

Lung ventilation during treadmill locomotion in a terrestrial turtle, *Terrapene carolina*

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Summary

The limb girdles and lungs of turtles are both located within the bony shell, and therefore limb movements during locomotion could affect breathing performance. A mechanical conflict between locomotion and lung ventilation has been reported in adult green sea turtles, *Chelonia mydas*, in which breathing stops during terrestrial locomotion and resumes during pauses between bouts of locomotion. We measured lung ventilation during treadmill locomotion using pneumotach masks in three individual *Terrapene carolina* (mass 304–416 g) and found no consistent mechanical effects of locomotion on breathing performance. Relatively small tidal volumes (2.2 ± 1.4 ml breath⁻¹; mean \pm s.d., $N=3$ individuals) coupled with high breath frequencies (36.6 ± 26.4 breaths min⁻¹; mean \pm s.d., $N=3$ individuals) during locomotion yield mass-specific minute volumes that are higher than any previously reported for turtles (264 ± 64 ml min kg⁻¹; mean \pm s.d., $N=3$ individuals). Minute volume was higher during locomotion than during recovery from exercise ($P < 0.01$; paired *t*-test), and tidal volumes measured during locomotion were not significantly different from values measured during brief pauses between locomotor bouts or during recovery from exercise ($P > 0.05$; two-way ANOVA). Since locomotion does not appear to conflict with breathing performance,

the mechanism of lung ventilation must be either independent of, or coupled to, the stride cycle. The timing of peak airflow from breaths occurring during locomotion does not show any fixed phase relationship with the stride cycle. Additionally, the peak values of inhalatory and exhalatory airflow rates do not differ consistently with respect to the stride cycle. Together, these data indicate that *T. carolina* is not using respiratory-locomotor coupling and limb and girdle movements do not contribute to lung ventilation during locomotion. X-ray video recordings indicate that lung ventilation is achieved via bilateral activity of the transverse (exhalatory) and oblique (inhalatory) abdominal muscles. This specialized abdominal ventilation mechanism may have originally circumvented a mechanical conflict between breathing and locomotion in the ancestor of turtles and subsequently allowed the ribs to abandon their role in lung ventilation and to fuse to form the shell.

Movies available on line.

Key words: *Terrapene carolina triunguis*, North American three-toed box turtle, Emydidae, breathing mechanism, respiration, functional morphology, exercise physiology, hypaxial musculature.

Introduction

In this study we investigate whether limb movements hinder or assist lung ventilation during turtle locomotion. A peculiar aspect of turtle morphology is that both pectoral and pelvic limb girdles are located inside the bony shell – equivalent to having our shoulder blades and hips inside our rib cage (Fig. 1). The rigid turtle shell contains a relatively fixed volume, thereby causing the air within the lungs to be displaced whenever axial or appendicular elements move within the shell. This constant volume constraint suggests that during locomotion, limb movements could affect breathing performance.

The breathing mechanisms of turtles have been of enduring interest to scientists for more than three centuries (e.g.

Malpighi, 1671; Townson, reprinted in Mitchell and Morehouse, 1863; Gans and Hughes, 1967). A hyobranchial pumping mechanism was proposed (most notably by Agassiz, 1857) to function like the buccal pump of fishes and amphibians, forcing air into the lungs under positive pressure, but numerous experimental investigations have found that the oscillatory throat movements do not contribute to lung ventilation in turtles at rest (Mitchell and Morehouse, 1863; Francois-Franck, 1908; Hansen, 1941; McCutcheon, 1943; Gans and Hughes, 1967; Gaunt and Gans, 1969; Brainerd, 1999; Druzisky and Brainerd, 2001; Landberg et al., 2001, 2002a). Experimental studies have found two main breathing mechanisms in resting turtles: (1) the action of sheet-like

muscles such as the oblique and transverse abdominis, diaphragmaticus, and striatum pulmonale muscles (Fig. 1A; Mitchell and Morehouse, 1863; Hansen, 1941; McCutcheon, 1943; George and Shaw, 1954, 1955, 1959; Shaw, 1962; Gans and Hughes, 1967; Gaunt and Gans, 1969) and (2) a limb-pump ventilation mechanism (Fig. 1B,C; Gans and Hughes, 1967; Gaunt and Gans, 1969).

The transverse abdominis (TA) and oblique abdominis (OA) muscles alternate bilateral muscle activity to produce exhalation–inhalation breathing cycles in turtles at rest (McCutcheon, 1943; Gans and Hughes, 1967; Gaunt and Gans, 1969; Currie, 2001). These abdominal muscles are considered the primary ventilation mechanism of turtles because they are present in all extant turtle species (George and Shaw, 1959; Shaw, 1962) and have been found to be active consistently during lung ventilation (Gans and Hughes, 1967; Gaunt and Gans, 1969). The OA is a paired, thin, cup-shaped muscle attaching along the rear carapacial and plastral margins in the inguinal limb pockets, anterior to each hindlimb and just deep to the skin (Fig. 1A). At rest, this muscle curves into the body cavity; when contracted, it flattens to move the flank postero-ventero-laterally, which reduces intrapulmonary pressure and produces inhalation when the glottis is open.

The paired transverse abdominis (TA) lies deep to the oblique abdominis (OA). It attaches to the inside of the carapace and is cupped around the posterior half of each lung (Fig. 1A). As the TA contracts, intrapulmonary pressure increases, producing exhalation when the glottis is open. The convex sides of the TA and OA face each other and are attached by connective tissue at their apices. When one muscle (the agonist) contracts and flattens, the antagonist is stretched into a highly curved position from which it can contract to reverse the motion.

Despite close anatomical approximation to the pelvic girdle and hindlimbs, the TA and OA are considered abdominal muscles because they are innervated by spinal projections (from the 6th and 7th vertebrae of the carapace) that branch off before the pelvic enlargement of the spinal cord (Bojanus 1819, reprinted 1970; Currie and Gonsalves, 1997). These muscles are often called ‘respiratory muscles’, but we reject this term because many vertebrate muscles perform more than one function, and the functions of muscles may change during evolution (Carrier and Farmer, 2000; Deban and Carrier, 2002). The importance of avoiding functional names for the abdominal muscles was illustrated recently when they were found to be active in the absence of breathing during underwater locomotion of the red-eared slider (*Trachemys scripta*; Currie, 2001).

In non-locomoting turtles, movements of the limbs and girdles have been shown to contribute to ventilation as well as to the redistribution of air into different parts of the lungs (Francois-Franck, 1908; Gans and Hughes, 1967; Gaunt and Gans, 1969; Spragg et al., 1980). Authors

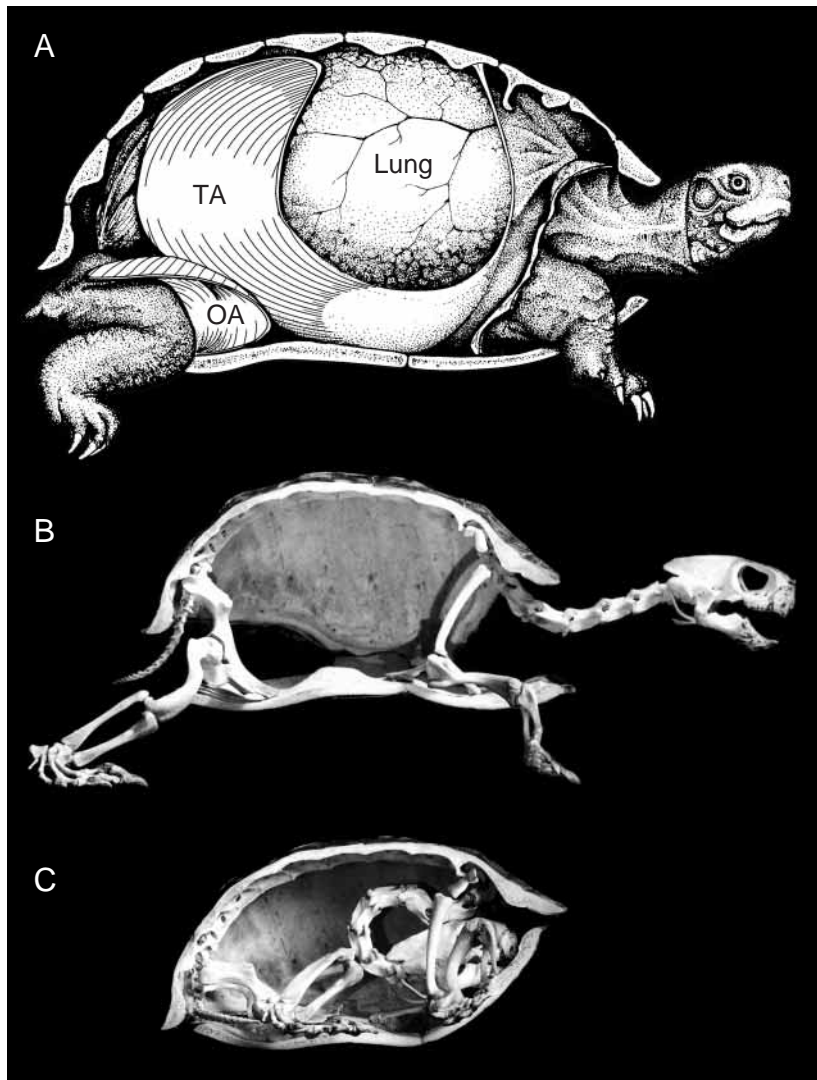


Fig. 1. Lateral view of *Terrapene carolina* illustrating the two main lung ventilation mechanisms of turtles. Half the shell has been removed to reveal the internal morphological relationships between the lungs, abdominal muscles and skeletal elements. (A) Illustration of the abdominal muscles and lungs of *T. carolina*. The paired transverse abdominis (TA) muscles wrap around the posterior portion of the lungs and produce exhalation by compressing the lungs as they contract. The cup-shaped oblique abdominis (OA) muscles actively produce inhalation as they flatten and expand the inguinal flank postero-ventero-laterally. (B) Photograph of the skeleton with limbs and neck fully extended. Because the shell contains a fixed volume, the lungs can be filled with air when the head and limbs are protracted. (C) Air can be forced out of the lungs when the limbs and head are retracted into the shell. Our recordings show that when *T. carolina* is in this fully retracted position, some air remains in the lungs and breathing is possible with the use of the abdominal muscles.

have variously speculated that this limp pump is the main ventilation mechanism (e.g. Pope, 1939), that breathing is an obligatory consequence of locomotion (Orenstein, 2001), and even that turtles must locomote to breathe at all (Tauvery, 1701; cited in Gans and Hughes, 1967). Because the volume within the turtle shell is nearly constant, retraction of the pectoral or pelvic limb and girdle elements into the shell drives air out of the lungs while protraction of limb elements creates subatmospheric pressures, which can produce inhalation (Fig. 1; Gans and Hughes, 1967; Gaunt and Gans, 1969). The muscles of the pectoral (testoscapularis, testocoracoideus and pectoralis) and pelvic (atrahens and retrahens pelvim) limbs and girdles that have been shown to be active during ventilation in resting turtles are also recruited for limb movement during locomotion (Gans and Hughes, 1967; Gaunt and Gans, 1969). If these muscles are used for both breathing and locomotion, might locomotion either interfere with or assist breathing?

Experimental evidence from adult female green sea turtles, *Chelonia mydas*, suggests that locomotion may interfere with breathing performance (Prange and Jackson, 1976; Jackson and Prange, 1979). During terrestrial locomotion, *C. mydas* stops breathing during bouts of locomotion and resumes breathing during pauses in locomotion. Jackson and Prange (1979) suggested that the use of limb musculature for both locomotion and breathing prevents the two behaviors from being performed at the same time.

Mechanical interactions between locomotion and breathing in extant tetrapods are of particular interest because lung ventilation has been hypothesized to conflict with locomotion in the common ancestor of amniotes (Carrier, 1987a). The primitive amniote locomotor pattern includes lateral undulation, which requires unilateral activity of axial musculature. Locomotion and ventilation come into mechanical conflict because costal ventilation requires bilateral activity of those same muscles (Carrier, 1987b, 1991). Birds, mammals and crocodylians have circumvented this constraint through the independent evolution of body postures and/or ventilatory mechanisms that partially decouple breathing from locomotion (Carrier, 1987a; Farmer and Carrier, 2000). In some lizards, the gular pump serves as an accessory mechanism to supplement lung ventilation while costal musculature is in use for locomotion (Owerkovicz et al., 1999, 2001). If limb movements interfere with breathing in turtles, alternative ventilation mechanisms such as the gular pump might be employed during locomotion. Previous studies have shown conclusively that gular oscillations do not contribute to lung ventilation in resting turtles (e.g. McCutcheon, 1943; Druzisky and Brainerd, 2001), but none of these studies measured ventilation during locomotion.

The respiratory and locomotor functions of vertebrates are often highly integrated and many vertebrates couple breathing and locomotion (Bramble and Carrier, 1983; Bramble, 1989; Bramble and Jenkins, 1989; Simons, 1996; Boggs et al., 1997; Carrier and Farmer, 2000; Boggs, 2002). During the locomotor cycle of mammals and birds, there are moments of acceleration

and deceleration as well as sagittal flexion and/or movements of the sternum. These mechanical consequences of locomotion can produce cyclic loading regimes within the thoracic cavity (e.g. Boggs et al., 1997). Birds and mammals may breathe at a particular point in the locomotor cycle so that the forces generated by locomotion can contribute to (Alexander, 1989; Boggs et al., 1997; Bramble and Carrier, 1983; Suther et al., 1972; Young et al., 1992) or avoid negative interaction with (Funk et al., 1993) pressure changes necessary for ventilation. In locomotor-respiratory coupling, components of the breathing cycle are predicted to maintain a fixed phase relationship with the stride cycle (Simons, 1999).

The goals of this investigation were to determine whether the box turtle, *Terrapene carolina* breathes during locomotion, and if so: (1) does locomotion alter breathing performance (i.e. tidal volume, breath frequency and/or minute volume); (2) are ventilation and locomotion temporally coupled; (3) are airflow rates directly affected by the stride cycle; and (4) are lung ventilation mechanisms the same as in resting animals (limb-pump and abdominal muscles), or is locomotion the impetus for an accessory mechanism such as the gular pump? Additionally, information about breathing performance during locomotion in box turtles may help to interpret the evolution of lung ventilation mechanisms in relation to the turtle's unique morphology.

Materials and methods

Morphology

Morphological descriptions are based on dissections and skeletal preparations of *Terrapene carolina carolina* L. specimens deposited in the Massachusetts Museum of Natural History at the University of Massachusetts, Amherst. The specimen photographed in Fig. 1B and C was skeletonized in a *Dermestes* beetle colony and the right half of the shell was removed following parasagittal sectioning. The bones of the limbs remained articulated by their natural ligamentous connective tissue and were dried in position after being degreased in ammonia. The connective tissue was rehydrated by immersion in water and the limbs were repositioned and dried in the second pose. The supra- and episcapular bones were photographed separately and digitally added to the image.

Experimental animals

Three individual *Terrapene carolina triunguis* Agassiz 1857 were used in treadmill locomotion experiments (304, 420 and 305 g and 11.4, 12.4 and 11.9 cm carapace length for individuals 01–03, respectively). Animals were housed individually in ~150 liter terraria containing deep sandy soil, structure to hide under and a water dish for soaking. They were fed earthworms, crickets and/or vegetables twice a week, and kept at 26±4°C on a 14 h:10 h light:dark cycle. Several attempts were made to run experiments during winter months but the animals were torpid and refused to locomote. Therefore, all experiments analyzed for this study were conducted during the summer months.

Mask construction

Turtles lack narial valves and can breathe through either the nares or mouth, so both were included in the pneumotach mask (Winokur, 1982). To avoid interference with vision or hearing, the mask was trimmed back from the eyes and tympanic membranes. During construction, attachment and removal of the mask, a padded restraint collar was fitted snugly around the turtle's neck to prevent withdrawal of the head into the shell. When complete, the mask was attached to the animal with surgical adhesive (cyanoacrylate). The seal of the mask was tested by gently blowing into the port after the adhesive was applied. The mask was removed immediately after the experiment without apparent harm to the underlying keratinized skin. The small mass of the mask (~3 g) should not have affected locomotion (Marvin and Lutterschmidt, 1997; Wren et al., 1998).

The pneumotach masks were custom built for each experiment from high viscosity, rubber-based dental impression material (Henry Shein Co., Port Washington, NY, USA) and required two stages of construction before being glued to the animal's head (Fig. 2). During the first stage, the mask covered the nares while the animal breathed through the mouth. Modeling clay (~0.2 ml) was placed over the nares and rounded to the size of the breathing port (Fig. 2A). When removed, this clay created open space in the mask for air to flow through. Dental impression material was applied over the clay and around the eyes (Fig. 2B). When set, the mask was removed, cured and trimmed back away from the eyes and mouth, and a plastic port was inserted through a hole punched in the tip of the mask. During the second stage of mask construction, the animal breathed through the port while clay was placed over the area where the upper and lower beaks (maxillary and mandibular tomia) meet, and from the apex of the maxillary beak up to the nares (Fig. 2C). Dental impression material was applied over the clay, jaws and entire head (except the nares). The previously constructed mask was placed over the uncured material and pressed into place ensuring solid contact at all points. After the composite mask was removed, cured, trimmed and the clay removed, the mask was ready to be glued to the animal on the day of the experiment.

The pneumotach itself was constructed from 53 μm nylon mesh screen secured between two cylinders made from

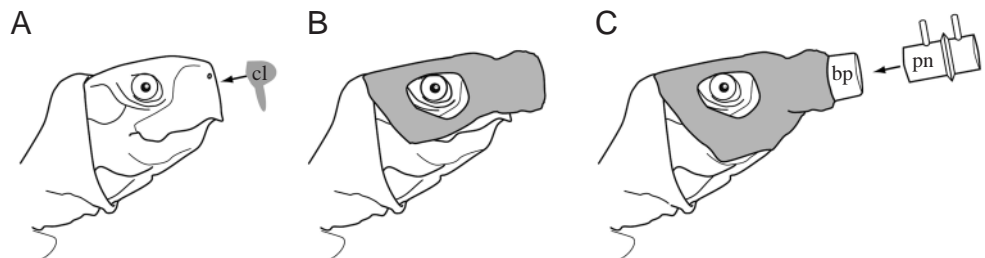
~0.6 cm long pieces of 1 ml syringe (Fig. 2C). The walls of the cylinder on each side of the screen were pierced by short (0.5 cm) pieces of metal tubing (an 18-gauge hypodermic needle). The pneumotach was inserted into the plastic breathing port (6 mm inner diameter) embedded in the mask (Fig. 2C), and could be removed during experiments for inspection and cleaning. The deadspace created by the mask (the sum of pneumotach, breathing port and channels in the mask left after removing the clay) was approximately 0.5 ml. The mask was not ventilated with flowing fresh air because the dead space inside the mask was smaller than normal tidal volume. If the combined tracheal and bronchial dead space is estimated as 0.61 ml kg^{-1} (Perry, 1978), the anatomical and dead space created by the mask add up to approx. 0.75 ml. The pneumotach was calibrated before each experiment using known airflow rates and volumes and was found to produce linear responses to flow over the ranges recorded from the animals ($r^2 > 0.99$).

Data acquisition

Locomotion experiments were conducted in a Plexiglass chamber enclosing a low-speed motorized treadmill. A mirror was placed above the treadmill at a 45° angle, so that experiments filmed from the side recorded both lateral and dorsal views. The pneumotach was connected to a differential pressure transducer (Validyne DP103-06, Northridge, CA, USA) via thin plastic tubing (PE 160). Data from the pressure transducer passed through a carrier demodulator (Validyne CD-15) and were recorded using SuperScope 2.1 software on a Macintosh computer. A real-time image of the pressure trace from the computer was displayed on a television screen with a simultaneous image of the treadmill chamber from a video camera. The video and computer images were synchronized with a video overlay device (TelevEyes Pro, Dedham, MA, USA) and recorded at 30 frames s^{-1} on S-VHS videotapes for frame-by-frame analysis.

A target experimental temperature of 30°C was chosen to maximize voluntary locomotion (Adams et al., 1989; Gatten, 1974), and temperature was controlled by a small space heater placed just outside the experimental chamber. When an animal stopped locomotion, it would be carried backward on the treadmill belt and typically resumed locomotion when it neared the heat source. Occasionally, however, the animals would rest

Fig. 2. Pneumotach mask construction. (A) A small amount of clay (cl) is placed over the turtle's nares. (B) Dental impression material is applied over the clay and face (avoiding lower jaw; turtle breathes through the mouth during this phase). The mask is then removed and trimmed and the breathing port (bp) is inserted.



(C) Clay is placed over the mouth, dental impression material is reapplied (over the mouth but not the nares; turtle breathes through the nares during this phase) and the previous mask is pressed into place. Once the composite mask has cured and been trimmed, it is attached with surgical adhesive on the day of the experiment and the pneumotach (pn) is inserted into the breathing port.

close to this heat source, causing rapid increases in cloacal temperatures. Cloacal temperatures, checked at least once every hour, varied between 25 and 35°C and probably fluctuated more than core body temperature.

The treadmill experiments were organized into four parts: (1) acclimation to the mask, treadmill chamber and experimental temperature; (2) pre-exercise; (3) locomotion; and (4) recovery (post-exercise). Stages 1, 2 and 4 were periods of 1 h each, while the locomotion part of the experiment varied between 2 and 3 h, depending on the animals' performance.

Locomotion was voluntary during the experiments, thus locomotor speed and actual amount of time spent walking during the 'locomotion' segments was variable. Treadmill speed was manually adjusted using a variable-speed control dial to match the animals' chosen locomotor speed. After a series of strides, the animals typically rested and then spontaneously resumed locomotion again after a few seconds (e.g. Fig. 3A). Otherwise, turtles were stimulated to resume locomotion by starting the treadmill belt underneath them, being carried on the treadmill belt back toward the heat source or finally having their shells gently tapped against the back wall.

X-ray video recordings were made (separately from the previous experiments) to compare ventilatory airflow with movements of the inguinal flank. Ventilatory airflow was recorded simultaneously with lateral view X-ray and light video images at 30 frames s^{-1} . In order to visualize movements of the inguinal flank for kinematic analysis, a small piece of metal wire (1 mm diameter \times 5 mm long) was glued to the skin anterior to the right hindlimb, at the most dorso-cranial extension of the limb pocket, just superficial to the region where the apexes of the oblique and transverse abdominal muscles come together. When the abdominal muscles are at rest (during apnea), this marker was just medial to the carapacial margin at the 7th marginal scute. Inguinal limb-pocket kinematics were measured by digitizing movements of the metal marker in the X-ray video relative to a point on the rear margin of the carapace, and calibrated by measuring the carapace height (in pixels) on screen and setting that equal to the actual carapace height (in cm).

Data analysis

A 'bout' of locomotion was defined as a sequence of continuous locomotion containing at least ten strides. 54 locomotor bouts from individual 01 were analyzed to quantify the relationship between locomotor speed and stride length, stride frequency, tidal volume and breath frequency. Distance traveled during a locomotor bout was calculated from video recordings (to the nearest 0.05 m) by counting the number of evenly spaced marks that the turtle passed on the treadmill belt. Locomotor speed was calculated for each bout by dividing this distance by the duration of the bout. The number of strides per bout was counted to the nearest half stride and average stride length and frequency were calculated by dividing the number of strides per bout by the distance traveled or duration of the bout respectively.

For all three individuals, single 20 min periods of locomotion were selected for analysis on the basis of locomotor consistency and duration. Within these intervals, however, the turtles would spend variable amounts of time resting between bouts of locomotion. In order to determine if these pauses might be acting as very short periods of recovery, we categorized each breath as either occurring during a pause or during locomotion, and analyzed these categories separately. For comparison, 20 min periods of breathing immediately before and after the locomotion trials were analyzed as pre-exercise and recovery respectively. The same 20 min periods of pre-exercise, locomotion and recovery were used in analyses of minute volume, tidal volume, breath frequency and phase.

In our tidal volume analysis, every breath was individually measured to calculate a mean tidal volume (\pm S.D.) for four behaviors: pre-exercise, locomotion, pauses and recovery. A two-way analysis of variance (ANOVA; StatView 5.0.1) tested for differences in tidal volume by including all of the 3325 measured breaths while accounting for within- and between-individual variation. Tukey's *post-hoc* test was used to test for pairwise differences between the four behaviors and the three individuals.

Minute volume ($ml\ min^{-1}$) and breath frequency ($breaths\ min^{-1}$) were calculated for each of the four behaviors (pre-exercise, locomotion, pauses and recovery) by dividing the sum of exhaled volumes or number of breaths by the duration of the sample period (20 min exactly for pre-exercise and recovery and the proportion of the 20 min period spent locomoting or in pause during the locomotion segment). Because these variables are measured over one long time period, there is no variance associated with the values. Paired *t*-tests (StatView 5.0.1) were used to make comparisons between the four behaviors (paired by individual).

We used phase analysis to quantify the temporal relationship between breathing and locomotion. For each individual, the first ten locomotor bouts containing at least ten breaths were selected for phase analysis. Maximum left hindlimb extension (MHE) was the kinematically distinct point in the stride cycle chosen to anchor the time measurements of the stride cycle (0°), and was defined as the video frame in which both knee and ankle extension were greatest (this corresponds to the end of stance for that limb). The duration of each stride was normalized to 360° and peak inhalatory and exhalatory airflow from each breath in a locomotor bout were plotted relative to when they occurred in the locomotor stride cycle (Simons, 1999). Raleigh's test of circular uniformity (Zar, 1996) was used to determine whether breath peaks were randomly distributed relative to the stride cycle. We analyzed each of the 30 bouts separately and analyzed the combined breaths from the ten bouts of each individual together.

Airflow rate analysis was designed to test whether the magnitude of peak exhalatory and inhalatory airflow rates vary with respect to the stride cycle. We determined that peak inhalatory and exhalatory airflow rates of turtles breathing at rest were not statistically different from each other ($P > 0.05$ for

all three individuals; three separate unpaired *t*-tests with 50 exhalations and 50 inhalations measured per individual during recovery). If the stride cycle had no effect on airflow rates, the magnitude of inhalatory and exhalatory airflow peaks during locomotion would also be expected to be the same. If, however, limb movements during locomotion caused pressure changes around the lungs, the magnitude of peak inhalatory and exhalatory airflow at different points in the stride cycle would be expected to differ. For example, positive pressures created by limb movements would be expected to add to exhalatory airflow rates and subtract from inhalatory airflow rates, making exhalations larger than inhalations. For each of the three individuals, the locomotor stride cycle was divided into 18 (20°) bins and the mean peak airflow rate was plotted for inhalations and exhalations occurring in each bin. The means were considered significantly different if the 95% confidence limits did not overlap within a bin.

Results

Morphology

The plastron of *Terrapene carolina* is hinged between the hyo- and hypoplastra such that the front and back halves are connected to each other and to the carapace only through ligamentous connective tissue. Both halves of the plastron can be raised to meet the carapace and thereby entirely conceal the limbs, head and tail of the animal (Fig. 1C). The lungs are extremely large organs situated between the limb girdles in the dorsal region of the highly domed carapace (Fig. 1A). The large neck retractor muscles run between the two lungs, and when the neck is retracted it lies between them as well. Our airflow recordings show that large exhalations (up to 45 ml for a 300 g animal) accompany head and limb retraction and

plastral adduction. However, the high domed shell allows for a large residual lung volume, and our X-ray video and airflow recordings reveal that lung ventilation still occurs in this fully retracted position.

Both the pectoral and pelvic girdles have highly mobile connections to the shell that may permit a wide range of movement during locomotion (compare Fig. 1B,C). The pelvic girdle articulates with the spine on either side *via* two small unfused ribs and has considerable freedom to rotate mediolaterally, dorsoventrally and even to translate cranio-caudally (Bramble, 1974). The epipubis occupies the ventero-cranialmost position of the pelvis and can translate mediolaterally during locomotion and dorso-ventrally during plastral adduction. Each triradiate half of the pectoral girdle lies inside the shell as it does in all turtles. It articulates with the carapace *via* two small sesamoid ossifications within the suprascapular cartilage (Walker, 1973). These supra- and episcapular bones are interconnected by ligaments that allow the pectoral girdle to translate anteroposteriorly during plastral adduction and abduction (Bramble, 1974). The presence of both these bones is unique to *Terrapene* and they have been hypothesized to lock the scapula passively in place when the plastron is abducted and the pectoral girdle is protracted (Bramble, 1974).

Right and left pairs of antagonistic abdominal muscles are present in *Terrapene carolina* (Fig. 1A). The thin, sheet-like domes of the oblique abdominis (OA) and transverse abdominis (TA) muscles are cupped in opposite directions, with the convex sides of each muscle juxtaposed at their apices. The OA lies just under the skin of the inguinal leg pocket (cranial to the hindlimb). The OA has broad attachment to the edges of the shell from the 10th to the seventh peripheral bones of the carapace, ventrally over the bridge, and from the

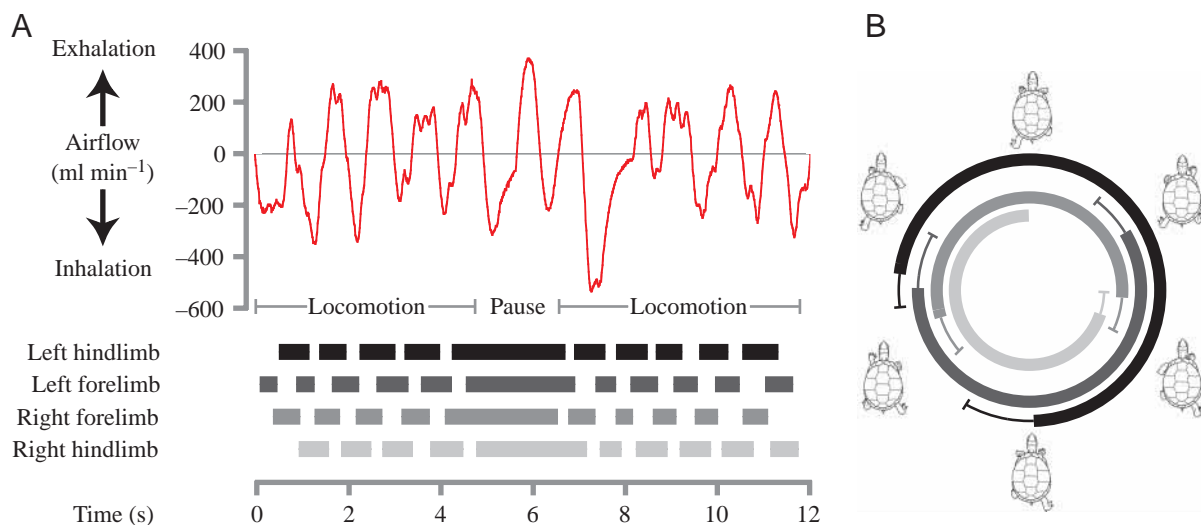


Fig. 3. Footfall diagrams of *Terrapene carolina* (individual 01) from bouts of treadmill locomotion. (A) Limb support (solid bars) and ventilatory airflow (red trace) during two bouts of locomotion. Note the short pause between the bouts of locomotion. (B) Polar diagram showing the relative timing of limb support (mean \pm s.d.). Each solid bar represents a different limb and is shown in the same shade of grey as in the previous panel. Each stride cycle (from Fig. 3A) is normalized to 360° so that the end of left hindlimb support is always at 0° (top of circle) and the stride cycle proceeds clockwise.

hypoplastron halfway to the caudal limit of the ziphiplastron. The origin of the TA describes an 'L' shape on the inner surface of the carapace. It runs parasagittally near the neural bones from the seventh to the fourth costal plates, turning 90° to continue ventro-laterally down the length of the fourth costal plate to the seventh peripheral plate. From the origin, the fibers of the TA travel posteroventrally around the caudal portion of the lungs, and then in an anteroventral arc under the internal organs to insert on a broad connective tissue aponeurosis. This connective tissue sheet continues from the plastral hinge cranio-dorsally around the anterior extent of the viscera to insert posterior to the pectoral girdle. In other turtle species, there may be muscular investment (diaphragmaticus) of the anterior portion of this connective tissue sheet, but this was not found in any of the *T. carolina* specimens examined in this study. There was also no indication of a striatum pulmonale muscle.

Airflow measurements and locomotion

Lung ventilation occurs almost continuously during treadmill locomotion (Fig. 3; to view video clips of turtle breathing during locomotion, refer to the online version of this article: <http://jeb.biologists.org/>). Small buccal oscillations (<0.4 ml) were recorded during locomotor and non-locomotor behavior, and were distinguished from lung ventilations by expansion and contraction of the throat region (visible in video recordings). Gular pumping for lung ventilation would be evident in airflow traces as small inhalations followed by little or no exhalatory airflow (Owerkowicz et al., 2001; Druzisky and Brainerd, 2001). No such airflow pattern was ever observed in *Terrapene carolina*, indicating that gular pumping for lung ventilation does not occur in this species (or in any other turtle studied to date; Mitchell and Morehouse, 1863; Hansen, 1941; McCutcheon, 1943; Gans and Hughes, 1967; Gaunt and Gans, 1969; Druzisky and Brainerd, 2001).

During treadmill experiments, the three individual *Terrapene carolina* all showed similar patterns of short (approximately 10–30 s) voluntary locomotor bouts interspersed with brief pauses (approximately 2–60 s). During the 20 min periods of locomotion selected for analysis, the animals spent between one half and two-thirds of the time actually locomoting (61, 48 and 66%, respectively, for individuals 01–03).

Analysis of 54 locomotor bouts from individual 01 revealed that mean voluntary locomotor speed on the treadmill was 0.10 m s⁻¹ and the range (0.074–0.124 m s⁻¹) was relatively narrow (Fig. 4). Only data from individual 01 are presented here, but results were similar from individuals 02 and 03 except that the range of speeds was even smaller. Stride frequency and stride length were both strongly and positively correlated with speed (Fig. 4A). The slope of the relationship between stride frequency and speed was 7.7 times greater than the slope of stride length versus speed, indicating that increases in stride frequency accounted for 88% of increases in speed while increases in stride length accounted for the remaining 12% (Fig. 4A). Breath frequency and tidal volume were only weakly correlated with speed (Fig. 4B).

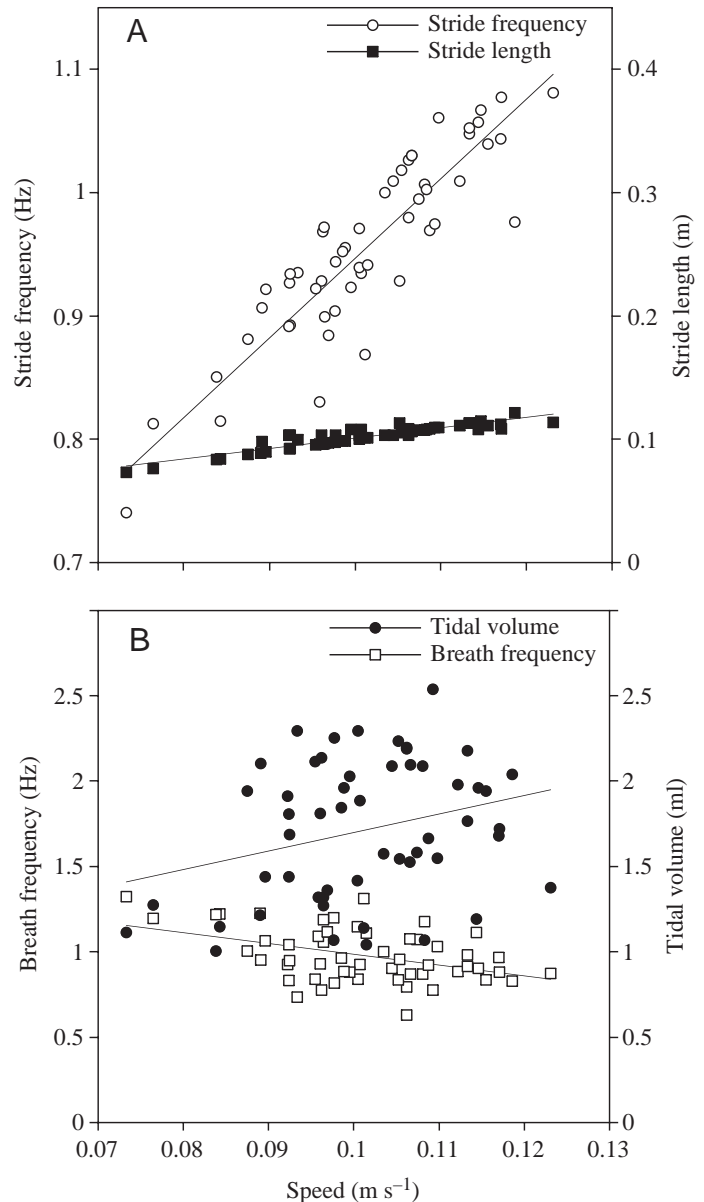
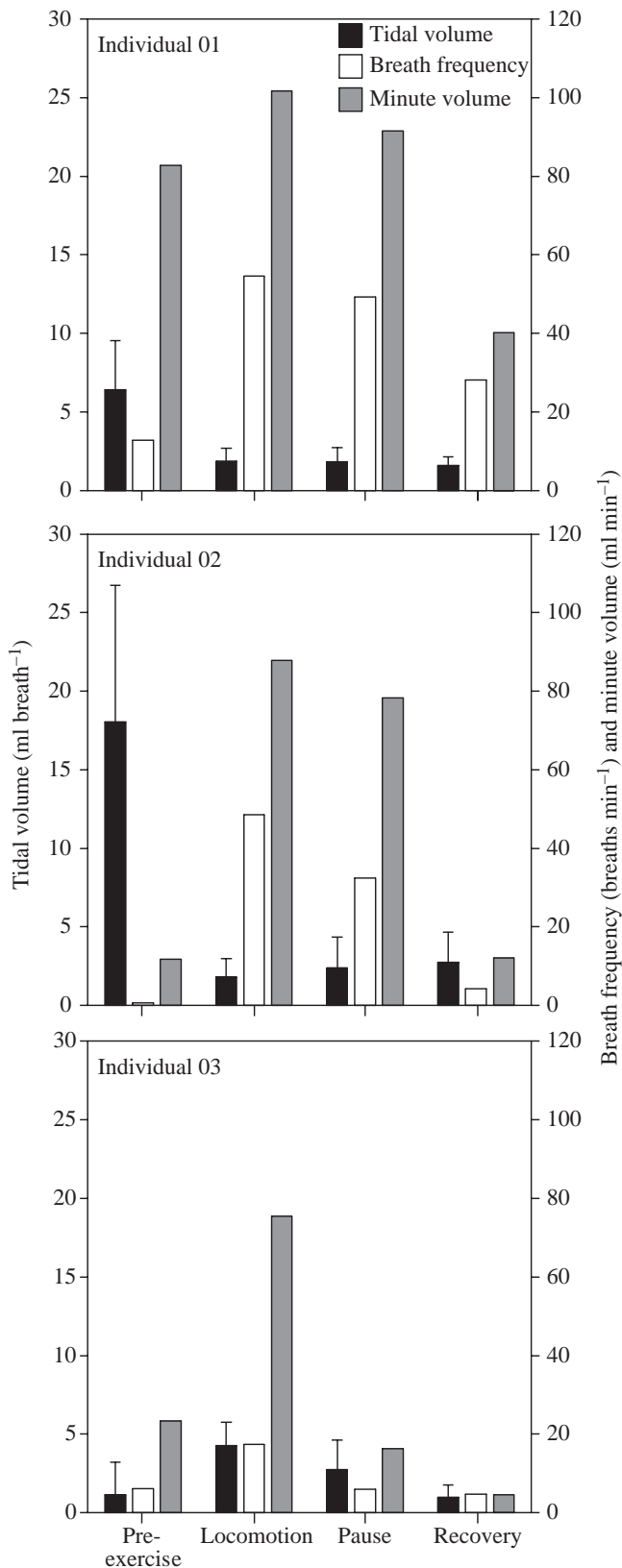


Fig. 4. Stride frequency, stride length, breath frequency and tidal volume versus speed for *Terrapene carolina* ($N=54$ locomotor bouts from one individual). (A) Stride frequency (open circles; $y=6.47x+0.3$, $r^2=0.79$, $P<0.0001$) and stride length (filled squares; $y=0.843x+0.017$, $r^2=0.851$, $P<0.0001$) versus speed. (B) Tidal volume (filled circles; $y=10.9x+0.611$, $r^2=0.08$, $P<0.0377$) and breath frequency (open squares; $y=-6.36x+1.62$, $r^2=0.19$, $P<0.001$) versus speed.

During locomotion, breathing frequency and stride frequency were negatively but only weakly correlated ($y=-1.03x+1.96$, $r^2=0.264$, $P<0.0001$). Tidal volume and stride length ($y=19.06x-0.232$, $r^2=0.206$, $P<0.001$) were also weakly but positively correlated (graphs not shown).

Expired volumes ranged from 1 ml to over 40 ml. The largest exhalations occurred when the animals were accidentally startled and the head and limbs were retracted into the shell. Mean tidal volumes during locomotion, pauses and

recovery were small (range 1.0–4.3 ml breath⁻¹; Fig. 5) and not significantly different from each other (two-way ANOVA; $P>0.05$). Tidal volumes during pre-exercise were relatively



large and significantly different from locomotion, pause and recovery values (Fig. 5; two-way ANOVA with Tukey's *post-hoc* tests; $P<0.0001$ for all three pairwise comparisons).

In all three individuals, the highest breath frequency (breaths min⁻¹) was recorded during locomotion (Fig. 5), but no statistically significant differences between behaviors (pre-exercise, locomotion, pauses and recovery) were detected (paired *t*-tests; $P>0.05$).

Mean minute volumes (ml min⁻¹) were high during all four behaviors (Fig. 5). However, minute volumes during locomotion were exceptionally high (range 75–102 ml min⁻¹) and significantly different (paired *t*-test; $P=0.0037$) from recovery values (range 5–40 ml min⁻¹).

Polar plots of the temporal distribution of peak inhalatory and exhalatory airflow relative to the stride cycle show no fixed phase relationship (Fig. 6). Inhalations and exhalations were analyzed separately for each of the ten locomotor bouts from three individuals. Raleigh's test of circular uniformity (Zar, 1996) revealed that breaths were uniformly distributed in 55 bouts ($P>0.05$) and five sequences had statistically non-random distributions of breaths relative to the stride cycle ($P<0.05$). However, when the ten sequences from each individual were combined, inhalations were randomly distributed relative to the stride cycle in all three individuals (Fig. 6A). Exhalations were uniformly distributed for individuals 02 and 03 and non-uniformly distributed for individual 01 ($P<0.001$; Fig. 6B).

To determine whether limb movements affect airflow rates during locomotion, mean airflow rates ($\pm 95\%$ confidence intervals) were calculated for inhalations and exhalations occurring within 20° intervals of the stride cycle (Fig. 7). If the confidence intervals overlapped within a bin, the means were considered statistically indistinguishable. Very few statistically significant differences were found between inhalatory and exhalatory peak airflow rates and the differences that we did find were not consistent across the three animals studied. In individual 03, peak exhalation and peak inhalation were not statistically different at any point in the stride cycle (Fig. 7C). In individual 02, peak exhalation was greater than peak inhalation in two bins between 270° and 360° (Fig. 7B) and in individual 01, peak exhalation was greater than peak inhalation in two bins between 180° and 270° (Fig. 7A).

In order to determine whether the abdominal muscles could be the mechanism responsible for breathing during locomotion, we used X-ray video recordings to track the movement of the

Fig. 5. Tidal volume (ml breath⁻¹, mean \pm s.d.), breath frequency (breaths min⁻¹) and minute volume (ml min⁻¹) during 20 min periods of pre-exercise, locomotion, pauses between locomotor bouts and recovery from exercise in three individual *Terrapene carolina*. Tidal volume during locomotion is not significantly different from tidal volume during pauses or during recovery (two-way ANOVA, $P>0.05$). Breath frequency values are not significantly different between behaviors (paired *t*-test, $P>0.05$). Minute volume during locomotion is significantly higher than during recovery (paired *t*-test, $P=0.0037$).

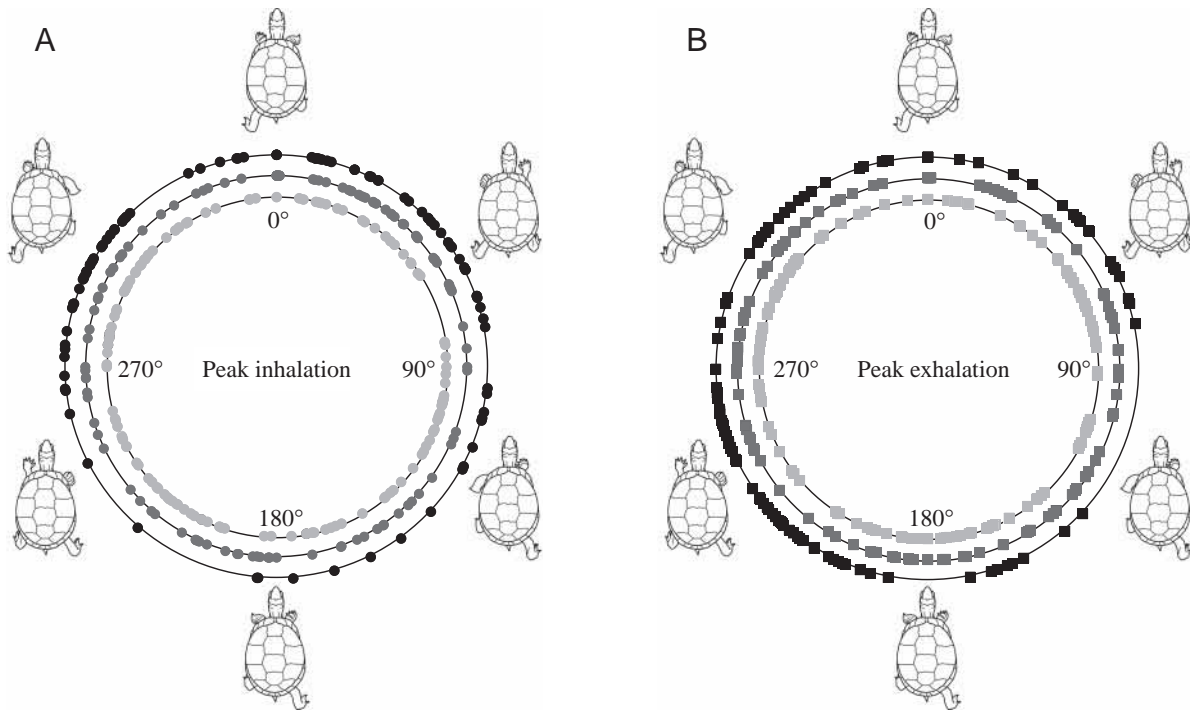


Fig. 6. Polar plots of the phase relationship between peak ventilatory airflow and the locomotor stride cycle for three individual *Terrapene carolina* (individual 01, black; individual 02, dark grey; individual 03, light grey). The stride cycle begins at maximum extension of the left hindlimb (0°) and continues clockwise around the polar diagram. (A) Timing of peak inhalation relative to the stride cycle in ten locomotor bouts for each of the three individuals (inhalations: $N=134$, 117 and 127 breaths for individuals 01–03, respectively). (B) Timing of peak exhalation ($N=132$, 117, and 130 breaths for individuals 01–03 respectively). Inhalations from all three individuals and exhalations from individuals 02 and 03 were randomly distributed relative to the stride cycle (Raleigh's test of circular uniformity, $P>0.05$). Exhalations from individual 01 showed a statistically significantly non-uniform distribution (Fig. 6B, black squares; Raleigh's test of circular uniformity, $P<0.001$).

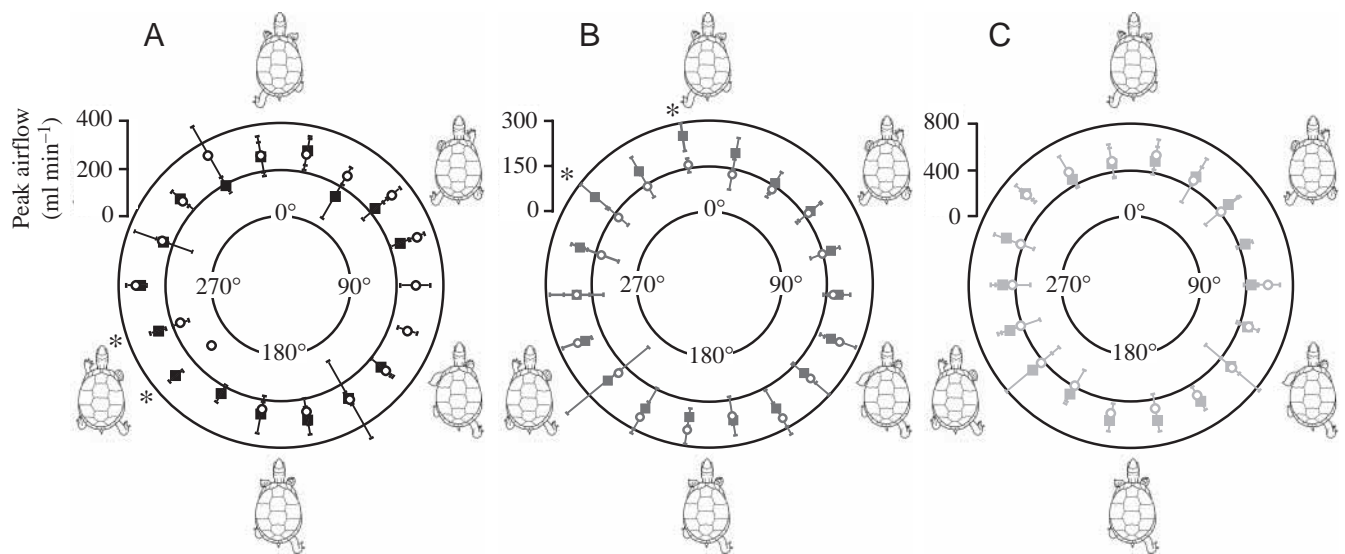


Fig. 7. Polar plots showing the mean magnitude of peak inhalatory and exhalatory airflow of breaths occurring at different points in the stride cycle for *Terrapene carolina*. (A) Individual 01, (B) individual 02 and (C) individual 03. The magnitude of peak inhalatory (circles) and peak exhalatory (squares) airflow from the breaths in Fig. 6 were averaged into 20° bins and plotted (means \pm 95% confidence limits) onto the stride cycle. Magnitude of peak airflow increases with the radius of the plot. The number of breaths varies for each bin (range 0–25) and can be estimated by comparison with the distribution in Fig. 6. Mean values of peak airflow rate are considered statistically significantly different (*) if the 95% confidence intervals do not overlap within a bin.

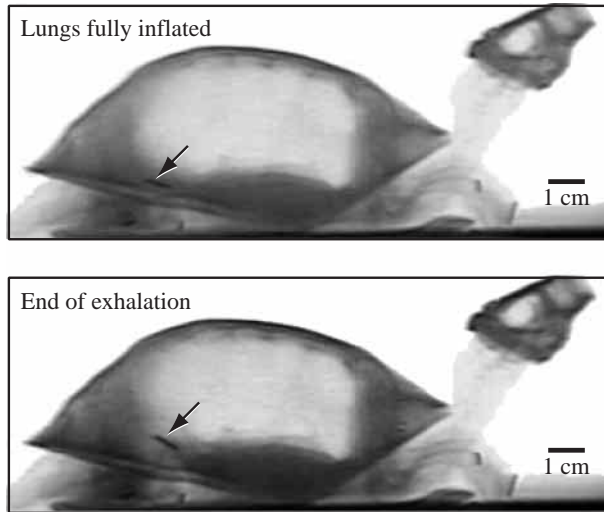


Fig. 8. Still frames from an X-ray video recording of lung ventilation at rest in *Terrapene carolina* (individual 03). Simultaneous pneumotachographic airflow measurements were also recorded and synchronized with the X-ray video (Fig. 9). The lungs appear as a large light area in the middle of the body, and the pneumotach mask appears dark in this radio-positive lateral view. A small metal marker (arrow) has been glued to the skin of the inguinal flank just superficial to the oblique abdominis (OA) and transverse abdominis (TA) muscles. The upper frame shows the position of the metal marker when the animal has fully inflated lungs. The lower frame shows the metal marker at the end of exhalation. X-ray video clips with simultaneous pneumotachographic airflow recordings of *Terrapene carolina* breathing at rest and during locomotion can be viewed on line as part of this article (<http://jeb.biologists.org/>).

inguinal flanks during breathing. A small metal marker was glued to the skin of the inguinal flank just superficial to the oblique and transverse abdominis muscles on the right side of the body (Fig. 8). The Δy coordinate (dorso-ventral component of flank movement) was measured and plotted with simultaneous recordings of ventilatory airflow from the pneumotach mask (Fig. 9). When the turtle was not locomoting, exhalation was accompanied by dorsal movement of the marker, and the marker moved ventrally during inhalation. These movements are not likely to be passive deflections of the inguinal flank; acting passively, they would be expected to move down (and laterally) during exhalation (when pressure is greatest inside the pleuroperitoneal cavity) and up (and medially) during inhalation (when pressure is lowest inside the pleuroperitoneal cavity). During locomotion, inguinal flank movements were similar to those at rest (up during exhalation and down during inhalation); however, kinematic analysis was obscured by motion artifact caused by pitch, roll and yaw during locomotion. X-ray video clips of *Terrapene carolina* breathing at rest and during locomotion can be viewed on line as part of this article (<http://jeb.biologists.org/>).

Discussion

Mechanical interactions between breathing and locomotion

Evidence of a mechanical conflict between breathing and locomotion has been found in green sea turtles (*Chelonia mydas*; Jackson and Prange, 1979) and in some species of lizards (Carrier, 1987a,b; Wang et al., 1997; Owerkowicz et al., 1999). When female *C. mydas* return to land to deposit their

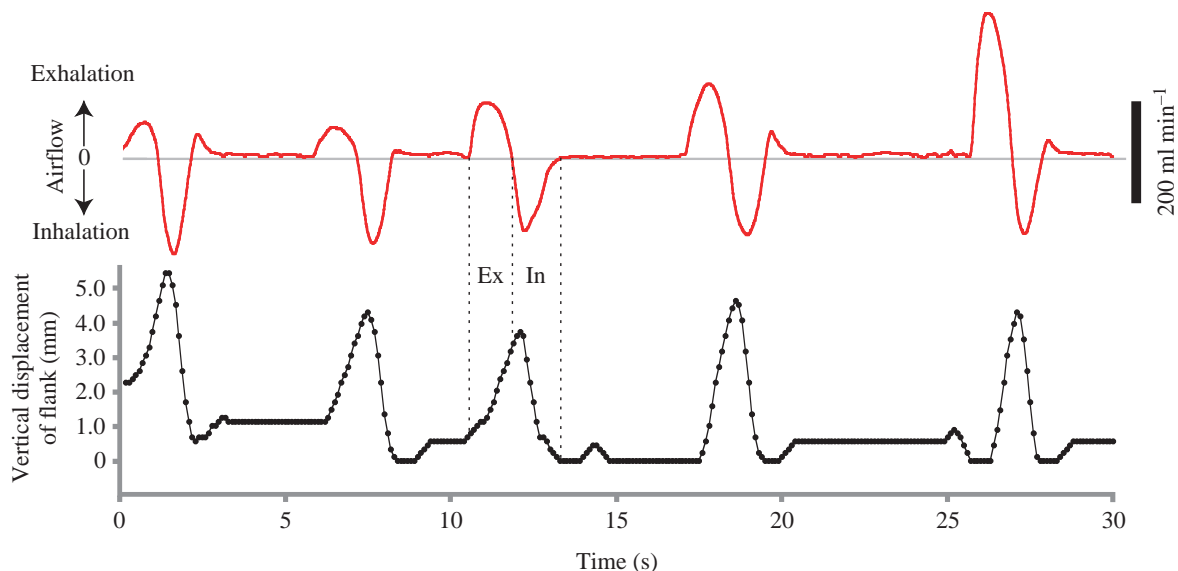


Fig. 9. Ventilatory airflow and inguinal flank displacement in *Terrapene carolina* (individual 03) during breathing at rest. The upper trace shows five exhalation/inhalation cycles separated by short periods of apnea. The lower trace shows vertical displacement (Δy coordinate) of a marker glued to the skin of the inguinal flank just superficial to the oblique abdominis and transverse abdominis muscles measured from X-ray video recordings (see Fig. 8). Exhalation (Ex) occurs as the inguinal flank moves dorsally. Inhalation (In) occurs as the inguinal flank moves ventrally.

eggs, they use a bilaterally synchronous gait to haul themselves up the beach. Lung ventilation ceases during locomotion, but then resumes during pauses between bouts of locomotion. Jackson and Prange (1979) suggested that breathing during locomotion may be impossible because some limb muscles are known to be recruited for both breathing and locomotion (Gans and Hughes, 1967). In contrast to green sea turtles, we found that box turtles *Terrapene carolina* breathe almost continuously during locomotion. Tidal volumes in *T. carolina* are not significantly different during locomotion, brief pauses in locomotion and recovery, and minute volumes are largest during locomotion (Fig. 5).

Most lizard species locomote intermittently with low tidal and minute volumes during high-speed bursts of locomotion and high tidal and minute volumes during pauses and recovery (Carrier, 1987b). When lizards are forced to locomote steadily, minute volume generally decreases as speed increases, and the highest minute volumes are recorded during recovery from exercise (Wang et al., 1997). Breathing performance declines with increasing speed because axial muscles used for breathing require a bilateral motor pattern while those same muscles must be activated unilaterally to bend the body during locomotion (Carrier, 1991). Monitor lizards circumvent this mechanical conflict by using a gular pump to inflate the lungs during locomotion (Owerkowicz et al., 1999, 2001). In the present study, we hypothesized that *Terrapene carolina* might use gular pumping for lung ventilation during locomotion if it experiences the apparent mechanical conflict observed during locomotion in *Chelonia mydas*. However, in agreement with all previous experimental studies of turtle ventilation mechanisms, we found no evidence for the use of a gular pump during locomotion in *T. carolina*.

Because the thoracic cavity undergoes cycles of pressurization with each stride and with each breath, breathing and locomotion are often coordinated in mammals and birds (e.g. Simons, 1996; Boggs, 2002). The shell of *Terrapene carolina* contains a nearly fixed volume, and therefore we hypothesized that a cyclic pressure regime may be imposed on the lungs as the limbs are protracted and retracted during locomotion. Whether limb and girdle movements comprise the main (limb-pump) lung ventilation mechanism or whether another breathing mechanism is synchronized to its rhythm, the breathing and stride cycles were predicted to show phase coupling. However, our results show that peak inhalatory airflow for all three individuals and peak exhalatory airflow for two out of three individuals were randomly distributed with respect to the stride cycle (Fig. 6). We conclude that *T. carolina* does not couple breathing and locomotion and limb movements do not contribute to lung ventilation during locomotion.

Even though the timing of breaths relative to the stride cycle was found to be random, the airflow rates could still be affected by limb movement during locomotion. When the turtles were at rest, inhalations and exhalations were symmetrical and did not differ statistically in peak airflow rates. During locomotion, net retraction of the limbs during a given part of the stride cycle

might increase peak exhalatory airflow rates and decrease peak inhalatory rates of breaths that happen to fall in that part of the stride cycle. Contrary to this hypothesis, however, we found few statistical differences between mean peak inhalatory and exhalatory airflow rates; the observed differences occurred at different parts of the stride cycle in different individuals (Fig. 7). Furthermore, because *Terrapene carolina* uses an alternating (symmetrical) gait, effects of limb movement on intrapulmonary pressure would be expected to cycle twice with each stride (see Fig. 3B). Contrary to this prediction, we found no cases in which statistical differences within individuals were mirrored on the opposite side of the stride cycle. Together, these results on the timing and magnitude of breaths relative to the stride cycle indicate that locomotion has no consistent, measurable mechanical effect on breathing in *T. carolina*.

Given the apparent independence of the breathing and stride cycles of *Terrapene carolina*, the lung ventilation mechanism must be mechanically separate from the locomotor system. At rest, *T. carolina* uses the transverse and oblique abdominal muscles to breathe (see Figs 1, 8, 9 and supplemental video clips). Since we found neither diaphragmaticus nor striatum pulmonale muscles in this species and no evidence for the use of a limb or gular pump mechanism, the abdominal muscles are the most likely mechanism for breathing during locomotion. Turtles rotate about all three orthogonal axes during locomotion, thereby making quantitative measurements of flank movements from two-dimensional X-ray videos difficult. However, X-ray videos show clearly that, when our study animals breathed during locomotion, the inguinal flanks moved in phase with the ventilatory cycle and independently from the stride cycle (see supplemental video clip).

The kinematics of locomotion in *Chelonia mydas* and *Terrapene carolina* differ substantially and may help explain differences in their breathing performance. When locomoting on land, adult *C. mydas* lift the body and push it forward by retracting both front limbs simultaneously (Wyneken, 1997). As pointed out by Jackson and Prange (1979), the bilaterally synchronous motor pattern presumably needed to produce this gait is also used during limb-pump lung ventilation (Gans and Hughes, 1967). Terrestrial locomotor movements in *C. mydas* may therefore generate large intrapulmonary forces. If the glottis were open during the support phase of the stride cycle, limb movement otherwise producing forward thrust could instead be producing exhalation, and deflation of the lungs could result in medial rotation of both halves of the pectoral girdle. *Chelonia mydas* may therefore cease breathing during locomotion because the pressurized lungs are used as a support platform to stabilize limb movements during locomotion (pneumatic stabilization: Simons, 1996; Kidd and Brainerd, 2000). In contrast to the bilaterally synchronous gait of *C. mydas*, *T. carolina* employs the more typical lateral sequence diagonal couplet walk used by most turtles (Walker, 1971; Zug, 1971; Fig. 3). In this alternating (symmetrical) gait, one (slightly staggered) diagonal pair of

limbs is extended while the other (also staggered) pair is flexed and retracted. The balanced effect of these paired limb movements on internal shell volume, combined with the independence of the abdominal muscles from the locomotor muscles, may sufficiently explain the absence of any consistent measurable effect of locomotion on ventilation in box turtles.

Despite not measuring any consistent effect of locomotion on breathing, we still consider it possible, even likely, that locomotion has momentary, net effects on internal shell volume. It seems unlikely that locomotion is so tightly regulated that every movement of the limbs on the left side is accompanied by perfectly synchronized and exactly opposite counter-action on the right side. Additionally, left and right limb pairs are 180° out of phase, but each limb spends more time in contact with the ground and applying a rearward-directed force than it does in recovery or forward-directed movement (duty factor >0.5; Fig. 3B). There are therefore two moments in each stride cycle when both right and left members of each limb pair are moving backwards. The unilateral abdominal motor pattern that Currie found in swimming turtles (Currie, 2001, 2003) is one potential mechanism that may counteract the effects that limb movements probably have on the lungs.

The speeds observed in this study are slow – even for turtles. These speeds are typical for *Terrapene carolina* (Muegel and Claussen, 1994; Marvin and Lutterschmidt, 1997); however, the interactions that we hypothesized between locomotion and breathing may be more apparent in faster turtles e.g. *Chrysemys picta* (Zani and Claussen, 1994, 1995), *Terrapene ornata* (Adams et al., 1989; Claussen et al., 2002; Wren et al., 1998) and *Trachemys scripta* (Landberg et al., 2002b).

Breathing patterns

Minute volume was substantially higher during locomotion than during recovery from exercise and not significantly different from pauses during locomotion, indicating that *Terrapene carolina* is meeting (if not exceeding) its aerobic metabolic demands during locomotion. Surprisingly, the high minute volumes during locomotion were achieved by reducing breath size (and duration) while increasing breath frequency. Previous studies have found that turtles increase breath frequency and decrease tidal volume with increases of temperature and metabolic rate (Altland and Parker, 1955; Glass et al., 1979). The relatively small tidal volumes associated with locomotion could be a response to increased metabolism, but they may also minimize the mechanical interactions between limb movement and breathing.

Pre-exercise breathing values in this study were recorded shortly (~1–2 h) after the pneumotach mask was attached to the animal and may not be entirely characteristic of ‘rest’ (Glass and Wood, 1983). Turtle breathing at rest is typically characterized by several large breaths clustered into bouts that are separated by variable length non-ventilatory periods (Milsom and Jones, 1980). Tidal volume during pre-exercise

was higher than during locomotion, pause and recovery (two-way ANOVA; $P < 0.0001$), but not as large as reported in other studies of box turtles at rest (e.g. Altland and Parker, 1955). The turtles in our study showed pre-exercise breath frequencies that were high (8.4 ± 9.0 breaths min^{-1} ; mean \pm s.d. for $N=3$ individuals) compared to published data showing that *Terrapene carolina* breathes 4–5 times min^{-1} at around 30°C (Altland and Parker, 1955), *T. ornata* breathes 1.5 times min^{-1} at 25°C (Glass et al., 1979) and *Trachemys scripta* breathes 1–2 times min^{-1} at 30° (Jackson, 1971; Jackson et al., 1974). We interpret the high frequency and small (relative to other studies) pre-exercise tidal volumes to be due to the presumed stress associated with the masking procedure or experimental conditions.

Evolutionary considerations

Extant lizards exhibit a mechanical conflict between simultaneous ventilation and locomotion because axial muscles are used in a unilateral activation pattern to bend the body from side to side during locomotion, while many of those same muscles require a bilaterally synchronous motor pattern to expand the thoracic cavity during breathing (Carrier, 1987a,b, 1991). Extant turtles would not be subject to this constraint because their ribs are fused to form part of the shell, and therefore do not contribute to either locomotion or ventilation. However, the shell-less ancestor of turtles probably did rely on axial bending during locomotion and rotation of the ribs during breathing. In the absence of another breathing mechanism, this hypothetical ancestor of turtles would have experienced a mechanical conflict between locomotion and ventilation. We hypothesize that the specialized ventilatory functions of the abdominal muscles in extant turtles were favored by natural selection because they permitted breathing during locomotion in the lineage that led to turtles. This accessory ventilation mechanism would then have become the primary lung ventilation mechanism as the ribs abandoned their ventilatory function and fused into the shell.

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