

## Darkness induces mobility, and saturation deficit limits questing duration, in the tick *Ixodes ricinus*

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### Summary

The behaviour of *Ixodes ricinus* nymphs was recorded in 10-day experiments using computer-assisted video-tracking, in the absence of any host stimuli. These ticks switch spontaneously from questing in a desiccating atmosphere to quiescence in a water-saturated atmosphere after dark. Quantification of both questing and quiescence duration demonstrates that questing duration is inversely related to saturation deficit whereas quiescence duration is not. Distance walked after quiescence increased with desiccating conditions, while the distance walked after questing remained unchanged.

Almost all locomotor activities of *I. ricinus* occurred during darkness under either a 14 h:10 h L:D or a 8 h:4 h L:D cycle. We established that all life stages of *I. ricinus* are equipped to sense shifts in light intensity with bilaterally placed strings of photoreceptors. This permits *I. ricinus* to use onset of darkness to trigger mobility when desiccation risk is reduced in nature.

Key words: questing, quiescence, tick, *Ixodes ricinus*, dark, desiccation, saturation deficit, photoreceptor, behaviour.

### Introduction

Although ticks are commonly perceived to spend their lives clinging from the skin of animals and humans, these ectoparasites pass most time developing in the litter zone and in search of a blood meal. *Ixodes ricinus* L. (Acari: Ixodidae), the most common European tick, is the main vector of various agents of medical and veterinary importance such as viruses (e.g. tick-borne encephalitis and looping-ill viruses), bacteria (i.e. Lyme borreliosis spirochaetes), rickettsiae (i.e. *Anaplasma* spp.) and protozoa (i.e. *Babesia divergens* and *Babesia microti*). This tick species feeds on a large range of vertebrate hosts including mammals, birds and reptiles (Aeschlimann, 1972).

The life span of free-living stages of ticks (larvae, nymphs and adults) is limited by the fixed energy reserves they possess at emergence. To find a blood meal, *I. ricinus* first climbs onto low vegetation to quest for a passing vertebrate host. During 'questing', ticks lose water (Lees, 1946), which they normally regain by descending at intervals to the litter zone (Lees, 1946; Milne, 1950; Lees and Milne, 1951) where they actively reabsorb water vapour from the atmosphere (Rudolph and Knülle, 1979; Gaede and Knülle, 1997; Kahl and Alidousti, 1997) during a period called 'quiescence' (Lees and Milne, 1951). In addition to water sorption, movements up and down the vegetation also require energy. These ectoparasites would therefore be expected to display behavioural and physiological adaptations that minimize energy use to facilitate prolonged questing activities.

Most studies on *I. ricinus* behaviour have focused on field

observations and have been limited to observing questing ticks. To gain more insight into hitherto unobserved tick behaviours, we developed a computerized video-tracking system for continuous recording of tick behaviours under controlled climatic conditions and in the absence of any host stimuli. We studied factors governing the alternation of questing and quiescence and the duration of these states by continuously following the behaviours of individual *I. ricinus* nymphs.

### Materials and methods

#### Computerized video-tracking system

Individual *I. ricinus* L. nymphs that had been collected in the field (Neuchâtel, Switzerland) and held for a minimum of 48 h in a water-saturated atmosphere before testing were placed in vertical plastic channels (100 mm×5 mm×1.2 mm, height×width×depth) delimited by a nylon mesh (500 mm) on one face. Ticks were left overnight in the channel before the observation started. Ten such parallel channels were cut into a single plastic block for simultaneous observation of individuals. Water was provided from a wet cotton wick at the bottom of each channel (simulating a water-saturated condition in the litter zone). The whole system was placed in a climate chamber where light, temperature and humidity were controlled. The carbon dioxide level in the climatic chamber showed no variation over 24 h (measured with a Binos1 Leybold-Heraeus IR analyser; resolution 2 p.p.m.). A light cycle was provided by fluorescent light (Osram Lumilux de

lux L36W/12-950 daylight tubes; >10 kHz). Maximum light intensity was 1400 lux and minimum light intensity was 0 lux, with gradual transitions at dawn and dusk (for details, see below).

Constant infrared illumination (950 nm) enabled video observations independent of lighting conditions. Images were acquired every 3 s using up to four Philips ToUCam PRO webcams with their built-in infrared-block filter replaced with a Kodak 87 infrared-pass filter. The position of ticks in the vertical channels was determined on the acquired images by a homemade tracking software ('camtud') running on a Linux computer, resulting in time-coded tracks. This set-up could follow the position of up to 40 nymphs simultaneously with a spatial resolution of 0.5 mm, independent of light conditions (the error rate in position detection was inferior to 1/10 000, as determined by following a mechanically driven moving object).

Time-coded tracks were further analysed by splitting them into three event types: (1) questing (ticks immobile for more than 2 h at a position higher than 2 cm above the wet cotton wick; i.e. outside the humid zone of the wet cotton, as measured with a Vaisala Humitter 50Y humidity sensor), (2) quiescence (ticks immobile for more than 2 h within 2 cm of the wet cotton) and (3) walking (ticks that moved more than 3 cm or changed their position by more than 1.5 cm within the channels). For each event, we analysed the start time, end time and duration. A few events for which the start or the end time could not be precisely determined were discarded. Actographs of tick movements recorded every 3 s were also generated from the time-coded tracks.

#### *Experimental conditions*

Tick behaviour was observed under three climatic conditions: 25°C, 60% relative humidity (RH); 25°C, 85% RH; and 15°C, 85% RH; corresponding to saturation deficits of 9.3 mmHg, 3.5 mmHg and 1.9 mmHg (1 mmHg=133.3 Pa), respectively, as calculated by Randolph and Storey (1999). Saturation deficit integrates temperature and relative humidity to derive a measure of the drying power of the atmosphere. Ticks were observed for 10 days under a light cycle of 14 h:10 h L:D at the three climatic conditions. In addition, the ticks that were observed at 9.3 mmHg saturation deficit under the 14 h:10 h L:D cycle were then left for 5 days in complete darkness and subsequently observed under a 8 h:4 h L:D cycle for 10 days. During the 14 h:10 h L:D cycle, light increased from 08.00 h to 10.00 h to reach a maximum of 1400 lux and decreased from 20.00 h to 22.00 h to reach a minimum of 0 lux. During the 8 h:4 h L:D cycle, light increased from 08.00 h to 10.00 h and from 20.00 h to 22.00 h and decreased from 14.00 h to 16.00 h and from 02.00 h to 04.00 h.

#### *Statistics*

Questing and quiescence event durations were not normally distributed, so we used the nonparametric Jonckheere test (Siegel and Castellan, 1988) to evaluate the null hypothesis of independence against the alternative hypothesis of a

quantitative relationship between saturation deficit and event duration. The Rayleigh test (Batschelet, 1981) for cyclic data was used to evaluate the null hypothesis of a uniform distribution for start and end of behavioural events over the day. Calculations were made using R for Linux (Ihaka and Gentleman, 1996).

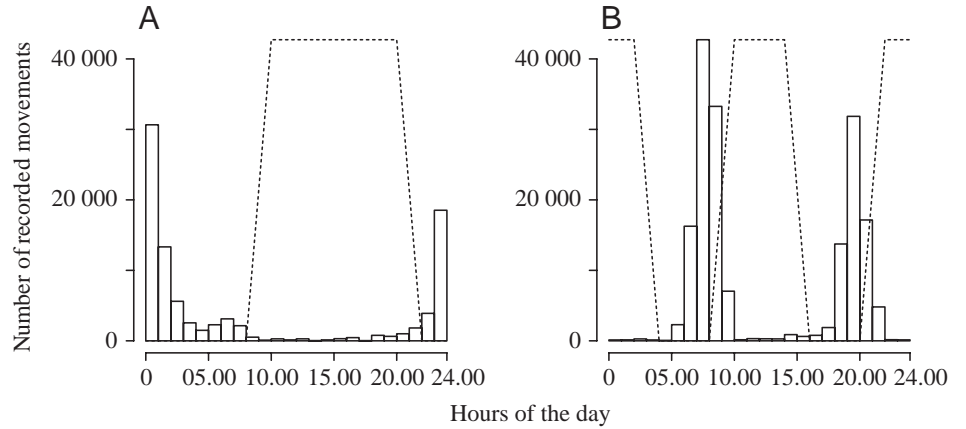
#### *Light and electron microscopy*

Laboratory reared *I. ricinus* were examined for the presence of photosensitive cells by light and electron microscopy. Six larvae, four nymphs, two adult females and three adult males were cut longitudinally in half and immediately fixed for 4 h at 4°C in paraformaldehyde and glutaraldehyde, prepared according to Karnovsky (1965), using a 0.1 mol l<sup>-1</sup> cacodylate buffer (pH 7.4) with 4% sucrose added. Specimens were washed four times in the same buffer and postfixed for 1 h at room temperature in 1% buffered OsO<sub>4</sub>. After three washes with the cacodylate buffer, specimens were dehydrated for 10 min in three solutions of increasing ethanol concentration (30–70%). For intermediate block contrasting, specimens were held for 2 h in darkness in 2% uranyl acetate diluted in 75% ethanol (Philis and Cromroy, 1977). Dehydration was terminated in 90% ethanol, 90% acetone and then in three successive baths of absolute acetone. Finally, the samples were embedded in Spurr's resin and polymerised for 24 h at 60°C. Semi-thin sections (500 nm) and thin serial sections (100–150 nm) were obtained on a Reichert Ultracut S microtome. Semi-thin sections were stained with toluidine blue and observed by light microscopy at a magnification of 400×. Thin sections were mounted on copper grids, poststained with uranyl acetate and lead citrate and observed with a Philips CM 100 transmission electron microscope at 60 kV.

#### **Results**

We were first interested to know whether *I. ricinus* nymphs show any spontaneous alternation between questing and quiescence behaviours. For this, 38 nymphs were observed in three different 10-day experiments at 25°C, 60% RH (saturation deficit, 9.3 mmHg) in a 14 h:10 h L:D cycle. Recordings showed that questing, walking and quiescence events alternated in the absence of any host stimuli. The probability of a tick switching to quiescence after questing was 93% (25/27 events). The converse, i.e. the probability of switching to questing after quiescence, was only 21% (25/121 events). In other words, quiescence was often interrupted by walking events, which did not necessarily lead to questing, whereas questing was primarily interrupted to start quiescence. Overall, walking events represented only 6.6% of the experimental duration. During these events, nymphs repeatedly walked up and down the vertical channels, reaching a median distance of 0.38 m per walking event, with a maximum of 9.65 m for one event. After quiescence, the distances walked were significantly longer (median=0.43 m, maximum=9.7 m) than after questing (median=0.17 m, max=2.92 m) (Wilcoxon test,  $P < 5 \times 10^{-10}$ ). When no water source was provided at the

Fig. 1. Frequency plots of walks by *I. ricinus* nymphs recorded at intervals of 3 s over 10 days with a photoperiod of (A) 14 h:10 h L:D ( $N=58$  nymphs) and (B) 8 h:4 h L:D ( $N=49$  nymphs) at 25°C and 60% relative humidity. Light cycles are indicated by the broken lines, with full intensity beginning at 10.00 h in A and at 10.00 h and 22.00 h in B.



bottom of the channels at 9.3 mmHg, questing and quiescence behaviour ceased or did not occur, and the ticks walked between 5 m and 31 m until they died within 12–83 h (three nymphs, data not shown).

During these experiments, tick movements recorded in the actographs were not uniformly distributed during the day (Rayleigh test,  $P<0.001$ ; Fig. 1A) but occurred preferentially during darkness, with only 6% of movements occurring during the period of maximum light intensity. It was thus hypothesised that dropping light intensity could trigger tick mobility. To test this, we changed the photoperiod from a 14 h:10 h L:D cycle to a 8 h:4 h L:D cycle; temperature and relative humidity remained unchanged (25°C and 60% RH; saturation deficit, 9.3 mmHg). Tick walking still occurred predominantly during darkness, i.e. twice in 24 h (Fig. 1B). Only 1% of movements occurred during the period of maximum light intensity (Rayleigh test,  $P<0.001$ ).

Since ticks lose water during questing, the duration of questing bouts should be related to desiccating conditions. To test this hypothesis, we followed different groups of ticks under three different saturation deficit conditions: at 9.3 mmHg ( $N=58$  ticks), 3.5 mmHg ( $N=50$  ticks) and 1.9 mmHg ( $N=27$  ticks) under a 14 h:10 h L:D cycle. In these experiments, the duration of questing events was inversely related to saturation deficit: means of 19.4 h, 25.9 h and 39.8 h at 9.3 mmHg, 3.5 mmHg and 1.9 mmHg, respectively (Jonckheere test,  $P<0.05$ ;  $N=33$ ,  $N=44$  and  $N=66$ , respectively; Fig. 2; 1 mmHg=133.3 Pa). A negative relationship between questing duration and saturation deficit was also observed when another group of ticks was successively exposed to saturation deficits of 9.3 mmHg and 3.5 mmHg (data not shown). By contrast, the duration of quiescence was not related to saturation deficit (means of 28.6 h, 17.2 h and 20.6 h at 9.3 mmHg, 3.5 mmHg and 1.9 mmHg, respectively; Jonckheere test,  $P=0.93$ ,  $N=148$ ,  $N=106$  and  $N=68$ , respectively; Fig. 3). The durations of quiescence events were not normally distributed but strongly skewed in favour of events of short duration (83% of events were below the mean at 9.3 mmHg).

In these experiments, we also examined walking to ascertain whether ticks walked preferentially during darkness under the different climatic conditions. Indeed, walks associated with the start and end of quiescence and questing events occurred mainly

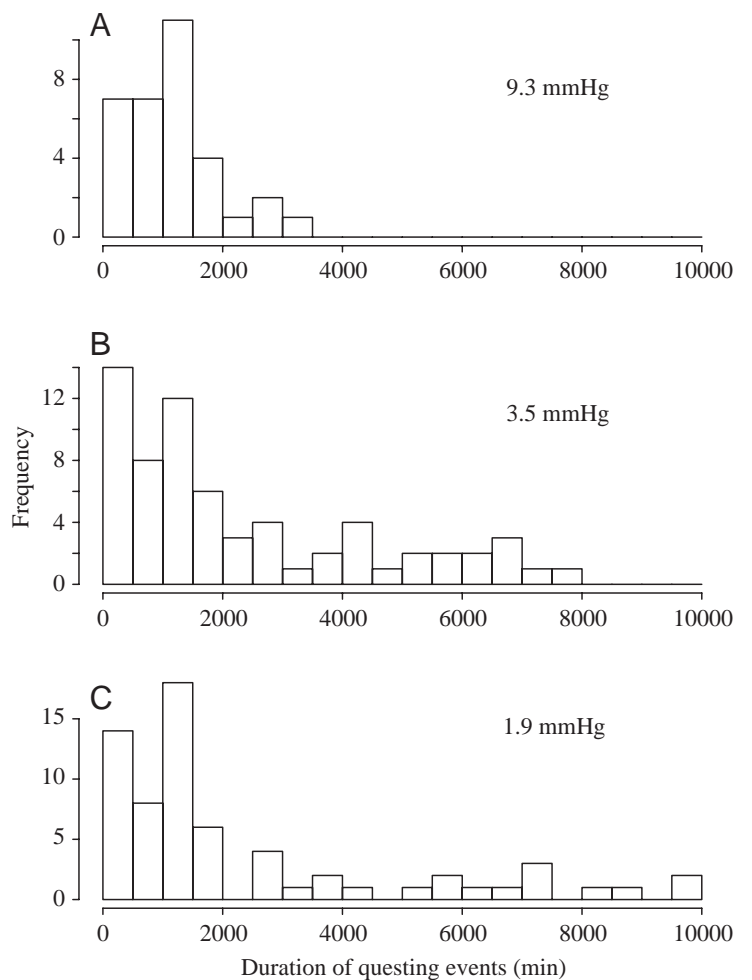


Fig. 2. Frequency plots of the duration of questing events by *I. ricinus* nymphs at saturation deficits of (A) 9.3 mmHg, (B) 3.5 mmHg and (C) 1.9 mmHg (1 mmHg=133.3 Pa). Questing duration decreases with increasing saturation deficit (Jonckheere test,  $P<0.05$ ; see text for details).

during darkness under all three climatic conditions (Fig. 4; Rayleigh test,  $P < 0.005$  for the start and end times of all event types under all conditions). The distance walked after questing interruption was not related to saturation deficit (Jonckheere test,  $P = 0.28$ ) and reached a median of 13 cm and a maximum of 2.92 m. By contrast, the distance walked after quiescence interruption was positively related to saturation deficit (Jonckheere test,  $P < 10^{-6}$ ; median distance at 1.9 mmHg=20.5 cm, at 3.5 mmHg=26.9 cm and at 9.3 mmHg=42.6 cm) and reached a maximum of 9.65 m at 9.3 mmHg. This means that the dryer the conditions, the further nymphs walked after quiescence. By contrast, independent of saturation deficit, nymphs interrupting questing walked similar distances before entering quiescence.

The mean walking speed of nymphs during walking events was influenced by temperature. At 25°C, this speed reached a median of 0.97 cm min<sup>-1</sup> (50% of observations between 0.56 cm min<sup>-1</sup> and 1.53 cm min<sup>-1</sup>), either at 60% or at 85% RH (Wilcoxon test,  $P = 0.35$ ), and was similar after quiescence and questing (Wilcoxon test,  $P = 0.38$  at 60% RH and  $P = 0.14$  at 85% RH). At 15°C, which is closer to the temperature encountered by ticks at night, the mean speed was reduced compared with that at 25°C, either after questing (Wilcoxon test,  $P < 10^{-5}$ ) or after quiescence (Wilcoxon test,  $P < 10^{-15}$ ). However, at 15°C, nymphs walked faster after questing (median 0.67 cm min<sup>-1</sup>; 50% of observations between 0.48 cm min<sup>-1</sup> and 0.84 cm min<sup>-1</sup>) than after quiescence (median 0.43 cm min<sup>-1</sup>; 50% of observations between 0.31 cm min<sup>-1</sup> and 0.56 cm min<sup>-1</sup>; Wilcoxon test,  $P < 10^{-6}$ ). This shows that the walking speed of nymphs is reduced at lower temperature, but this reduction is dependent on whether nymphs are interrupting questing or quiescence.

Since *I. ricinus* movements occurred preferentially during darkness, we suspected the presence of photoreceptors in this species, as previously suggested for other ticks (Binnington, 1972). Examination of semi-thin sections of larvae, nymphs and adults by light microscopy revealed two rows of 20–21 photosensitive cells located dorsolaterally in a pearl-string fashion in all three life stages of *I. ricinus* (Fig. 5A). The morphology of these cells was examined in thin sections by electron microscopy (Fig. 5B), which revealed cells containing a major rhabdomere, attached to the hypodermis (Fig. 5C). Axons from the photoreceptor cells join to form the optic nerves that pass antero-dorsally to reach the synganglion rostrally, parallel to the cheliceral nerves.

### Discussion

Our understanding of tick questing behaviour is limited by the difficulty of observing ticks without disturbing them. As humans are potential hosts for *I. ricinus*, the presence of an observer may influence tick behaviours (McMahon and Guerin, 2002). Due to the small size of *I. ricinus* nymphs, their movements are particularly difficult to study, so most studies on *I. ricinus* questing behaviour have focused on adults (Gigon,

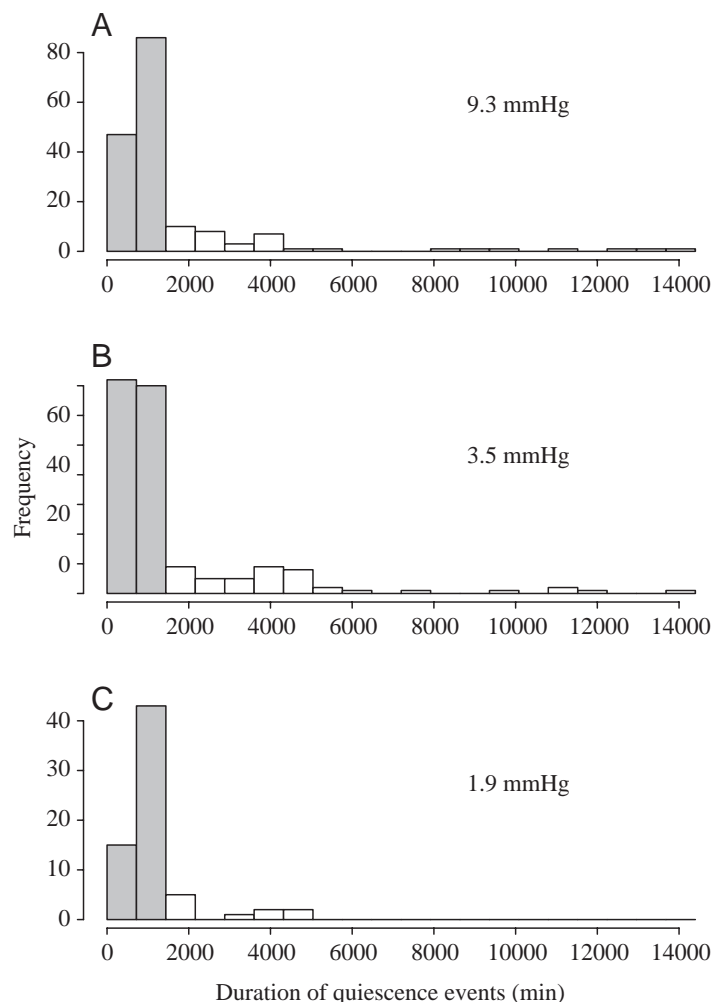


Fig. 3. Frequency plots of the duration of quiescence events by *I. ricinus* nymphs at saturation deficits of (A) 9.3 mmHg, (B) 3.5 mmHg and (C) 1.9 mmHg (1 mmHg=133.3 Pa). Most events are shorter than 24 h (grey bars). Quiescence duration is not related to saturation deficit (Jonckheere test,  $P = 0.93$ ; see text for details).

1985). Tick behaviours last many hours or even days and so continuous observation of single individuals over several days is hardly possible without automation. Furthermore, humans are not able to see ticks in the dark. For these reasons, we developed an automated video-tracking system to record the movements of *I. ricinus* nymphs continuously, independent of any potential host stimuli and under both light and dark conditions. Using this system, we show that questing and quiescence behaviours of *I. ricinus* alternate in the absence of any host stimuli. Lees (1946) had already observed that *I. ricinus* alternates between questing periods on vegetation and quiescence in the litter zone in his direct observations on adult *I. ricinus*. Here, we show that the alternation of these behaviours occurs spontaneously in nymphs in the absence of any intervention by an observer. In addition, we were able to follow ticks that left their questing site, something that is not possible in nature.



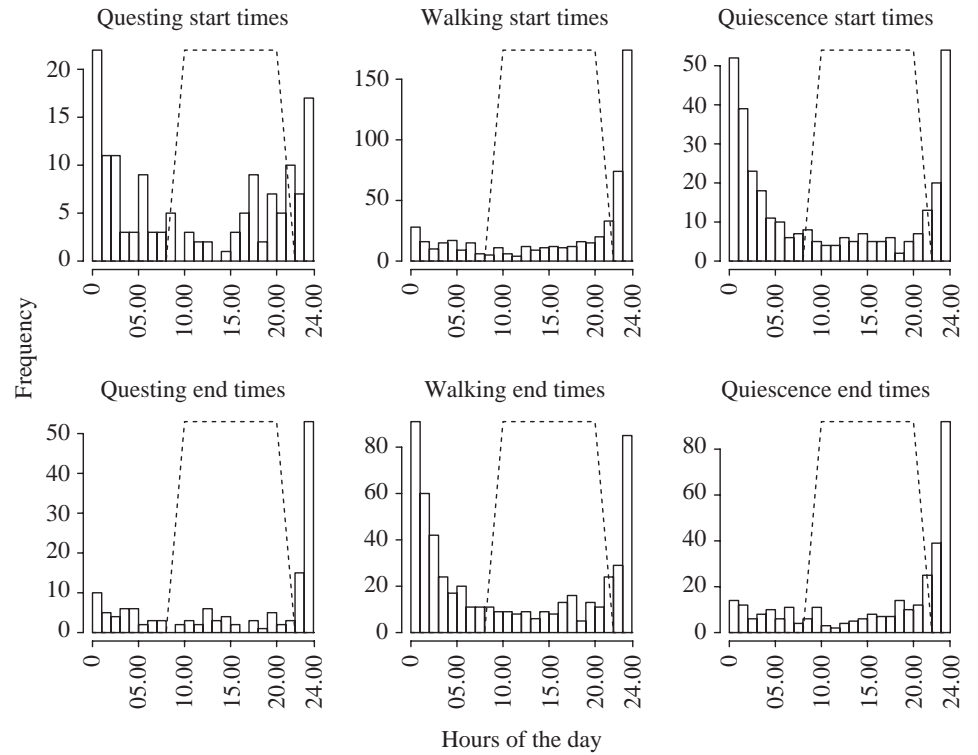


Fig. 4. Pooled frequency plots of *I. ricinus* nymphal questing, walking and quiescence start (top) and end (bottom) times over 10 days in a 14 h:10 h L:D cycle under three climatic conditions (see text). The light cycle is indicated by the broken lines, with full intensity beginning at 10.00 h. Totals of 322 quiescence, 143 questing and 541 walking events were recorded for 95 nymphs (see text for further details).

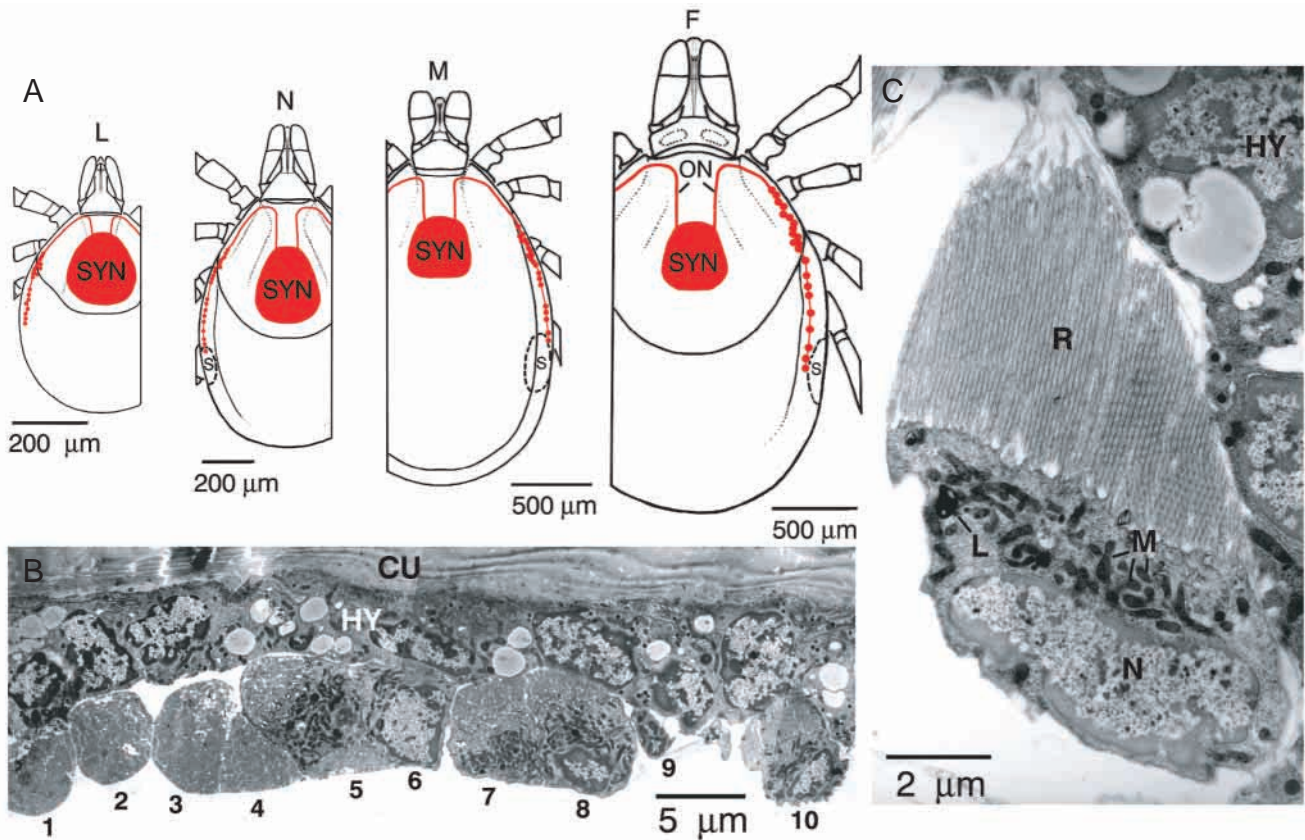


Fig. 5. Photoreceptor cells of the tick *I. ricinus*. (A) Optical and electron microscopical examination of larval (L), nymphal (N), adult male (M) and adult female (F) *I. ricinus* revealed 20–21 bilaterally arranged photoreceptors, each placed dorsolaterally posterior to coxa 2; axons from the photoreceptor cells form the optic nerves (ON) that pass antero-laterally to reach the synganglion (SYN) rostrally; S, spiracle. (B) Photoreceptors (1–10) of a larva, located immediately below the cuticle (CU) and the hypodermis (HY). (C) A larval photoreceptor cell containing a major rhabdomere (R), attached to the hypodermis (HY), lysosome (L), mitochondria (M) and nucleus (N).

Questing was almost always followed by quiescence in the humid zone of our observation channels. Furthermore, the walking speed to reach the humid zone was higher than the walking speed to leave it at 15°C, and the distance walked to reach the humid zone was shorter than the distance walked after leaving it. This demonstrates that ticks need to hydrate after questing. Indeed, *I. ricinus* is very susceptible to desiccation and loses water during questing on vegetation (Lees, 1946). In the humid zone, ticks restore their water content by practicing water sorption (Rudolph and Knülle, 1979; Gaede and Knülle, 1997; Kahl and Alidousti, 1997). Recent studies have suggested an influence of saturation deficit on the questing duration of *I. ricinus* in both quasi-natural arenas (Randolph and Storey, 1999) and the field (Perret et al., 2000; Randolph et al., 2002). Here, we demonstrate that the duration of questing is indeed limited by saturation deficit. When conditions are less desiccating, ticks will quest for longer periods. The proportion of questing individuals in the tick population is influenced by the prevailing saturation deficit. Longer periods of questing may increase the host-finding probability and thus influence tick population dynamics (Randolph et al., 2002). Abrupt declines in the density of questing ticks have been shown to coincide with abrupt increases in saturation deficit at field sites in both Switzerland (Perret et al., 2000) and the UK (Randolph et al., 2002). This demonstrates the important role of saturation deficit on tick populations in different ecozones occupied by *I. ricinus*.

Although desiccation may dictate when ticks should interrupt questing to move down the vegetation to rehydrate (quiescence), the factors driving interruption of quiescence remain largely unknown. We did not investigate this, but we discovered that quiescence was often interrupted by walking events that did not necessarily lead to questing. We suggest that some of these movements represent activities that enable ticks to find a favourable questing site in nature. Good questing sites are those where the probability of host encounter is high during the questing bout permitted by the prevailing climatic conditions. It has been shown that another *Ixodes* tick species, *I. scapularis*, chooses questing sites where chemostimuli have been left by hosts (Carroll et al., 1998). In the present study, we observed that the distance walked after quiescence increased with saturation deficit. *I. ricinus* nymphs were ready to repeatedly walk long distances (up to 9.65 m) during the night. Although the walks recorded in our experimental set-up occurred in vertical channels, we assume that at least some of these movements could represent horizontal walks if the ticks were not confined to the vertical channels. This suggests that *I. ricinus* nymphs can undertake extensive displacement in search of a questing site with appropriate microclimatic conditions.

Most tick walking occurred during darkness under all our climatic conditions. Preference for questing interruption during darkness was described in the field by Lees and Milne (1951), who suggested it to be due to an immediate response to temperature drops at night. Our data show that a drop in light

intensity is sufficient to trigger mobility in *I. ricinus* and need not necessarily be accompanied by shifts in temperature or RH. Similarly, Carroll et al. (1998) observed that *I. scapularis* moves preferentially during darkness. In our experiments, we show that even when the L:D cycle was changed to a 12 h period, the ticks continued to move during darkness. In addition, we show that, irrespective of the climatic conditions we applied, not only interruption of questing but also most walking occurs during darkness.

When active, respiration and thus spiracle opening increases in ticks (Lighton et al., 1993), resulting in increased water loss (Rudolph and Knülle, 1979; Knülle and Rudolph, 1982). In addition, the time needed to search for suitable questing and quiescence loci is unpredictable for a tick in nature. By undertaking movements during less desiccating conditions, *I. ricinus* minimizes water loss and the energy costs to reabsorb it. Since darkness generally coincides with less severe desiccating conditions in the field, *I. ricinus* uses darkness to trigger mobility when desiccation risk is lowest. As a wide range of tick hosts show peak activity just after dark (Hausser, 1995), tick mobility during this period may increase the probability of finding a host either by direct encounter or by orientation to host stimuli by mobile ticks (Carroll et al., 1998; McMahon and Guerin, 2002).

Unlike other ticks (Phillis and Cromroy, 1977; Kaltenrieder et al., 1989), *I. ricinus* does not have eyes with corneas. So, how can *I. ricinus* perceive the changes in light intensity that trigger its movements after dark? Although the existence of photosensitive cells in a variety of tick species, including *Ixodes holocyclus*, was suspected by Binnington (1972), none was described for *I. ricinus*. In the present study, we found 20–21 cells containing a rhabdomere, located dorsolaterally behind coxa 2 on each side of larvae, nymphs and adult *I. ricinus*. These cells most probably play a role in the perception of shifts in light intensity that trigger tick movements during darkness. They may also be implicated in the perception of changes in photoperiod that trigger morphogenetic diapause (Belozarov, 1982). Since photosensitive cells were found in all three life stages of *I. ricinus*, we expect nocturnal preference for movements in larvae and adults as well. Beyond avoidance of water loss, synchronised movements during darkness would carry a further advantage for adult ticks; since mating in *I. ricinus* occurs mostly off the host (Graf, 1978), synchronised mobility of adults with sundown could serve to increase encounters between sexes.

*I. ricinus* has a very wide geographical distribution, occurring throughout Europe and extending to North Africa. The climatic, photoperiod and vegetational conditions experienced by this tick species, as well as its host range, are extremely heterogeneous, which has diverse effects on tick populations. Therefore, individual *I. ricinus* populations may have developed particular physiological and behavioural adaptations to optimize energy use in a particular ecozone. The video-tracking system developed for this study might help to estimate such local adaptations in populations of *I. ricinus* from diverse climatic and photoperiod conditions.

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