $V_T$ would be predicted to be extremely energetically expensive as the optimal combination is no longer maintained (Milsom, 1984; Vitalis and Milsom, 1986a). Increasing $f_{R_{inst}}$ would also be expensive as one steers away from the optimal combination; therefore, as long as periods of apnea exist, the most efficient route of increasing ventilation is by shortening the $T_{NVP}$ (Glass and Johansen, 1976; Glass et al., 1985). This allows the optimal combination of $V_T$ and $f_{R_{inst}}$ to be preserved within an episode to maintain the work of taking each breath at minimum. The overall $f_R$ increases to increase total ventilation, not $f_{R_{inst}}$.

Hypoxia and hypercapnia are respiratory stimuli, both of which increase total ventilation; however, the routes via which this final response takes place differ between the two gas mixtures. Analyzing the components of $f_R$ reveal that although overall $f_R$ increases in both situations, hypoxia decreases the number of breaths per episode while
increasing the number of breathing episodes whereas hypercapnia increases both the number of breaths in each episode and the number of breathing episodes (e.g. Frankel et al., 1969; Glass et al., 1978, 1983; Milsom and Jones, 1980; Milsom and Chan, 1986). Again, in both cases \( f_{R_{\text{inst}}} \) remains relatively constant to maintain minimal work of breathing.

Several theories attempt to explain why turtles and few other species breathe episodically, claiming the episodic breathing pattern to be an adaptive phenomenon for those animals that do not require continuous breathing to meet their metabolic demands. Interspersing breathing at optimum volume and rate with apnea may be the mechanically and energetically efficient approach (Milsom, 1991; Del Negro et al., 2009) by minimizing the work involved. Episodic breathing has also been considered a strategy for water conservation and enhanced gas exchange by maximizing the potential to increase the gas diffusion gradient by the end of an apnea (the Chthonic hypothesis) (Quinlan and Gibbs, 2006). Lastly, episodic breathing has been proposed to a means to minimize tissue damage from \( O_2 \)-derived free radicals by minimizing the tissue \( O_2 \) levels (the “oxidative damage hypothesis”) (Hetz and Bradley, 2005; Bradley, 2006).

1.5. Oxidative cost of ventilation

There are oxidative costs associated with the work performed to power respiration. In reptiles, both inspiration and expiration are active processes (Gans and Hughes, 1967) during which the respiratory muscles work and consume oxygen to inflate and deflate the lungs. Calculating this oxidative cost of ventilation in reptiles and more specifically, turtles, has proven to be a challenge and is a topic of controversy today.
1.5.1. Turtles

Kinney and White (1977) first estimated the oxidative cost of breathing in the Florida Cooter, *Pseudemys floridana*, at three different temperatures using a method employing “unidirectional ventilation”. By calculating the difference in O₂ consumption during spontaneous resting ventilation and zero ventilation (spontaneous breathing was suppressed by supplying fresh gas directly to the lungs by producing a unidirectional flow of gas through the respiratory system), they estimated the relative oxidative cost of breathing to be approximately 20% of total resting metabolism at 20°C. This suggested that a rather large portion of total metabolism was dedicated to the act of breathing.

A second investigation conducted by Jackson, Singer and Downey (1991) almost two decades later re-examined the estimation of oxidative cost of breathing in the painted turtle, *Chrysemys picta* using the “regression method”, a method frequently adopted in studies estimating the oxidative cost of breathing in various reptiles and mammals (see Frappell et al., 1992; Wang and Warburton, 1995; Skovgaard and Wang, 2004). With this method, a respiratory stimulus (i.e. hypoxia or hypercapnia) is used to promote an increase in breathing. The increase in ventilation and the simultaneous increase in O₂ consumption are measured. The relationship between the two variables is determined and subsequently used to calculate the oxygen consumption that should occur when ventilation falls to zero by regression. Using this method with hypercapnia (5% CO₂), Jackson et al. (1991) calculated the oxidative cost of breathing to be approximately 1% of total metabolism at 25°C, a much smaller value than that estimated by Kinney and White (1977). With the huge discrepancy in numbers, the cost and efficiency of turtle ventilation still remains to be determined and the controversy is yet to be resolved.
1.5.2. Other reptilian species

Wang and Warburton (1995) estimated the oxidative cost of breathing in the American alligator, *Alligator mississippiensis*. Separate exposures to hypoxic and hypercapnic gas mixtures delivered via a mask in spontaneously breathing alligators triggered an increase in ventilation and corresponding changes in O$_2$ consumption. According to the data, the estimated relative cost of ventilation was approximately 13% of resting metabolism when breathing increased due to hypoxia but -1.5% of resting metabolism when breathing increased due to hypercapnia at 23°C.

A similar study of the tegu lizard (*Tupinambis merianae*) conducted with similar protocols also produced inconsistent results (Skovgaard and Wang, 2004). Only severe hypoxia elicited any responses in ventilation whereas hypercapnia acted as a stronger respiratory stimulus throughout. Similar to the study by Wang and Warburton (1995), exposures to hypoxia indicate a relatively high cost of 17% of total metabolism while hypercapnia yielded a very small negative cost (less than -1%) of breathing at 25°C.

The inconsistent results of both studies only added to the cost of breathing controversy.

1.6. Aim of the thesis

The aim of this study was to measure the oxidative cost of breathing in a single group of turtles using the two different methods, the unidirectional ventilation and the regression methods. In addition, a third method (a hybrid method of the two) was employed in an attempt to resolve why the two different methods have produced such different results in the past. Superimposing the two methods should eliminate any possible differences due to methodology. Furthermore, using known mechanical cost
values and muscle efficiency values from the literature, a theoretical oxidative cost was calculated to act as a point of comparison for the experimental results from all three methods. The ultimate goal of this thesis was to determine the true oxidative cost of breathing in turtles.
Figure 1.1. An illustration of the steps in ventilation. (A) Expiration: As the transverse abdominus muscle contracts, volume decreases to increase pressure and push air out. (B) Inspiration: Oblique abdominus contracts to expand the body cavity and increase volume. The drop in pressure pulls air into the lungs (adapted from McCutcheon, 1943).
Figure 1.2. Minute work at various levels of ventilation. The gray bar represents the range within which turtles are naturally found to be breathing (reprinted with permission from Vitalis and Milsom, 1986b).
Figure 1.3. Work per breath at constant minute ventilation across the breathing frequency range. This illustrates how work per breath changes as breathing frequency and tidal volume change and the contributions from elastic and non-elastic resistive forces (reprinted with permission from Vitalis and Milsom, 1986a).
2. Determining ventilatory cost: comparison of three methods

2.1. Introduction

Studies of the cost and efficiency of ventilation in turtles have produced contradictory results. The two key investigations by Jackson et al. (1991) and Kinney and White (1977) calculated cost of ventilation to be 1 and 20% of total metabolism respectively, a huge discrepancy in numbers. These two studies used two different methods to calculate oxidative cost of breathing in turtles: a unidirectional ventilation method and a regression method. Although the two methods vary, they should, in principle, produce the same results.

The unidirectional ventilation method involves measuring the oxygen consumption of an artificially ventilated animal. By artificially maintaining normal arterial Po2 and Pco2 levels, active ventilation is suppressed (Peterson and Fedde, 1968; Ray and Fedde, 1969; Fedde and Peterson, 1970; Roberts, 1975; Roberts and Ballintijn, 1988). Oxygen consumption is initially measured in a spontaneously breathing animal (\( \dot{V}_{O_2}^{\text{(spontaneous)}} \)) and then compared to the oxygen consumption levels of a turtle artificially ventilated via the lungs (\( \dot{V}_{O_2}^{\text{(artificial)}} \)). Providing gas inflow through one lung cannula and measuring the changes in gas composition from the collected gas outflow via a cannula in the other lung, oxidative cost without the act of ventilation can be calculated by subtracting \( \dot{V}_{O_2}^{\text{(artificial)}} \) from \( \dot{V}_{O_2}^{\text{(spontaneous)}} \):

\[
\dot{V}_{O_2}^{\text{(spontaneous)}} - \dot{V}_{O_2}^{\text{(artificial)}} = \dot{V}_{O_2}^{\text{(resp. muscles)}}
\]

(Kinney and White, 1977; Morgan and Milsom, unpublished).
Using this method Kinney and White (1977) estimated the oxidative cost of breathing in the Florida Cooter, *Pseudemys floridana*, at three different temperatures, and calculated the cost of breathing to be approximately 20% of total metabolism at 20°C.

The regression method involves measuring oxygen consumption, or metabolic rate, at various levels of minute ventilation stimulated by either hypoxia or hypercapnia. Since cost of breathing cannot be measured directly, one must rely on the relationship between O₂ consumption and ventilation (oxygen-uptake ventilation relationship) to estimate costs. The relationship between minute ventilation and metabolic rate is plotted and fitted with a regression line, its slope acting as an indication of the relative metabolic cost of breathing. The regression is extrapolated to zero to estimate oxygen consumption without ventilation, from which point any additional oxygen consumption as ventilation increases can be attributed to the act of breathing. Ultimately, the ratio of this additional oxygen consumption from ventilation to the total metabolism provides the estimated value of the relative oxidative cost of breathing. The regression method is commonly used for calculating cost of breathing in reptiles and mammals (see Frappell et al., 1992; Wang and Warburton, 1995; Skovgaard and Wang, 2004), including the study by Jackson et al. (1991), in which the calculated oxidative cost of breathing was approximately 1% of total metabolism at 25°C.

The two studies used different methods and it is possible that this is where the discrepancy in the calculated costs arises. Both methods operate under two assumptions: that all other metabolic functions other than ventilation remain constant throughout the trials and that the correlation between O₂ consumption and ventilation is linear; however,
neither may be the case. In a few studies, it appears that a non-linear curve better represents the oxygen uptake-ventilation relationship than a linear one (see Wang and Warburton, 1995; Skovgaard and Wang, 2004) where oxidative cost is initially greater when starting from zero ventilation and then plateaus as ventilation increases further. It is plausible that it costs less to simply increase ventilation in an already ventilating animal than to start from zero. It is also known that both hypoxia and hypercapnia, as used in the regression method, can suppress metabolism (Busa and Nuccitelli, 1984; Wang et al., 1993). This begs the question whether the two studies using different methods of calculating oxidative costs only have different results because they refer to different stages of a potentially non-linear relationship under different degrees of metabolic suppression.

The objective of the present study, therefore, was to estimate the cost of breathing using the two previously used methods in a single group of animals in an attempt to establish the source of the discrepancy in the oxidative cost values in the literature. We hypothesized that the UDV and the regression methods would produce different results as a result of a non-linear oxygen uptake-ventilation relationship. In addition, we also used a hybrid method, which employ exposure to elevated CO₂ levels during unidirectional ventilation, and hypothesized that this method would produce estimates that do not differ from the values obtained using the regression method alone.
2.2. Materials and methods

2.2.1. Animals

Adult red-eared sliders of both sexes were obtained from three commercial suppliers (Lemberger Company (Oshkosh, Wisconsin, USA), Sullivan Company Inc. (Nashville, Tennessee, USA) and Niles Biological Inc. (Sacramento, California, USA)). They were housed in a rectangular semi-natural outdoor pond with mud and moss available for burrowing. They had access to a dry terrestrial environment for basking and experienced the natural changes in environmental temperature and photoperiod throughout the year. All turtles were fed on a mixture of trout chow (Aquamax Grower 500, 5D05) and dry pellets for freshwater turtles (Mazuri fresh water turtle diet G190) three times a week during the warmer seasons (spring, summer and fall) and only once a week during winter (turtles eat less as their metabolism declines with environmental temperature).

Prior to experiments turtles were brought indoors and held at room temperature (20 – 23.5°C) for one to two weeks in a 90 gallon aquarium with access to a dry platform for basking. They were exposed to full spectrum lights which were set by a timer on a 12 hour light, 12 hour dark photoperiod and were fasted to avoid the confounding effects of digestion on metabolism for a minimum of five and up to seven days. All animals were weighed prior to each experimental treatment.

2.2.2. Series 1 (regression method)

Preparation: Before conducting any experiments, turtles were sedated with either isoflurane or 100% CO₂ to reduce their resistance to handling. A collar was placed around the neck and attached to the rostral carapace to prevent the head from being
retracted and a mould was made of the turtles’ head using dental impression material (Jeltrate - Alginate Impression Material (fast set, Dentsply Caulk, Dentsply International Inc., Milford, Delaware)). Turtles were then allowed to recover from the anesthetic for 24 to 48 hours.

Custom-fitted masks were then made for each turtle as described by Glass et al. (1978) and modified by Wang and Warburton (1995). A plaster cast of the head was made from a head mould using Plaster of Paris mixed with water in a 2:1 ratio. The mixture poured into the mould was dried for 30 minutes to an hour and once the plaster replica of the turtle’s head was ready for use, a 2.5” x 2.5” sheet of thermo-forming material (clear-mouthguard, 0.040”, Henry Schein Inc. Melville, New York) softened over a heating pad was stretched over the cast to form a tight-fitting mask. A hole was cut in the mask at the site of the nostrils. A pneumotachograph was then mounted on top of the mask at this site with tubes leading to a differential pressure transducer (Model DP103-18, Validyne Engineering Corp, USA) connected to an amplifier (Model 7P122E, Grass Instruments, USA) for measuring tidal volume and breathing frequency.

On the day of the experiment, each turtle was instrumented with a mask, sealed onto the head with 3M™ ESPE™ Impregum™ F Impression Material. A gas mixer (Cameron Instrument Company – GF-3/MP) placed upstream of the turtle produced different gas mixtures at a rate of 1 L/min. Gas was drawn from this stream through a t-tube and past the pneumotachograph connected to the mask of the turtle by a suction pump at a rate of 350 to 500 ml/min. A second pneumotachograph, was used to confirm the total gas flow past the turtle. This gas mixture, which included the expired gas from
the turtle, was drawn through a drying column (drierite) to O₂ (Raytech O₂ analyzer) and CO₂ (Beckman CO₂ analyzer) gas analyzers (Fig. 2.1).

Protocol: Turtles were first exposed to humidified air and allowed to adjust to the experimental set-up for several hours. Once they appeared calm and the breathing pattern appeared relatively stable (these animals are episodic breathers and the breathing pattern is never regular while breathing air), respiratory flow traces were recorded for at least one hour for analysis. Following this, eight turtles (0.49 ± 0.03 kilograms body weight) were immediately exposed to 7% O₂ and further exposed to stepwise decreases in O₂, in one percent increments from 7 to 4% O₂. In all cases recordings were made for 30 minutes after breathing reached a new steady state, which could take up to one hour.

The following day, each of these turtles, plus five additional turtles (total n = 13 (0.56 ± 0.05 kilograms body weight)) were exposed to progressive hypercapnia. Experiments were run in a similar fashion to the hypoxia experiments, increasing CO₂ sequentially from 2 to 6% in one percent increments. Again, traces were recorded for 30 minutes after breathing reached a steady state at each level.

2.2.3. Series 2 (unidirectional ventilation (UDV) method)

Protocol: In this series, six turtles (0.61 ± 0.10 kilograms body weight) had each lung surgically cannulated, one cannula to serve as an inlet for air and the other as an outlet to establish unidirectional artificial ventilation. For the surgery, turtles were first placed in a sealed box saturated with isoflurane. This achieved mild if not full induction of anesthesia, at which point the turtles were intubated and artificially ventilated with air mixed with 4% isoflurane. Following induction, 1cm by 1cm rectangular holes were cut into the shell, on both the left and the right side of the fourth dorsal scute along its lateral
borders. This was approximately where the posterior end of the lungs could be found.

Once the lung tissue was exposed, a small hole was cut into each lung and a cannula (PE-190) was inserted and held in place with a purse-string suture. The rectangular pieces of shell were secured back into place using Flexacryl (ethyl methacrylate acrylic resin) producing an air-tight, waterproof seal. A collar was then placed around their necks and attached to the rostral carapace to prevent the head from being retracted and a mould was made of the turtles head using dental impression material as in Series 1. Turtles were allowed to recover from the anesthesia and surgery for a minimum of 48 hours before conducting experiments.

Protocol: On the day of the experiment, turtles were fit with the custom mask and set up as described in Series 1. Again, O2 consumption and ventilation were measured first during spontaneous ventilation on air. Following this, humidified air was pushed through the lungs at a flow rate controlled by two rotameters starting at 100 ml/min with the mask sealed to prevent the turtles from breathing through their nostrils or mouth. Flow rate through the lungs was increased in 10 ml/min increments until spontaneous breathing ceased (usually between 100 to 120 ml/min) (Fig. 2.2). The outflow from the lungs was then collected in a balloon for 20 minutes and analyzed for both O2 and CO2 composition. A balloon was used to collect the gas rather than sampling them directly with the gas analyzers in consideration of the relatively low flow rate across the lungs in comparison to the sampling rate of the gas analyzers. The analyzers were most accurate at set flow rates that exceeded the flow rate of unidirectional ventilation, which would have resulted in an excess amount of gas being pulled through the lungs.
2.2.4. Series 3 (the “hybrid” method)

**Preparation:** Experiments were performed on the same animals used in Series 2.

**Protocol:** Once zero ventilation was achieved during UDV, each turtle was exposed to progressive stepwise increases in hypercapnia in the unidirectional ventilation gas from 3% (roughly the CO₂ level reported in the lungs of turtles during moderate periods of apnea (Burggren and Shelton, 1979; Shelton and Boutilier, 1982; Herman and Smatresk, 1999)) to 7% CO₂ in one percent increments. Again, traces were recorded for 20 minutes after breathing reached a steady state at each level of CO₂. As spontaneous breathing resumed with the presence of CO₂, turtles breathed in and out through the lung cannulae. Tidal volume, breathing frequency and ventilation were calculated using the respiratory airflow trace obtained from a pneumotachograph placed upstream of the inlet cannula and the gas outflow was again collected into a balloon for 20 minutes (Fig. 2.3).

2.2.5. Theoretical estimation

Using the Work per breath ($W$) vs. pump ventilation ($V_p$) graph derived from artificially ventilated turtles (Vitalis and Milsom (1986a)), work values corresponding to various volume-frequency combinations were estimated. The relationship between work, tidal volume and instantaneous frequency was plotted as a three-dimensional mesh plot using MATLAB (version R2008a). MATLAB produced three independently interpolated mesh plots: V4, Linear and Cubic interpolations. The V4 plot was discarded for interpolating obviously false and inaccurate results (i.e. improbable negative work values) whereas the Linear and Cubic plots yielded realistic values very similar to one another. Only one plot was required for further analysis and therefore the Linear plot was randomly chosen to be used further for estimating work values associated with each
volume-frequency combination. Tidal volume and instantaneous frequency values obtained experimentally from the test animals were superimposed on the mesh plot and the corresponding work values were determined.

The work values in units of ml cm H2O/min (as presented in Vitalis and Milsom (1986a)) were converted into units of ml O2 consumed per unit time according to Frappell et al. (1998). The converted work values in units of ml O2/min/kg were considered to be the mechanical cost for the work performed during the act of ventilation in an artificially ventilated system. Knowing mechanical cost and using a skeletal muscle efficiency value of 10 percent from the literature (Otis et al., 1950), oxidative cost of breathing was calculated theoretically.

It should be noted that the pump used to produce artificial ventilation in Vitalis and Milsom (1986a) produced an inhalation only by actively inflating an anesthetised turtle but allowing it to passively deflate without the assistance of the pump. Because turtle ventilation involves an active inhalation and an active exhalation, the work output estimated here only represents half of the amount of work performed by a conscious animal. Assuming equal work distribution for inhalation and exhalation, the work values should be doubled in order to take into account the work that would have been performed to generate a breath.

2.2.6. Data analysis

Sample length variation: To verify the accuracy of the respiratory variables calculated from the breathing traces, normoxic traces of three turtles (90 to 180 minutes in length, depending on the turtle) were divided into 60, 30 and 15 minute portions and each sample was used to re-calculate ventilation and O2 consumption. This revealed how
the two variables depend heavily on the segment used for the calculations and its length, reflecting the irregularity of the episodic breathing patterns. The ventilation and O\textsubscript{2} consumption values re-calculated for each turtle were plotted together on a linear regression plot using Sigmaplot (version 10.0; Systat Software Inc.). By adding a best-fit line, the slope and the R\textsuperscript{2} value could be determined.

*Series 1:* Signals from both pneumotachographs and both gas analyzers were amplified using Gould amplifiers and recorded to computer at 240 Hz per channel using a Windaq data acquisition system (DI200; DataQ Instruments, Akron, OH, USA). From these signals it was possible to calculate breathing frequency (f\textsubscript{R}) and tidal volume (V\textsubscript{T}) as well as the rates of oxygen consumption (V\textsubscript{O}\textsubscript{2}) and carbon dioxide production, reported as STPD (standard temperature and pressure, dry). O\textsubscript{2} consumption and CO\textsubscript{2} production were obtained by integrating the area under the O\textsubscript{2} and CO\textsubscript{2} curves and corrected according to Withers (1977) for a system using a H\textsubscript{2}O absorbent but no CO\textsubscript{2} absorbent. The raw traces collected during normoxia were analyzed (90 to 180 minutes in length) to generate the resting air value for each individual turtle. For data collected during the hypoxia and hypercapnia exposures, raw traces were analyzed for 20 minutes as stated above to obtain total ventilation and O\textsubscript{2} consumption then separately plotted into regression-hypoxia and regression-hypercapnia O\textsubscript{2} consumption vs. ventilation plots for the estimation of oxidative cost of breathing, including a best-fit line and the values for both the slope and the r\textsuperscript{2} value. All the calculated ventilation (V\textsubscript{E}) and V\textsubscript{O}\textsubscript{2} values were also averaged across the gas compositions to produce O\textsubscript{2} consumption vs. ventilation plots of the mean values for both regression-hypoxia and regression-hypercapnia data.
**Series 2**: Prior to ventilating the lungs via UDV, the resting values for both \( V_{E} \) and \( V_{O_2} \) during normoxia were determined in a similar fashion to Series 1. With the onset of UDV, which suppresses spontaneous breathing to zero, there was no calculation required for determining ventilation. \( V_{O_2} \) at zero ventilation was calculated from the volume and composition of the gas collected in a balloon over the 20 minute period. Knowing both the total volume and the gas composition of a balloon filled over a known time period, the difference in the percentage of O\(_2\) before and after traveling through the lungs was mathematically converted into the total volume of O\(_2\) consumed over that same period of time. Similar to the graphs in Series 1, \( V_{E} \) and \( V_{O_2} \) values were plotted as O\(_2\) consumption vs. ventilation including three groups of data: 1) control values during normoxia of spontaneously breathing turtles before surgery (collected during Series 1), 2) control values during normoxia of spontaneously breathing turtles after surgery (collected during Series 2) and 3) \( V_{O_2} \) during UDV. The graph included the best-fit line, mean data for each group and the values for both the slope and the R\(^2\) value.

**Series 3**: Since the same group of turtles was used for both Series 2 and 3, the same resting air values were also used in Series 3. As spontaneous ventilation resumed with the progressive increase in CO\(_2\), the pneumotachograph upstream of the inlet cannula recorded the breathing flow in and out of the turtle. The same methods from Series 1 were used to calculate ventilation and O\(_2\) consumption.

**Statistical analysis**: To determine whether hypoxia and hypercapnia exposures elicited significant effects on the different variables, the t-test was conducted to compare
the resting variables during normoxia to the variables measured during each gas exposure individually. $P \leq 0.05$ was applied and data is presented in mean ± S.E.M.
Figure 2.1. A schematic diagram of the experimental set-up for the regression method. Flow meter (FM), differential pressure transducer (DP) and gas analyzers (GA).
Figure 2.2. A schematic diagram of the experimental set-up for the unidirectional ventilation method. The section in gray is bypassed during UDV to provide flow through the lungs and the mask is completely sealed off.
Figure 2.3. A schematic diagram of the experimental set-up for the hybrid method. The set-up is identical to the one used during UDV except with increased CO₂ levels in the gas going to the lungs.
2.3. Results

2.3.1. Overall response to respiratory stimuli

Exposures to hypoxia and hypercapnia induced different changes in the overall breathing pattern of turtles. With hypoxia, there was a trend towards a reduction in the number of breaths per episode (Fig. 2.4A) and increase in the mean episode frequency (number of episodes per hour) (Fig. 2.4B) although neither change was statistically significant. With hypercapnia, on the other hand, the number of breaths per episode stayed fairly constant throughout the experiment (Fig. 2.4A) while the mean episode frequency increased significantly from normocapnia starting at 3% CO$_2$ exposure up to 6% CO$_2$ exposure (Fig. 2.4B).

The mild effects of hypoxia on the breathing pattern led to a very small increase in the overall mean $f_R$ (Fig. 2.5A) and mean $V_T$ (Fig. 2.5B), which ultimately resulted in a small increase in minute ventilation ($\dot{V}_E$) (Fig. 2.5C). $O_2$ consumption ($\dot{V}_{O_2}$) decreased initially when first exposed to hypoxia at 7% $O_2$ but then increased gradually throughout the remainder of the experiment back to a value similar to the normoxic value (Fig. 2.5D). None of the changes were significant.

In hypercapnia both mean $f_R$ and $V_T$ increased significantly starting from 4% CO$_2$ (Fig. 2.5A, B). Mean frequency of $1.38 \pm 0.27$ min$^{-1}$ at normoxia increased to as high as $6.62 \pm 1.13$ min$^{-1}$ at 5% CO$_2$. From the maximum value, $f_R$ decreased slightly to $5.31 \pm 1.14$ min$^{-1}$ as the level of CO$_2$ increased to 6% (Fig. 2.5A). $V_T$ steadily increased from $13.01 \pm 3.29$ ml kg$^{-1}$ to as high as $31.84 \pm 5.40$ ml kg$^{-1}$ (Fig. 2.5B). The overall increase in both $f_R$ and $V_T$ resulted in an exponential increase in $\dot{V}_E$ from $14.52 \pm 2.56$ ml min$^{-1}$ kg$^{-1}$
at rest to as high as $194.53 \pm 50.62 \text{ ml min}^{-1} \text{ kg}^{-1}$ at 5% CO$_2$ (Fig. 2.5C). $V_{O_2}^\cdot$ initially increased from normocapnia to 2% CO$_2$ but then gradually decreased for the remainder of the hypercapnic exposure (Fig. 2.5D).

2.3.2. Sample length variation

Re-calculating $V_E^\cdot$ and $V_{O_2}^\cdot$ using segments of different durations (the whole trace (1.5 to 3 hours), 60, 30 and 15 minute samples) from normoxic traces produced a wide range of values as demonstrated for three individuals in Fig. 2.6. For the turtle that exhibited the widest range of values, $V_E^\cdot$ was as low as 0 and as high as $23.84 \text{ ml min}^{-1} \text{ kg}^{-1}$ whereas $V_{O_2}^\cdot$ also spanned from 0 to $1.42 \text{ ml min}^{-1} \text{ kg}^{-1}$ (Fig. 2.6A). The three animals re-examined here had different mean values of ventilation from $3.32 \pm 0.64$ to $17.73 \pm 0.94 \text{ ml min}^{-1} \text{ kg}^{-1}$, representing both the lower and higher end of the spectrum of resting $V_E^\cdot$ and $V_{O_2}^\cdot$ values of all the test animals (Fig. 2.7). The O$_2$ consumption vs. ventilation plots of the three individual turtles each presented their own slope, ranging from 0.0102 to 0.0623 ml O$_2$ per ml air.

The resting values of all individuals in normoxia varied tremendously (Fig. 2.7). They had a mean $V_E^\cdot$ of $12.98 \pm 2.34 \text{ ml min}^{-1} \text{ kg}^{-1}$ and mean $V_{O_2}^\cdot$ of $0.52 \pm 0.07 \text{ ml min}^{-1} \text{ kg}^{-1}$ with a linear regression slope of 0.024 ml O$_2$ per ml air and a R$^2$ value of 0.5682.

2.3.3. Mean data

Plotting $V_{O_2}^\cdot$ as a function of $V_E^\cdot$ using mean resting values from Series 1 (resting values for turtles pre-surgery; n = 5 for turtles used in both Series 1 and 2), mean resting
values from Series 2 (resting values from the same turtles post-surgery) and mean \( \dot{V}_{O_2} \) at zero ventilation during UDV (same group of turtles) produced a positive regression with a slope of 0.0103 ml O\(_2\) per ml air and \( R^2 \) value of 0.9908 (Fig. 2.8A). Plotting mean \( \dot{V}_E \) and \( \dot{V}_{O_2} \) values calculated across each hypoxia trial (Figure 2.8B) produced very similar results (a slope of 0.0096 ml O\(_2\) per ml air). On the other hand, plotting mean \( \dot{V}_E \) and \( \dot{V}_{O_2} \) values calculated across each hypercapnia trial (Figure 2.8C) produced a negative slope of -0.0005 ml O\(_2\) per ml air.

Combining all the mean values of UDV, hypoxia and hypercapnia experiments into a single plot with a best-fit line generated a hyperbolic curve with the UDV and hypoxia data along the steep portion of the curve and the hypercapnia data along the plateau and falling portion of the curve (Fig. 2.9).

2.3.4. Individual data

Separately plotting the individual data set from each experiment into its own \( \dot{V}_{O_2} \) vs. \( \dot{V}_E \) plot revealed much variation in the oxygen uptake-ventilation relationship amongst the different groups. A \( \dot{V}_{O_2} \) vs. \( \dot{V}_E \) plot combining 1) resting values from Series 1 (black circles), 2) resting values from Series 2 (gray triangles) and 3) \( \dot{V}_{O_2} \) during UDV (squares) (three trials in which ventilation was always below 30 ml min\(^{-1}\) kg\(^{-1}\)) produced a positive regression slope of 0.0136 ml O\(_2\) per ml air and \( R^2 \) value of 0.2954 (Fig. 2.10A).
Progressively increasing the level of CO₂ via UDV (hybrid method) restored spontaneous ventilation to approximately 60 ml min⁻¹ kg⁻¹ and \( V'_{O_2} \) from 0.16 to 1.69 ml min⁻¹ kg⁻¹ (Fig. 2.10B). The slope of this relationship was 0.0245 ml O₂ per ml air.

Hypoxia exposures (n = 6) had only a modest effect, increasing \( V'_E \) to approximately 30 ml min⁻¹ kg⁻¹ from rest. The slope of the linear regression between \( V'_{O_2} \) vs. \( V'_E \) was nearly identical to the slope for the data obtained with the hybrid method at 0.0223 ml O₂ per ml air with a \( R^2 \) value of 0.4856 (Fig. 2.10C).

Hypercapnia produced a stronger effect than hypoxia, \( V'_E \) reaching levels almost as high as 500 ml min⁻¹ kg⁻¹. The first group of data with \( V'_E \) less than 100 ml min⁻¹ kg⁻¹ included values from normocapnia up to 3% CO₂ exposure whereas the second group with \( V'_E \) greater than 100 ml min⁻¹ kg⁻¹ included values from 4% to 6% CO₂ exposures (Fig. 2.10D, E). Figure 2.10D with \( V'_E < 100 \text{ ml min}^{-1} \text{ kg}^{-1} \) demonstrated the effects of low CO₂ exposures on \( V'_E \) and \( V'_{O_2} \) as mean \( V'_E \) increased to 39.76 ± 5.48 ml min⁻¹ kg⁻¹ and \( V'_{O_2} \) to 0.61 ± 0.06 ml min⁻¹ kg⁻¹. The slope of the regression was calculated to be 0.0047 ml O₂ per ml air, less steep than those of the normoxia and hypoxia graphs. Data also appeared to be more scattered with a \( R^2 \) value of 0.1867. Figure 2.10E where \( V'_E > 100 \text{ ml min}^{-1} \text{ kg}^{-1} \) illustrates the more pronounced effects of high CO₂ exposures as mean \( V'_E \) and \( V'_{O_2} \) increased to a greater degree at 254.46 ± 30.35 ml min⁻¹ kg⁻¹ and 0.71 ± 0.09 ml min⁻¹ kg⁻¹ respectively. The data still appeared scattered (\( R^2 = 0.2708 \)) with a regression slope of 0.0011 ml O₂ per ml air, again less steep than the previous graphs.
The varying degree of steepness of the slopes was more evident when all regressions were plotted together in Figure 2.10F.

All the individual data obtained across all three series were plotted together in Figure 2.11, each data set in a different colour (black – UDV; orange – hybrid; red – hypoxia; green – hypercapnia data with $\dot{V_E} < 100$ ml min$^{-1}$ kg$^{-1}$; blue – hypercapnia with $\dot{V_E} > 100$ ml min$^{-1}$ kg$^{-1}$). The hypercapnia data set as a whole was split into two groups using $\dot{V_E} = 100$ ml min$^{-1}$ kg$^{-1}$ as an arbitrary dividing line to point out the difference in the slopes of $\dot{V_{O_2}}$ vs. $\dot{V_E}$ when ventilation is less than and greater than 100 ml min$^{-1}$ kg$^{-1}$.

2.3.5. Theoretical calculations

The three-dimensional mesh plot derived from work done by Vitalis and Milsom (1986a) provided work values at various volume-frequency combinations (Fig. 2.12). By overlapping the experimental $V_T$ and instantaneous $f_R$ values obtained during the present study (denoted by yellow diamonds) with the mesh plot, the difference in the effect of hypoxia and hypercapnia on the two variables was evident. Hypoxia had little effect on the variables (Fig. 2.12A) whereas hypercapnia induced a noticeable increase in $V_T$ (Fig. 2.12B). $V_T$ under hypoxic exposures spanned only from 1.64 to 13.84 ml whereas under hypercapnic exposures it spanned from 1.32 to 32.61 ml, three times the range observed in hypoxia.

The corresponding work values at each volume-frequency combination were converted into ml O$_2$ consumed per unit time and plotted against $\dot{V_E}$ to generate a theoretical linear regression plot (Fig. 2.13). The slope of the theoretical regression was
0.0003 ml O$_2$ per ml air, closest to the least steep experimental slope of 0.0011 ml O$_2$ per ml air observed during high CO$_2$ exposures.
Figure 2.4. Mean values of the (A) number of breaths per breathing episode and (B) the episode frequency (episodes per hour). Red denotes hypoxia results and blue denotes hypercapnia results. Significant changes are observed only in episode frequency during 3% to 6% CO₂ exposures (asterisks indicate significant differences) (t-test; P < 0.05).
Figure 2.5. Mean values of (A) overall frequency, (B) tidal volume, (C) minute ventilation and (D) O₂ consumption of turtles breathing different gas mixtures. Red denotes hypoxia results and blue denotes hypercapnia results. Hypoxia did not elicit any significant effects in any variables. Neither hypoxia nor hypercapnia induced significant increases in O₂ consumption (asterisks indicate significant differences) (t-test; P < 0.05).
Figure 2.6. Re-evaluation of ventilation and $O_2$ consumption using segments of different durations (the whole trace, 60, 30 and 15 minute samples) from normoxic traces. Each regression plot represents an individual turtle. Values are presented as means ± S.E.M.
Figure 2.7. Control values of resting, spontaneously ventilating turtles during normoxia (n=12). The values vary between individual turtles tremendously, from $V_E$ of 3.67 to 27.83 ml/min/kg and $V_O_2$ of 0.16 to 1.10 ml/min/kg. Values are presented as means ± S.E.M.
Figure 2.8. Three different groups of mean data. (A) Mean resting values from Series 1 and 2 and mean O\textsubscript{2} consumption during UDV. (B and C) Regression plots for mean data averaged across gas composition. The two gases produced two very regression slopes.
Figure 2.9. The three different groups of mean data combined into one. The initial steep increase in O₂ consumption plateaus as ventilation increases further (best-fit line: double rectangular 5 parameter hyperbola).
Figure 2.10. $O_2$ consumption vs. ventilation plots for individual data. (A) UDV, (B) hybrid, (C) regression-hypoxia, (D) regression-hypercapnia (ventilation < 100ml/min/kg), (E) regression-hypercapnia (ventilation >100ml/min/kg) and (F) the slopes alone (converging point of the slopes = mean air value). Values are presented as means ± S.E.M.
Figure 2.11. All individual data plotted together. Different colours denote different experimental treatments. Black – UDV method; orange – hybrid method; red – hypoxia in regression method; green and blue – hypercapnia in regression method (best-fit line: double rectangular 5 parameter hyperbola).
Figure 2.12. Three-dimensional mesh plots of work vs. instantaneous frequency vs. tidal volume. (A) Hypoxia produced a mild response in comparison to (B) hypercapnia, spanning across a wider range in work and volume.
Figure 2.13. A theoretical O₂ consumption vs. ventilation plot derived from the three-dimensional mesh plots. Values are presented as means ± S.E.M.
2.4. Discussion

2.4.1. Overall response to respiratory stimuli

The values for all components of the breathing patterns recorded in the present study of resting, spontaneously breathing turtles fell within the field of values previously recorded in *Trachemys scripta elegans* and other turtle species (Table 1). Values for \( V_T \), \( f_R \), \( V_E \) and \( V_{O_2} \) all fell in the mid-range of values recorded in other studies. Hypoxia and hypercapnia stimulated breathing to various degrees, hypoxia always acting as a mild stimulus and hypercapnia as a stronger one (Table 1). Values for all four respiratory variables recorded in this study during hypoxia fell in the lower range of reported values. In contrast, values for the same variables recorded during hypercapnia fell in the upper range (Table 1).

2.4.2. Relative versus absolute cost

The oxygen uptake-ventilation relationship maps the changes that occur in \( V_{O_2} \) as \( V_E \) increases. The two studies in the literature that estimated the oxidative cost of breathing in turtles calculated the relative cost of breathing proportional to total metabolism from these relationships (see Kinney and White, 1977; Jackson et al., 1991). Relative costs, however, can vary tremendously as a function of the other metabolic processes that are occurring simultaneously in the animal. The y-intercept of a \( V_{O_2} \) vs. \( V_E \) regression is \( O_2 \) consumption at zero ventilation and represents the non-ventilatory metabolic rate. If the non-ventilatory metabolic rate fluctuates, then the estimated relative costs of breathing will also vary widely. As a result, turtles with higher
“non-ventilatory” metabolism on average will appear to have a lower relative cost of ventilation.

Inaccurate estimation of resting $V_E$ and $V_{O_2}$ in spontaneously breathing turtles can also drastically alter the estimated relative costs. Figure 2.6 demonstrates the variation in resting values that can occur within individuals. This variation exists not only within individuals but between individuals as well (Fig. 2.7). In the present study, we eliminated this confounding factor by calculating the absolute oxidative cost of breathing, or the slope of the relationship between $O_2$ consumption and ventilation: i.e. the amount of $O_2$ consumed per unit of air ventilated. When this was done, however, we still found that the slopes of the $O_2$ consumption vs. ventilation plots from the present study, for both individual and mean data, were highly variable.

2.4.3. Mean data

Unidirectional airflow through the lungs completely suppressed spontaneous ventilation and decreased $V_{O_2}$ from resting values. The slope of the UDV plot of $V_{O_2}$ versus $V_E$ suggested that the absolute cost of breathing was 10.3 ml $O_2$ per L air (0.0103 ml $O_2$ per ml air). This value is slightly higher than double the value calculated from the data in the study by Kinney and White (1977) (4.7 ml $O_2$ per L air; see Table 2). Note that in the present study, the mean resting values taken before and after the cannulation surgery were significantly different but fell on the same regression line. This suggests that the surgery did not alter the oxygen uptake-ventilation relationship but rather suppressed both variables proportionately. The most likely explanation for this is that although all animals were given 48 hours to recover from surgery and anesthesia,
their metabolism was low and the isoflurane was not completely eliminated from their system.

Hypoxia and hypercapnia used during the regression method elicited very different changes in $V_e$ and $V_{O_2}$ and led to different estimates of the absolute cost of breathing. The hypoxia plot suggested a cost of 9.6 ml O$_2$ per L air, a cost estimate nearly identical to the estimate based on the UDV plot. In contrast, the hypercapnia plot produced a negative estimate of the cost of breathing of -0.5 ml O$_2$ per L air (Table 2). This was similar to the inconsistent results obtained from past investigations on various species of reptiles and mammals (Table 3). Paradoxically, the regression obtained from the hypercapnia plot generated a cost estimate that implies that it is energetically cheaper to increase $V_e$ than to not breathe. Similar results have been obtained in alligators, snakes and mammals breathing hypoxic mixtures as well as lizards and mammals breathing hypercapnic gas mixtures (see Table 3).

It should be noted that the data in the hypoxia and hypercapnia plots extend over very different ventilation ranges. To observe the oxygen uptake-ventilation relationship simultaneously across the entire $V_e$ range presented by the three mean plots, all mean data are combined into a single plot in Figure 2.9. In this plot, the UDV and hypoxia slopes depict an initial steep increase in $V_{O_2}$ as $V_e$ increases, which then peaks and decreases with further increase in $V_e$ due to hypercapnia. Overall, the relationship between the two variables for all data was best described by a polynomial regression rather than a linear one. This hyperbolic curve suggests two things: first, that initiating ventilation from zero is an expensive process, which requires a steep increase in $V_{O_2}$ as
\( V_E \) initially increases and second, that as the drive to breathe increases further, the oxygen uptake-ventilation relationship changes.

One explanation for the secondary change in the shape of this overall relationship is that although initiating breathing from zero is relatively costly, increasing ventilation further once the respiratory system is already in motion is less expensive as the inertial forces have been overcome and the momentum of one breath can help initiate the following breath. This gives rise to the hyperbolic shape of the curve. While past studies of the cost of breathing in various reptiles using the regression method assume that the oxygen uptake-ventilation relationship was linear, the data frequently appeared to be better represented by a second order or higher regression (see Wang and Warburton, 1995; Skovgaard and Wang, 2004). These studies also exhibit a steep initial increase in \( V_{O2} \) that plateaus with further increase in \( V_E \), just as in the present study.

While this would explain a decrease in the slope of the relationship between \( V_{O2} \) and \( V_E \) at higher levels of ventilation, it will not explain the fall in \( V_{O2} \) at higher levels of \( V_E \). A possible explanation for this might be a reduction in non-ventilatory metabolism due to the acidosis brought on by the hypercapnia (Busa and Nuccitelli, 1984; Wang et al., 1993). We also cannot dismiss the fact that high levels of CO₂ have known anesthetic effects, which could further induce metabolic suppression. The level of CO₂ used during the present study was certainly high enough to be able to reduce the non-ventilatory metabolism and transform the oxygen uptake-ventilation relationship due to either of these effects. \( V_E \) would no longer be the only factor influencing \( V_{O2} \) and
ultimately the inhibitory effects of CO₂ on metabolism may override any increases in \( V_{O_2} \) due to the increased ventilation and lead to an overall reduction in metabolism.

A third and most likely explanation acknowledges both possibilities with high CO₂ suppressing metabolism to decrease the slope of an oxygen uptake-ventilation relationship that was already non-linear.

2.4.4. Individual data

Given the intra-individual variability described in section 2.4.2, we also examined plots derived from all individual data rather than just mean data.

The relationship between \( \dot{V}_{O_2} \) and \( \dot{V}_{E} \) derived from the UDV data as well as the regression data from the hypoxia and the hybrid trials had similar, steep slopes (13.6, 24.5 and 22.3 ml O₂ per L air respectively). In all three trials the range of \( \dot{V}_{E} \) was less than 50 to 70 ml min\(^{-1}\) kg\(^{-1}\). For the oxygen uptake-ventilation relationship obtained from the hypercapnia regression trials over the lower \( \dot{V}_{E} \) range (< 100 ml min\(^{-1}\) kg\(^{-1}\)), the slope of the \( \dot{V}_{O_2} \) versus \( \dot{V}_{E} \) relationship was 4.7 ml O₂ per L air; however, as ventilation increased further (up to 500 ml min\(^{-1}\) kg\(^{-1}\)), the slope decreased to 1.1 ml O₂ per L air. In short, the greater the range of \( \dot{V}_{E} \), the lower the estimate of the absolute cost of ventilation (Fig. 2.10).

Collectively plotting all the individual data recorded from Series 1, 2 and 3 as a single group of data produced results similar to those obtained when this was done for the mean data with the exception that now there was no secondary dip in the hyperbolic
curve of best fit (Fig. 2.11). Again, a cluster of data at the lower range of ventilation gave rise to a steep positive slope but the slope decreased over the higher range of ventilation.

The results of this analysis are consistent with those of the mean data; there is a non-linear relationship between $\dot{V}_E$ and $\dot{V}_{O_2}$ that may be suppressed at higher levels of $\dot{V}_E$ by the effects of elevated CO₂.

Assuming the O₂ consumption vs. ventilation relationship is non-linear, the estimated oxidative costs from the UDV and the regression methods should differ as originally hypothesized. One method suppresses spontaneous ventilation over the lower, steeper portion of the $\dot{V}_{O_2}$ versus $\dot{V}_E$ relationship while the other stimulates $\dot{V}_E$ over the higher range of the same relationship. The UDV method starts at resting $\dot{V}_E$ and suppresses all spontaneous breathing to achieve zero ventilation whereas the regression method starts at resting $\dot{V}_E$ and increases ventilation to levels as high as 500 ml min⁻¹ kg⁻¹.

Therefore, the steeper slope between zero and resting ventilation in the UDV trial will estimate higher costs than the regression method. This is consistent with the results of the present study and also the studies by Kinney and White (4.7 ml O₂ per L air) and Jackson et al. (0.3 ml O₂ per L air); a non-linear O₂ consumption vs. ventilation relationship could explain the discrepancy in results between the two studies that employ two different methods.

CO₂-induced metabolic suppression could still also be a plausible explanation for the discrepant results. The inverse correlation between CO₂ levels and the estimated oxidative cost from the present study was obvious: the higher the CO₂, the lower the cost. The calculated oxidative costs from the hypercapnia-regression experiments decreased
from 4.7 to 1.1 ml O₂ per L air as the level of CO₂ increased, which could in part reflect a reduced metabolism. This could also be what is responsible for the low cost estimated by Jackson et al. (1991).

2.4.5. Theoretical estimate

The theoretical estimate of the oxidative cost of breathing was very low at 0.3 ml O₂ per L air, identical to Jackson’s estimate and closest to the hypercapnia-regression results from the present study when \( V'_E \) exceeded 100 ml min⁻¹ kg⁻¹ (1.1 ml O₂ per L air).

Unlike the experimental results, the theoretical data suggest that the oxygen uptake-ventilation relationship should be linear. The mechanical cost of breathing increased in a very linear fashion and thus the calculated theoretical cost of breathing did so as well over the entire \( V'_E \) range from zero to 500 ml min⁻¹ kg⁻¹. The transition from no breathing to only some breathing was not more expensive than the transition from some breathing to more breathing. Overcoming inertial forces did not increase the work and cost involved with initiating breathing.

Furthermore, the theoretical data suggest that metabolic suppression may not be occurring either. Although experimental data support the idea that lower oxidative costs at high \( V'_E \) could be due to the reduction in overall metabolism from CO₂ exposures, the low cost estimates obtained from the theoretical data (similar or less than the experimental estimate from the high CO₂ data) imply that the true cost is in fact low.

As one would expect, this produces a conundrum. If metabolic suppression by high CO₂ is not acting to reduce the slope of a non-linear oxygen uptake-ventilation relationship, a different explanation is needed. The only other explanation that could
account for the differences in values obtained at low versus high levels of respiratory drive is the change in breathing pattern as turtles go from breathing episodically to breathing continuously.

Diverse breathing patterns were recorded with each method in the present study ranging from very sporadic to continuous, depending on the level of $V_E$. As overall $V_E$ increased, irregular bursts of breaths transformed into regular breathing episodes and finally into consistent, continuous breathing with the periods of apnea eliminated.

At lower levels of $V_E$ (i.e. resting, control levels), the breath-hold or the apnea length is highly variable (Milsom and Johansen, 1975; Burrgren and Shelton, 1979; Ackerman and White, 1979; Milsom and Chan, 1986). One might expect a strong correlation between an apnea length and the size of either the following or preceding breathing episode. This would either be to pay off the O₂ debt accumulated during an apnea or to store O₂ for the coming period of apnea; however, several researchers have failed to find such a correlation (Fig. 2.14) (Burrgren and Shelton, 1979). Instead, it appears that the lengths of both breathing episodes and apneas are more tightly linked to behaviour and O₂ debts accumulated are paid off over longer periods of time. Given this, correlating the amount of O₂ extracted from each breath with metabolic rate becomes extremely difficult and the oxidative costs of ventilation calculated from irregular patterns could under or overestimate $\dot{V}_{O₂}$ (see Figs. 2.6, 2.7). Since most irregular breathing patterns were recorded when $V_E$ was low (i.e. less than 50 to 70 ml/min/kg), this may have given rise to the steep slopes of the $\dot{V}_{O₂}$ versus $V_E$ relationships and led to overestimates of the absolute cost of breathing under these conditions (the UDV,
hypoxia-regression and hybrid-regression trials). In contrast to the irregular bursts of breathing at low $V_e^*$, breathing episodes and apneas became more regular and consistent in length at high $V_e^*$. In the hypercapnia-regression trials with $V_e^* < 100$ ml/min/kg, the estimated cost of breathing was lower as overall breathing became more uniform. Breathing was still very episodic, however, and the cost estimates higher than when $V_e^* > 100$ ml/min/kg. Exposure to higher levels of CO2 stimulated $V_e^*$ up to 500 ml/min/kg, a level sufficiently high enough to produce evenly spaced breathing episodes separated by very short, equal lengths of apnea. In the present study, breathing appeared continuous during 5 and 6% CO2 exposures. Under these conditions, the amount of O2 extracted with each breath should have evened out and the calculated costs based on these consistent breathing traces should be more reliable and accurate. Of note, the low oxidative costs of breathing derived from the study of Jackson et al. (1991) in turtles used 5% CO2 to stimulate ventilation up to approximately 500 ml/min/kg, which most likely would have achieved continuous breathing with no apnea.

2.4.6. Applications of the research findings

Recalculating the relative cost of ventilation from the literature: The theoretical O2 consumption vs. ventilation plot was derived from measurements of the mechanical work of breathing in turtles. The theoretical $V_{O2}^*$ values estimate the amount of O2 consumed for the act of breathing alone. Using the slope of this relationship (0.3 ml O2/L air) and resting $V_e^*$ and $V_{O2}^*$ values reported for different turtle species from the literature, the theoretical relative oxidative costs of breathing can be calculated. When this was
done (see Table 4) for resting values of turtles breathing air, the relative cost of breathing was consistently less than 1% of total metabolism. As total $V'_E$ increased with increasing respiratory drive (either low O$_2$ or high CO$_2$), a greater portion of total metabolism was required to support breathing; roughly 2% at the lower levels of drive associated with hypoxia and consistently up to 6% for studies producing higher levels of respiratory drive using hypercapnia.

**Optimal Breathing Patterns:** As mentioned previously, it is believed that turtles breathe with an optimum combination of tidal volume and instantaneous breathing frequency that minimizes the mechanical work of breathing and that this combination is retained fairly constant within breathing episodes (Vitalis and Milsom, 1986a). To determine just how much of a difference this might make to overall metabolic costs, the minute work of breathing values in Figure 1.2 were converted to corresponding oxidative cost values based on our theoretical estimates (Fig. 2.15). From this figure, one can see that any alteration from the optimum combination of $V_T$ and $f_{Rinst}$ increases work exponentially (Vitalis and Milsom, 1986b). While this was true at any level of $V'_E$, the significance was ventilation-dependent. The additional energy expenditure required to power respiration at less optimal combinations at a total ventilation of 100 ml/min/kg was insignificant; however, at ventilations of 200 or 300 ml/min/kg, O$_2$ consumption almost doubled across the $V_T/f_{Rinst}$ range indicating that maintaining an optimal tidal volume-breathing frequency combination can significantly minimize the work and cost associated with breathing at higher levels of respiratory drive.
Figure 2.14. (A) Examples of various episodic breathing patterns. There is no correlation between the length of an apnea and the following or preceding breathing episode. (B) Fluctuations in femoral artery ($P_a$) gas tensions. Shaded vertical bars indicate bouts of breathing (adapted from Burggren and Shelton, 1979).
Figure 2.15. $O_2$ consumption vs. instantaneous frequency at three different levels of ventilation. The white bar indicates the range of instantaneous frequency that is optimal for minimizing work and $O_2$ consumption during respiration.
<table>
<thead>
<tr>
<th>Species</th>
<th>Respiratory gas</th>
<th>$V_T$ (ml kg$^{-1}$)</th>
<th>$f_R$ (min$^{-1}$)</th>
<th>$\dot{V}$ (ml min$^{-1}$ kg$^{-1}$)</th>
<th>$\dot{V}_{O_2}$ (ml min$^{-1}$ kg$^{-1}$)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>12.56 ± 3.04</td>
<td>1.37 ± 0.25</td>
<td>14.08 ± 2.38</td>
<td>0.55 ± 0.08</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>1.0 ± 0.1</td>
<td>0.1</td>
<td>1.94 ± 0.32</td>
<td>0.07 ± 0.01</td>
<td>Bagatto &amp; Henry (1999)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>0.90 ± 0.07</td>
<td>0.09</td>
<td>2.5 ± 0.32</td>
<td>0.01 ± 0.01</td>
<td>Crawford Jr. et al. (1976)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>0.47 ± 0.15</td>
<td>0.04</td>
<td>0.92 ± 0.15</td>
<td>0.01 ± 0.01</td>
<td>Belkin (1968)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>36 ± 4</td>
<td>3.6</td>
<td>2.6 ± 0.42</td>
<td>0.2 ± 0.01</td>
<td>Hitzig (1982)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>38 ± 8 (ml)</td>
<td>0.82 ± 0.2</td>
<td>31 ± 4</td>
<td>0.6 ± 0.1</td>
<td>Hitzig &amp; Nattie (1982)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>20 ± 3 (ml)</td>
<td>0.95 ± 0.1</td>
<td>19 ± 4</td>
<td>0.6 ± 0.1</td>
<td>Hitzig &amp; Nattie (1982)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>23 ± 5 (ml)</td>
<td>1.03 ± 0.2</td>
<td>24 ± 6</td>
<td>0.7 ± 0.1</td>
<td>Hitzig &amp; Nattie (1982)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>6.9 ± 1.2</td>
<td>2.0 ± 0.07</td>
<td>13.8</td>
<td>0.7 ± 0.1</td>
<td>Vitalis &amp; Milsom (1986)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>18.5 ± 7.0</td>
<td>1.6 ± 0.8</td>
<td>25.6 ± 12.9</td>
<td>0.65 ± 0.28</td>
<td>Jackson (1971)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>8.8* (ml)</td>
<td>1.40 ± 0.15</td>
<td>12.3 ± 1.3 (ml/min)</td>
<td>0.69 ± 0.08</td>
<td>Jackson (1973)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>Air</td>
<td>15.7 ± 2.1</td>
<td>1.6 ± 0.2</td>
<td>23.8 ± 3.4</td>
<td>0.7 ± 0.08</td>
<td>Jackson et al. (1974)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Air</td>
<td>38 ± 8 (ml)</td>
<td>0.82 ± 0.2</td>
<td>31 ± 4</td>
<td>0.6 ± 0.1</td>
<td>Stockard &amp; Gatten Jr. (1983)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Air</td>
<td>11</td>
<td>2.0</td>
<td>20</td>
<td>0.61</td>
<td>Glass et al. (1983)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Air</td>
<td>10.7 ± 1.2</td>
<td>1.90 ± 0.27</td>
<td>17.5</td>
<td>0.60</td>
<td>Glass et al. (1985)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Air</td>
<td>9.4 ± 3.1</td>
<td>1.9 ± 0.3</td>
<td>16.7 ± 4.0</td>
<td>0.58 ± 0.10</td>
<td>Silver &amp; Jackson (1985)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>Air</td>
<td>13.5 ± 0.9</td>
<td>1.8 ± 0.2</td>
<td>24.8 ± 3.4</td>
<td>0.7 ± 0.2</td>
<td>Milsom &amp; Jones (1980)</td>
</tr>
<tr>
<td><em>Pseudemys floridana</em></td>
<td>Air</td>
<td>14</td>
<td>28.5</td>
<td>0.9</td>
<td>0.60</td>
<td>Kinney et al. (1977)</td>
</tr>
<tr>
<td><em>Pseudemys floridana</em></td>
<td>Air</td>
<td>28.5</td>
<td>0.9</td>
<td>0.60</td>
<td>0.60</td>
<td>Kinney &amp; White (1977)</td>
</tr>
<tr>
<td><em>Pelomedusa subrufa</em></td>
<td>Air</td>
<td>33</td>
<td>1.6</td>
<td>39.8</td>
<td>0.7 ± 0.2</td>
<td>Burggren et al. (1977)</td>
</tr>
<tr>
<td><em>Pelomedusa subrufa</em></td>
<td>Air</td>
<td>15.5 ± 2.4</td>
<td>1.4 ± 0.2</td>
<td>22.4 ± 5.6</td>
<td>0.8 ± 0.2</td>
<td>Glass et al. (1978)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------------</td>
<td>----------------------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>0% O₂</td>
<td>68 ± 11 (ml)</td>
<td>1.15 ± 0.30</td>
<td>78 ± 12</td>
<td>Hitzig &amp; Nattie (1982)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-3% O₂</td>
<td>9.40 ± 1.06</td>
<td>1.29 ± 0.14</td>
<td>9.53 ± 1.10</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>3% O₂</td>
<td>14.9* (ml)</td>
<td>2.58 ± 0.25</td>
<td>38.4 (ml/min)</td>
<td>Jackson (1973)</td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>4% O₂</td>
<td>6.2 ± 0.8</td>
<td>3.0 ± 0.4</td>
<td>18.6</td>
<td>Vitalis &amp; Milsom (1986)</td>
<td></td>
</tr>
<tr>
<td><strong>Chrysemys picta bellii</strong></td>
<td>3% O₂</td>
<td>19</td>
<td>3.2</td>
<td>60</td>
<td>Glass et al. (1983)</td>
<td></td>
</tr>
<tr>
<td><strong>Pelomedusa subrufa</strong></td>
<td>5% O₂</td>
<td>35</td>
<td>2.0</td>
<td>51.3</td>
<td>Burggren et al. (1977)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypercapnia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>4-6% CO₂</td>
<td>34.89 ± 2.34</td>
<td>7.32 ± 0.66</td>
<td>254.46 ± 30.35</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>3-5% CO₂</td>
<td>6.8 ± 0.4</td>
<td>4.2 ± 1.3</td>
<td>28.3</td>
<td>Vitalis &amp; Milsom (1986)</td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>6% CO₂</td>
<td>47.3 ± 4.3</td>
<td>4.6 ± 0.3</td>
<td>215 ± 21.6</td>
<td>Jackson et al. (1974)</td>
<td></td>
</tr>
<tr>
<td><strong>Trachemys scripta</strong></td>
<td>8% CO₂</td>
<td>45 ± 22 (ml)</td>
<td>2.70 ± 0.3</td>
<td>122 ± 28</td>
<td>Hitzig &amp; Nattie (1982)</td>
<td></td>
</tr>
<tr>
<td><strong>Chrysemys picta bellii</strong></td>
<td>5% CO₂</td>
<td>17.5 ± 2.0</td>
<td>3.9 ± 0.6</td>
<td>65.5 ± 11.4</td>
<td>Milsom &amp; Jones (1980)</td>
<td></td>
</tr>
<tr>
<td><strong>Chrysemys picta bellii</strong></td>
<td>5.7% CO₂</td>
<td>22.3 ± 3.5</td>
<td>9.4 ± 2.6</td>
<td>235.8 ± 91.4</td>
<td>Silver &amp; Jackson (1985)</td>
<td></td>
</tr>
</tbody>
</table>

* All studies conducted at temperatures between 20 and 25°C.
Table 2.2. Comparison of calculated oxidative cost values between different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Oxidative cost (ml O₂ / ml air)</th>
<th>Ventilation range (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression (Mean data):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td>0.0096</td>
<td>0 - 30</td>
</tr>
<tr>
<td>Hypercapnia</td>
<td>-0.0005</td>
<td>0 - 200</td>
</tr>
<tr>
<td>Regression (Individual data):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td>0.0223</td>
<td>0 - 30</td>
</tr>
<tr>
<td>Hypercapnia (&lt;100ml/min/kg)</td>
<td>0.0047</td>
<td>0 - 100</td>
</tr>
<tr>
<td>Hypercapnia (&gt;100ml/min/kg)</td>
<td>0.0011</td>
<td>100 - 500</td>
</tr>
<tr>
<td>Jackson et al. (1991)</td>
<td>0.0003</td>
<td>0 - 500</td>
</tr>
<tr>
<td>UDV (Mean data):</td>
<td>0.0103</td>
<td>0 - 30</td>
</tr>
<tr>
<td>UDV (Individual data):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinney and White (1977)</td>
<td>0.0136</td>
<td>0 - 30</td>
</tr>
<tr>
<td>Regression – UDV Hybrid:</td>
<td>0.0245</td>
<td>0 - 60</td>
</tr>
<tr>
<td>Theoretical estimate:</td>
<td>0.0003</td>
<td>0 - 500</td>
</tr>
</tbody>
</table>
Table 2.3. Comparison of calculated oxidative cost values using the regression method taken from the literature

<table>
<thead>
<tr>
<th>Species</th>
<th>Respiratory gas</th>
<th>Oxidative cost (ml O₂ / ml air)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tupinambis merianae</em></td>
<td>6% O₂</td>
<td>0.006</td>
<td>Skovgaard &amp; Wang (2004)</td>
</tr>
<tr>
<td><em>Alligator mississippiensis</em></td>
<td>2.8% O₂</td>
<td>-0.0008</td>
<td>Wang &amp; Warburton (1995)</td>
</tr>
<tr>
<td><em>Natrix rhombifera</em></td>
<td>5% O₂</td>
<td>-0.001</td>
<td>Gratz (1979)</td>
</tr>
<tr>
<td><em>Amphisbaena alba</em></td>
<td>3% O₂</td>
<td>0.006</td>
<td>Abe &amp; Johansen (1987)</td>
</tr>
<tr>
<td><em>Dasypus novemcincus</em></td>
<td>8% O₂</td>
<td>-0.02</td>
<td>Boggs et al. (1998)</td>
</tr>
<tr>
<td><em>Phyllostomus discolor</em></td>
<td>8% O₂</td>
<td>0.05</td>
<td>Walsh et al. (1996)</td>
</tr>
<tr>
<td><em>Spermophilus lateralis</em></td>
<td>7% O₂</td>
<td>0.002</td>
<td>Barros et al. (2001)</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>10% O₂</td>
<td>0.001</td>
<td>Saiki et al. (1994)</td>
</tr>
<tr>
<td><em>Lasiorhinus latifrons</em></td>
<td>8% O₂</td>
<td>-0.009</td>
<td>Frappell et al. (2002)</td>
</tr>
<tr>
<td>Hypercapnia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tupinambis merianae</em></td>
<td>6% CO₂</td>
<td>-0.002</td>
<td>Skovgaard &amp; Wang (2004)</td>
</tr>
<tr>
<td><em>Alligator mississippiensis</em></td>
<td>9.3% CO₂</td>
<td>0.005</td>
<td>Wang &amp; Warburton (1995)</td>
</tr>
<tr>
<td><em>Natrix rhombifera</em></td>
<td>6% CO₂</td>
<td>0.003</td>
<td>Gratz (1979)</td>
</tr>
<tr>
<td><em>Amphisbaena alba</em></td>
<td>6% CO₂</td>
<td>0.004</td>
<td>Abe &amp; Johansen (1987)</td>
</tr>
<tr>
<td><em>Dasypus novemcincus</em></td>
<td>7% CO₂</td>
<td>-0.006</td>
<td>Boggs et al. (1998)</td>
</tr>
<tr>
<td><em>Phyllostomus discolor</em></td>
<td>7% CO₂</td>
<td>0</td>
<td>Walsh et al. (1996)</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>5% CO₂</td>
<td>-0.002</td>
<td>Seifert &amp; Mortola (2002)</td>
</tr>
<tr>
<td><em>Lasiorhinus latifrons</em></td>
<td>5% CO₂</td>
<td>-0.003</td>
<td>Frappell et al. (2002)</td>
</tr>
</tbody>
</table>
Table 2.4. Comparison of theoretically estimated relative oxidative cost of breathing for semi-aquatic turtles from the literature

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature (°C)</th>
<th>Respiratory gas</th>
<th>Relative oxidative cost (% total $\dot{V}_{O_2}$)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trachemys scripta</em></td>
<td>20 - 23</td>
<td>Air</td>
<td>0.03</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>20</td>
<td>Air</td>
<td>0.3</td>
<td>Jackson (1971)</td>
</tr>
<tr>
<td><em>Trachemys scripta</em></td>
<td>20</td>
<td>Air</td>
<td>0.3</td>
<td>Jackson, Palmer &amp; Meadow (1974)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>20</td>
<td>Air</td>
<td>0.2</td>
<td>Glass, Boutilier &amp; Heisler (1983)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>20</td>
<td>Air</td>
<td>0.1</td>
<td>Glass, Boutilier &amp; Heisler (1985)</td>
</tr>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>20</td>
<td>Air</td>
<td>0.1</td>
<td>Silver &amp; Jackson (1985)</td>
</tr>
<tr>
<td><em>Pseudemys floridana</em></td>
<td>22</td>
<td>Air</td>
<td>0.3</td>
<td>Kinney &amp; White (1977)</td>
</tr>
</tbody>
</table>

- Hypoxia
  - *Chrysemys picta bellii* 20 3% O$_2$ 2 Glass et al. (1983)

- Hypercapnia
  - *Trachemys scripta* 20 4-6% CO$_2$ 6 Present study
  - *Trachemys scripta* 20 6% CO$_2$ 6 Jackson et al. (1974)
  - *Chrysemys picta bellii* 20 5.7% CO$_2$ 6 Silver & Jackson (1985)
3. Conclusion

3.1. Summary

Determining the oxidative cost of breathing in turtles has been a topic of interest for decades. The two key studies in the past that have attempted to answer this question produced contradictory results and this controversy had been left to be resolved for more than a decade. The present study thoroughly examined the oxygen uptake-ventilation relationships and the changing breathing patterns of red-eared sliders under various circumstances (i.e. re-using the two methods from the past studies (hypoxia/hypercapnia exposures, unidirectional ventilation via the lungs) and incorporating a third hybrid method of the two).

As originally hypothesized, a non-linear oxygen uptake-ventilation relationship could be a plausible explanation for the discrepant data in the original studies as well as in the present study. However, this was not consistent with the theoretical relationship that suggested that the increase in oxygen uptake was linear with increasing ventilation.

The theoretical regression also suggested that metabolic rate suppression from high CO₂ exposure was unlikely since the theoretical oxidative cost was similar to or less than the estimate from the hypercapnia-regression trials.

The only explanation that was compatible with all of the data (experimental and theoretical) was that the spectrum of breathing patterns observed during different experimental trials, from irregular episodes to continuous breathing, was responsible for the discrepant results. This suggests that breathing pattern, rather than methodology itself, was the source of the discrepancy and the key factor to be considered in determining the true oxidative cost of ventilation.
3.2. Shortcomings and future directions

Studying ventilation in turtles or any other episodic breathers should come with a caveat; using short segments of irregular breathing traces to calculate ventilatory costs does not produce consistent, reliable results. Oxidative cost estimates based on longer periods (i.e. several hours) of breathing produce more consistent and accurate costs of breathing.

Regardless of the conclusions drawn from this study, there are several shortcomings to the research. During the hypoxia-regression trials, hypoxia only acted as a mild respiratory stimulus and did not significantly stimulate breathing. Also, recordings used to estimate costs during the hypoxia exposures were only 20 minutes long and based on the conclusions of this study, 20 minutes of breathing trace is insufficient in length for an accurate estimation. These experiments should be repeated with longer recordings, preferably several hours, at greater levels of hypoxia. The same is true for the UDV and hybrid experiments. Breathing traces used in these analyses were again only 20 minutes long and should have been longer.

Although the theoretical data indicate that metabolic rate suppression during CO₂ exposures is unlikely, conducting experiments to confirm this will be the next logical step. By artificially ventilating an anesthetised, non-breathing turtle with the same hypercapnic gas mixtures used in the regression method, any alteration in metabolic rate can be tracked without stimulating ventilation. Despite the theoretical data, high levels of CO₂ are known to suppress metabolism; therefore, a precise documentation of any changes in metabolic rate when exposed to high CO₂ is an essential step to solidifying any arguments from this study.
3.3. Overall conclusion

From the outcomes of this study, the challenges of working with episodic breathers are evident. Two factors should always be taken into consideration; first, the length of recordings and second, the regularity of the breathing pattern used for estimating oxidative costs of ventilation. Calculations based on episodic breathing containing irregular periods of apnea may either underestimate or overestimate O₂ consumption and lead to inaccurate cost estimates. Consequently, we discovered that apparent differences due to methodology were likely due to differences in breathing patterns. Oxidative costs based on periods of more continuous breathing yielded values compatible with theoretical estimates and costs of breathing in turtles appear to be similar to those of mammals (0.3 ml O₂/L air). Ultimately, the results of this study, I believe, have formulated a new solution to a question that was left to be answered for decades and have provided a new starting point for investigations-to-come in the future.
Bibliography


