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EVOLUTION OF REPELLENT TOLERANCES IN REPRESENTATIVE ARTHROPODS[1]

L. C. RUTLEDGE,[2] R. K. GUPTA[3] AND Z. A. MEHR[4]

ABSTRACT. Eight commercial insect repellents were tested against *Ornithodoros parkeri* (Acari: Argasidae), *Dermacentor variabilis* (Acari: Ixodidae), *Aedes aegypti* (Diptera: Culicidae), and *Xenopsylla cheopis* (Siphonaptera: Pulicidae). Patterns of tolerance to the test materials were distinctive for each test species. Levels of tolerance were coded as character state 0 (sensitive), 1 (intermediate), or 2 (tolerant) and mapped on a cladogram reflecting the accepted classification of the test species. Character state 0 was regarded as primitive, as indicated by the ontology of repellent tolerances in ticks. *Aedes aegypti* was least evolved and *X. cheopis* was most evolved in tolerance to repellents. Multiple parallelism of the arachnid and *X. cheopis* lines occurred in the evolution of the observed tolerances.

INTRODUCTION

Many repellents, including dimethyl phthalate, butopyronoxyl, deet, ethyl hexanediol, and dibutyl phthalate, are broadly effective against arthropods (World Health Organization 1984). However, the degree of effectiveness may differ at class, order, or family level. For example, dimethyl phthalate, butopyronoxyl, MGK Repellent 11, and MGK Repellent 326 are considered to be most effective against flies and mosquitoes, whereas dibutyl phthalate is considered to be most effective against mites (Metcalf 1966, Painter 1967). These conclusions are clouded by the variant methods used in testing repellents against unlike taxa (Dethier 1956).

The present study determined the comparative tolerances of representative arthropods to commercial repellents with the use of similar test methods and demonstrated how the observed patterns of sensitivity and tolerance might have evolved in geologic time. The study was conducted from 1976 to 1986 at the former Letterman Army Institute of Research, Presidio of San Francisco, CA.

MATERIALS AND METHODS

Test materials: Repellents tested were dimethyl phthalate, butopyronoxyl (butyl 3,4-dihydro-2,2-dimethyl-4-oxo-2*H*-pyran-6-carboxylate), MGK Repellent 11® (1,5a,6,9,9a,9b-hexahydro-4a(4*H*)-dibenzofurancarboxaldehyde), Citronyl® (3-acetyl-2-(2,6-dimethyl-5-heptenyl)-oxazolidine), deet (*N,N*-diethyl-3-methylbenzamide), ethyl hexanediol (2-ethyl-1,3-hexanediol), MGK Repellent 326® (di-*n*-propyl 2,5-pyridinedicarboxylate), and dibutyl phthalate (di-*n*-butyl phthalate).

All repellents were obtained commercially as technical grade materials. All are or have been employed in commercial repellent formulations. Except for dimethyl phthalate and dibutyl phthalate, all are chemically unrelated.

Test species: Species tested were *Ornithodoros parkeri* Cooley (Acari: Argasidae), University of California at Berkeley strain; *Dermacentor variabilis* (Say) (Acari: Ixodidae), USDA Livestock Insects Research Laboratory strain; *Aedes aegypti* (Linn.), University of California at San Francisco strain; and *Xenopsylla cheopis* (Rothschild) (Siphonaptera: Pulicidae), USDA Medical and Veterinary Entomology Research Laboratory strain. *Ornithodoros parkeri* and *D. variabilis* were tested as nymphs. *Aedes aegypti* was tested as nulliparous females 5–15 days old. *Xenopsylla cheopis* was tested as adult males and females selected at random.

The test species were selected on the basis of taxonomic diversity, medical importance, and ease of rearing. Data reported for *O. parkeri* and *X. cheopis* were previously published, in part, by Mehr et al. (1984, 1986). Data reported for *D. variabilis* and *Ae. aegypti* have not been previously published.

Test method: Repellents were tested on generic 7–10-day-old white laboratory mice. In conducting the research, investigators adhered to National Institutes of Health Publication 85-23, *Guide for the care and use of laboratory animals.*

Tests were conducted as described by Rutledge et al. (1994b). Five mice were treated with ethanol (control) and 4 serial dilutions of the test material in ethanol (treatments). Repellents were applied with a pipet over the whole mouse to point of runoff. The percent concentration of repellent applied was used as the unit of dose. Treatments were assigned to individual mice at random using a table of random numbers.

Treated mice were transferred to a test cage containing the test species. Test cages for *O. parkeri, D. variabilis,* and *X. cheopis* were glass jars, 20 cm in diameter and height, containing 50 (*O. parkeri*)

[1] Opinions and assertions herein should not be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Use of trade names does not imply official endorsement or approval of the products named.

[2] 11 Circle Way, Mill Valley, CA 94941-3420.

[3] Department of Entomology, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

[4] Headquarters, Defense Logistics Agency, 8725 John Kingman Road, Suite 2533, Fort Belvoir, VA 22060-6219.

or 75 (*D. variabilis* and *X. cheopis*) ticks or fleas. A substrate of ground corncobs was provided for *X. cheopis* to facilitate dispersal of the test insects. The test cage for *Ae. aegypti* was a 30 × 30 × 30-cm aluminum frame mosquito cage modified by replacing the floor with additional screening. The cage contained 100 female mosquitoes and was supported 10 cm above the surface of the work table to permit unrestricted circulation of air during the test.

After introduction of the treated mice into the test cage, the number of test insects or ticks biting each mouse was recorded every 2 min for 20 min (*O. parkeri, Ae. aegypti,* and *X. cheopis*) or cumulatively (once only) at the end of 4 h (*D. variabilis*). Totals for each mouse were obtained at the end of the 20-min or 4-h recording session.

A range of doses bracketing the median effective dose (ED_{50}) was determined in preliminary tests, and additional tests were then conducted using that range. In some cases, overlapping ranges were tested to obtain additional independent estimates of the ED_{50} or to obtain a more reliable single estimate than that provided by data obtained at the initial range. Tests were replicated 8–36 (*O. parkeri*), 4–7 (*D. variabilis*), 3–10 (*Ae. aegypti*), or 2–15 (*X. cheopis*) times at each range of doses tested. On completion of testing, totals over all replicates were obtained for the control and each treatment.

Statistical analysis: Treatment totals were converted to percentages of the respective control totals for analysis. Replacement values for 0 and 100% observations were calculated as 100 × (0.5/control) and 100 × ([control − 0.5]/control), respectively (Armitage 1971). Response (percentage of the control) and applied dose (percent concentration) were transformed to the probit and logarithmic scales, and the dose–response equation was determined as described by Goldstein (1964) for data with graded (nonquantal) responses.

The ED_{50}s from the dose–response analyses were analyzed as logarithms by 2-way ANOVA. Main effects (test materials and test species) and interaction (test materials × test species) were tested by variance ratio (*F*-test). Differences among means were tested by Fisher's (protected) least significant difference (Steel and Torrie 1980). This analysis can be regarded as a higher nodes analysis of 4 species, representing 4 families, 3 orders, and 2 classes of arthropods (Harvey and Pagel 1991).

Cladistics: The tolerance of each test species to each test material was coded on a scale of 0 to 2 based on results of the ANOVA. Character state 0 (sensitive) was assigned to species that differed significantly from 1 or more species having higher ED_{50}s. Character state 2 (tolerant) was assigned to species that differed significantly from 1 or more species having lower ED_{50}s. Character state 1 (intermediate) was assigned to species that differed significantly from 2 or more species having lower *and* higher ED_{50}s and to species that did not differ significantly from any other species.

Character states were mapped on a cladogram reflecting the accepted phylogeny to illustrate how the observed patterns of sensitivity and tolerance might have evolved in geologic time. Studies in ticks have shown that tolerance to repellents increases progressively through the egg, larva, nymph, and adult stages (Dremova and Smirnova 1970, Estrada Pena et al. 1982). On this basis, character state 0 was considered to be plesiomorphic (primitive) and character state 2 was considered to be apomorphic (advanced). Accordingly, the cladogram was rooted on a hypothetical ancestor having the lowest observed character state for each test material (polarization by ontogeny).

Character states were mapped in accord with Wagner parsimony as described by Forey et al. (1992). Under Wagner parsimony, transformation of character state 0 to character state 2, or vice versa, proceeds via character state 1 by terminal addition or deletion. Character state transformations were delayed to maximize the proportion of homoplasy accounted for by parallelism and minimize the proportion accounted for by reversals (Forey et al. 1992). This technique (delayed transformation optimization) was adopted to reconcile the cladogram with the ontogenetic sequence in ticks.

RESULTS

Analysis of variance: Mean ED_{50}s obtained in the tests ranged from 0.001% (butopyronoxyl against *Ae. aegypti*) to 8.947% (dibutyl phthalate against *X. cheopis*) (Table 1). The coefficient of determination obtained in the ANOVA was 0.94 ($df = 11$, $P < 0.05$), indicating that the model employed accounted for 94% of the variation observed among ED_{50} values.

Differences among means of test species were statistically significant ($F = 21.16$; $df = 3, 11$; $P < 0.05$). Means of the test species are shown at the bottom of Table 1 with results of Fisher's (protected) least significant difference test. *Aedes aegypti* was significantly more sensitive to repellents than were *D. variabilis, O. parkeri,* and *X. cheopis. Dermacentor variabilis* was significantly more sensitive to repellents than *O. parkeri* and *X. cheopis.*

The statistical interaction of test materials and test species was also significant ($F = 3.02$; $df = 21, 11$; $P < 0.05$). Means of test species by test material are shown in the body of Table 1 with results of Fisher's (protected) least significant difference test. *Aedes aegypti* was significantly more sensitive to dimethyl phthalate, butopyronoxyl, deet, ethyl hexanediol, and dibutyl phthalate than were *D. variabilis, O. parkeri,* and *X. cheopis. Dermacentor variabilis* was significantly more sensitive to dimethyl phthalate, MGK Repellent 326, and dibutyl phthalate than were *O. parkeri* and *X. cheopis. Ornithodoros parkeri* was significantly more

Table 1. Median effective doses of 8 commercial repellents for *Aedes aegypti, Dermacentor variabilis, Ornithodoros parkeri,* and *Xenopsylla cheopis* as determined on laboratory mice.[1]

Repellent	*Ae. aeg.*	*D. var.*	*O. par.*	*X. che.*
Dimethyl phthalate	0.006a (2)	0.307b	0.006a	0.948b
Butopyronoxyl	0.001a	0.131b	0.136b	0.794b (2)
MGK Repellent 11	0.120a (2)	0.024a	0.080a	0.102a
Citronyl	0.056a (2)	0.020a	0.270a	0.128a
Deet	0.006a (2)	0.110b	0.847b	0.150b
Ethyl hexanediol	0.015a	0.383b	2.360b	0.560b
MGK Repellent 326	0.389ab	0.051a (2)	0.670ab	1.397b
Dibutyl phthalate	0.322ab (2)	0.131a (3)	3.500bc	8.947c (3)
Mean	0.029a	0.093b	0.309c	0.565c

[1] Entries are means of 1–3 independent determinations with number of determinations shown in parentheses if greater than 1. Means were calculated as the antilogarithm of the mean logarithm. Means in same row not followed by same letter differ at the 5% level of significance. Unit of dose is percent concentration of repellent.

sensitive to dimethyl phthalate than were *D. variabilis* and *X. cheopis.*

Cladistics: Table 2 shows the character states assigned to each test species. The intermediate state (character state 1) was ancestral for MGK Repellent 11 and Citronyl. The sensitive state (character state 0) was ancestral for all other test materials.

Figure 1 shows how the observed character states might have evolved in geologic time. The arachnid/acarid branch diverged from the insect branch with increasing tolerance to butopyronoxyl, deet, and ethyl hexanediol. The argasid/*O. parkeri* and ixodid/*D. variabilis* branches subsequently diverged with increasing tolerance to MGK Repellent 326 and dibutyl phthalate (*O. parkeri*) and dimethyl phthalate (*D. variabilis*).

The insect branch diverged from the arachnid/acarid branch with increasing tolerance to MGK Repellent 326, after which the dipteran/culicid/*Ae. aegypti* branch remained unchanged, whereas the siphonapteran/pulicid/*X. cheopis* branch evolved increasing tolerance to dimethyl phthalate, butopyronoxyl, deet, ethyl hexanediol, MGK Repellent 326, and dibutyl phthalate.

Figure 1 indicates multiple parallelism in the evolution of the repellent tolerances of *O. parkeri* and *D. variabilis* in the arachnid branch and *X. cheopis* in the insect branch. The apomorphic state evolved independently in *O. parkeri* and *X. cheopis* for butopyronoxyl, deet, ethyl hexanediol, and dibutyl phthalate. The apomorphic state evolved independently in *D. variabilis* and *X. cheopis* for dimethyl phthalate, butopyronoxyl, deet, and ethyl hexanediol.

Because of parallelism, differences in tolerance were greater within the insect branch than between the insect and arachnid branches. In the *Ae. aegypti* branch, the plesiomorphic state was retained in every case but one (MGK Repellent 326, intermediate state). In the *X. cheopis* branch, the apomorphic state was evolved in every case but 2 (MGK Repellent 11 and Citronyl, intermediate state).

Table 2. Character states assigned to *Aedes aegypti, Dermacentor variabilis, Ornithodoros parkeri,* and *Xenopsylla cheopis* for comparative tolerance to 8 commercial repellents.[1]

Repellent	*Ae. aeg.*	*D. var.*	*O. par.*	*X. che.*
Dimethyl phthalate	A^0	A^2	A^0	A^2
Butopyronoxyl	B^0	B^2	B^2	B^2
MGK Repellent 11	C^1	C^1	C^1	C^1
Citronyl	D^1	D^1	D^1	D^1
Deet	E^0	E^2	E^2	E^2
Ethyl hexanediol	F^0	F^2	F^2	F^2
MGK Repellent 326	G^1	G^0	G^1	G^2
Dibutyl phthalate	H^0	H^0	H^2	H^2

[1] Each character state code consists of a letter denoting a repellent and a superscript denoting the sensitive (0), intermediate (1), or tolerant (2) state for that repellent. See text for explanation of method of determining character states.

DISCUSSION

Preliminary tests showed that the number of *D. variabilis* feeding at 20 min was only 27% of the number feeding at 4 h. To accommodate the relatively slow feeding rate of *D. variabilis,* a 4-h test procedure was adopted in lieu of the 20-min test procedure used for *O. parkeri, Ae. aegypti,* and *X. cheopis.* Although the difference in test methods complicates exact interpretion of the test data, there is reason to expect the unadjusted 4-h test data to be approximately equivalent to the 20-min test data.

In terms of sampling, data collected by one–zero time sampling (as in tests against *D. variabilis*) approximate data collected by instantaneous time sampling (as in tests against *O. parkeri, Ae. aegypti,* and *X. cheopis*) when the sample interval is short compared with the duration of the behavior recorded (Martin and Bateson 1993). In tests against *D. variabilis,* the sample interval was only 4 h compared with ≈6 days required to complete feeding.

In terms of pharmacokinetics, because the dose of repellent applied decays exponentially with time (Rutledge et al. 1985), loss of repellent is greatest in the period immediately following application, i.e., in the period common to the 20-min and 4-h test procedures. Loss of repellent is relatively small

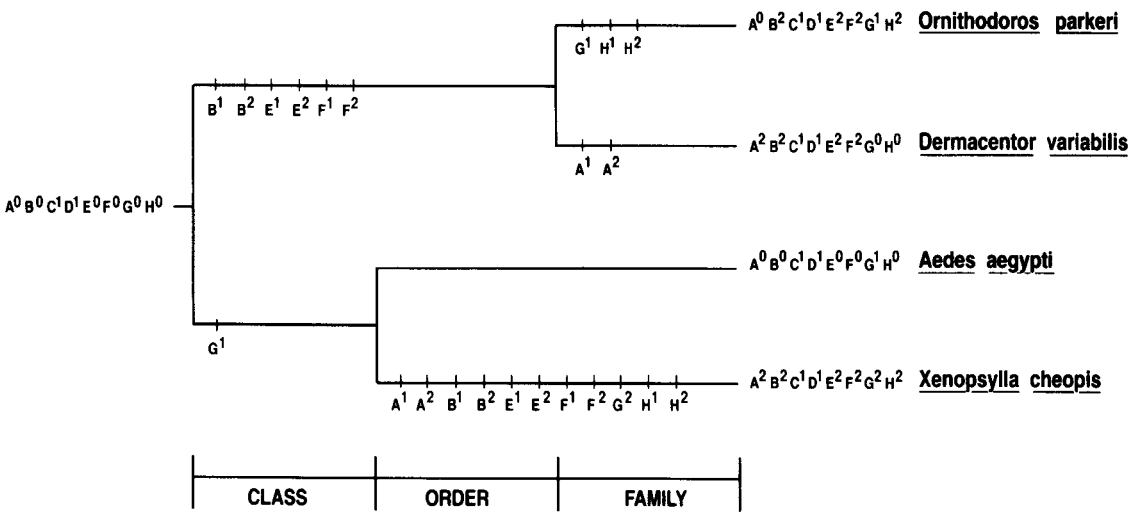

Fig. 1. Cladogram for 4 species of arthropods tested against 8 commercial repellents. See Table 2 for explanation of character state codes.

in the period from 20 min to 4 h, which occurs in the 4-h test only.

On the basis of these considerations, we reported unadjusted test data for *D. variabilis* in lieu of computing values adjusted for the longer test session. Bias in the unadjusted data, if any, is upward. In that case, the significance of differences between *D. variabilis* and more sensitive species would be weakened, whereas the significance of differences between *D. variabilis* and more tolerant species would be strengthened.

An anonymous reviewer raised the question of genetic change in the test arthropods during the period of colonization. However, genetic changes in laboratory colonies do not necessarily affect the traits under study. Genetic variability and life table characteristics were similar in laboratory colonies and field populations of *Ae. aegypti* (Carmelina and Machado-Allison 1976). Life table characteristics of colonized *Glossina morsitans* Westwood (Diptera: Muscidae) were unchanged after 40 generations of inbreeding (Jordan 1980). Accordingly, pending determination of the tolerances of natural populations of species used in the study, speculation on what might be or what could be does not seem useful.

An anonymous reviewer objected to the use of ontogenetic information in constructing cladograms, stating that "Repellency is inversely proportional to degree of sclerotization, which of course increases ontogenetically. But Haeckel was wrong! Ontogeny and phylogeny are disparate phenomena." In fact, however, repellents act in the vapor state on the olfactory organs (Steward and Atwood 1963), and the degree of sclerotization has little or nothing to do with the process. (Certain dual-action insecticides/repellents, notably the pyrethroids and thiocyanates, are irritant on contact. These materials were not included in the study.)

In addition, Haeckel's theory of recapitulation has been replaced by von Baer's biogenetic law, which provides for peramorphosis (recapitulation) through mechanisms of acceleration, predisplacement, and hypermorphosis. According to Harvey and Pagel (1991), the most useful techniques for rooting cladograms are ontogeny, paleontology, and outgroup comparison, of which only the first was available in the present study.

The ability to respond to chemical stimuli is thought to be universal in living things. Because well-developed chemotaxes occur even in primitive taxa (Adler 1975), arthropod chemotaxes may be supposed to be highly evolved. The existence in arthropods of complex organs of chemoreception (Welsch and Storch 1976) and multiple factors affecting repellent tolerances (Rutledge et al. 1994a) supports this view.

If the arthropod chemotactic system evolved at the phylum level or higher, chemotactic systems of the constituent classes can be expected to exhibit similarities attributable to a common origin (homology). For example, both ticks and insects detect repellents by smell. The olfactory organs are located on the tarsi of the first pair of legs (Haller's organ of ticks) or on the antennae (insects). As segmental appendages, the legs of ticks and antennae of insects are serially homologous (homotypic). The olfactory sensilla of ticks and insects are cytologically and histologically similar (Welsch and Storch 1976).

Although the mode or modes of action of repellents are still uncertain, research has centered on the mechanism of interference with the reception of host kairomones (Davis 1985). At least one host

kairomone, carbon dioxide, is known to attract ticks, mosquitoes, fleas, and other bloodsucking arthropods. Certain olfactory sensilla are known to react to carbon dioxide and repellents (Davis and Bowen 1994, Sutcliffe 1994).

In the present study, the observed differences of effectiveness of 8 repellents among classes, orders, and families of arthropods were differences of degree only (Table 1). A review of the literature by Rutledge et al. (1978) indicated that deet was known, at that time, to be effective at one level or another against more than 20 genera of arthropods. The apparently universal susceptibility of diverse arthropods to the same repellent compounds is prima facie evidence of the basic similarity of arthropod chemotactic systems.

Patterns of sensitivity and tolerance to the test materials were identical in tests against MGK Repellent 11 and Citronyl (no significant differences among species) and in tests against butopyronoxyl, deet, and ethyl hexanediol (*Ae. aegypti* < *D. variabilis* = *O. parkeri* = *X. cheopis*), even though the test materials were chemically unrelated (Table 1).

On the other hand, patterns of sensitivity and tolerance differed significantly for dimethyl phthalate and dibutyl phthalate, even though the two molecules differ only in the dialkyl substituent of the phthalic acid diester (Table 1). *Ornithodoros parkeri* and *Ae. aegypti* were more sensitive to dimethyl phthalate than were *D. variabilis* and *X. cheopis*. *Dermacentor variabilis* was more sensitive to dibutyl phthalate than were *O. parkeri* and *X. cheopis*. *Aedes aegypti* was more sensitive to dibutyl phthalate than was *X. cheopis*.

Patterns of similar responses to dissimilar materials and dissimilar responses to similar materials observed in the study are consistent with the weak correlation of chemical structure and repellent efficacy reported in prior studies (Garson and Winnike 1968, Skinner and Johnson 1980). The observed noncorrelation of chemical structure with biological activity suggests that repellent tolerances may be nonadaptive. This is to be expected because, to our knowledge, none of the test materials occurs in nature except deet, which occurs in female pink bollworm moths, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Jacobson and Jones 1974).

On this basis, the observed patterns of sensitivity and tolerance to the test materials can be hypothesized to have evolved by random drift of selectively neutral mutations (Kimura 1983). Figure 1 can be rewritten for the neutral model by accelerated transformation optimization and polarization on character state 1. However, the random drift model does not agree with the ontogenetic sequence in ticks or with the one-sided pattern of divergence in the insect branch (Fig. 1).

Accordingly, Fig. 1 was constructed to agree with the Darwinian model of evolution. Because the test materials do not occur in nature, the basis for selection of the observed tolerances is problematic. One possible explanation is that of pleiotropy, the phenomenon by which individual genes exert multiple, apparently unrelated, phenotypic effects. In this case, selection for repellent tolerance would be incidental to selection for traits having greater adaptive value. Geneticists believe that many, perhaps all, genes are pleiotropic.

In conclusion, we believe that the unifying principle of evolution is just as relevant in repellent research as it is in other fields of study. This report represents the first published attempt to elucidate the evolution of repellent tolerances in the Arthropoda, and we have tried to make it as factual and objective as possible. Although we do not expect Fig. 1 to be correct in every detail and principle, it is based on quantitative, experimental data and accepted principles of evolutionary biology. Future research is needed to determine the necessary modifications, major and minor, and to extend current knowledge to include additional species and repellents.

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