IN VIVO TEST METHOD for REPELLENTS against the HARD TICK, *IXODES RICINUS* (ACARI: IXODIDAE), MAIN VECTOR of LYME BORRELIOSIS and TBE in EUROPE

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Abstract The present paper describes a test procedure with human volunteers used for a comparative investigation of tick repellents. Eight commercially available repellents were tested against the tick *Ixodes ricinus* (L.) and several test criteria described. Ticks were placed on a copper plate situated on an arm or leg treated with repellent. While the arm or leg was kept vertically, it was observed whether or not a tick entered treated skin and walked a distance of at least 5 cm. No repellent was able to prevent more than 50% of the ticks from entering repellent-treated skin. Two repellents prevented >90% of the ticks from walking over the skin. Taking this as the test criterion, the other six products either repelled lower proportions of the ticks or showed no repellent effect at all. In the majority of repellents, ticks that managed to cross treated skin walked predominantly downwards. This behavior was contrary to the control, in which ticks preferred to walk upwards, and might be indicative of a somewhat weaker repellent effect. Only one of the products tested showed no sign of repellency at all. The described procedure thus proved able to discriminate repellencies of a range of products and may additionally be used for dose-efficacy studies.

Key Words Repellent tick *Ixodes ricinus* tick-borne diseases medical entomology host-seeking behaviour

INTRODUCTION

Ticks are important vectors transmitting pathogenic microorganisms to vertebrate hosts worldwide (Sonenshine, 1993; Estrada-Pena and Jongejan, 1999). In many parts of temperate Asia, America, and Europe, tick-borne diseases rank among the most frequent arthropod-borne diseases of humans (Korenberg and Kovalevskii, 1999; Shapiro and Gerber, 2000). To avoid infection by such tick-borne pathogens, vector tick-bites have to be prevented. A practicable way is the use of repellents for personal protection (Brown et al., 1997). Both an increasing knowledge of the occurrence and distribution of tick-borne pathogens (Wicki et al., 2000; Štépánová-Tresová et al., 2000; Stromdahl et al., 2001; Scoles et al., 2001) and an increasing public awareness drive an ongoing interest in the development of tick repellents.

Development of repellents needs testing of candidate substances in suitable bioassays. A variety of bioassays are available (Schreck, 1977). The simplest test used for ticks involves a tick-walking arena with untreated and repellent-treated parts (e.g., Dremova and Smirnova, 1970), where repellency is indicated by the percentage of ticks entering the treated zone compared to a control. Such an assay can be performed with virtually any tick species, but it is the least demanding one, as the motivation of ticks to enter a treated zone is very probably low. An improvement often used is the adoption of vertically placed rods, tubes, or strips of fabric, parts that are partially covered by a repellent (Carroll et al., 1989; Kaaya et al., 1995; Mwangi et al., 1995; Ndungu et al., 1995; Lwande et al., 1999). Ticks exhibiting the so called ambush strategy for host-seeking (Waladde and Rice, 1982) climb a vantage point where they wait for a passing-by host. Ticks walking up vertically a rod or a tube in such an assay are supposed to look for a vantage point, thus demonstrating their readiness to go for a host. However, it does not necessarily imply a high

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motivation for the tick to climb a particular test rod. This test, however, would be advantageous in order to screen for substances with high repellent activity. In order to increase the tick´s actual motivation for contacting a host, and thus the force the repellent has to counteract, the so-called Moving-Object (MO) bioassay was developed (Dautel et al., 1999). This test system uses warmth and movement as host-associated attractive stimuli, and explores the behavioural sequence involved when a tick changes to a passing-by host. This assay is inherently restricted to tick species (and developmental stages) exhibiting the mentioned ambush strategy.

However, no in vitro test system developed so far can precisely predict the efficacy a repellent will show on the host. Here it beomes part of a complex pattern of volatile and contact host stimuli, and additionally exhibits interactions with skin chemistry. Therefore, a critical test using the vertebrate species to be protected still produces the most reliable results. With human volunteers, field tests as well as laboratory investigations have been performed. In field tests, the repellent may be applied to clothes (Lane, 1989; Evans et al., 1990) or to skin (Schreck et al., 1995; Solberg et al., 1995). The latter method puts higher demands on the repellent, because it has to counteract not only short-distance attractants such as warmth or skin volatiles but also contact attractants of the skin. Field tests may yield meaningful results, when performed at the right season and in an area with sufficient tick questing activity. However, weather conditions are known to alter such questing activity (Randolph and Storey, 1999; Perret et al., 2000; Schulze et al., 2001) and nonhomogeneous habitats used for control and test trials can influence the results. Additionally, a standardized walking behaviour of volunteers in the field is important, since it is less the tick who approaches humans, but man who picks up the ticks.

In laboratory tests, parameters mentioned above are under better control. Currently, a certain test procedure described by the Environmental Protection Agency is recommended for U.S. regulatory affairs (see www.epa.gov). The procedure briefly involves application of a repellent on a vertically positioned arm, leaving an area at the bottom of the arm untreated. Ticks are placed 2 cm below the repellent border and are monitored for entering/not entering the treated zone.

Once on a host, the natural behavior of ticks (particularly such species feeding for prolonged periods) is to search for a suitable feeding site, thereby showing a tendency to walk upwards. The repellent in the mentioned assay has to counteract this tendency. Since the tick‘s motivation to walk up may not be as high as the motivation to enter a host at all, a modified procedure was developed with the aim to increase the demands on the tick repellent to be evaluated. The present paper focuses on test criteria critical for the evaluation of repellent action and discusses their respective meaningfulness. The data presented are part of a study conducted for a consumer care organization. Since the aim of the paper is not to judge the efficacy of particular products, eight commercial tick repellents were arbitrarily chosen in order to include products based on different chemical classes and spanning the whole range of efficacy.

MATERIALS and METHODS

Ticks

Nymphs of a laboratory colony of *I. ricinus* were used at an age of 1 year post ecdysis. The ticks were kept in a climate chamber set at the mean weekly outside temperature and photoperiod. Ticks were free of *Borrelia* and TBE virus. Three days prior to experiment, all ticks were randomized and subsequently kept at 15°C in darkness.

General Test Procedure

Tests were performed in a glasshouse at 19-22°C and 35-60% RH. The test persons washed either their lower tibia or forearm with perfume-free soap, dried it with a towel, and then applied the repellent on the arm/leg according to product labels. Application was done outdoors in order

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to minimize repellent concentration of the air inside the test room. The total amount of repellent applied was determined by weighing the repellent container before and after application. From these data, the respective amount applied per area of skin was computed (mg cm^{-2}). The skin area to be treated was measured by determining the length of the arm/leg at its respective front and back as well as its circumference at 5 equidistant points across the length.

A circular plate of copper (thickness: 0.1 mm; diameter: 3 cm) was applied in the centre of the treated skin area, using forceps and white vaseline. Care was taken not to touch the copper plate by hand. With the aid of a stencil (paper with an appropriate circle cut out), a circle of 13 cm diameter was marked around the copper plate. By this procedure, the prospective test area remained untouched.

During a test, the arm or leg was held vertically. Fifteen min. after repellent application, two ticks were placed on the plate. These were observed for a maximum of 5 min. and it was protocolled whether: ticks entered the treated skin area or not; ticks fell down from plate or skin; ticks walked a distance of at least 5 cm, reaching the mark of the circle, and the direction (either up, down, or horizontally) the ticks had walked when crossing the marked circle.

Every five minutes, two new ticks were placed on the plate until a total of 12 ticks was observed (time block 15 to 45 min. after repellent application). This procedure was repeated every hour until 3 ticks had crossed at least 5 cm of skin. In this case, the hourly block was completed in order to determine the actual repellency rate. In case of continuous efficacy, the test was abandoned after 6 hours. At the end of a trial, the skin was again washed with perfume-free soap.

Control Tests

Prior to product test, a control run with 12 ticks was performed under identical conditions, but without repellent applied. This control served to demonstrate sufficient activity of the ticks at the test day (at least 10 of the 12 ticks should cross 5 cm of skin within 5 min.) and to make sure that there was no residual repellent activity in cases where tests had been performed on that arm or leg previously (more than 48 h).

Determination of Test Criteria

Duration of efficacy. The time point after repellent application when the third tick had passed at least 5 cm of treated skin, was arbitrarily chosen as the end point of efficacy. However, since the test started 15 min. after application, the third tick could cross treated skin not before 20 min. after application. Since this would mean an efficacy period of 20 min., although the repellent was not active, the observed protection time was adjusted according to the following criteria: a time of 20 min. was subtracted from the efficacy period in cases where at least 10 of the 12 ticks tested within a one-hour block crossed the treated skin; the efficacy period was increased by 20 min. in cases where only one or two ticks crossed the treated skin within the last test block.

In cases where the repellent duration lasted longer than 6 hours, the maximum test period, the efficacy time was arbitrarily determined to be 380 min. Mean values containing such data are therefore underestimated.

Quantitative repellency. Quantitative repellency was determined based on: the number of ticks entering the treated skin, and on the number of ticks crossing at least 5 cm of treated skin.

Repellency (R) was computed by the formula: $R = 100 - N_R/N * 100$; whereby N_R is the number of ticks entering or crossing treated skin, and N the respective tick numbers of the control trial. This relative repellency, however, was only computed for the first hour after application, a time period when all test persons were included in the analysis.

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Tick walking direction. The walking direction of ticks crossing 5 cm of skin was determined at the point when traversing the marked circle as either upwards, downwards, or horizontally. For statistical evaluation, the number of ticks walking upwards vs. not upwards (horizontally or downwards) was compared with the respective control by a G-test (see below).

Time course of tick activity. The time period between placing the tick on the copper plate and the moment when a tick entered the skin was measured using a stopwatch, both in the control and the test trials.

Test Persons

Test persons were three women and three men, who were fully informed and voluntary. Test persons were advised neither to use any perfume nor to drink coffee or tea or take other drugs on days of experiments. Certain parameters, such as the mean amount of repellent applied and the mean duration of repellent efficacy, were evaluated for each test person individually in order to find out whether or not individual differences existed.

Products tested

Eight repellents, commercially available in Germany, were selected. Every product was tested by the same six persons and each volunteer used a new package. The sequence of tests was determined by random for each test person. Two of the products used were based on mixtures of essential oils (hereinafter called Essoil1, a pump spray, and Essoil2, an oily formulation), two on coconut (called Coco1 and Coco2, two lotions), two on N,N-diethyl-m-toluamide (Deet), (called Deet1, a spray, and Deet2, a lotion), and two on other synthetic chemicals (called Synth1 and Synth2, two lotions).

Data Analysis

Differences in the duration of repellency between products or test persons as well as between time periods were investigated using oneway ANOVA followed by the conservative Scheffé-Test. A p-value <0.05 was regarded significant. The repellency of a product was tested by the G-test (Sokal and Rohlf, 1981) which was written in an Excel spreadsheet. Statistical tests were done with SPSS for Windows v.2.0.

RESULTS

Duration of Efficacy

Table 1 shows the mean durations of efficacy of the different products based on the criterion of a maximum of three ticks crossing treated skin. There were marked differences between test products. Only two of the selected products showed a mean efficacy of 4 hours or more. Since in single persons the efficacy proved to last longer than 6 hours, the mean values for these two products are even underestimated. Only in these products was a minimum duration of efficacy of approx. 2 hours in all individual volunteers observed. In all other products, there was at least one person for whom the efficacy lasted less than half an hour. Despite different mean values, the post hoc test did not detect significant differences (Table 1). The mean amount of repellents applied according to label instructions was different in the single products (Table 1). However, this did apparently not confer with duration of efficacy.

Quantitative Repellency

When the repellency was tested quantitatively, all but one product (Coco1) showed at least a certain degree of repellent action (G-test, Figure 1, see Table 2). Figure 1 shows the repellency of the products in relation to their respective controls within the 15-45 min period after applica-

Table 1. Adjusted mean duration of efficacy of commercial tick repellents tested with nymphs of *I. ricinus*

	Duration of efficacy [min.]			Significance	Amount of repellent applied [mg cm^{-2}]
Product	Mean ± SD	95% conf. interval			(Mean ± SD)
Coco2	333 ± 105	223	443	a	0.94 ± 0.44
Synth1	241 ± 115	120	362	a	0.79 ± 0.63
Synth2	90 ± 61	27	155	b	0.65 ± 0.45
Deet1	59 ± 29	28	90	b	1,15 ± 0.62
Deet2	50 ± 37	12	88	b	0.88 ± 0.25
Essoil1	23 ± 3	20	25	b	1.35 ± 0.42
Essoil2	21 ± 11	9	33	b	2.35 ± 1.59
Coco1	15 ± 15	0	31	b	1.29 ± 0.55

Significant differences between durations are signed by different letters (Scheffé-Test).

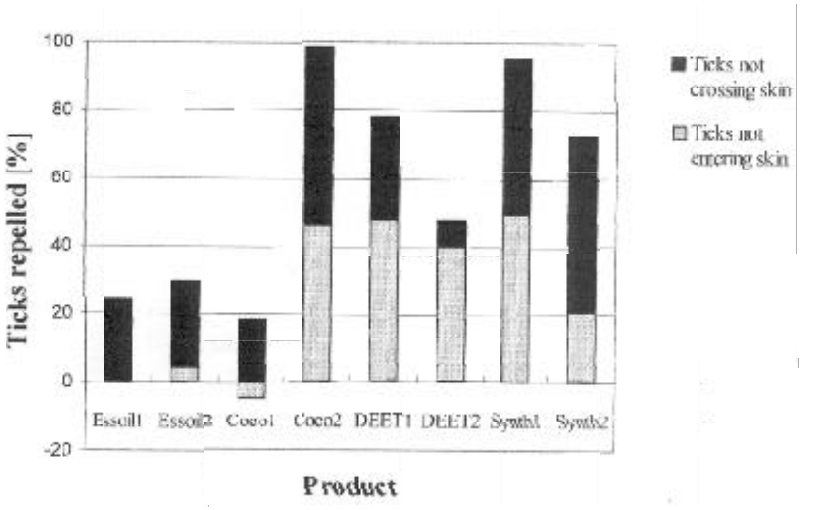

Figure 1. Repellency of the investigated products, expressed as the combined percentage of ticks not entering treated skin and the percentage of ticks, once on the skin, not traversing a distance of at least 5 cm compared to the respective control. In Coco1, more ticks walked onto the skin than in the control.

tion. A repellent either had the effect, that ticks did not enter treated skin, or, if ticks indeed had entered the skin, they were unable to cross it properly. Taken together, only two products prevented a total of >90% of the ticks from walking a distance of 5 cm over treated skin. Two other products showed a repellency of 70-80% whereas in all remaining products >50% of the ticks crossed the skin already within the first observation period. Remarkably, none of the products could prevent more than 50% of the ticks from entering treated skin. After treatment with Essoil1 or Coco1, as many ticks or even more than in the control walked onto the skin, respectively.

From Figure 2 it is apparent, that most of the ticks that did not cross treated skin either fell down from the plate or from the skin, a behaviour that was observed only in 2.8% of the control ticks (n=576).

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Table 2. Results of the G-tests evaluating the proportion of ticks traversing repellent treated skin at 15-45 min. after application vs. control (untreated skin, same person).

Product	G-Value	Significance
Essoil1	11.9	p<0.001
Essoil2	19.3	p<0.001
Coco1	2.8	n.s.
Coco2	137.5	p<0.001
Deet1	91.7	p<0.001
Deet2	26.4	p<0.001
Synth1	141.2	p<0.001
Synth2	61.5	p<0.001

n.s.: not significant

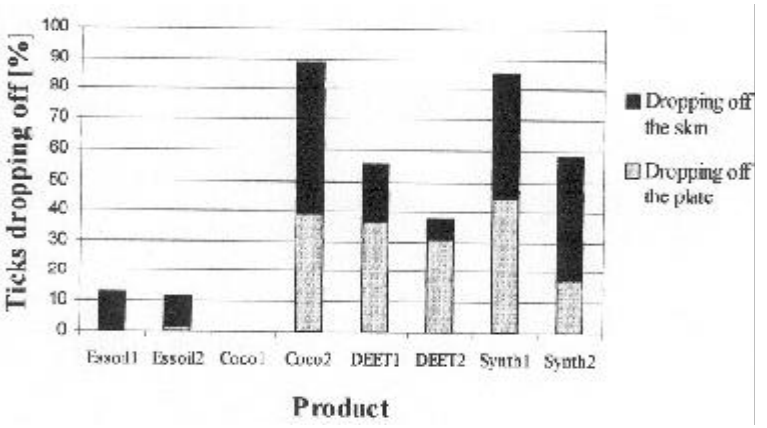

Figure 2. Percentages of ticks either dropping off the plate or off the skin in the course of the repellent test.

Tick Walking Direction

Control ticks showed a strong tendency to walk upwards, i.e., negatively geotactic, on untreated skin (Table 3). Taken together, 83% of the control ticks (n=518) walked up, 6.7% walked to either side, and 10.3% walked down. In contrast, ticks walking on treated skin preferred to walk downwards on the host. This was most apparent in products containing Deet as well as in Synth1 and Essoil2, less apparent in Essoil1, and no significant effect was observed in the remaining products (only single ticks were included in Coco2 and Synth1). Table 4 shows, that with increasing time after application of Synth1, the proportion of ticks walking upwards, increases.

Time Course of Tick Activity

Table 5 shows the time periods that elapsed between placing the tick on the plate and tick entering of the skin. There was no significant difference between control and repellent in two products, whereas in four repellents the ticks entered skin significantly later after application than

Table 3. Number of ticks (n) traversing 5 cm of skin and the respective percentage walking either upwards, downwards or horizontally, before repellent application (Control) as well as at 15-45 min. after application

Product	Application	n	Tick walking direction			G-value/ Significance
			up	side	down	
Essoil1	Control	66	86.4	10.6	3.0	4.6
	Repellent	50	68.0	16.0	16.0	P<0.05
Essoil2	Control	68	75.0	4.4	20.6	16.8
	Repellent	48	35.4	4.2	60.4	P<0.001
Coco1	Control	61	78.7	13.1	8.2	0.3
	Repellent	51	74.6	7.8	17.6	n.s.
Coco2	Control	64	82.8	9.4	7.8	0.6
	Repellent	1	100.0	0.0	0.0	n.s.
Deet1	Control	68	94.1	4.4	1.5	51.8
	Repellent	15	0.0	6.7	93.3	P<0.001
Deet2	Control	61	80.3	3.3	16.4	58.7
	Repellent	30	0.0	10.0	90.0	P<0.001
Synth1	Control	68	92.6	0.0	7.4	9.1
		3	0.0	0.0	100.0	P<0.01
Synth2	Control	62	74.2	8.1	17.7	0.3
	Repellent	17	64.7	5.9	29.4	n.s.

Significant differences in the percentages of ticks walking up vs. not walking up between control and repellent treatment were evaluated for each product by the G-test.

Table 4. Number of ticks (n) traversing treated area and the respective percentage walking either upwards, downwards or horizontally, at certain time periods after application of repellent Synth1

Time [min.] after application	n	Tick walking direction		
		up	side	down
30	3	0	0	100
90	6	17	17	67
150	9	22	0	78
270	8	38	12	50
330	1	100	0	0

in the respective control. In two products (Synth1 and Coco2), this effect was not so clear, since significant differences were inconsistently observed only later after application, when certain persons were excluded from the trial, because of the end of efficacy.

Test Persons

Comparing single test persons, differences concerning the mean repellent duration averaged over all test products are apparent (Table 6), but statistically not significant (ANOVA, p>0,05). Table 6 also shows that certain persons tended to apply more repellent than others. The applied mean amount, however, did not conply with the overall duration of the repellent effect.

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Table 5. Time period between placing a tick on the copper plate and the time the tick entered the skin measured for the control (untreated skin) and at different time periods after application of repellent products

Product	Time [min.] after application	Number of ticks	Time period [s] until tick entered skin	Significance
Essoil1	Control	58	16.5 ± 16.0	a
	30	59	25.2 ± 30.2	a
Essoil2	Control	71	20.7 ± 21.9	a
	30	67	52.7 ± 59.1	b
Coco1	Control	66	25.9 ± 42.2	a
	30	70	33.4 ± 55.9	a
Coco2	Control	70	23.4 ± 24.9	a
	30	38	35.9 ± 49.6	ab
	90	43	29.8 ± 27.7	ab
	150	32	43.1 ± 57.6	ab
	270	31	53.6 ± 56.5	b
	330	33	25.9 ± 30.0	ab
Deet1	Control	71	17.2 ± 22.0	a
	30	35	43.4 ± 56.3	b
	90	29	23.3 ± 25.2	ab
	150	12	13.8 ± 14.5	ab
Deet2	Control	72	31.8 ± 44.9	a
	30	43	65.6 ± 71.6	b
	90	10	75.5 ± 56.7	ab
Synth1	Control	72	22.9 ± 28.8	a
	30	36	30.9 ± 51.6	ab
	90	25	62.8 ± 55.0	b
	150	24	42.3 ± 45.9	ab
	270	25	73.4 ± 77.6	b
	330	8	65.0 ± 85.6	ab
Synth2	Control	72	22.9 ± 30.6	a
	30	54	57.6 ± 69.0	b
	90	37	60.1 ± 59.4	b
	150	22	50.7 ± 56.8	ab

Significant differences (ANOVA followed by Scheffé-test) between control and test within a single product are shown by different letters.

DISCUSSION

A good repellent assay should challenge a product in a situation as close as possible to practice conditions, i.e., to the intended use. Thus it should test the critical properties a repellent must exhibit. In order to prevent infection by tick-borne pathogens, it should theoretically suffice if a repellent just induces a tick not to bite. If a chemical could render human skin unacceptable as a blood source, there would be no need to repel the tick in a strict sense, i.e., inducing it to move away from the repellent source. This, however, would be insufficient under practice conditions. Using such a (hypothetic) chemical, the complete body, including the head would have to be treated, because otherwise, a tick could walk across the body until it finds an untreated area and bite there. Such whole body treatments are unlikely to be accepted by the consumer. As a consequence, a repellent must have the property to prevent ticks from accessing untreated skin. This

Table 6. Adjusted mean duration of efficacy [min.] of all investigated products in single test persons

Person	Amount of repellent applied [mg/cm²] (Mean ± SD)	Mean duration of efficacy [min.] (Mean ± SD)
A	1.11 ± 0.63	110.9 ± 125.6
B	1.71 ± 1.08	92.8 ± 122.9
C	1.00 ± 0.66	95.6 ± 133.8
D	1.20 ± 0.69	100.3 ± 122.7
E	1.59 ± 1.16	112.6 ± 134.1
F	1.15 ± 0.81	67.9 ± 82.4

could be achieved either by preventing the tick from clinging to the body at all, or by inducing the tick to drop off once it landed on treated (exposed) skin. The described protocol performs a rigorous test for this situation and proved able to discriminate the repellent efficacy of a range of test products developed for use on human skin.

The ratio of the described assay is as follows: Ticks placed on the copper plate are on the one hand exposed to certain host stimuli such as body warmth and very likely also to volatile skin kairomones, but on the other are still not yet on the host itself. In this situation, a tick has to make a choice between staying off the host and walking onto treated skin. If, alternatively, the tick had been placed not on the plate but on untreated skin surrounded by treated skin, the choice would be less rigorous for the tick, namely untreated versus treated skin.

In the present test, none of the chosen products was able to prevent all ticks from entering treated skin. Apparently, none of the repellents had a (distance) effect sufficient for that task. Once on the skin, it was further observed whether a tick could walk vertically a distance of 5 cm or not. A tick unable to walk properly would drop off the vertical area. As shown, the great majority of ticks not crossing the area dropped off (compare figures 1 and 2), whereas only single specimens either walked back to the plate or remained stationary. That is, besides the criteria of entering/not entering skin and crossing/not crossing a certain distance, the dropping off of ticks should be a suitable criterion for repellent tests. Such criteria are not subject to individual error and may be protocolled by the volunteer.

Another criterion, albeit for a more subtle repellent effect, may be the direction the tick walks on treated skin. The data show that ticks placed on a human body tend to walk upwards. This tendency was reversed by some products, suggesting that ticks not forced to drop off still might have the motivation to leave the host. If this interpretation is right, then a substantial proportion of ticks walking down might be indicative of a somewhat weaker repellent effect. This is in accordance with the observation that the proportion of ticks walking down decreased with increasing time passed after application of repellent, i.e., with decreasing concentration of the repellent (Table 4). However, it has to be kept in mind that the ticks crossing treated skin are already selected, representing those specimens that are least repelled, and a comparison of such ticks with the control may be problematic.

A further criterion that might be indicative for a certain repellent effect is the time period recorded between placing the tick on the copper plate and the time point when the tick entered the skin. This time period, however, seems not to be a reliable indicator, since in certain products like Synth1 and Coco2 there was no significant difference detectable between the first test period (15-45 min. after application) and the control, although these products showed a clear repellent activity. Therefore, this time parameter should be of only very limited value, if at all.

The present investigation found numerical differences in the mean durations of product efficacies between the single volunteers. Averaged over all products, this mean efficacy period was almost twice as high in the person, where the products showed the longest activity compared to the person where the respective period was shortest. Although the differences were not statistically significant, it cannot be excluded that a real difference exists, bearing in mind that only six persons were tested (see Rutledge and Gupta, 1999) and that individual differences in the attractivity of organisms for other blood-sucking arthropods are well known (Steelman et al., 1991; Schofield and Sutcliffe, 1997).

Another observation was the individual amount of repellent applied per test person. In practice, the consumer will not use a fixed quantity of repellent, but apply it according to the individual feeling. Here, discrete differences may exist not only between individual test persons but also between different formulations of the same repellent chemical. These differences should be incorporated into a repellent test, since they could have an important impact on the protection times evaluated. Figure 3 shows the adjusted efficacy periods determined for single volunteers in relation to the repellent amount applied. Due to the limited number of data points, no regression analysis was performed. However, the figure suggests that there may be interesting relationships between efficacy time and amount of repellent, not necessarily positively correlated. Thus, such a study could also help clarify dose-response relationships.

The valuable information that can be gained using human test persons outweighs some inherent drawbacks, such as the need for volunteers and the associated labor costs. A further specific requirement necessary to avoid health hazards in volunteers (and the experimenter) is the need for uninfected ticks. Such ticks need to be laboratory-reared and must have sufficient motivation to feed, which can be verified, e.g., by feeding trials on animals. Even very hungry ticks, however, do not bite immediately when placed on the skin, and no such tick bites were observed in the course of the experiments. Nevertheless, care should be taken that ticks do not enter any protected skin area, and all volunteers were advised to remove the copper plate instantly, if any tick happened to crawl beneath it.

In summary, the described test procedure investigates critical properties of a tick repellent and produces results that allow to judge the protective power of a test product in terms of quantitative repellency and duration of efficacy. Additionally, more subtle repellent effects are discernable and dose-response relationships should be recognizable.

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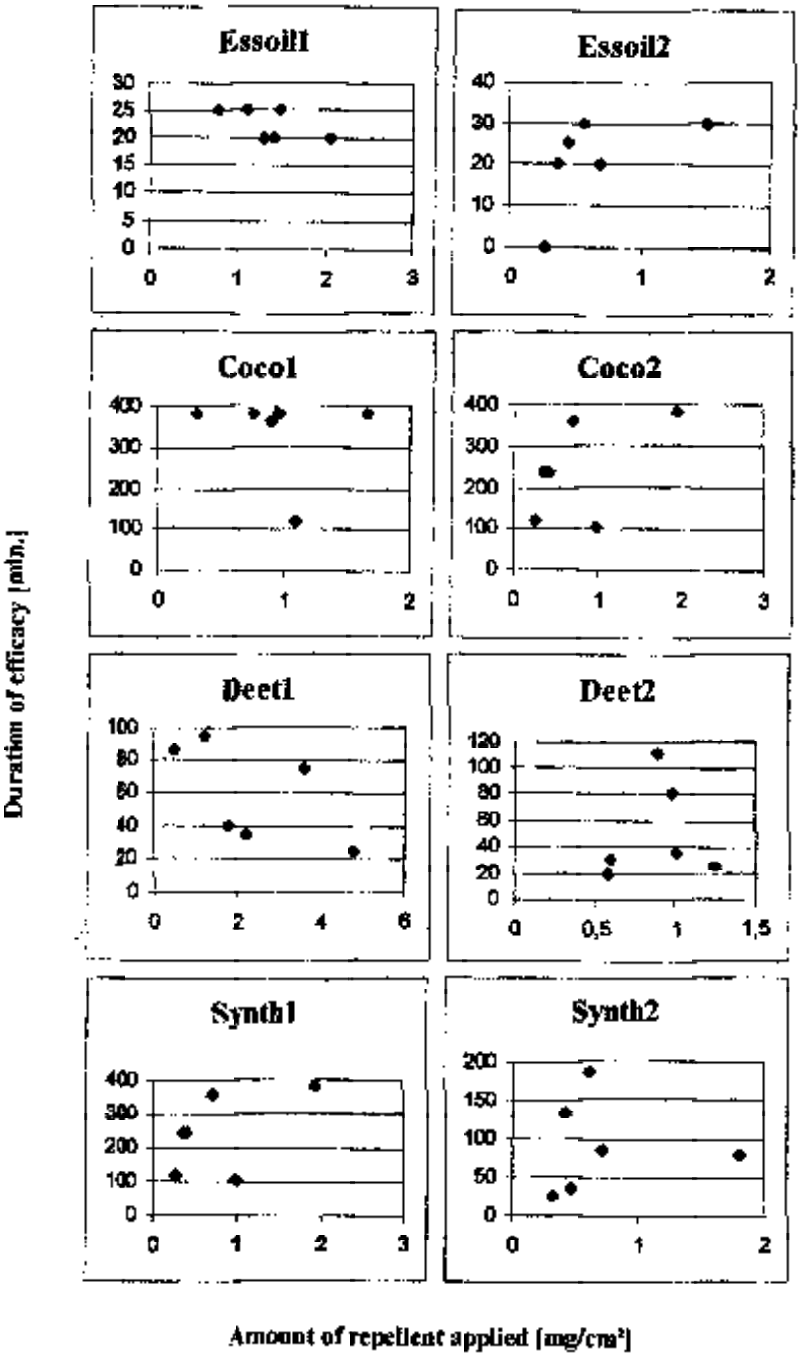

Figure 3. Adjusted duration of efficacy [min.; Y-axis], based on a maximum of 3 ticks traversing 5 cm of repellent-treated skin, and amount of repellent applied [mg/cm²; X-axis] in eight repellents tested. Data points represent values of individual volunteers.

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