

Determination of the physiological age of *Rhipicephalus appendiculatus* (Acari: Ixodidae)

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Abstract

A non-destructive method to determine the physiological age of *Rhipicephalus appendiculatus* is described. The ratio of two readily available parameters of the tick was used: body weight over scutal length. Because the latter is constant throughout life and because of the high correlation between body weight and the nutritional status of starving ticks, the ratio between the two provides a biomarker for the physiological condition of the tick in relation to the environmental factors. Two hundred laboratory ticks were followed over a period of one year at two different levels of relative humidity. Three distinct phases of physiological age indices were observed, both in male and female ticks: a steep decline immediately after moulting independent of humidity, a period of constant ratios found only when the ticks were kept at the higher humidity, and a period of gradual decline depending on the humidity. It was shown that the proposed method provides an easy and quick method to evaluate the physiological age of laboratory tick stocks. Preliminary results of the application of this method in the study of the age structure of field populations are discussed.

Keywords: *Rhipicephalus appendiculatus*, ticks, physiological age, diapause

Introduction

The use of physiological age has gained importance in eco-physiological studies of ixodid tick species, possibly stimulated by its successful application in malaria epidemiology and mosquito control (Uspensky 1984). Determining the physiological age aims to provide an estimate of life expectancy of individual ticks, to characterise the age status of a group of ticks and to predict their response to external factors that influence survival, their susceptibility to environmental factors and infectivity of pathogens (Uspensky 1995). Specifically, in reference to unfed instars of ixodid ticks, physiological age refers to an index of cumulative irreversible nutrient-reserve-linked morphological and physiological changes caused by vital activity, which inevitably raises the probability of death (Uspensky 1984, 1995). In nature, conservation of body water and judicious energy utilisation greatly contribute to longevity of unfed ticks (Lees 1964, 1969). Despite sophisticated water conserving characteristics (e.g. lipid epicuticular layer), sporadic respiration and excretion of uric acid (Knülle & Rudolph 1982; Wharton 1985; Needham & Teel 1986, 1991), ticks sooner or later experience body water and solute imbalances. Passive and active access to water from unsaturated air, from metabolism and imbibition soon becomes necessary for water balance. At humidity levels above the Critical Equilibrium Activity (CEA), passive uptake accounts for most of the water vapour sorption, with active uptake providing the difference (Freda & Needham 1984). This situation entails lower energy utilisation because the active uptake mechanism is required to operate less frequently (Needham & Teel 1986), but some energy is expended when transferring water against a diffusion gradient from the environment to the haemolymph where it occurs at a higher water activity of about 0.99 (Needham & Teel 1986). Depletion of energy reserves and an increase in cuticular permeability due to nutrition reserves and lipid utilisation in other physiological processes requiring energy, eventually results in a raised CEA value with time (Lees 1946, 1964; Williams *et al.* 1986), and failure to actively absorb water follows.

At stable tolerable ambient water activity below the CEA, a near-equilibrium water mass is reached as water being lost through transpiration ultimately equals the water acquired passively. This balance may induce intolerable changes in the haemolymph properties and ticks must osmoregulate to keep the haemolymph solute and water concentrations at levels allowing adequate cell functioning. Failure to osmoregulate due to insufficient ambient water or diminished energy reserves result in higher haemolymph solute concentration that ultimately leads to tick mortality (Needham & Teel 1986). Haemoglobin and its metabolites, probably the major nutrient reserve for adult ticks, decline over a period of starvation (Tatchell 1964; Cook 1973; Jaworski *et al.* 1984; Williams *et al.* 1985, 1986). Some of the vital activities that are energy driven in starving ticks are osmoregulation, locomotion and respiration.

Haemolymph, the main water reservoir in ticks (Hsu & Sauer 1975), changes insignificantly during desiccation (Shih *et al.* 1973). No significant seasonal changes in water content of starving ticks have been reported (Williams *et al.* 1986) and, as explained before, a virtual water equilibrium is attained between the tick and a sub-saturated environment. It thus seems likely that the progressive weight decline of starving ticks is primarily due to energy expenditure. Weight then correlates with the energy-reserve status of starving ticks and can thus be used as a biomarker of its physiological condition at any given time. The simultaneous use of another quantitative attribute such as scutal length, though itself not a variable character, provides an index that may estimate a tick's physiological age. This method is furthermore non-destructive and free from the subjective shortfalls characteristic of estimation methods that use qualitative indicators (Uspensky 1995). This paper describes the use of this new quantitative method for the determination of physiological age of laboratory reared and field-captured *Rhipicephalus appendiculatus* Neumann, the principal vector of *Theileria parva*, the causative agent of East Coast fever and one of the most important cattle diseases in eastern, central and southern Africa.

Materials and methods

Physiological age ratio

The ratio, used to describe the physiological status of a tick or a tick population sampled at a given time and place, was calculated as follows:

$$\text{physiological age index} = \frac{\sqrt[3]{\text{weight (g)}}}{\text{scutal length (mm)}}$$

This ratio was used as an indicator for the physiological age because scutal length does not change during the life span of a tick and because there is a positive linear relationship between nymphal engorgement weight and the adult tick size (Chiera *et al.* 1985). Thus, scutal length represents at all times the extent of repletion of the tick when it fed as a nymph and changes in weight represent the extent of nutritive reserve utilisation up to the time of measurement. Weight, essentially a three-dimensional value (correlated with length, width and depth of the tick), was transformed into a linear value by calculating its cube root. The ratio of the transformed weight value to the corresponding scutal length gives an index of nutrition utilisation in relation to the original amount obtained at repletion of the nymph: higher ratios indicate physiologically younger ticks and vice versa.

The scutum was measured with a precision measuring ocular (Schraubenmicrometer okular, Wild 15xSK, precision 0.0017mm for females and 0.0033mm for males) mounted on a dissection stereo microscope, by immobilising the individual ticks on double-sided sticking tape (Tesa[®]). The maximum distance between the tip of the scapular process and the distal end of the male and female scutum was taken as tick size index (Chaka *et al.* 1999). The weight of the laboratory ticks was determined on a Sartorius balance (precision 0.1mg) within two hours of ticks being taken out of their experimental conditions. Field captured ticks were weighed the same day of sampling.

Field collected ticks

On 13 January 1997, 91 adult *R. appendiculatus* were collected from an open grassland area of about 600m by 300m at an altitude of 980m near Wafa Village (13°35'S, 32°29'E, Eastern Province of Zambia). Ticks were picked by local herd boys in the morning between 08:00 hours to 09:30 hours from the blades, stems and flowers of grass plants. All collected ticks were placed together in one tightly sealed sample bottle and transferred to the laboratory for age determination. Individual body weights and scutal lengths were measured and ratios calculated as explained above.

Laboratory reared ticks

The laboratory colony of *R. appendiculatus* originated from engorged females collected in Eastern Province of Zambia in 1992. Routinely, ticks are fed as larvae, nymphs and adults on tick-naïve rabbits and the engorged ticks are incubated at 27±1°C, 87±2% relative humidity (RH) and total darkness. The newly hatched or moulted ticks are transferred to a climate room maintained at 22.5±0.5°C (STAEFA Temperature Control), 87±2% RH (STULTZ Ultrasound Humidifier) and a light-dark regimen of 14h:10h.

TABLE 1. Number of *R. appendiculatus* male and female surviving at 22.5°C as a function of ambient relative humidity (%RH) and days after moulting.

Sex	%RH	Days after moulting													
		15	30	44	58	72	86	100	114	128	157	212	289	361	414
Female	72	50	50	50	48	44	29	14	3	1					
	87	50	50	49	49	49	49	49	49	49	47	46	35	13	2
Male	72	50	50	50	47	43	38	32	16	8					
	87	50	50	50	50	49	49	49	49	49	49	47	40	15	2

Experimental setup

On 23 October 1996, a random sample of 1000 nymphs was taken from the above laboratory colony and applied to the ears of a tick-naive New Zealand White rabbit. From the fourth day after application, the ear bags were emptied daily and the engorged nymphs were divided into two groups. The first group was allowed to moult at $27\pm 1^{\circ}\text{C}$, $87\pm 2\%\text{RH}$ and total darkness, the second group at $27\pm 1^{\circ}\text{C}$, $72\pm 2\%\text{RH}$ and total darkness. After the moult, 50 males and 50 females were randomly selected from each of the two groups and placed at $22.5\pm 0.5^{\circ}\text{C}$ and their respective humidity. The day length was set at 14h:10h (light:dark) to avoid diapause which could influence the physiological ageing. Every fortnight until the fourth month after moulting, the individual body weights and scutal lengths were measured. Thereafter, the recordings were done every month or every two months. Mortality was recorded just before each measurement.

Statistical analysis

The statistical analysis of the physiological age ratios was carried out in Stata 7.0 (StataCorp. 2001). The locations of the pivot points in the trend lines were determined using a standard generalised linear model (GLM) with Gaussian error and its canonical link. To account for the repeated measurements, the actual analysis was done by means of the generalised estimating equation (GEE) method of Liang & Zeger (1986), using an exchangeable within-group correlation structure and including the previously obtained pivot points. Females and males were analysed separately as their ratios are not comparable. The relationship between survival and physiological age ratio was determined by means of a GLM with binomial error and its canonical link.

Results

The number of ticks measured and weighed at each recording is presented in Table 1.

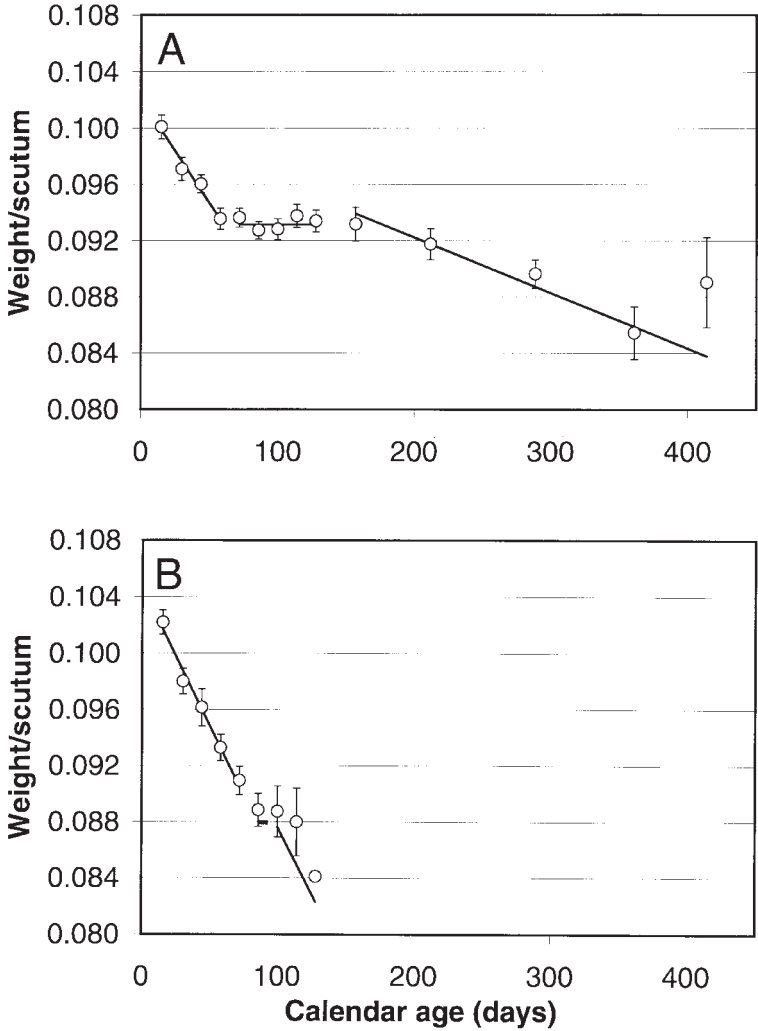


FIGURE 1. Females of *R. appendiculatus* at 87% RH (A) and 72% RH (B): ratio cube root weight to scutal length (ordinate) as a function of days since moulting (abscissa). Line for estimate and circles with bars for average ± 1.96 s.e. observations

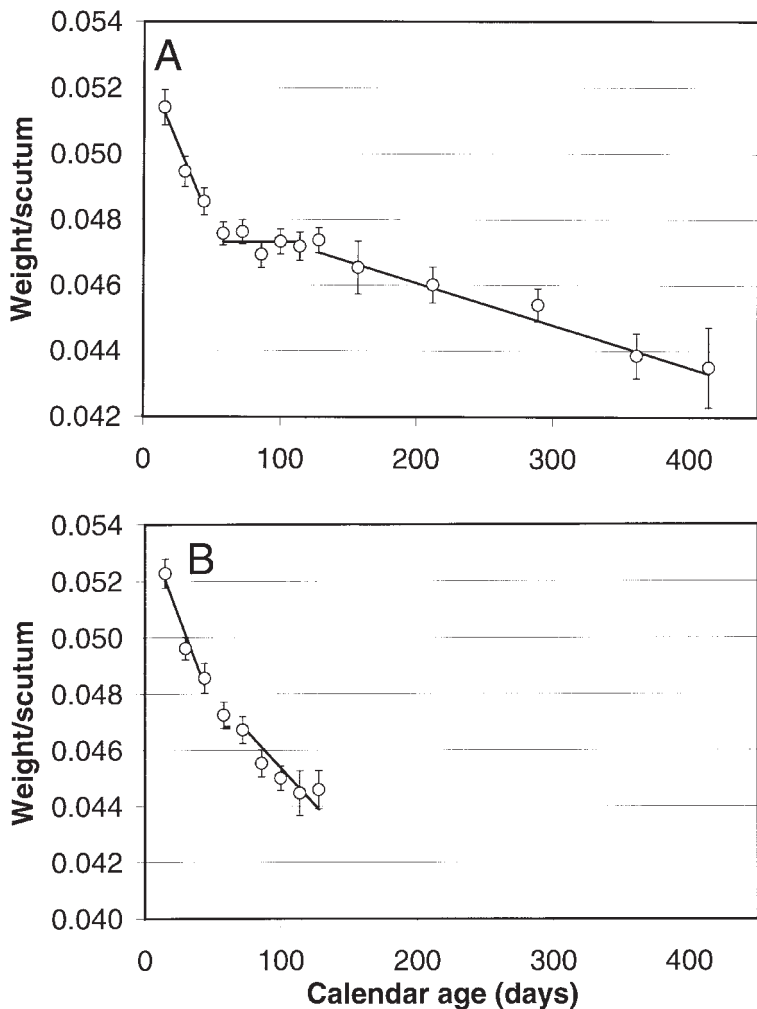


FIGURE 2. Males of *R. appendiculatus* at 87% RH (A) and 72% RH (B): ratio cube root weight to scutal length (ordinate) as a function of days since moulting (abscissa). Line for estimate and circles with bars for average ± 1.96 s.e. observations

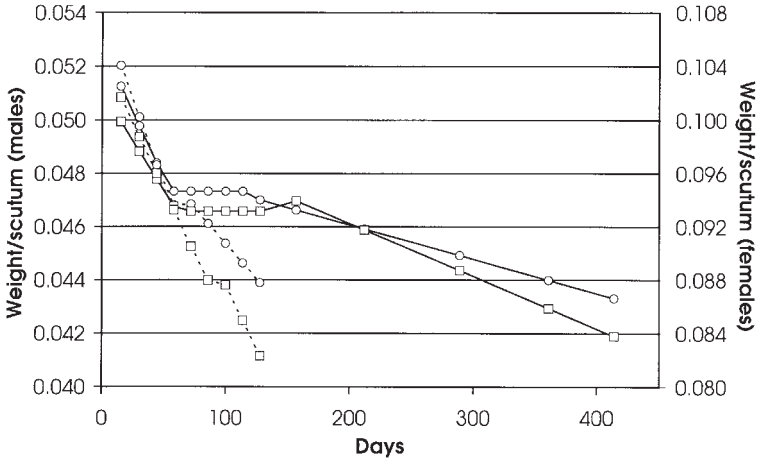


FIGURE 3. *R. appendiculatus*: ratio cube root weight to scutal length as a function of relative humidity and days since moulting. Square for female; circle for male; solid line for 87% RH; broken line for 72%RH.

The average observed ratios and estimated trend lines derived from the GLM and GEE models are plotted in Figures 1-2. Figure 3 shows the similarity between the estimated trends for males and females. The results of the GEE analysis indicate a decrease with time of the ratio in both males and females. Three periods can be discerned: a steep decline immediately after moulting, independent of humidity; a period of constant ratios for the ticks kept at 87% RH (reduced to a point for ticks kept at 72% RH), and a further decrease dependent on the humidity at which the ticks were kept (steeper at 72% RH).

The relationship between proportion surviving and physiological age ratio is given in Figure 4. No significant effect of relative humidity could be found in either sex. The corresponding logistic relationship between survival and ratio is:

Male ticks:
$$\text{survival} = 1 - \frac{1}{1 + e^{-77.13 + 1721.80 \cdot \text{ratio}}}$$

Female ticks:
$$\text{survival} = 1 - \frac{1}{1 + e^{-50.02 + 577.19 \cdot \text{ratio}}}$$

During the field collection, 41 males and 50 females were taken. The average ratio for the male ticks was calculated as 0.045 (S.E. = 0.0004) and 0.090 (S.E. = 0.0007) for the females. Using the above logistic regressions, 0.60 of the original male cohort was still alive (95% confidence interval: 0.31 - 0.83), whereas 0.87 of the original female cohort had survived until the date of collection (95% confidence interval: 0.76 - 0.94).

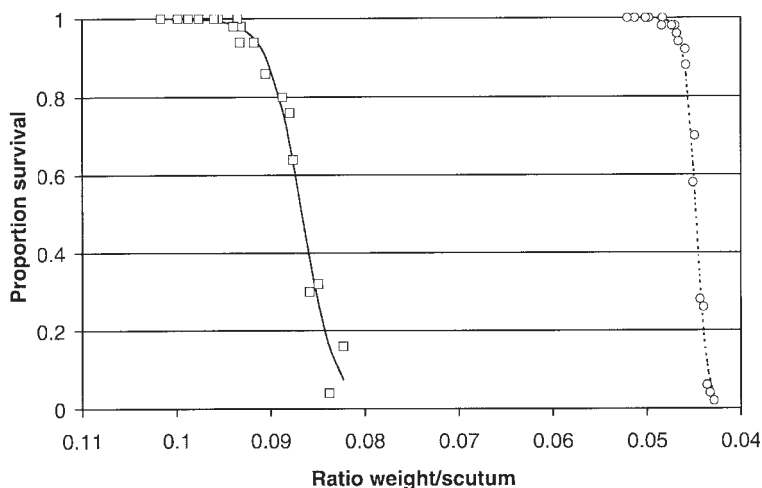


FIGURE 4. *R. appendiculatus*: proportion female (square for observed; solid line for estimate) and male (circle for observed; broken line for estimate) adults surviving as a function of the ratio of cube root weight to scutal length.

Discussion

The rapid steep decline of the physiological age indices observed during the initial few weeks after moulting in both sex groups and at both humidity levels is probably due to the initial scutal lengths and weights being recorded when the ticks were still undergoing their cuticular hardening process. The ticks were susceptible to evaporative water loss during the first few days because the cuticle is highly permeable to water immediately after ecdysis, returning to the impermeable state seen prior to moulting or in older individuals within a short period (Needham & Teel 1986). Because the hardening process is temperature dependent, hardening took longer at the moderate temperature of 22.5°C and it is not surprising that it had the same duration in both sex groups, irrespective of the ambient relative humidity. It would appear that, although newly moulted ticks have the capability to actively absorb water within hours after the moult, having lost that capability during the process of separation of the cuticle from the hypodermal cells of the integument (apolysis) in the pharate phase (Kahl & Knülle 1988), efflux of water through the cuticle during the hardening phase proceeded at a higher rate than ticks were able to actively absorb water. The voiding of faecal materials and nitrogenous excretory end-products (guanine and purine) observed to take place within a few weeks post-moult in all instars of ixodid ticks (Knülle & Rudolph 1982) would have amplified the weight loss during this period.

Keeping the ticks at 87% RH enabled male and female ticks to maintain their respective physiological age indices for between 2-4 months. This relative humidity offers ambient water activity slightly above the CEA value of 0.85 which according to Knülle & Rudolph (1982) is the threshold ambient water activity at which young ticks with undiminished energy reserves are able to maintain a steady state by active uptake of atmospheric water vapour for extended periods. Fluctuations in the average physiological age indices during this period could have been due to the irregular fluctuations in body water maintenance that has been observed to occur in individual ticks maintained at water

activities above their CEA (Rudolph & Knülle 1978; Knülle & Rudolph 1982). The eventual failure to actively absorb water, which occurs because of a raised CEA value in older ticks due to diminishing energy reserves (Lees 1946, 1964; Jaworski *et al.* 1984; Williams *et al.* 1986), probably occurred fairly uniformly among ticks maintained at 87% RH because of a narrow difference between the ambient water activity and the CEA value of *R. appendiculatus*, despite the fact that individual ticks held under identical conditions expend food reserves at different rates (Uspensky 1995). The failure to actively absorb water, after about two and four months in males and females respectively, and hence the increased energy demand to maintain the required haemolymph osmolarity, resulted in the period of decreasing physiological age indices. The decrease was moderate because, at 87% RH, the amount of ambient water available was fairly high, requiring less energy to maintain the balance, and because ticks have the capability to tolerate significant changes in their haemolymph constituents (Needham & Teel 1986), which further reduced the energy demands for osmoregulation in ticks maintained at this near optimal condition.

At the more desiccating condition of 72% RH and 22.5°C, a faster ageing was observed after the hardening in both sexes of ticks. No period of constant index was observed. The absence of active water absorption together with lower ambient water activity caused the ticks to expend considerably more energy for osmoregulation.

The value of the use of the proposed index in age grading of *R. appendiculatus* is shown by the very good relationship between the ratio and the survival curve in both male and female ticks. The fact that humidity level does not significantly influence this relationship further strengthens the indication that the ratio does indeed reflect the true physiological age of the ticks.

The average ratios obtained for the field collected ticks indicated that the male sub-population had a higher physiological age than the female sub-population (relatively lower ratio and thus lower proportion surviving in the males). Although this is obviously a single point in time at one particular locality, our own unpublished observations at that

locality indicate that the ratio of male to female *R. appendiculatus* collected from vegetation declines as the rainy season progresses. Similar faster mortality in males has been observed elsewhere (Newson *et al.* 1984). A biological explanation is not obvious at present, although it has been noted that male *R. appendiculatus* do not show the same diapausing behaviour as found in females (Berkvens *et al.* 1995) and diapause is associated with lowered metabolic activity (Tauber *et al.* 1986).

In eastern Zambia an unusual phenology of *R. appendiculatus* has been observed particularly at lower altitudes. A second peak in adult tick numbers is recorded on the host at the start of the dry season during years with normal or above normal rainfall (Berkvens *et al.* 1998). This second cohort is thought to be a second generation, transmitting *Theileria parva*, the causative agent of East Coast fever, immediately after moulting, when the infection prevalence in the tick is still high (Newson *et al.* 1984; Walker & Fletcher 1985; Billiouw *et al.* 1999). Periodic estimates of the tick population age structure provides a way to settle the question of this purported multigenerational phenology, as a second generation at the start of the dry season should show up as an increase of the ratio.

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