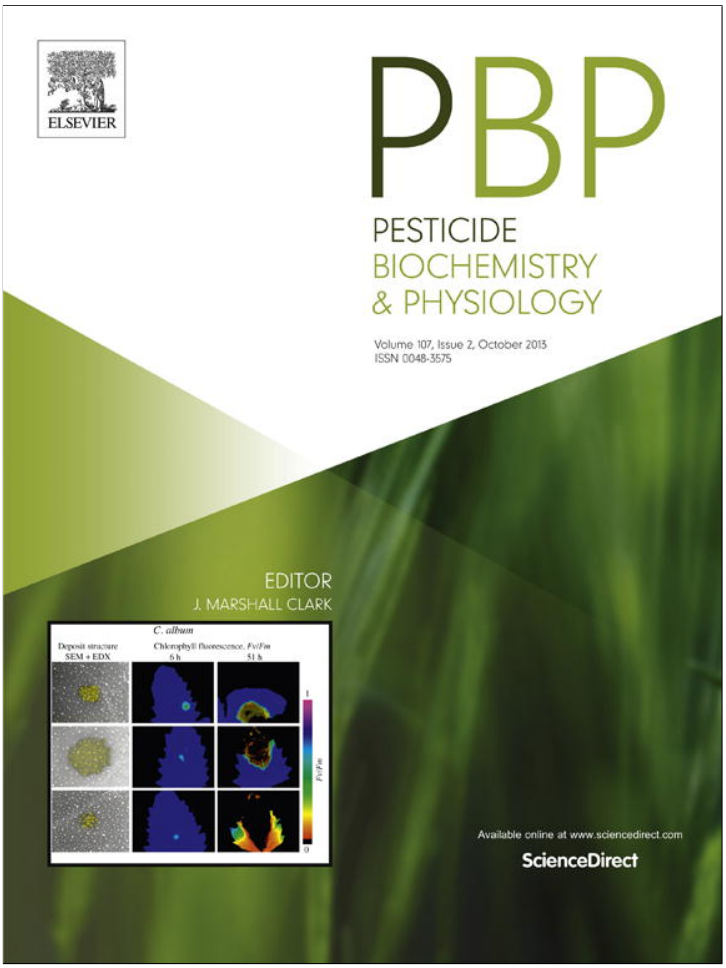

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A standardised *in vivo* and *in vitro* test method for evaluating tick repellents

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ABSTRACT

The threat of transmission of Lyme borelliosis and tick-borne encephalitis by ixodid ticks has resulted in an increasing number of tick repellents coming onto the market. To allow proper evaluation of the efficacy of different types of compounds and their formulations, there is a need for standardised methods for testing ticks repellents. Ticks show a marked negative geotactic response following contact with a potential host, i.e., they climb up in order to locate attachment and feeding sites, whereas exposing ticks to repellents induces positive geotaxis, i.e., ticks walk downwards or drop off the treated host or substrate. We describe here complementary tests that employ these geotactic responses to evaluate repellents: one *in vitro* on a warm glass plate and the other on the lower human leg (shin). The compounds tested were DEET, EBAAP, icaridin, capric acid, lauric acid, geraniol, citriodiol, citronella essential oil and lavender essential oil, all non-proprietary ingredients of widely distributed tick repellent formulations.

In controls on both the warm glass plate and the human leg, the majority of *Ixodes ricinus* nymphs walk upwards. By contrast, in both the *in vitro* and *in vivo* tests, effective doses of repellents cause ticks to either walk downwards or fall off the substrates, termed here "affected ticks". The ED75 values for affected ticks on the human leg indicate that the test products can be divided into three groups: (1) icaridin, EBAAP, DEET and capric acid with values between 0.013 and 0.020 mg/cm^2, (2) citriodiol and lauric acid with values between 0.035 and 0.058 mg/cm^2, and (3) geraniol, citronella oil and lavender essential oil with values between 0.131 and 1.58 mg/cm^2. The latter three products can be considered as less effective repellents. The tests on the warm glass plate resulted in very similar efficacy rankings for the products tested *in vivo*, and the ticks' behavioural responses also corresponded closely to those observed on the treated human leg. The ED75 values on the glass plate ranged from half to one sixth needed on the leg. The warm glass plate test thus provides a reliable alternative to human subjects for an initial evaluation of new repellents, and is particularly appropriate for testing products with still to be determined human toxicity and dermatological effects.

1. Introduction

The threat of transmission of Lyme borelliosis, tick-borne encephalitis and other diseases by ixodid ticks has resulted in increasing interest in tick repellents. These products aim to interfere with the tick-host interaction to protect the public, forestry personnel and other professionals from tick bites. Repellent products can be applied directly to a person's skin, clothing or shelter. However, there is a need for standardised methods for testing tick repellents to permit valid evaluation of the efficacy of different types of compounds and their formulations. While tick repellents will eventually require testing on humans or animals, it would be most appropriate to have an *in vitro* method for testing products that complements the *in vivo* situation. An *in vitro* test method for testing tick repellents with a strong predictive value for the *in vivo* situation would be ideal for initial screenings to identify potential new products. A further advantage of a validated *in vitro* method is its practical value for testing products of unknown human and animal toxicological and dermatological profiles.

Ticks show negative geotaxis on encountering a potential host, i.e., they climb up in order to locate attachment and feeding sites on the human body (behind the knees, pubic area, armpit, neck, head and ear zones). Exposing ticks to repellents causes positive geotaxis, i.e., ticks walk downwards or drop off the host or other treated substrate. This response has been used to test anti-tick products *in vivo* [1–2] and *in vitro* [3]. We describe here a test using a warm glass plate and another test on human skin, using the shin area of the lower leg. These are very practical methods and closely simulate the situation in the field, as most ticks make initial contact on the lower leg. Both tests use ticks' geotactic responses to

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evaluate the efficacy of the repellents. Ten products were tested: DEET, EBAAP, icaridin, capric acid, lauric acid, geraniol, citriodiol, citronella essential oil, and lavender essential oil – all non-proprietary ingredients of widely distributed tick repellent formulations, and neem plant oil. We show how the dose-dependent responses of the tick *Ixodes ricinus* L. to repellents *in vivo* closely correspond to its responses to the same products *in vitro*. In addition we show that the ticks' behavioural responses were closely associated to the chemical nature of the test products and these responses were the same in both the *in vivo* and *in vitro* tests.

2. Materials and methods

2.1. Ticks

Nymphs are easily overlooked because of their small size and it is this development stage that is most prevalent in the transmission of disease to humans. For this reason, *I. ricinus* L. nymphs were used for all tests. Nymphs were collected in a forest north of Neuchâtel using the dragging method in spring. They were stored at 14 °C and 100% relative humidity for at least three weeks before the tests and were tested within three months. Nymphs were only used once and were killed afterwards by deep freezing. Test protocols were carried out with the appropriate safety regulations and were compliant with the relevant biosafety and ethical authorisations.

2.2. Products tested

Ten products were tested:

1. N,N-Diethyl-3-methylbenzamide or DEET, an amide (Pestanal® >97% Riedel-de-Haen, Seelze, Germany);
2. 3-(N-acetyl-N-butyl)-aminopropionic acid ethyl ester or EBAAP, a N-disubstituted ß-alanine derivative, (IR3535® 98%, Merck KGaA, Darmstadt, Germany);
3. A 1:1 mixture of the two racemic diastereomers of 1 piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester or icaridin, classified as a substituted carbamate or a piperidine, synonyms picaridin, bayrepel, KBR3023, (Saltidin®; >97% Saltigo, Leverkusen, Germany);
4. Capric acid (decanoic acid) >99%, Fluka, Buchs, Switzerland;
5. Lauric acid (dodecanoic acid) >99% Fluka, Buchs, Switzerland;
6. Geraniol, a terpenoid (98% Sigma–Aldrich, Steinheim, Germany);
7. Citriodiol®, a refined essential oil of *Eucalyptus citriodora* (Hook.) K.D. Hill & L.A.S. Johnson, containing >64% of a mixture of the cis and trans isomers of 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol, synonyms p-menthane-3,8-diol, PMD (menthoglycol, Citrifine International, Yeadon, UK);
8. Citronella, the essential oil of *Cymbopogon winterianus* Jowitt (Citronella oil Java, Art. No. 736-340, Düllberg Konzentra GmbH & Co, Hamburg, Germany);
9. The essential oil of lavender, *Lavandula hybrida* (grosso herb oil, Art.No. 740-550, Düllberg Konzentra);
10. Neem oil (Margosa neem extract containing 97% carboxylic acids and 0.25% azadirachtin, Terra Nostra GmbH, Geisenheim, Germany).

Each product was tested on the lower leg (shin) of test subjects and on a heated glass plate at doses of 0.005, 0.01, 0.05, 0.1, 0.5 and 0.8 mg/cm^2 diluted in ethanol (p.a., >99.8% Merck). Each dose was applied in the same volume of ethanol (360 µl) and spread evenly with a fingertip covered by a latex glove over a 130 mm diameter circle on the shin (see below) or on the glass plate, both held horizontally for this purpose.

2.3. In vivo test on the lower human leg (shin)

The effects of test products on the behaviour of *I. ricinus* nymphs were examined in an 8-person test panel (sex ratio 1:1). A circle 130 mm in diameter was drawn on a shaved area to one side of the upper shinbone on the lower leg and marks were made on the circumference to indicate the horizontal (Fig. 1). The circular area was treated with a given dose of the test product. After 10 min, an aluminium foil disc (30 mm diam.) was stuck with paper glue to the centre on the treated area, taking care that the edges of the aluminium disc were pressed flush with the skin. 15 min after applying the test product, with the lower leg held vertically, nymphs were placed, two at a time with a fine sable-hair paintbrush onto the aluminium disc and observed for a maximum of 5 min. This procedure was repeated until the responses of 12 nymphs had been observed for each product dose. The effect of each test product was assessed on all ticks within 45 min of applying the product. In a control experiment, the behaviour of 12 *I. ricinus* nymphs was recorded on a leg treated with ethanol, at the beginning of each experimental day.

To evaluate the repellent effect of the test compounds the following behaviours were recorded (Fig. 2):

1. The number of ticks that walked downwards and that left the treated zone below the horizontal line;
2. The number of ticks that fell off the aluminium disc or the treated zone around it;
3. The number of ticks that did not leave the aluminium disc, which were immobilised on the treated zone around it, or had not left these zones within 5 min;
4. The number of ticks that walked up and left the treated zone above the horizontal line;
5. Ticks that left the treated surface having walked parallel to the horizontal or changed their walking direction from down to up or vice versa on the treated zone before leaving it were classified under "other behaviours". Overall, such ticks did not make up more than 10% of the total.

Affected ticks are defined as those that did one of the following: walked down, fell outside the treated surface from either the central disc or from the treated surface (irrespective of walking direction) or did not leave the disc or the treated surface within 5 min.

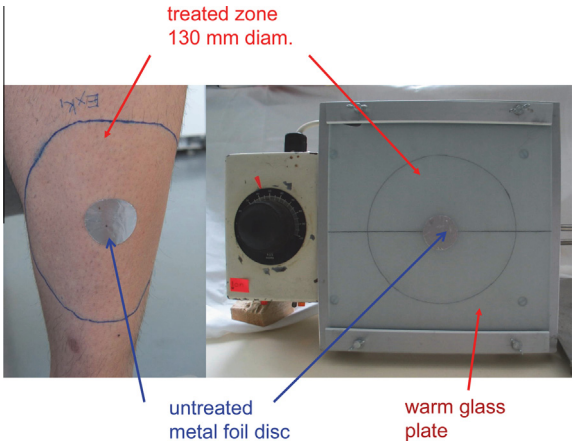

Fig. 1. Experimental setup employed for testing responses of *I. ricinus* to repellents *in vivo* on the skin (left) and *in vitro* on a heated glass plate (right).

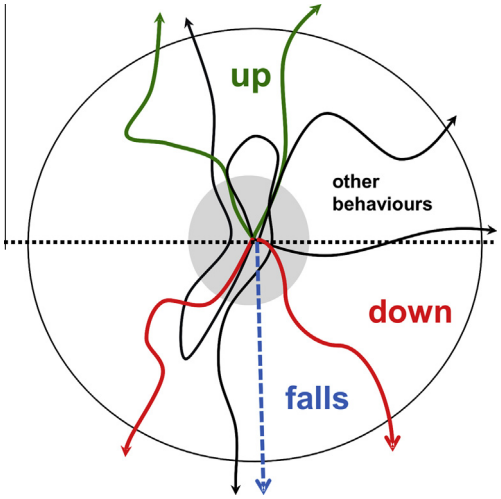

Fig. 2. Walking directions as quantified in both the *in vitro* and *in vivo* tests. Only ticks walking up (in green) or walking down below the horizontal (in red) were in included in the analysis of tick displacement directions. Ticks walking horizontally, or that changed their walking direction from up to down or vice versa before leaving the treated zone were classified as "other behaviours" (black traces). Ticks that fell off (in blue) or walked in any direction but did not leave the central disc or treated surface (not illustrated) were included under affected ticks.

2.4. In vitro test on a warm glass plate

The behaviour responses of individual *I. ricinus* nymphs to the test products were recorded *in vitro* on a heated sandblasted glass plate (200 × 200 mm, 4 mm thick). The plate was fixed to a thermostatically controlled aluminium hot plate of equal size to maintain a constant temperature of 34 °C at the glass surface. A 130 mm diameter circle was drawn on the glass and a horizontal line (Fig. 1) was drawn. The circular surface was treated with the test product. After 10 min, a disc of aluminium foil (30 mm diam.) was stuck with paper glue to the centre of the treated circle on the glass taking care that the edges of the aluminium disc were pressed flush to the glass surface. The whole set-up was tilted to an angle of 15° from vertical and away from the observer. 15 min after applying a given dose of a test product, nymphs were placed, two at a time, with a fine sable-hair paintbrush onto the aluminium disc and observed for a maximum of 5 min. This procedure was repeated until the responses of 12 nymphs had been observed for each product dose. This means that the effect of a test product on the ticks was assessed within 45 min of applying it.

In a control experiment, the behaviour of at least 12 *I. ricinus* nymphs was recorded on a surface treated with ethanol on each experimental day.

The categories of tick behavioural responses recorded on the warm glass plate were identical to those used to record tick responses *in vivo* and numbers of affected ticks were calculated in the same way (see Section 2.3 above).

2.5. Estimating risk reduction of a tick bite over time using the human leg (shin) test

An estimate of risk reduction of a tick bite over time was made using the human leg (shin) test by applying the proprietary anti-tick product Anti-Brumm® Zecken Stopp (Vifor SA, Villars-sur-Glâne) that contains 15% icaridin and 7.8% citriodiol. The lotion was applied at a median dose of 1.41 mg/cm^2 to the shins of 10 people and the behavioural responses of *I. ricinus* nymphs placed on an untreated disc in the centre of the treated area was recorded as described in section 2.3. Observations on groups of 12 nymphs were repeated at intervals of 1, 2, 3, 4, 6 and 8 h to estimate risk reduction over time.

2.6. Data analysis

The percentage of *I. ricinus* nymphs affected by each dose (log scale) of a test product was plotted for data collected *in vitro* and *in vivo*. The dose affecting 75% of ticks (ED75), the dose below which more than 10% of ticks walked up (ineffective dose or ID10) and their confidence intervals were calculated using an asymptotic four parameter log logistic regression model (LL.4, type = binomial; package DRC version 2.3-7 [4]) in R assuming an asymptotic devolution of the curve and fixing the left asymptote to the median level of controls. The LL.4 model was chosen because it allowed an asymptotic representation of observed responses as a function of dose and, in addition, provided a direct comparison of the effects on the nymphs of each product on the glass plate and the leg. A similar model was used to analyse the percentage of ticks walking up at different test product doses. To accommodate the maximum of the inverse U-shaped curves for ticks falling off from the leg treated with DEET and icaridin, a log linear model with 4 parameters with the left asymptote fixed to the control level was fitted using the modified Brain-Cousens log–logistic model for hormesis (package DRC version 2.3-7 [4]). This model is designed to fit data which show a maximum response at an intermediate concentration. In addition, the percentage of ticks walking down at different doses of test compounds was analysed using a general linearised model (GLM, family = quasibinominal) in R. For this analysis relevant dose levels were pooled (high and low) and used as a parameter in the model in addition to the product effect. Test compounds were grouped using contrasts. Median values were calculated for ticks falling off, walking down and affected *in controls.* To determine outliers in control groups used for the linear models, a binomial distribution of the affected proportion was assumed and observations outside the 95% confidence intervals in Quantile-Comparison Plots were selected (package "car" version 2.016 [5]) in R version 2.15 [6].

To estimate risk reduction of a tick bite following the application of the proprietary anti-tick product Anti-Brumm® Zecken Stopp, the probability of one tick walking up at two successive recording intervals on the same person was calculated and compared to the theoretical probability estimated as a Bernoulli trial, best suited to generate such probabilities. This theoretical probability was then compared to the observed numbers using the exact binomial test.

3. Results

3.1. Comparing the effects of test products in vivo and in vitro

On an untreated leg, 87% of ticks (of a total 1164) walked up, 1% fell off, 1% walked down and 10% showed other behaviours. All ticks left the 130 mm circle with most ticks walking up. On the untreated glass plate 83% of ticks (of a total 750) walked up, 2% fell from the glass plate, 2% walked down, 3% did not leave and 7% showed other behaviours. In both tests appetitive *I. ricinus* nymphs show negative geotaxis in order to reach a suitable attachment site.

The effects of repellent compounds on ticks were very similar in both the leg and warm glass plate tests. The action of icaridin on ticks, described below, provides a good example of this.

3.1.1. Tick responses to icaridin in vitro and in vivo

Despite the variability between individual persons, a clear picture emerges from the data recorded on the leg (Table 1 and Fig. 3). At doses higher than 0.05 mg/cm^2 hardly any ticks walk up-

Table 1
Behavioural responses of *I. ricinus* nymphs to increasing doses of 10 test products (see Section 2.2) on human skin (lower leg) compared to a warm glass plate (rounded to the nearest %): **affected ticks** (i.e. ticks falling off, ticks walking down and ticks not leaving the treated zone) and ticks **walking up** (see Section 2.3). Other non-conclusive behavioural responses are not included.

Product	Test method	Behaviour	Dose (mg/cm^2)							
			0.001 (%)	0.005 (%)	0.01 (%)	0.05 (%)	0.1 (%)	0.5 (%)	0.81 (%)	1 (%)
DEET	**Human leg**	**Affected**	**nt**	**30**	**56**	**88**	**90**	**84**	**87**	**nt**
		Falling off	nt	16	31	72	64	44	25	nt
		Walking down	nt	13	21	14	20	36	48	nt
		Not leaving	nt	1	4	2	6	4	14	nt
		Walking up	nt	46	11	0	0	1	0	nt
	Glass	**Affected**	**nt**	**25**	**86**	**100**	**85**	**72**	**83**	**nt**
		Falling off	nt	6	64	81	52	8	6	nt
		Walking down	nt	17	22	19	26	33	58	nt
		Not leaving	nt	2	0	0	7	31	19	nt
		Walking up	nt	44	0	0	2	6	4	nt
EBAAP	**Human leg**	**Affected**	**nt**	**34**	**58**	**88**	**89**	**83**	**90**	**nt**
		Falling off	nt	16	36	68	51	34	33	nt
		walking down	nt	13	18	16	35	30	39	nt
		Not leaving	nt	5	4	4	3	19	18	nt
		Walking up	nt	42	20	0	0	2	1	nt
	Glass	**Affected**	**13**	**58**	**94**	**83**	**98**	**88**	**61**	**nt**
		Falling off	6	39	79	75	78	3	6	nt
		Walking down	1	18	15	8	17	51	33	nt
		Not leaving	6	1	0	0	3	34	22	nt
		Walking up	64	4	2	0	3	0	8	nt
Icaridin	**Human leg**	**Affected**	**nt**	**41**	**67**	**91**	**92**	**92**	**91**	**nt**
		Falling off	nt	25	50	82	73	31	39	nt
		Walking down	nt	16	12	9	17	53	47	nt
		Not leaving	nt	0	5	0	2	8	5	nt
		Walking up	nt	32	15	5	0	0	0	nt
	Glass	**Affected**	**11**	**84**	**75**	**100**	**86**	**91**	**75**	**nt**
		Falling off	11	70	56	83	64	11	0	nt
		Walking down	0	14	19	17	22	72	66	nt
		Not leaving	0	0	0	0	0	8	9	nt
		Walking up	76	4	10	0	0	3	0	nt
Capric acid	**Human leg**	**Affected**	**25**	**35**	**50**	**92**	**99**	**96**	**nt**	**nt**
		Falling off	15	22	41	86	95	86	nt	nt
		walking down	3	2	6	4	2	10	nt	nt
		Not leaving	7	11	3	2	2	0	nt	nt
		Walking up	65	58	40	4	1	0	nt	nt
	Glass	**Affected**	**11**	**27**	**96**	**100**	**100**	**nt**	**83**	**nt**
		Falling off	3	8	96	100	100	nt	33	nt
		Walking down	0	6	0	0	0	nt	17	nt
		Not leaving	8	13	0	0	0	nt	33	nt
		Walking up	81	38	4	0	0	nt	0	nt
Lauric acid	**Human leg**	**Affected**	**nt**	**nt**	**5**	**70**	**72**	**89**	**89**	**97**
		Falling off	nt	nt	3	61	67	86	87	96
		Walking down	nt	nt	1	2	4	2	0	0
		Not leaving	nt	nt	1	7	1	1	2	1
		Walking up	nt	nt	88	25	23	6	8	3
	Glass	**Affected**	**16**	**48**	**81**	**92**	**100**	**nt**	**100**	**nt**
		Falling off	0	39	64	77	75	nt	100	nt
		walking down	8	6	11	8	17	nt	0	nt
		Not leaving	8	3	6	6	8	nt	0	nt
		Walking up	67	31	6	2	0	nt	0	nt
Citronella oil	**Human leg**	**Affected**	**nt**	**9**	**9**	**24**	**18**	**97**	**94**	**96**
		Falling off	nt	3	0	12	11	80	74	71
		Walking down	nt	1	1	6	3	16	20	23
		Not leaving	nt	5	8	6	4	1	0	2
		Walking up	nt	84	89	65	73	0	1	0
	Glass	**Affected**	**0**	**17**	**12**	**23**	**33**	**98**	**81**	**83**
		Falling off	0	0	0	17	25	53	36	17
		Walking down	0	0	8	3	8	39	39	58
		Not leaving	0	17	4	3	0	6	6	8
		Walking up	92	67	71	58	14	0	0	0
Citriodiol	**Human leg**	**Affected**	**nt**	**nt**	**7**	**88**	**98**	**96**	**98**	**98**
		Falling off	nt	nt	1	67	86	78	63	57
		Walking down	nt	nt	5	8	7	14	29	31
		Not leaving	nt	nt	1	13	5	4	6	10
		Walking up	nt	nt	79	4	0	1	0	0
	Glass	**Affected**	**8**	**15**	**17**	**100**	**100**	**100**	**92**	**nt**
		Falling off	0	15	13	92	83	75	67	nt
		Walking down	8	0	0	8	17	25	25	nt
		Not leaving	0	0	4	0	0	0	0	nt
		Walking up	75	73	42	0	0	0	8	nt
Geraniol	**Human leg**	**Affected**	**nt**	**nt**	**9**	**11**	**54**	**99**	**97**	**97**

(*continued on next page*)

Table 1 (*continued*)

Product	Test method	Behaviour	Dose (mg/cm^2)							
			0.001 (%)	0.005 (%)	0.01 (%)	0.05 (%)	0.1 (%)	0.5 (%)	0.81 (%)	1 (%)
		Falling off	nt	nt	1	4	41	80	66	57
		Walking down	nt	nt	7	6	12	15	29	34
		Not leaving	nt	nt	1	1	1	4	2	6
		Walking up	nt	nt	82	85	25	0	0	0
	Glass	**Affected**	**8**	**8**	**15**	**14**	**39**	**96**	**78**	**nt**
		Falling off	0	0	6	8	39	42	28	nt
		Walking down	0	8	3	4	0	44	39	nt
		Not leaving	8	0	6	2	0	10	11	nt
		Walking up	75	83	69	52	25	0	3	nt
Lavender oil	**Human leg**	**Affected**	**nt**	**nt**	**nt**	**11**	**3**	**56**	**74**	**89**
		Falling off	nt	nt	nt	6	2	43	66	70
		Walking down	nt	nt	nt	4	0	10	4	18
		Not leaving	nt	nt	nt	1	1	3	4	1
		Walking up	nt	nt	nt	82	90	31	18	10
	Glass	**Affected**	**0**	**nt**	**14**	**25**	**17**	**100**	**89**	**92**
		Falling off	0	nt	3	19	11	67	72	92
		Walking down	0	nt	8	0	3	33	17	0
		Not leaving	0	nt	3	6	3	0	0	0
		Walking up	92	nt	78	58	75	0	0	0
Neem oil	**Human leg**	**Affected**	**nt**	**nt**	**nt**	**nt**	**2**	**7**	**12**	**30**
		Falling off	nt	nt	nt	nt	1	6	5	6
		Walking down	nt	nt	nt	nt	0	0	5	14
		Not leaving	nt	nt	nt	nt	1	1	2	10
		Walking up	nt	nt	nt	nt	93	88	74	49
	Glass	**Affected**	**nt**	**nt**	**0**	**8**	**17**	**17**	**17**	**75**
		Falling off	nt	nt	0	0	17	0	0	0
		Walking down	nt	nt	0	8	0	17	17	58
		Not leaving	nt	nt	0	0	0	0	0	17
		Walking up	nt	nt	100	67	67	42	67	8

Note: nt = not tested.

wards and thereafter, as doses per cm^2 increase, the action mechanism of icaridin changes. Whereas the number of ticks falling off rises from 0.005 mg/cm^2 to peak at 0.05 mg/cm^2, the number walking down increases to peak at the higher dose of 0.5 mg/cm^2 icaridin (Table 1 and Fig. 3). In other words, ticks tend to fall off at the lower effective doses of icaridin and to walk down at the higher doses tested. At the inefficient dose of 0.0025 mg/cm^2 icaridin on the leg, 10% of ticks walk up (ID10, Tables 1 and 2; Fig. 3). Comparable tick behaviours were recorded as a function of icaridin dose on the warm glass plate: almost no ticks walked up at doses higher than 0.01 mg/cm^2, the number of ticks falling off climbs with increasing doses to peak at 0.05 mg/cm^2 icaridin and numbers walking down increase with dose to reach a maximum at the higher dose of 0.5 mg/cm^2 (Table 1 and Fig. 3).

3.1.2. ED75 values of products in vivo and in vitro

The ED75 values for affected ticks on human skin indicate that the test products can be divided into three groups: (1) icaridin, EBAAP, DEET and capric acid with ED75 values between 0.013 and 0.020 mg/cm^2, (2) citriodiol and lauric acid with values between 0.035 and 0.058 mg/cm^2, and (3) geraniol, citronella oil and lavender essential oil with values between 0.131 and 1.58 mg/cm^2 (Fig. 4). The same ranking was observed on the warm glass plate: (1) icaridin, EBAAP, DEET and capric acid with ED75 values between 0.002 and 0.006 mg/cm^2, (2) citriodiol and lauric acid with values between 0.01 and 0.02 mg/cm^2, and (3) geraniol, citronella oil and lavender essential oil with values roughly ten times higher at 0.16-0.27 mg/cm^2, (Table 2). The ED75 values are generally lower on the glass plate than on the leg, but by no more than by a factor of 6 for the most effective compounds (Table 2 and Fig. 4). The number of ticks walking up increases on both the glass plate and leg when the dose of the most effective repellent compounds is too low (<0.01 mg/cm^2; Table 1 and Fig. 5). Neem oil had little effect on the nymphs, except at the highest dose of 1.0 mg/cm^2 which affected 30% on the human leg and 75% on the warm glass plate (Table 1). It was therefore not possible to calculate an ED75 value for this product with sufficient accuracy.

3.1.3. Mode of action of test products

Some insights into the mode of action of the test products can be obtained by comparing the dose dependent effects of selected compounds from different chemical classes on tick behaviour *in vivo* and *in vitro*. The mode of action of the nitrogen-containing compounds icaridin, DEET and EBAAP contrasts to that of the carboxylic products, capric and lauric acid. At 0.01 mg/cm^2 icaridin already caused 50% or more nymphs to fall from both the leg and glass plate, and the proportion of ticks falling off increased for icaridin, DEET and EBAAP at 0.05 mg/cm^2 (with the exception of EBAAP that had already peaked at 0.01 mg/cm^2 on the glass plate; Table 1 and Fig. 3). Fewer ticks fell off both the glass plate and the leg at the higher doses of 0.5 and 0.8 mg/cm^2 of icaridin, DEET and EBAAP compared to those exposed to surfaces treated with 0.1 mg/cm^2 of these compounds. However this was compensated for by an increase in the numbers of ticks not leaving the treated zone or walking down. Overall, the GLM analysis of ticks walking down reveals that for DEET, EBAAP and icaridin the proportion of ticks responding in this way on the glass plate or leg increases significantly from low (0.05 and 0.1 mg/cm^2) to high (0.5 and 0.8 mg/cm^2) doses (GLM quasibinominal, analysis of deviance, $P < 0.0001$). This effect is strongly influenced by the chemical nature of the test compound ($P < 0.0001$) with icaridin showing the strongest increase in the number of ticks walking down (3 times that of DEET, $P < 0.02$, GLM quasibinominal). In contrast to this, ticks reacted primarily by falling off with increasing doses of capric and lauric acid (Table 1 and Fig. 3).

In the evaluation of the proprietary anti-tick product Anti-Brumm® Zecken Stopp over time, there was a gradual change in

Table 2
In vivo and *in vitro* ED75 and ID10 values for ten tick repellent products.

	ED75 (mg/cm^2)			ID10 (mg/cm^2)	ED75-ID10
	Human leg	Glass	Ratio	Human leg	
	Estimate	Estimate	Human leg/glass	Estimate	Dose range difference[a]
Icaridin	0.013	0.002	5.9	0.0025	0.01
EBAAP	0.012	0.005	2.4	0.0018	0.01
DEET	0.013	0.006	2.1	0.0006	0.01
Capric acid	0.020	0.006	3.3	0.0017	0.02
Citriodiol	0.035	0.020	1.7	0.0081	0.03
Lauric acid	0.058	0.010	5.6	0.0101	0.05
Geraniol	0.131	0.158	0.8	0.0734	0.06
Citronella oil	0.463	0.194	2.4	0.0640	0.40
Lavender oil	1.508	0.271	5.6	0.1728	1.34
Neem oil	na	na	na	na	na

[a] Dose range within which the difference between the ED75 and ID10 falls for each product. *Note*: na = not analysed

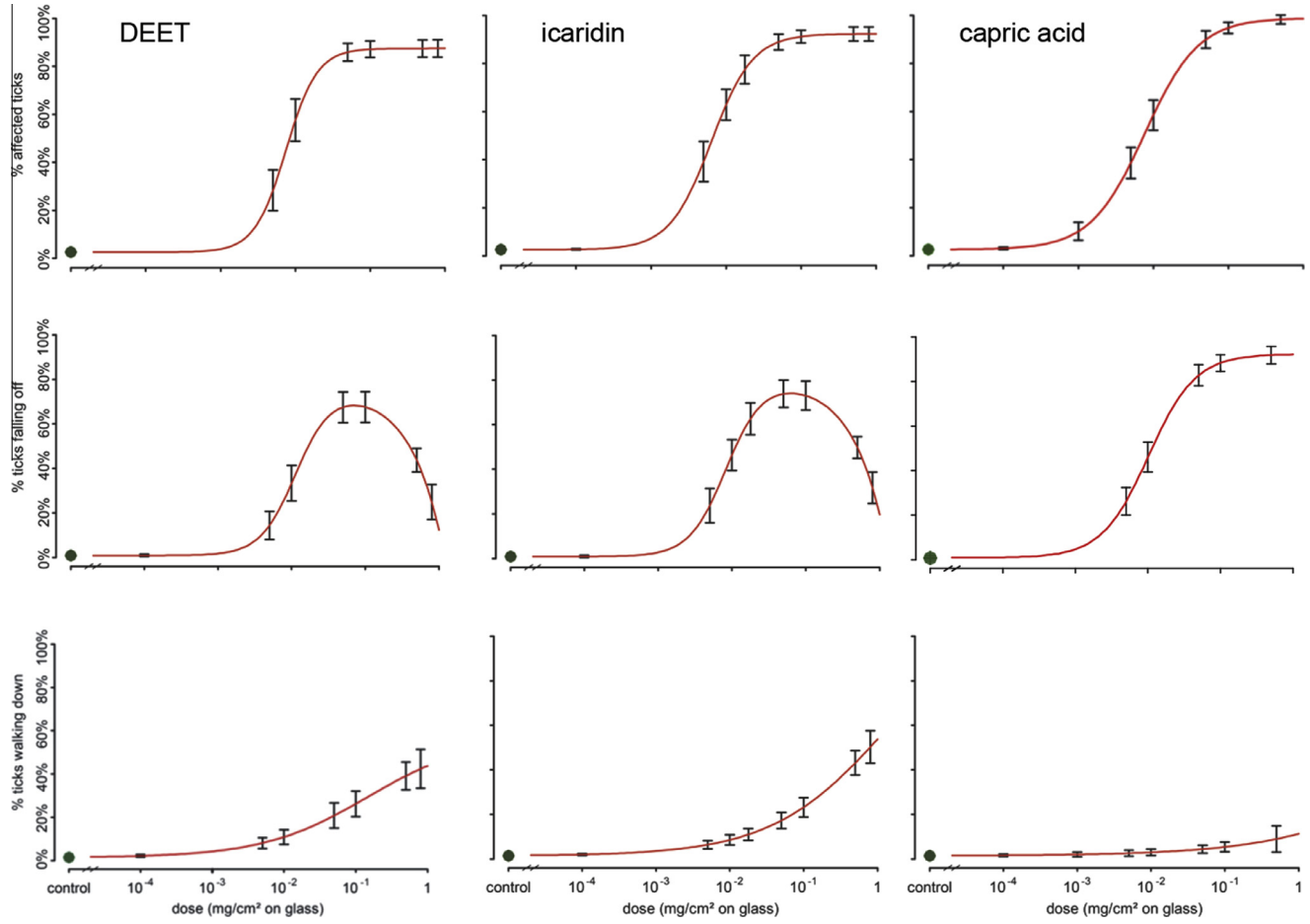

Fig. 3. Response of *I. ricinus* nymphs on the skin of 8 human subjects held near to upright to different doses (log scale) of DEET, icaridin and capric acid, under the behaviour criteria affected (top row), falling off (second row) and walking down (bottom row). Percentage points at each dose refer to the mean proportion of 12 ticks on 8 subjects showing the behavioural responses that occurred within 5 min of placing the nymphs in the centre of a treated zone (for details see Section 2, Materials and methods). Error bars indicate the mean model error at each dose. The green dot in the left lower corner of each plot is the response to the solvent control (ethanol).

the behavioural responses of the affected nymphs during the 8 h test period. Two thirds of affected ticks walked down (59%) at 15 min, but this gradually declined to 17.4% at 8 h. In contrast, the percentage of ticks falling off increased from 29% at 15 min to 52% at 8 h. The number of affected ticks which did not leave the disc or treated zone remained low throughout the test (4–8%). These observations are consistent with the behavioural responses described in the glass plate and leg assays where most ticks walk down when exposed to high doses of icaridin, i.e. when a repellent is first applied, but increasing numbers fall off at the lower concentrations, i.e. as the amount of the active ingredient diminishes over time.

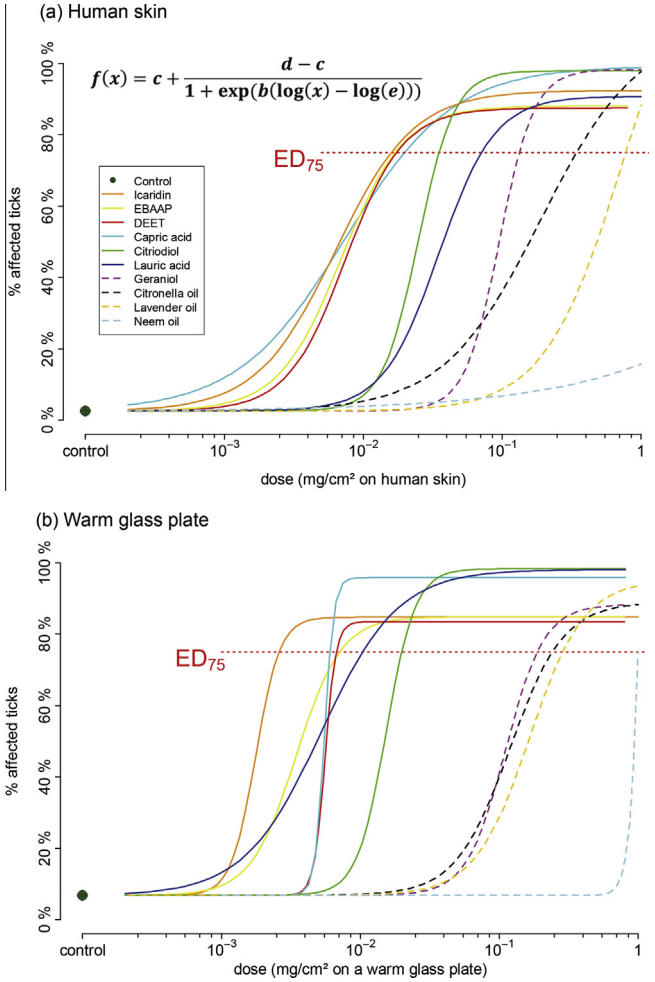

Fig. 4. Plots of affected *I. ricinus* nymphs on the human skin (a) and on a warm glass plate (b) as a function of dose (log scale) of 10 test products tested within 60 min of applying the products as fitted with an asymptotic log linear regression model (DRC package [4]). Response functions in a and b follow the formula inserted in (a) where *b = is proportional to the slope of the inflexion point at ED50, c = left asymptote, d = right asymptote, e = ED50.* The green dot in the left lower corner of each figure panel is the mean response to the solvent control (ethanol) over all products.

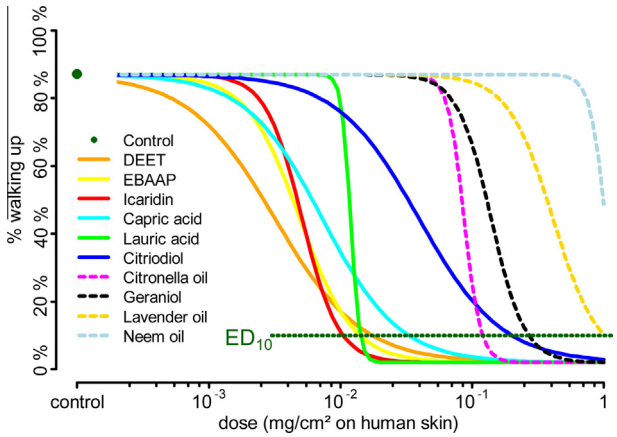

Fig. 5. Plots of *I. ricinus* nymphs walking up on the human skin as a function of dose (log scale) of 10 test products tested within 60 min of applying the products to the skin as fitted with an asymptotic log linear regression model (DRC package [4]) in which both asymptotes were fixed. The green dot in the left upper corner is the response to the solvent control (ethanol).

3.2. Estimating risk reduction of a tick bite over time for an anti-tick product

In the evaluation of tick repellents on humans a criterion used to measure the risk of incurring a tick bite is a record of a tick walking up across a treated area of skin, termed here an event. Risk reduction resulting from the application of an anti-tick product to human skin is considered to break down when a tick succeeds in walking up across a treated area of skin at two successive recording intervals on the same person, termed a "confirmed event" [7]. The probability of such a confirmed event being recorded evidently depends on the number of ticks used at each interval, the frequency at which such records are made on groups of ticks over time and the number of test persons on which records are made. We can consider this probabilistically in a Bernoulli trial with probability of "success" p_0 and probability of "failure" $1-p_0$. The probability that a (first) confirmed event occurs on the same person at time interval k is given by the formula:

$$P_k = p_0 P_k - 1 + p_0(1-p_0)P_k - 2; \quad \text{for all } k \geqslant 3 \qquad (1)$$

When the number of ticks (N) used per interval (k) remains constant and the number of recording intervals (n) is increased, the probability of occurrence of a confirmed event increases. Nevertheless, when the overall number of ticks ($N * n$) used remains constant and the number of recording intervals (n) is increased the probability of a confirmed event decreases, i.e. with increasing number of recording intervals, the number of ticks per interval is reduced and the probability drops in proportion to the number of ticks used per interval.

In our case, the distribution of events recorded on persons treated with Anti-Brumm® Zecken Stopp proved to be homogeneous throughout the 8 h test period (binominal lmer, $P > 0.7$; Table 3 and Fig. 6) at 1.7 events per interval (ticks walking up) with a standard error (SE) of ±1.17% over the 120 ticks used per interval on the 10 test persons. A confirmed event was recorded at the 6 h interval on one test person (Table 3). We can apply Eq. (1) to compute a probability of 2.5% for a confirmed event to occur by chance alone at this 6 h recording interval on a test person. This estimation has itself a big error factor (SE) due to the propagation of the relatively big SE (above) of an event occurring. Comparing the observed number of confirmed events for breakdown of risk reduction, i.e. once in 10 persons, to the theoretical value, extrapolated for 10 persons, shows no significance using the exact binomial test. Indeed, iterative application of the exact binominal test shows that at least 2 confirmed events would need to occur at the 6 h interval in the example provided (Table 3) to signal a significant change in risk reduction ($P = 0.02$). This did not occur in the case presented. Risk reduction cannot therefore be considered as being affected using one confirmed event as a criterion.

4. Discussion

Here we describe *in vivo* and *in vitro* tests for tick repellents that are based on geotactic responses of ticks. Lees [8] described the responses of *I. ricinus* to gravity, and demonstrated how hungry nymphs use negative geotaxis to climb glass rods and stay near the top in their quest for a suitable host, and will even locate the highest point on an inverted U-shaped rod. It has been frequently demonstrated how repellent products can induce the opposite, namely a positive geotaxis response in ticks [1–3]. In the controls, most of the ticks walked up the human leg and the glass plate, demonstrating their strong inclination to find an attachment site to feed. This behaviour was disrupted in both the *in vivo* and

Table 3
Numbers of ticks walking up (events based on "protection failure" US EPA, 2010 guidelines [7]) on the legs of each of 10 human subjects treated with the anti-tick product Anti-Brumm® Zecken Stopp. In this case the proprietary product reduced the risk of host colonisation for 6 h. The data correspond to ticks walking up plotted on Fig. 6. (For interpretation of the references to colour in this table, the reader is referred to the web version of the article.)

Test person	0.25 h	1 h	2 h	3 h	4 h	6 h	8 h
person 1	0	0	0	0	0	0	0
person 2	0	0	0	0	0	0	0
person 3	0	0	0	0	0	0	0
Person 4	1	0	0	0	0	0	2
Person 5	0	1	0	0	0	0	0
person 6	0	0	1	0	1	0	0
person 7	0	1	0	0	1	1	0
Person 8	0	0	1	0	0	0	0
person 9	1	0	0	0	0	0	0
person 10	0	0	0	0	0	1	0
Total	**2**	**2**	**2**	**0**	**2**	**2**	**2**

Unconfirmed event (yellow) - an isolated record of a tick walking up constitutes an unconfirmed event.
Confirmed event (red) - a record of a tick walking up at two consecutive recording intervals on the same test person

in vitro tests where adequate doses of effective anti-tick products induced nearly all ticks to either fall off or walk downwards. The strong complementarity between the tests on the human leg and the warm glass plate described here for repellent products is due to the fact that they both rely on the innate responses of ticks: negative geotaxis in controls and positive geotaxis as a reaction to anti-tick products. This study accounts for the response of *I. ricinus* to repellents, but we have also recorded similar response in *Ixodes scapularis*, the North American vector of Lyme disease and in *Ixodes persulcatus*, the taiga vector of tick-borne encephalitis to repellents applied to the warm glass plate (unpublished data).

4.1. Comparison of the effects of test products in vitro and in vivo

The positive geotactic response of *I. ricinus* nymphs to test products was dose dependent on both the warm glass plate and human leg. This permitted the calculation of an ED75 for each product for purposes of comparison. Based on this, active compounds can be divided into three groups depending on their ED75 values on the human skin: (1) icaridin, EBAAP, DEET and capric acid with values between 0.013 and 0.020 mg/cm^2, (2) citriodiol and lauric acid with values between 0.035 and 0.058 mg/cm^2, and (3) geraniol, citronella oil and lavender essential oil with values between 0.131 and 1.58 mg/cm^2. The latter three products can be considered as less effective repellents, and the effect of neem oil was too low to measure an effect. Product ranking was the same on the glass plate as on the human leg, with the exception of the ranking between lauric acid and citriodiol between the two tests. Such a high level of complementarity between the two test methods is particularly noteworthy when one considers the inherent variability of host-specific stimulants for ticks amongst the test persons in the *in vivo* trials. The ED75 values for the six most effective compounds on the leg vary from 2 to 6 times higher than on the warm glass plate, depending on the test product. This is not surprising considering how skin presents a matrix that can interact with repellents [9] and the relatively low vapour pressures of the products exposed on the glass plate at 34 °C without fixative. The effective dose levels for the non-proprietary repellents tested here serve as a guideline for the evaluation of consumer products containing these compounds as active ingredients.

4.2. Mode of action of products on ticks

The strong positive geotactic responses of ticks to repellents *in vivo* and *in vitro* serve to underline the relevance of this behavioural trait for evaluating repellents. Tick responses to the repellents tested on vertical or near vertical surfaces can be described stepwise dependent on repellent dose. At high doses (0.5 mg/cm^2 and above) of the most effective compounds, icaridin, EBAAP and DEET, ticks walk down the leg or the warm glass plate across the treated surface, i.e. against the convection current of rising warm air containing the repellent in the vapour phase (type 1 response). The response to citriodiol follows a similar pattern, but is not as marked. At median doses of these repellents (0.05 and 0.1 mg/cm^2) more ticks fall off after encountering the repellent vapours

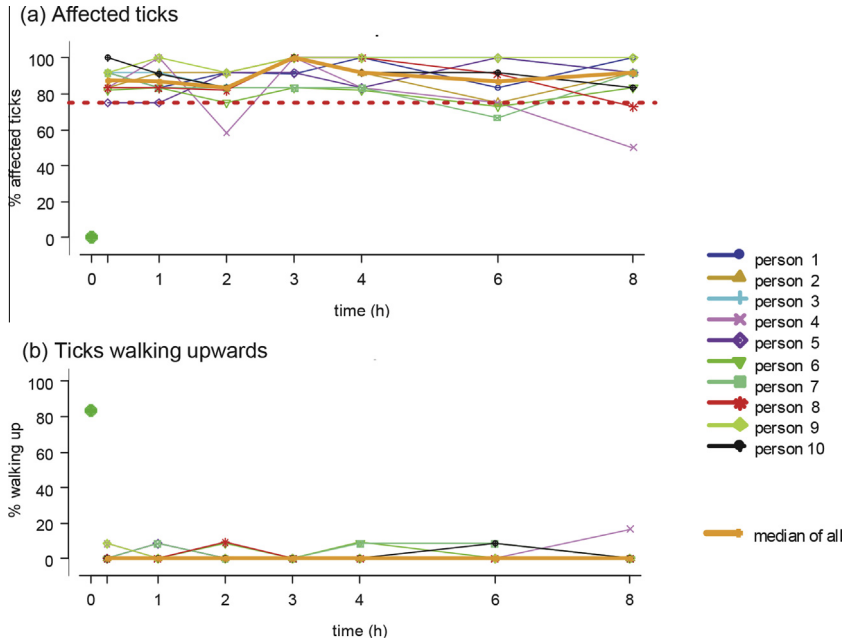

Fig. 6. Percentage of *I. ricinus* nymphs affected* (a) and walking up** (b) on the lower leg (shin) of 10 persons treated with the anti-tick product Anti-Brumm® Zecken Stopp as a function of time from application of the product. *See Section 2.3 for definition. **Ticks walking up contribute to "protection failure" (U.S. EPA guidelines, 2010 [7]) when recorded at successive observation intervals on the same leg (see Table 3). The data in (b) correspond to those in Table 3. The green dot in the lower or upper left corner of a and b is the control value (solvent only) for the 10-person panel.

on the disc or when walking on the treated surface (type 2 response). The same pattern was recorded as Anti-Brumm® Zecken Stopp evaporated from the skin of the 10-person panel over 8 h, as more ticks walked down when exposed to the freshly applied product but over half fell off in the second half of the 8-h test as the active ingredients evaporated. The carboxylic products (capric and lauric acid) induce mainly a type 2 response at all doses. Positive geotaxis (either crossing the treated zone downwards or falling off the disc or treated zone) is the opposite of tick behaviour on the untreated leg and warm glass plate where most ticks walk upwards and almost none fall off. The number of ticks walking upwards increases on both the glass plate and leg when the dose of the effective repellent compounds is too low (0.01 mg/cm^2 or lower). This negative geotactic response is a clear indication of poor or no repellent activity.

The findings show that effective doses of the carboxylic acids induce a reflex reaction in *I. ricinus* to fold its legs and fall off. This is also the primary mechanism of escape from doses of DEET, EBAAP and icaridin up to 0.1 mg/cm^2, whereas at higher doses, these products operate by inducing more ticks to walk downwards, off the treated surface, or less frequently by immobilising them. Immobilisation and walking downwards are indicative of an inhibition of the falling reflex. Walking downwards is illustrative of the ability of *I. ricinus* to descend from a perch under adverse conditions – a well-known behavioural response in this species when exposed to unsuitable environmental conditions [10].

4.3. Evaluation of the in vivo and in vitro tests

The *in vivo* and *in vitro* tests described here, requiring no specialised equipment, can be used to predict the dose range at which repellent products are likely to affect tick behaviours. The fact that ticks are initially placed within the treated zone in both tests facilitates recording graded responses of the ectoparasite to different doses of products tested. By contrast, in other tests developed for anti-tick products on vertical surfaces (*in vivo* and *in vitro*) tick responses are recorded on encounter with the border of the treated area after approaching it from below [1,2,11]. However, such tests do not allow the ticks to respond to the ascending repellent vapours from a warm substrate. For example, ticks fell in roughly equal numbers from the central disc as from the treated zone around it at the ED75 dose of DEET on both the leg and warm glass plate, indicating that repellent vapours are sufficient to cause the ticks to respond. The effective doses for the non-proprietary repellents tested here serve as a guideline for the evaluation of consumer products containing these compounds as active ingredients. Carroll et al. [2] concluded that a vertical test provided a sensitive tool for testing tick repellents and showed that 90% of *I. scapularis* nymphs were repelled by DEET at 95 μg/cm^2 on a piece of filter paper held vertically. In contrast, DEET is shown here to affect 75% of *I. ricinus* nymphs on the warm glass plate at 6 μg/cm^2, i.e. a dose more than an order of magnitude lower. While the two tests are on vertical or near vertical surfaces they differ in a number of respects, notably species tested, the substrates on which DEET was exposed and ED criteria. The higher sensitivity of *I. ricinus* nymphs to repellents in the warm glass plate test described here is most probably related to the exposure of the ticks on the central disc to the rising current of repellent in the vapour phase that caused many nymphs to fall from the disc [1,2,11]. There is a need for a standardised bioassay for tick repellents that can substitute for tests on human volunteers [12]. The glass plate test presented here, running at a temperature close to that on human skin, provides an alternative to humans for the initial evaluation of products of unknown human toxicity and dermatological effects.

4.4. Estimating risk reduction of tick bite over time using the human leg (shin) test

The proprietary product Anti-Brumm® Zecken Stopp continued to affect 75% of ticks 8 h after it was applied to the legs of the 10-person test panel. It can therefore be concluded that Anti-Brumm® Zecken Stopp would greatly reduce the risk of a tick bite for this length of time. This is not surprising considering that each person applied about 7 times the combined ED75 doses of icaridin and citriodiol that Anti-Brumm® Zecken Stopp contains. However, 2 ticks walked up in 6 of the 7 recording intervals. Indeed, the number of ticks walking up remained constant over time, suggesting that there is no relationship between the walking up response and the amount of evaporation of the product from the skin. Using the Bernoulli trial, we show here that the number of ticks walking up is no different to what could be expected to occur by chance. Shortening the recording intervals to periods of less than 1 h while keeping the number of ticks constant would even decrease the probability of a tick walking up at two successive intervals on the same person, a so-called confirmed event. Our calculations show that a test panel of 10 people, using 5–12 ticks per interval, as is standard practise in *in vivo* trials, provides an insufficient estimator to permit the use of just one confirmed event to distinguish between what occurs at random from what occurs as a consequence of using the repellent product.

Acknowledgments

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