Papers

Evaluation of pulmonary function in European land tortoises using whole-body plethysmography

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The aim of this study was to evaluate the use of whole-body plethysmography as a non-invasive method to determine the respiratory parameters and profiles in two tortoise species belonging to the genus *Testudo*. Pulmonary functions and volumetric parameters were determined in 10 adults of *Testudo hermanni* and in seven *Testudo marginata* animals, using whole-body plethysmography. A profile pattern was regularly observed: an inspiratory flow peak, an expiratory peak, an apnoea phase and a second expiratory peak, previous to the beginning of the next respiratory cycle. Positive and significant correlation was observed between the inspiratory time, weight and length of the tortoises. Larger tortoises showed a higher time of inhalation. The peak of inspiratory flow was correlated with the sex, being longer in the females. *T. marginata* had an inspiratory time longer than that of *T. hermanii*. In *T. hermanii*, differences related to the sex were observed in the tidal volume, peak inspiratory flow, peak expiratory flow, expiratory flow of 50 per cent and enhanced pause, which could be related to the smaller size of males. The results suggest that additional information on new technologies currently used in pet medicine or even in human medicine should be developed and adjusted as alternative ways to support the rehabilitation of turtles and tortoises.

Introduction

The Hermann's tortoise (*Testudo hermanni*) and the marginated tortoise (*Testudo marginata*) are European chelonians legally protected throughout their range, and classified in the International Union for Conservation of Nature (IUCN) Red List as near-threatened due to the high mortality from fires and destruction of their habitat along the Mediterranean forests (van Dijk and others 2004). Each year, tortoises are found sick or burned, and are rescued and relocated to rehabilitation centres in many of the European countries. On the island of Majorca, Spain, more than 3500 tortoises died in a large fire that affected more than 200 ha (Serra 2010). The impact of fire and mechanical destruction of the habitat of *T. hermanni* populations have been studied in detail in northern Greece (Hailey 2000), France (Couturier and others 2010) and in Spain (Martínez-Silvestre and Soler Massana 2000, Franch and others 2001, Couturier and others 2010). To evaluate the effect of the smoke on the respiratory function of rescued tortoises, an understanding of normal respiration parameters is needed.

Compared with mammals, the respiratory profile in reptiles is unusual because they have irregular respiratory cycles due to variable pauses between each movement (White 1978, Perry and Sander 2004). This represents a great challenge for veterinarians because even the simplest method employed to verify respiratory function, such as assessing the breathing frequency, is not accurate in these circumstances (Hernandez-Divers and others 2002). Reptiles use buccopharyngeal movements for olfactory function and these can be misinterpreted as respiratory movements (Druzisky and Brainerd 2001). Currently, diagnosing respiratory problems in chelonians is carried out with the use of several methods, such as physical examination, cytology, microbial culture, radiography, CT and endoscopy (Hernandez-Divers and others 2005).

Whole-body plethysmography is a very sensitive technique that verifies lung measurements. It can be used to detect lung pathology that might have been missed with conventional pulmonary function tests. It is often employed in comparative physiology studies because it avoids excessive handling of the animals. In flow plethysmography, airway resistance is measured by two manoeuvres, and the record of the respiratory mechanics is obtained based on the interchange of air between the body and the chamber in which the animal is placed. The interchange induces pressure variation in the volume of air retained in the chamber, which is then measured.

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The aim of this study was to evaluate the use of whole-body plethysmography as a non-invasive method to determine the respiratory parameters and profiles in two species belonging to the genus *Testudo*, *T. hermanni* and *T. marginata*.

Materials and methods

Pulmonary functions and volumetric parameters were determined in 10 Hermann's tortoises (five males (150–172 mm long, bodyweight 593–830 g), five females (193–230 mm long, bodyweight 892–1718 g)) and seven marginated tortoises (five males (246–337 mm long, bodyweight 2250–5200 g), and two females (240 and 285 mm, and bodyweight 2450 and 3650 g, respectively)) using whole-body plethysmography. A transparent chamber of plexiglass for plethysmography was used (Buxco Electronics Unrestrained WBP plethysmograph PLY4219, 25 cm × 51 cm × 30 cm) with a continuous flow of stable air (BFL0250; Buxco) at 10 litres/minute. The airflow provided ventilation inside the chamber, avoiding accumulation of carbon dioxide and maintaining suitable ranges of temperature and humidity. The air entrance aperture was positioned on a wall of the principal chamber, and it was fitted with a pneumotacograph of 35 mm diameter (Halcyon TM; Halcyon Dive Systems), whereas the exit in the opposite wall was at 30 mm from the chamber floor. One of the differential pressure transduction poles (TRD5715; Buxco) was positioned inside the principal chamber, and the other one was connected to a referential chamber that was balanced with the atmospheric pressure through a small conduit (1.5 mm). The transducer was provided with a DIN-compatible connector with pre-amplification memory cards MAX2270, MAX2275, MAX2285 and MAX2295, all with calibration sets of signals and a range of external gain. The chamber also had a probe with temperature and humidity sensors (TRD5716; Buxco) designed for Buxco model PLY4200 plethysmography chambers. This device was connected to the probe TRD5715 that transmitted the signal using a cable to the pre-amplifier. The data were processed using the software Biosystem XA V.2.10.1 for profile analyses of the respiratory movements. Before making each record, the pressure signal was calibrated by injecting 50 ml of air inside the chamber. The temperature of the room ranged from 24.8° to 25.2°C, and relative humidity ranged from 41 to 43 per cent.

The tortoises studied were from the Catalonian Reptile and Amphibian Rescue Center, Masquefa, Barcelona, Spain. Only healthy tortoises were used. The animals were transported in plastic boxes to the Hospital Veterinari Molins, where the examinations were carried out. Biometrical values of weight and (stretched) carapace length, respiratory frequency and body temperature were verified before the examination. A digital probe (Patient Monitor PM-8000Vet; Mindray Bio-Medical Electronics) was used for body temperature measurement. Respiratory frequency was registered with the tortoise placed on an inverted cup, avoiding contact of the animal's limbs with the table. Three successive counts were performed based on the sound of air exhalation confirmed by the movement of a fine piece of paper placed in front of the nares. The tortoises were individually placed in the plethysmography chamber, and after five minutes of adaptation the procedure of data recording was done. For each animal, four records of three minutes each were performed and then the mean was calculated. Based on the real-time graphic presented by the program a regular and repeated pattern profile of exhalation and inhalation was identified. Due to the high sensitivity of the device, the real respiratory movements used in the calculations were based on this pattern, and anomalies produced by motion inside the chamber and external interferences in the room were removed. The influence of species, size, weight and sex on the different respiratory parameters, including respiratory frequency (F), tidal volume (TV), minute ventilation (MV), inspiratory time (Ti), expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), expiratory flow at 50 per cent volume (EF50) and enhanced pause (Penh), were analysed.

Data were statistically analysed using the Action 1.0 software package, and statistical significance was accepted at $P<0.05$. Data normality was verified using the Kolmogorov-Smirnov test. Nonparametric statistical tests (Pearson correlation and Wilcoxon test) were used due to non-normally distributed data.

Results

The chamber and plethysmography system, which was designed for cats and other small mammals, was sensitive to gas changes in the tortoises when calibrated to 50 ml. The tortoises did not show any signs of discomfort inside the chamber and usually remained quiet. A tryphasic profile was regularly observed: a PIF, a PEF, an apnoea phase and a second PEF, previous to the beginning of the next respiratory cycle (Fig 1). The interval between the first inspiratory movement and the second one was considered one respiratory cycle. Another profile of short and irregular respiration was seen in the three smallest tortoises (*T. hermanii* males). A period of apnoea as long as two minutes and 10 seconds was recorded in a female tortoise. The mean values of each studied parameter for each species are presented in Tables 1 and 2.

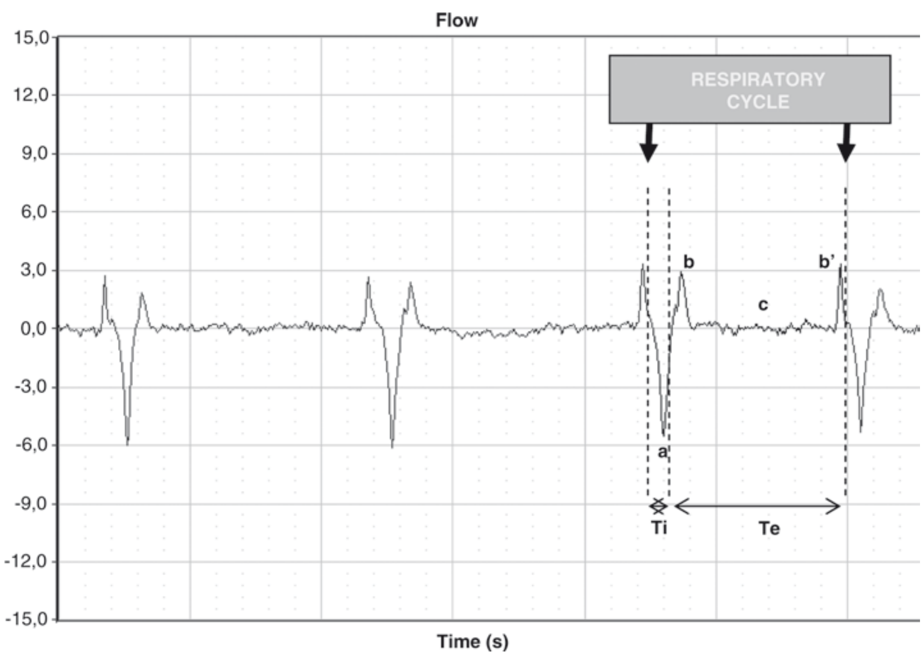

FIG 1: Respiratory profile of tortoises based on whole-body plethysmography. The beginning and end of one complete respiratory cycle is indicated by the arrows. (a) Inspiratory flow peak, (b) Expiratory peak, b' Second expiratory peak, (c) Apnoea phase, Ti Time of inspiration, Te Time of expiration

TABLE 1: Respiratory parameters (mean±sd, range) of healthy *Testudo hermanii* and *Testudo marginata* based on whole-body plethysmography

	T. hermanii (n=10)	*T. marginata* (n=7)
F (breaths/minute)	17.75±5.15 (10.90–26.73)	13.72±5.19 (8.70–24.55)
TV (ml/minute)	33.61±21.65 (10–82.20)	39.10±18.09 (18.17–62.61)
MV (ml/minute)	571.92±269.72 (180.87–1033.37)	502.31±228.73 (291.93–932.70)
Ti (seconds)*	0.42±0.05 (0.34–0.51)	0.63±0.15 (0.46–0.83)
Te (seconds)	5.38±1.42 (2.65–8.16)	5.98±2.13 (3.27–9.28)
PIF (ml/minute)	151.4±87.56 (49.85–336.71)	145.46±54.41 (80.72–235.02)
PEF (ml/minute)	103.32±45.71 (39.18–188.92)	123.64±38.51 (77.74–181.55)
EF50 (seconds)	2.29±0.97 (0.92–3.41)	2.19±0.82 (0.61–3.25)
Penh	12.68±5.78 (4.30–23.10)	10.44±4.19 (6.69–19.14)

*EF50 Expiratory flow at 50 per cent volume. Parameter with statistically significant difference between species (P=0.0002)
EF50 Expiratory flow at 50 per cent volume, F Respiratory frequency, MV Minute ventilation, PEF Peak expiratory flow, Penh Enhanced pause, PIF Peak inspiratory flow, Ti Inspiratory time, TV Tidal volume

TABLE 2: Respiratory parameters (mean ± sd, range) of males and females of Testudo hermanii and Testudo marginata based on whole-body plethysmography

	T. hermanii		*T. marginata*	
	Males (n=5)	Females (n=5)	Males (n=5)	Females (n=2)
F (breaths/minute)	17.09±5.95 (11.19–26.74)	14.65±4.54 (8.7–21.74)	14.39±5.73 (11.19–29.55)	12.03±4.72 (8.70–15.37)
TV (ml/minute)*	26.57±14.99 (9.99–62.61)	49.16±19.08 (28.27–82.20)	33.38±17.92 (18.17–62.61)	53.40±10.24 (46.16–60.64)
MV (ml/minute)	419.16±147.27 (180.87–645.75)	720.55±264.39 (339.67–1033.37)	438.31±154.94 (291.93–645.75)	662.32±382.37 (391.94–932.70)
Ti (seconds)	0.50±0.13 (0.39–0.81)	0.51±0.18 (0.34–0.83)	0.58±0.14 (0.46–0.81)	0.76±0.09 (0.69–0.83)
Te (seconds)	5.54±1.82 (2.64–9.28)	5.76±1.69 (3.68–8.16)	6.07±2.16 (3.27–9.28)	5.74±2.91 (3.68–7.80)
PIF (ml/minute)*	107.85±43.99 (49.85–191.08)	207.68±68.87 (140.34–336.71)	128.57±45.53 (80.72–191.08)	187.68±66.95 (140.34–235.02)
PEF (ml/minute)*	94.26±38.50 (39.18–164.23)	136.58±38.50 (99–188.92)	116.99±34.34 (77.74–164.23)	140.27±58.37 (99.00–181.55)
EF50 (seconds)*	1.86±0.87 (0.61–3.25)	2.81±0.59 (1.82–3.41)	2.07±0.94 (0.61–3.25)	2.51±0.48 (2.17–2.85)
Penh*	13.82±5.15 (8.61–23.10)	8.83±3.77 (4.30–14.84)	11.73±4.35 (8.61–19.14)	7.23±0.76 (6.69–7.76)

*EF50 Expiratory flow at 50 per cent volume. Difference statistically significant (P<0.05) between male and female T. hermanii
F Respiratory frequency, MV Minute ventilation, PEF Peak expiratory flow, Penh Enhanced pause, PIF Peak inspiratory flow, Te Expiratory time, Ti Inspiratory time, TV Tidal volume

A positive and significant correlation was observed between the inspiratory time, the weight (P=0.0003) and length (P=0.002) of the tortoises. Larger tortoises showed a higher time of inhalation. The PIF was correlated with the sex (P=0.002), being higher in the females.

When data were compared between species, only the inspiratory time showed significant a difference. *T. marginata* had a Ti higher than *T. hermanii* (Table 1).

Considering only the males and females of *T. hermanii*, differences related to their sex were observed in the TV, PIF, PEF, Penh and EF50 (Table 2). Due to the low number of females of *T. marginata* (n=2), the mean by sex of this species was not statistically compared.

Discussion

Most previous studies to check the ventilatory drive in tortoises were performed at least 25 years ago. Some of them used invasive techniques and did not have clinical application (Gans and Hughes 1967, Crawford and others 1976, Benchetrit and Dejours 1980, Vitalis and Milsom 1986). The major challenge to accessing the respiratory parameters in a healthy or sick tortoise is to find a way to measure it. Tests of pulmonary function have been developed for use in small laboratory animals, and include spirometry, mechanics, distribution of ventilation, gas exchange and ventilatory control (O'Neil and Raub 1984). More recently, whole-body plethysmography has been often employed in comparative physiology studies because it avoids excessive handling of the animals. However, the use of this technique may be limited in small or heterothermic animals because the signal captured by the device is produced based on differences in pressure inside the chamber, which are directly correlated with gradients of body and chamber temperatures, and humidity (Chaui-Berlinck and Bicudo 1998).

Because of their heterothermic condition, the measurement of respiratory events in reptiles requires sensitive and rapidly responding equipment. In the present study, the authors verified a difference of around 3°C between body and chamber temperature, and the plethysmograph was able to capture a standard signal in agreement with statements by Malan (1973). Due to the high acuity of the device, values recorded during oscillations produced by limb movements of the tortoise, or even interference caused by the operating personnel inside the room were not considered. The signal produced by rhythmic movements of the throat (gular moviments) were captured and not considered, because previous papers have demonstrated that in turtles the open/close movement of the glottis did not coincide with the pumping gular movements (Hansen 1941, McCutcheon 1943, Druzisky and Brainerd 2001).

A triphasic breathing pattern represented by an exhalation–inhalation–exhalation is known in some species of reptiles (Randall and others 1944). The mechanism of lung ventilation in the tortoise *Testudo graeca* was extensively explained by Gans and Hughes (1967). The authors verified the changes of intrapulmonary pressure during the respiratory movements, and verified that pressures changes were triphasic in form, consisting of an initial increase in pressure followed by a fall to a level of 7 cmH2O below atmospheric pressure, and again returning to atmospheric level or usually slightly above. During the pause between individual ventilation cycles, any overshoot gradually declined to the baseline. Controversially, the respiratory rhythm does not appear to be composed of brief periods of ventilation activity followed by prolonged pauses, as supposed by many authors, and it was attributed to differences in the species investigated. In the present study, the authors have identified an inspiration–expiration–apnoea phase, and a second expiration after the apnoeic plateau being different from the profiles described previously for Russian tortoises, *Testudo horsfieldi* (Benchetrit and Dejours 1980) and for the leopard tortoise, *Testudo pardalis* (currently *Stigmochelys pardalis*) (Glass and others 1978). In both papers, the authors describe an expiratory phase immediately followed by the inspiratory one and then by an apnoeic plateau. The authors considered apnoea when there was no movement of the muscles of respiration, and the volume of the lungs remained unchanged. The second peak expiratory described in the present study, which was considered to belong to a same respiratory cycle, was not cited by previous authors. Unlike that described for *T. horsfieldi* in the present work, the expiratory phase was always longer than the inspiratory one (measured by Te), and breath holding was not preceded by an inspiration. According to Glass and others (1983), previous studies have failed to establish the existence of clear 'thresholds' for initiating breath holding that could be associated with different methodologies to access the respiratory function in chelonians. In sea turtles, it is known that there is a good correlation between breath-hold length and the end-inspiratory lung volume over a range of 35 per cent of resting lung volume (Milson and Johansen 1975). As a consequence of a progressive decline in respiratory quotient in the lungs during the apnoea plateau, CO2 storage volume not excreted via extrapulmonary routes must be eliminated. The second peak expiratory described in the present study may account for Shaw and Baldwin's (1935) observation that agree with a respiratory cycle terminated by a partial 'exhalation'.

Most lung physiological studies in turtles and tortoises have determined the pulmonary volume (Milson and Johansen 1975, Crawford and others 1976, Vitalis and Milsom 1986). In these studies, anatomical dead space was estimated, but tidal volumes were not usually presented due to missing an accurate method (Funk and others 1986). Although not using whole-body plethysmography, tidal volume is known in *T. pardalis*. It ranged from 4.7 to 10 ml/kg in tortoises weighing around 2.9 kg (Glass and others 1978). Crawford and others (1976) verified lung volume, pulmonary blood flow, and CO-diffusing capacity during pump-ventilation in an aquatic turtle, *Trachemys* (formerly *Pseudemys) scripta elegans* and in a terrestrial turtle, *T. graeca*. The mean resting lung volumes, determinated by argon dilution, were similar in both species, being 160 ml/kg and 170 ml/kg, respectively. The dead space averaged 0.6 ml/kg in *T. scripta elegans* and 2.6 ml/kg in *T. graeca*. The values reported by those authors were lower than those described in the present study, which, except for the methodological questions, could be related to differences due to species, or even because of the small sample size used in those works.

Druzisky and Brainerd (2001), studying the buccal oscillation and lung ventilation in four specimens of big-headed turtles (*Platysternon megacephalum*), recorded TV between 2.513 and 10.482 ml/kg and respiratory frequency of 65–90 movements. TV around 6.9 ml/kg and respiratory frequency as low as two breaths/minute were also described for other semi-aquatic turtles, *Pseudemys* (currently *Trachemys*) *scripta* (Vitalis and Milsom 1986). Compared with these data, the present study has reported higher TV and respiratory frequencies, which could be justified as differences in the physiological mechanisms due to the usage of aquatic environment and methodological differences in the studies.

Gans and Hughes (1967) documented pauses in the breathing drive in *T. graeca* that lasted between four seconds and 23 minutes. These authors related the great oscillation on the apnoea timing to the level of disturbance that the animals had been subjected to. Three tortoises that moved constantly in the chamber providing an inaccurate record were excluded. All tortoises used in the present study remained very quiet; the maximum time of apnoea recorded occurred in one female *T. hermanii*, and was approximately two minutes. The higher value of Ti in *T. marginata* could be associated with the larger size of the species, which have a very compact carapace, with the posterior end having a saw-like formation, flanged outward like a bell. Differences in the TV, PIF, PEF and EF50 observed between male and female *T. hermanii* could be also justified by sexual dimorphism in the species, in which males are smaller than females.

TV is usually used in mammals and calculated by means of volume of air exchanged and weight of the animal. In tortoises, this parameter could be influenced by the disproportional weight, density and shape of the plastron and carapace in relation to the whole body of the animal. Therefore, in chelonians, TV should be interpreted with caution mainly when comparing between species with differences in size and shape of the carapace.

According to Bates and Irvin (2003), Penh is a unit-less index of airway hyper-reactivity. This parameter is not a pure measure of resistance, but a mathematical artifice that has been criticised by some authors (Lundblad and others 2002, Mitzner and Tankersley 2003). Plethysmograph manufacturers claim, however, that it is the only commercial option for studying a wide range of pulmonary parameters in conscious, unrestrained animals. Interestingly, this test could have importance in case of tortoises experiencing smoky conditions, in which the bronchial and tracheal oedema could be responsible for changes in the standard values.

Comparing the parameters recorded in the present study with those verified in healthy cats (Garcia-Guash 2008), the differences in the respiratory patterns are clear. Cats, like other mammals, due to their homeothermic condition, present a relatively higher respiratory frequency, although similar values of tidal volume as tortoises. The most notable difference observed is in the Ti, Te, PIF and PEF parameters. Te and Ti in cats are very close, while in tortoises, Te is approximately 10 times higher than Ti, characterised by a long exhalation period. The values of Penh in cats are lower than in tortoises, which could be attributable to the differences in the morphology of the lungs and low respiratory tract between mammals and reptiles, especially the S-shaped trachea present in chelonians belonging to the suborder Criptodira. Ancillary diagnostic tests in reptilian medicine have been improved in the last decade, mainly concerning imaging diagnostic methods (Valente and others 2006a, b, 2007, 2008) and clinical pathology (Delgado and others 2011). In conclusion, the authors would encourage the development and evaluation of this technique as a major benefit to tortoises and turtles in pet ownership, rehabilitation and rescue centres, zoos and other institutions.

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