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Thyme is of the essence: Biochemical polymorphism and multi-species deterrence

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ABSTRACT

Populations of the common thyme (*Thymus vulgaris*, Labiatae) are characterized by a complex genetic and biochemical polymorphism. Individual plants manufacture a single dominant monoterpene which gives them a characteristic smell and taste, and the genetic determination of these chemotypes involves a series of five loci interacting with each other in epistatic fashion. We tested the hypothesis that multi-species herbivory and allelopathy are associated with this monoterpene polymorphism. We compared the relative attractiveness and deterrence of various chemotypes by offering either thyme plants or artificial diets laced with monoterpenes to six herbivores: two molluscs, two insects and two mammals. In addition, we summarize experiments by us and others in which bacteria, fungi and seeds of the grass *Brachypodium phoenicoides*, a common competitor of *T. vulgaris*, were exposed to the individual monoterpenes. The results show that the monoterpenes have differential deterrence value against these various species. We conclude that a combination of selective herbivory and allelopathy contributes to the maintenance of this polymorphism.

Keywords: genetic polymorphism, herbivory, Mediterranean ecosystems, monoterpene variation, *Thymus vulgaris*.

INTRODUCTION

Disease, parasitism and herbivory can play important roles in the origin and maintenance of complex genetic polymorphisms. This theme was first developed by Ford (1942) and Haldane (1949). Since then, a vast literature has accumulated on the subject. The primary emphasis in most studies has been the relatively straightforward interaction between hosts and single species of disease organisms, parasites or herbivores (see reviews by Anderson and May, 1982; Denno and McClure, 1983; O'Brien and Everman, 1988; Marquis, 1991; Fritz and Simms, 1992). In natural situations, however, a given species can be a host to many species of consumers of widely different taxonomic affiliations that have very different biological attributes, and therefore potentially different host requirements (Linhart, 1989, 1991; Maddox and Root, 1990; Strauss, 1991).

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These multi-species interactions lead to the hypothesis tested in this study: that the variety of consumer taxa *per se* may influence the evolution and maintenance of variation in host defences, since a potential host may be exposed either simultaneously or sequentially to multi-species – and thus multi-directional – selection as a result of herbivore and parasite activity. The consequences of such complex interactions are especially amenable to investigation in plants, given: (1) their place at the base of most food chains, and the concomitant diversity of invertebrates, fungi and mammals that feed on any given plant species; and (2) the diversity of defences used by plants against herbivores and parasites.

Among the variety of plant traits that may reduce the amount of damage inflicted by herbivores, secondary compounds are known to provide effective chemical defences (Hay *et al.*, 1988b; Bryant *et al.*, 1991; Harborne and Tomas-Barberan, 1991; Marquis, 1991; Langenheim, 1994). Of particular interest in this context is the genetic variation in resistance to herbivore feeding (Dirzo and Harper, 1982; Berenbaum *et al.*, 1986; Simms & Rauscher, 1989; Mithen *et al.*, 1995) found in natural plant populations and hence the possibility that herbivory and parasitism may generate selective pressures influencing the evolution and maintenance of variation in resistance. When plant defences vary in a quantitative fashion, the specifics of the association between parasite selectivity and plant chemical variation are difficult to elucidate (e.g. Simms and Rauscher, 1989; Maddox and Root, 1990; Simms, 1990). For this reason, plants in which the synthesis of secondary compounds is known to be under the control of single loci are especially useful to address the hypothesis outlined above.

The aim of the present study was to determine the extent to which multi-species interactions, including herbivory, parasitism and allelopathy, are affected by variability in the biochemical constitution of a single plant species. The host plant chosen is the perennial *Thymus vulgaris* (Labiatae), which is characterized by a biochemically and genetically well-documented polymorphism in monoterpene synthesis (Passet, 1971; Vernet *et al.*, 1986). In *T. vulgaris*, individual genotypes synthesize a single, dominant monoterpene and as a result possess a specific chemical phenotype, or chemotype. The species is a known host for a range of herbivores, including molluscs (Gouyon *et al.*, 1983; Linhart and Thompson, 1995), insects (Suire, 1951) and mammals (Harant and Jarry, 1987); it is also a potential host to bacteria and fungi (Vokou *et al.*, 1984). In addition, it is sensitive to competition from a variety of other plants, especially grasses such as *Brachypodium* in its native garrigue communities, which are xeric and nutrient-poor (Gouyon *et al.*, 1986; Harant and Jarry, 1987; Tarayre *et al.*, 1995). Here we test the hypothesis that these diverse interactions with herbivores and parasites are affected by the biochemical polymorphism in *T. vulgaris* via differential deterrence of the various monoterpene chemotypes. Specifically, we tested the prediction that the various chemotypes differ from each other in their deterrence of herbivores and influence upon growth of bacteria, fungi and a plant competitor.

This paper presents a combination of original and re-analysed published data to facilitate comparisons of the deterrent effects of the monoterpenes upon the diverse groups of taxa associated with *Thymus vulgaris*. We first present results from a series of food choice experiments using beetles (*Arima marginata*, Chrysomelidae), sheep (*Ovis aries*) and goats (*Capra hirtus*). We then compare these results with those of other, published experiments using molluscs (*Helix aspersa* and *Deroceras reticulatum*) and grasshoppers (*Leptophyes punctitatissimum*, Tettigoniidae), and with data on the effects of these chemotypes as agents of allelopathy on plants (Tarayre *et al.*, 1995) and on bacteria and fungi (Simeon de

Buochberg, 1976), to assess the relative deterrent effects of the different chemotypes when they are exposed to the diversity of herbivores, parasites and competitive species expected under natural conditions. Finally, we use this information to describe how these interactions may contribute to the maintenance of this multi-locus polymorphism in nature.

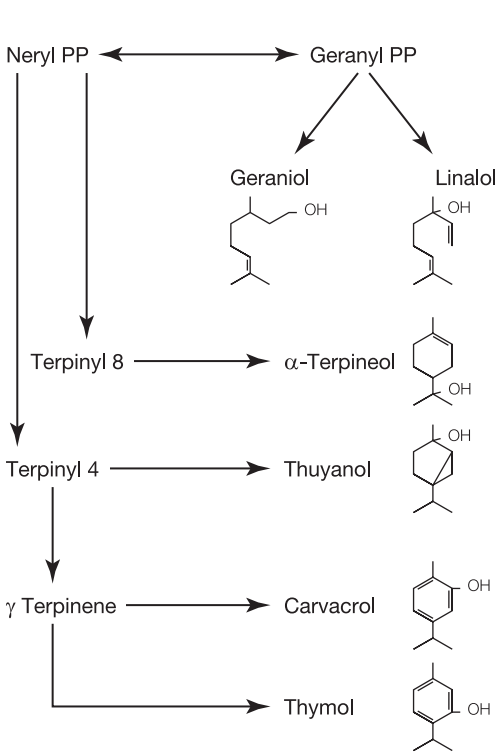
METHODS AND MATERIALS

The plant

Thyme (*Thymus vulgaris* L.) is an aromatic woody perennial of the western Mediterranean region. Its aroma is produced primarily by monoterpenes. A single plant produces predominantly a single monoterpene which gives the plant its characteristic taste and smell. Because of the importance of these monoterpenes in pharmacology and the manufacture of perfumes, both the biosynthetic pathway involved in their synthesis and the genetic control of this synthesis are well understood (Passet, 1971; Gouyon *et al.*, 1986; Vernet *et al.*, 1986). In the Languedoc region of the south of France, individual thyme plants produce as their dominant essence one of six monoterpenes: geraniol (G), linalol (L), alpha-terpineol (A), thuyanol (U), carvacrol (C) or thymol (T). Each plant thus has a specific chemotype which is unaffected by seasonality or changes in environmental conditions (Vernet *et al.*, 1986). The biosynthetic pathway of these monoterpenes is illustrated in Fig. 1. Two monoterpenes are acyclic (G, L), two are cyclic (A, U) and two are cyclic and phenolic (C, T). The genetic control of this pathway has been elucidated in a detailed series of Mendelian crosses and biochemical analyses of over 5600 progeny (Vernet *et al.*, 1986); it involves an epistatic series of at least five loci, presented in Fig. 2. At each locus, there is a pair of alleles, one dominant to the other. The epistatic effects are linear, so that: G > A > U > L > C > T. Therefore, a plant that has at least one dominant allele at the G locus produces a G chemotype regardless of its genetic composition at the other loci. A plant that is homozygous recessive at the G locus (i.e. gg) but with at least one dominant A allele will have chemotype A, again regardless of the genetic composition of the remaining loci in the series. The series continues in this manner; a plant which is homozygous recessive at all five loci has chemotype T. The only genetic complication in this pathway is that G may be controlled by two loci (Vernet *et al.*, 1986). The only physiological complication known is that plants belonging to the L chemotype only assume this chemotype at about 3 months of age. Prior to that age, they exhibit either a C or a T chemotype (Passet, 1971; Vernet *et al.*, 1986).

In natural populations, the frequencies of the six chemotypes vary markedly. Of the hundreds of populations analysed in southern France, some are monomorphic, but most are polymorphic, containing two or more chemotypes (Mazzoni and Gouyon, 1985; Gouyon *et al.*, 1986).

This thorough understanding of the polymorphism, in terms of its biochemistry, genetic basis and ecological distribution, and the fact that the monoterpenes involved have been shown to act as allelopathic agents and deterrents against herbivores (Gouyon *et al.*, 1983; Linhart and Thompson, 1995; Tarayre *et al.*, 1995), bacteria and fungi (Simeon de Buochberg, 1976; Vokou *et al.*, 1984; Fisher, 1991), provides an excellent setting for analysis of the role of multi-species selection by herbivores and potential parasites and competitors in the maintenance of a genetic polymorphism.



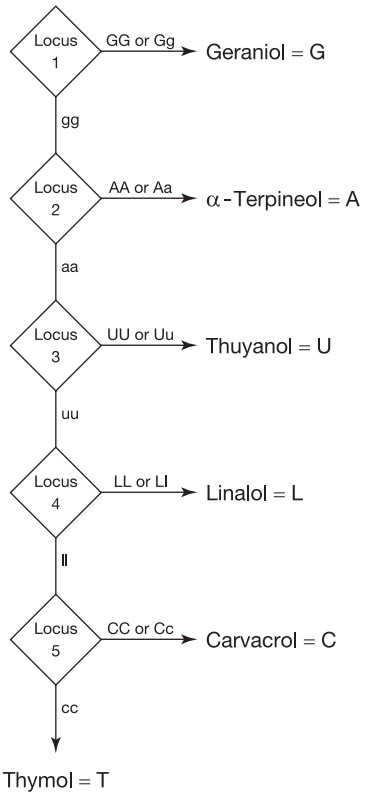


Fig. 1. (Left) The biosynthetic pathway of the six monoterpenes that characterize individual thyme plants in southern France (redrawn from Passet, 1971).

Fig. 2. (Right) The epistatic series of five loci that control the synthesis of thyme monoterpenes. A plant's chemotype is determined by the presence of a dominant allele at a given locus and by homozygous recessive alleles above that locus. See text for details (redrawn from Vernet *et al.*, 1986).

Herbivores and parasites

Thyme is host to a wide range of different herbivores and parasites. To date, the following have been identified: at least five species of Mollusca, 32 species of Lepidoptera, 17 species of Coleoptera, 30 species of Hemiptera, five species of Diptera, three species of Mammalia and several fungi. Some of these species are listed in published accounts (e.g. Suire, 1951); others we have documented ourselves (J.M. Parry, Y.B. Linhart and J.D. Thompson, in prep.). The herbivores used in experiments to date include: two molluscs, *Helix aspersa* (Helicidae) and *Deroceras reticulatum* (Limacidae); two insects, the beetle *Arima marginata* (Chrysomelidae) and the grasshopper *Leptophyes punctitatissimum* (Tettigoniidae); and two mammals, the domestic goat *Capra hirtus* and the sheep *Ovis aries*.

The intensity of predation and the associated potential for selection varies among these species. Some species are common in a variety of thyme habitats and have been associated with thyme for many millenia (e.g. Harant and Jarry, 1987). These include: *Helix*, a generalist herbivore; *Arima*, which is specialized on specific Labiatae, including *T. vulgaris*; and *Leptophyes*, whose feeding habits are poorly known but which was encountered only

on *T. vulgaris*. Other species are found on thyme only under certain conditions. For instance, in particularly humid sites, and usually close to some form of human activity, thyme will host the slug, *Deroceras*. In addition, some species of domesticated mammals (goats and sheep) have been introduced into thyme habitats. These species have a relatively short history of association with thyme habitats, going back (according to palaeontological evidence) roughly 4000 years or 1000–2000 thyme generations (Harant and Jarry, 1987). These mammals do, however, represent meaningful components of our studies of thyme–herbivore interactions because they can consume large numbers of plants and large quantities of biomass of individual plants, thereby generating significant fitness impacts on these plants. Also, they bear a close taxonomic relationship to the primary mammal that would have consumed thyme prior to human activity, the bouquetin, or ibex (*Capra ibex*) (M. Debusshe, personal communication).

The specific experiments we discuss in detail here involve *Arima*, *Capra* and *Ovis*. The beetle, *Arima*, is considered to be a specialist on certain Labiatae. It feeds on flowering stalks and young shoots, and damaging impacts of its feeding in France have been observed on natural populations and cultivated fields of lavender (*Lavandula* spp.), Hyssop (*Hissopus* sp.) and thyme. In the spring of 1991, it had such a significant impact on wild populations of thyme in the Drôme region (about 175 km northeast of Montpellier) that apiculturalists noted the almost complete absence of thyme flowers, an important food source for their bees. Individuals from a single population located near Grignan, Drôme were used in our experiments.

Goats of the 'Rove' breed used in the experiments are common, generalist browsers of woody vegetation such as *Juniperus* spp., *Quercus* spp. and thyme in the garrigue regions of southern France. Sheep of the 'Merinos d'Arles' breed used in these experiments are common grazers in the garrigue. Although preferring herbaceous vegetation to woody plants, they do eat the latter when food supply is limited. The amount of thyme eaten varies between herds in different regions, and can be considerable (R. Cordesse, P.H. Gouyon and G. Valdeyron, personal communication). Feeding can be highly selective: certain thyme plants are seldom, if ever, browsed, while adjacent plants can be browsed so heavily that short, new above-ground stems, which represent one season's growth, are connected to very large below-ground root systems, indicating that these plants are several years old, but were browsed repeatedly (C. Mazzoni and Y.B. Linhart, personal observations; J.D. Thompson and D. Manicacci, personal observations).

Experimental procedures

Rationale of herbivory experiments

A primary objective of this study was to test the prediction that the various chemotypes are differentially attractive to different herbivores under natural conditions. Hence, it was essential to test for selective herbivory by presenting an individual of a given animal species with a simultaneous choice of all six chemotypes known to occur in the study region. Such a design is more complex but more realistic than the commonly used binary choices offered to animals in choice trials. We used this design because a simultaneous choice of all chemotypes represents conditions much closer to what animals face, when they forage through complex vegetation, which usually consists of several thyme chemotypes, as well as other monoterpene-containing aromatic plants (e.g. *Rosmarinus*, *Origanum*, *Lavandula* and

Juniperus). Because of the olfactory complexity faced by these herbivores, a multi-chemotype scenario, although less tidy, is a more accurate, indeed a more conservative methodology, as it may decrease the likelihood that animals can identify specific odours or tastes in such complex settings. Our underlying assumption is that it is the specific monoterpene characterizing the chemotype which is responsible for the differential herbivory. Consequently, tests with all six herbivores involved live plants and, when possible, a second round of experiments using artificial food impregnated with a single monoterpene, thereby creating samples of food which differed only by the presence of this monoterpene.

Plant material

Ten individuals each of the six chemotypes of *Thymus vulgaris* were transplanted from field sites into a single greenhouse. Each chemotype was represented by plants from two to four field locations within a few kilometres of each other. Confirmation of the chemotype of each plant was performed by automated vapour phase chromatography (see Gouyon *et al.*, 1981, for details).

Clonal replicates of the 10 individuals of each chemotype were cultivated for experimental trials as follows. Young shoots, approximately 3 cm in length, were cut from the parent plant, the lower leaves removed and the basal part of the stem dipped in rooting powder. The cuttings, bearing 6–8 leaves at the time of planting, were then left for up to 1 month in trays filled with perlite, standing in a warm water bath in the greenhouse. A subsample of these clonal replicates was once again tested by chromatography to be sure they had the same chemotype as the parents and that they continued to synthesize monoterpenes despite their experimental treatments.

Monoterpenes were obtained by steam distillation from fresh leaf material.

Artificial diets

The nature of the artificial diet varied, depending on the herbivore used. For molluscs, they consisted of agar-based gelatin discs, first recommended by Whelan (1982) and modified by Linhart and Thompson (1995) to incorporate a known volume of the monoterpene essences. For the mammals, the artificial food consisted of a predetermined biomass of a processed straw–molasses mixture which is normally used as the daily diet for the animals employed in this study (R. Cordesse, personal communication). The volume of essence added was estimated based on the amount of essence that is bulk-distilled from a given biomass of thyme plant material. The two insect species could not be tested for their preferences on artificial diets because satisfactory diets have not been developed, and there were difficulties in keeping the animals alive under laboratory conditions beyond the many weeks required for us to develop a reasonable artificial diet.

Feeding experiments

In this paper, we detail the methods and results used in feeding trials with *Arima marginata*, *Capra* and *Ovis*. Details of the experimental procedure and results on the other species are presented elsewhere (Linhart and Thompson, 1995; Y.B. Linhart, L. Chaouni-Benabdallah, J.M. Parry and J.D. Thompson, in prep.) and are summarized here to facilitate interspecific comparisons.

For the invertebrate feeding trials (beetles, grasshoppers, molluscs), a minimum of 10 animals were used. Feeding trials were repeated, with different groups of animals, from the same or different populations. For goats and sheep, space and time constraints limited the testing to three individuals per species. Both goats and sheep had spent considerable time in the garrigue habitat and had the opportunity to feed on thyme, before being put into stalls at least 6 weeks before the trials were performed; during that period, they were not exposed to thyme prior to the onset of our experiment. Most of the feeding trials involved the simultaneous presentation of all six chemotypes; one feeding trial, however, as indicated below, involved tests of only two chemotypes, to determine more precisely the preference between given chemotypes.

In all tests, each chemotype was introduced in two randomized block-type replications of thyme plants or artificial diets (i.e. two 'gel cakes' for molluscs or two straw-filled pots for mammals). The conditions under which the feeding trials occurred consisted of circular arenas for the molluscs and *Arima*, rectangular trays for *Leptophyes*, and the habitual feeding stalls of the goats and sheep. Individuals of all species were kept separate in all trials.

Quantities of plant material or artificial diet offered reflected the volume of food that could be eaten in a day or less. For molluscs and insects, individual plants had one stem, were 4–6 cm long and typically had 6–10 leaves. The artificial food discs measured 8 mm and weighed 0.2 g, and contained the equivalent of 8.6 leaves of tissue. For sheep and goats, individual plants had 5–10 stems, were 20–30 cm high and typically had several hundred leaves. They were representative of the small adult plants these animals would encounter when feeding in the garrigues. The artificial food weighed 30 g. Each arena or tray contained one animal, and two plants, cakes or buckets of terpene-laced feed of each chemotype. For invertebrates, feeding was scored 24 h after the animals were introduced. Depending on the experiment, we recorded either the total number of leaves eaten, or the proportion of a cake consumed. Trials with sheep and goats consisted of introducing plants whose total branch length had been measured, or buckets with measured weights of the terpene-treated straw–molasses mixture. Feeding was scored after 2 h. We recorded length of remaining branches with leaves or weight of remaining food.

Statistical analyses

The design we used provided a choice of six chemotypes each replicated twice, for reasons described under 'Rationale' above. Following consultation with a biostatistician, we have analysed our results as described below. Analyses of consumption in all feeding trials were made by replicated tests of goodness of fit using the heterogeneity *G*-test outlined by Sokal and Rohlf (1981). In this method, the amounts eaten by each animal are treated as a replicate and the heterogeneity among these replicates (analogous to an interaction between animal and chemotype) is calculated as G_H. Then the pooled G (G_P) is calculated based on the summed chemotype consumption values over the animal replicate trials; the value represents heterogeneity of feeding choice, and indicates whether there is a significant difference among chemotypes in terms of volumes of food eaten. Additional comments about the inherent analytical difficulties of such complex designs are provided in Peterson and Renaud (1989), who discuss the lack of appropriateness of analysis of variance for these sorts of trials; Hay *et al.* (1988a), who discuss the suitability of tests such as the one we used; and Linhart and Thompson (1995), who describe the biological issues involved.

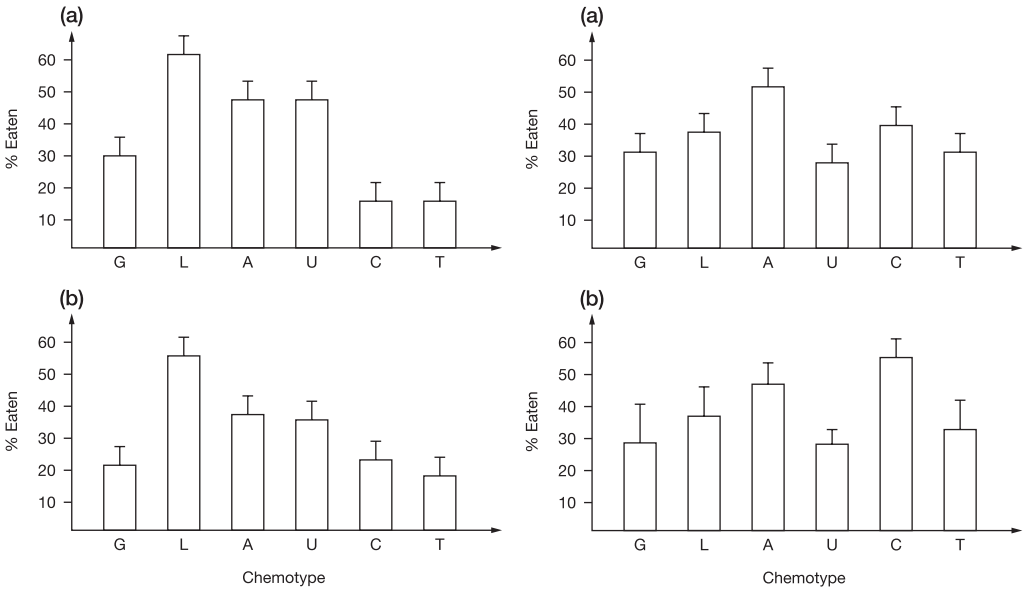


Fig. 3. (Left) Mean (±S.E.) percentage consumption by *Arima marginata* individuals offered the six chemotypes of *Thymus vulgaris* as plant cuttings. The six chemotypes are arranged in the sequence of their biosynthesis: G, geraniol; L, linalol; A, alpha-terpineol; U, thuyanol; C, carvacrol; T, thymol. (a) For run 1, $G_H = 5772.2$ (d.f. = 65, $P < 0.001$) and $G_P = 782.8$ (d.f. = 5, $P < 0.001$). (b) For run 2, $G_H = 964.0$ (d.f. = 55, $P < 0.001$) and $G_P = 322.0$ (d.f. = 5, $P < 0.001$).

Fig. 4. (Right) Mean (±S.E.) percentage consumption by *Capra hircus* individuals offered the six chemotypes of *Thymus vulgaris* as individual plants, or an essence-impregnated straw–molasses mixture. The six chemotypes are arranged in the sequence of their biosynthesis: G, geraniol; L, linalol; A, alpha-terpineol; U, thuyanol; C, carvacrol; T, thymol. (a) For whole plants, $G_H = 191.9$ (d.f. = 5, $P < 0.001$), $G_P = 187.7$ (d.f. = 5, $P < 0.001$). (b) For the artificial diet, $G_H = 7.3$ (d.f. = 10, $P > 0.50$), $G_P = 19.2$ (d.f. = 5, $P < 0.005$).

RESULTS AND DISCUSSION

This study involves a synthesis of original and published data. For this reason, we first present and discuss the outcomes of the feeding trials with the three herbivores for which original data are documented here, and then we summarize the data for the three herbivores for which the detailed data are published elsewhere. We then summarize the action of thyme monoterpenes as toxic growth inhibitors upon bacteria, fungi and other plants. Finally, we discuss the evolutionary significance of the diverse effects of these monoterpenes.

Chemotype choices by individual taxa

Arima marginata

The results of two feeding trials with live cuttings of thyme, using two populations of beetles collected at different times, are presented in Fig. 3. In both trials, the insects consumed higher amounts of linalol-containing plants, and to a lesser extent thuyanol, and avoided the two phenolics, carvacrol and thymol. This pattern appears to parallel the beetles' physio-

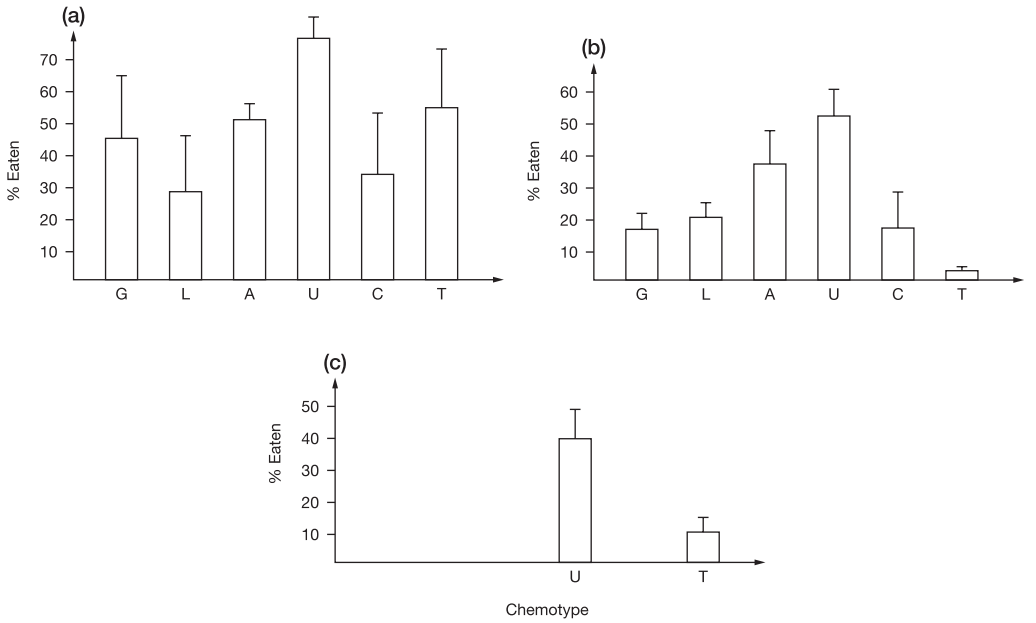


Fig. 5. Mean (±S.E.) percentage consumption by *Ovis aries* individuals offered the six chemotypes of *Thymus vulgaris* as individual plants, or an essence-impregnated straw–molasses mixture. The six chemotypes are arranged in the sequence of their biosynthesis: G, geraniol; L, linalol; A, alpha-terpineol; U, thuyanol; C, carvacrol; T, thymol. (a) For whole plants, $G_H = 161.9$ (d.f. = 5, $P < 0.001$), $G_P = 63.8$ (d.f. = 5, $P < 0.001$). (b) For the artificial diet, $G_H = 116.3$ (d.f. = 10, $P < 0.001$) and $G_P = 170.6$ (d.f. = 5, $P < 0.001$). (c) Comparison of U *vs* T, live plants; see text for details. $G_H = 23.9$ (d.f. = 2, $P < 0.001$) and $G_P = 47.9$ (d.f. = 1, $P < 0.001$).

logical capacities to tolerate various monoterpenes, since the results in our experimental trials reflect the observed concentration of *Arima* on Labiate species, which are characterized by the two preferred monoterpenes; that is, *Lavandula*, lavender (linalol), *Hissopus*, hyssop (thuyanol), and thyme (both chemotypes).

Capra hircus

The results of the feeding trials using live plants and essences are presented in Fig. 4. There were significant differences in the amounts of specific chemotypes eaten. It appears that goats fed selectively because the same three chemotypes eaten most in the real plant trial (C, A and L) were those eaten most in the artificial diets trial, although the sequence of preference differed somewhat, with A and C chemotypes being the most eaten in the plant and artificial diet runs respectively. When observed, animals repeatedly ($n \geq 15$ observations per chemotype) came within 5–10 cm of plants having the U and T phenotypes but moved away without feeding.

Ovis aries

The results of the feeding trials with thyme plants and essence-impregnated straw showed that U was eaten the most in both experiments (Fig. 5). However, the least eaten chemotype

Table 1. Relative deterrent values (*D*) of thyme chemotypes when exposed to herbivores, microorganisms and a competitor[a]

Biotic challenge		Thyme chemotype G	L	A	U	C	T	Significance
Herbivores								
Helix[b]	%	26	73	58	51	8	10	
	D	0.31	0.11	0.14	0.16	1.00	0.80	<0.001
Deroceras[c]	%	37	24	35	9	9	9	
	D	0.24	0.38	0.26	1.00	1.00	1.00	<0.001
Leptophyes[c]	%	39	32	38	36	42	52	
	D	0.82	1.00	0.84	0.89	0.76	0.62	<0.05
Arima	%	36	58	43	42	19	17	
	D	0.63	0.28	0.39	0.40	0.87	1.00	<0.001
Capra	%	32	38	52	28	40	32	
	D	0.94	0.86	0.67	1.00	0.83	0.94	<0.001
Ovis	%	45	27	51	77	33	54	
	D	0.60	1.00	0.53	0.35	0.82	0.50	<0.001
Microorganisms[d]								
Gram-positive	mg	0.8	0.6	0.9	1.8	0.5	0.4	
bacteria	*D*	0.49	0.73	0.42	0.12	0.77	1.00	<0.0001
Gram-negative	mg	1.1	1.6	1.9	1.8	1.5	0.6	
bacteria	*D*	0.64	0.29	0.09	0.14	0.36	1.00	<0.078
Fungi	mg	0.27	0.86	0.72	0.91	0.84	0.33	
	D	1.00	0.19	0.37	0.12	0.22	0.92	<0.0001
Competitor								
Brachypodium[e]	%	55	67	53	60	32	34	
	D	0.66	0.54	0.69	0.59	1.00	0.97	<0.0001
Mean deterrent	*D*	0.63	0.54	0.44	0.47	0.76	0.88	

[a] The values in the face of herbivory were calculated on the basis of consumption (standardized as % for this table) of whole plants. For each herbivore, the least consumed chemotype was given a *D* of 1, and relative *D* of other chemotypes was calculated on the basis of the differences in consumption relative to the least consumed (most repellent) chemotype. For bacteria and fungi, *D* is calculated on the basis of relative bacteriostatic and fungistatic activities (given in the table as mg terpene per ml of solution) of the chemotypes when placed in a growth medium for a given culture of an organism (Simeon de Buochberg, 1976). For the grass *Brachypodium*, the % indicates % seed germinated after 12 days in the presence of leaves of the relevant thyme chemotype. The highest bacteriofungistatic or allelopathic activity for a chemotype is given a $D = 1$; all other *D* values are derived from differences relative to the most toxic chemotype. G, geraniol; L, linalol; A, alpha-terpineol; U, thuyanol; C, carvacrol; T, thymol.

[b] Data from Linhart and Thompson (1995); [c] data from Y.B. Linhart, J.M. Parry, L. Chaouni-Benabdallah and J.D. Thompson (in prep.); [d] data from Simeon de Buochberg (1976); [e] data from Tarayre *et al*. (1995).

(T) in the essence trial was eaten to a much greater extent in the live plant experiment, but this was because one unusually small T plant was almost completely eaten. Because of this discrepancy, a second trial comparing U and T only was performed, in which U plants were consumed in significantly greater amounts than T plants (Fig. 5). The results of the live plant experiment are based on only two animals, as the third ate nothing. This result also explains the difference in degrees of freedom in the statistical test.

Other herbivores

The results obtained with other species are presented elsewhere in detail and are summarized in Table 1. All three species showed significant heterogeneity in their feeding patterns. The slug, *Deroceras*, showed a strong preference for the G chemotype both as live plants and in the artificial diet. The grasshopper, *Leptophyes*, preferred the T chemotype as live plants and could not be tested with an artificial diet as none was available (Y.B. Linhart, J.M. Parry, L. Chaouni-Benabdallah and J.D. Thompson, in prep.). The snail, *Helix*, preferred the L chemotype both as live plants and in artificial diets (Linhart and Thompson, 1995). The interspecific comparisons (Table 1 and Fig. 6) document an overall pattern of species-specific choice profiles depending on the taxon tested. In each of the taxonomic groups represented (molluscs, insects and mammals), the two species of a given group showed different preferences.

Bacteria and fungi

Bacteria and fungi also form part of thyme's biotic environment, especially in soils (Vokou *et al.*, 1984). No representatives of the groups directly associated with thyme in natural conditions have yet been identified, although the mildew fungus, *Oidium* (Fungi Imperfecti), can be damaging in greenhouses and moist microhabitats. For this reason, we can only get an indirect idea of chemotypic effects upon these taxa. The effects of *T. vulgaris* monoterpenes on the growth of various species of both bacteria and fungi have been demonstrated by Simeon de Buochberg (1976). The performances of all taxa were compared under controlled conditions against distilled extracts of all six monoterpenes. The main aim of her studies was to test for potential antibiotic properties of the monoterpenes, hence the taxa tested comprised those of public health interest. The species included 15 taxa of the Gram-positive bacteria, including *Staphylococcus* spp., *Sarcina*, *Streptococcus* spp., *Diplococcus* and *Bacillus*, and 12 species of Gram-negative bacteria, including *Escherichia coli*, *Klebsiella*, *Proteus*, *Moraxella* and *Neisseria*. The fungi tested included 11 *Aspergillus*, *Geotrichium*, *Saccharomyces*, *Candida* spp. and *Hansenula* sp. Her experimental results indicate a consistent effect of specific monoterpenes on bacteria as a group and fungi as a group (Table 1).

Simeon de Buochberg tested the effects of the individual monoterpenes upon growth of fungi and bacteria by adding measured volumes of the terpene to Petri dishes where the organisms were growing on defined media. She then reported the minimal bacteriostatic or fungistatic value volume of a given terpene which will prevent growth of a given organism. We used these values to calculate the mean bacteriostatic value for the 15 tests involving Gram-positive bacteria, the 12 tests involving Gram-negative bacteria and the 11 tests involving fungi. The significance of the differences between the most and the least effective chemotypes was tested with a *t*-test. All six chemotypes were also compared using the Ryan-Einot-Gabriel-Welch multiple range test (SAS, 1995).

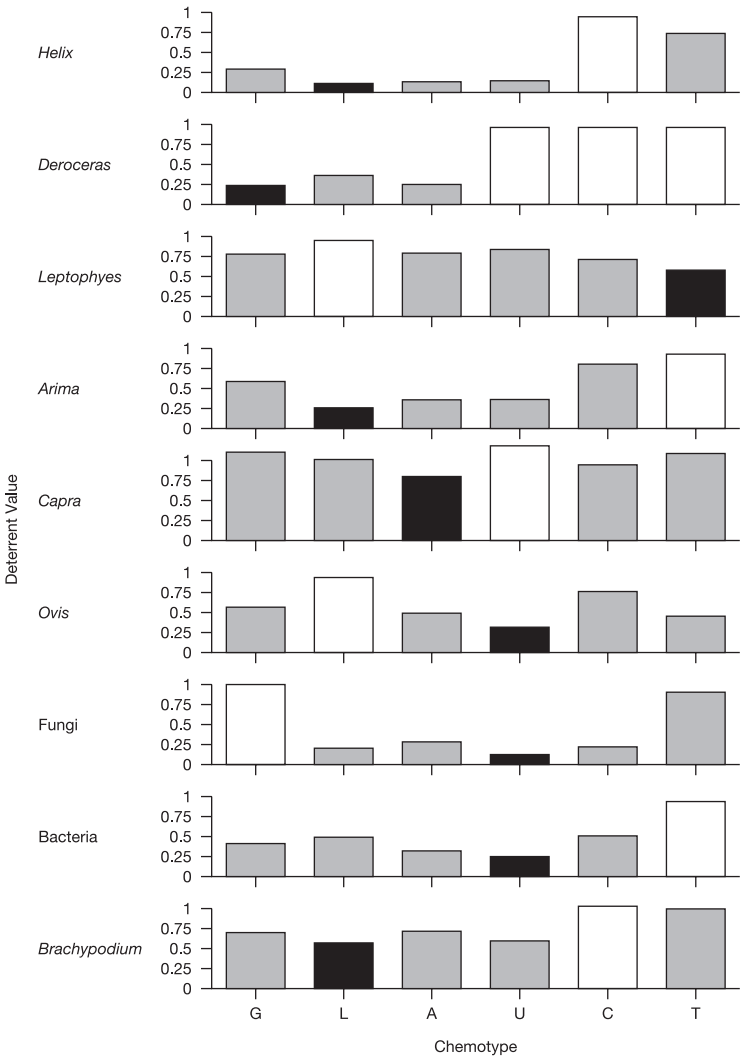


Fig. 6. Relative deterrent value of the six chemotypes when challenged by herbivory, microorganisms or competition. The herbivores are two molluscs (*Helix*, *Deroceras*), two insects (*Leptophyes*, *Arima*) and two mammals (*Capra*, *Ovis*). The microorganisms are 11 species of fungi, plus 15 species of Gram-positive bacteria and 12 species of Gram-negative bacteria. The competitor is a common native grass, *Brachypodium phoenicoides*. For all species, the chemotype which is least attractive to herbivores, or most toxic to microorganisms or plants, is given a deterrent value of 1. Other chemotypes are then compared to that one. The chemotype(s) with the highest deterrent value is shown as a white bar; the one with the lowest value is shown as a solid bar; and all intermediates are shown as stippled bars.

For Gram-positive bacteria, the most and least bacteriostatic values ($\bar{x} \pm$ S.E.) are T = 0.367 ± 0.033 mg per ml of medium and U = 1.800 ± 0.107 mg · ml^{-1}. The difference is highly significant ($P < 0.0001$), and the multiple-range test differentiates both U and T from

the chemotypes with intermediate effects. For Gram-negative bacteria, the most and least bacteriostatic chemotypes are T (0.615 ± 0.158 mg $\cdot$ ml^{-1}) and A (1.903 ± 0.592 mg $\cdot$ ml^{-1}); the differences between them are not statistically significant ($P = 0.078$) and the multiple-range test does not differentiate among the six chemotypes. The various monoterpenes have very different impacts upon fungi in that they stop colony growth at generally lower concentrations than is the case with bacteria. The most fungistatic chemotype is G (0.273 ± 0.037 mg $\cdot$ ml^{-1}) and the least fungistatic is U (0.909 ± 0.131 mg $\cdot$ ml^{-1}). The differences are highly significant ($P < 0.0001$) and the multiple-range test also shows significant differences between U, L, C and A as a group and T and G as a second group. The results suggest that the various chemotypes may have different impacts upon various groups of microorganisms that infect thyme above ground (e.g. the fungal mildew *Oidium*) or in the rhizosphere (Vokou *et al.*, 1984).

Other plant species

In these arid ecosystems where water and nutrients are limiting, all plants, including *Thymus vulgaris*, are very sensitive to the severe competition for water and nutrients (Gouyon *et al.*, 1986; Harant and Jarry, 1987; Tarayre *et al.*, 1995); inhibition of germination and subsequent allelopathy can be very advantageous as a mechanism to reduce competition. Monoterpenes, including those synthesized by thyme, have pronounced allelopathic effects. Indeed, it has been suggested that, in xeric plant communities, monoterpenes play a dominant role as allelopathic agents (Muller, 1970; Charlwood and Charlwood, 1991; Fisher, 1991). Comparisons of the six terpenes show that all reduce germination by the grass *Brachypodium phoenicoides*, the most common competitor of thyme communities. The phenolic (C, T) monoterpenes have a significantly more pronounced effect than do the others (Tarayre *et al.*, 1995).

Evolutionary significance of multi-species herbivory and allelopathy

The evolutionary consequences of parasitism and herbivory, in terms of the selective impacts of different herbivores on a single host species, are of particular relevance to our understanding of the maintenance of resistance polymorphisms in natural populations. Most studies have focused on the influence of single parasites or herbivores on a host, and have documented, either directly or in a correlative, circumstantial fashion, that parasites and herbivores can be important selective agents both in plants (see reviews in Denno and McClure, 1983; Agrios, 1988; Hay and Fenical, 1988; Burdon and Leather, 1990; Fritz and Simms, 1992; Hay and Steinberg, 1992) and in animals (Ford, 1942; Haldane, 1949; O'Brien and Everman, 1988; Combes, 1995). Recent research has begun to address the complexities of how different herbivore species may influence the evolution of resistance in a single host species (see reviews in Linhart, 1991; Marquis, 1991; Fritz and Simms, 1992) and the details of how such herbivores may exert specific selection pressures (Rauscher and Simms, 1989; Simms and Rauscher, 1989; Simms, 1990).

When feeding on thyme, all herbivores tested to date feed on some chemotypes more than on others (Table 1). These choices do not reflect any taxonomic pattern: there is no evidence that molluscs follow one pattern, insects another, and mammals another. This species-based pattern in herbivory has been termed 'species-specific host selection', and has been docu-

mented in several other multi-species interactions (Linhart, 1991; Mithen *et al.*, 1995). Our data indicate that such host selection may help explain how and why biochemical diversity is maintained in these populations.

To compare the effects of the different thyme monoterpenes on herbivore feeding, we present a comparative summary of herbivore feeding on real plants. We also include the relative growth rates of bacteria and fungi, and *Brachypodium* germination rates, to provide an approximation of the relative deterrent value, as an indirect estimate of the relative fitnesses of the six chemotypes (Fig. 6). By 'deterrent value', we mean the association between a given chemotype and herbivory, or reduction of bacterial growth, fungal growth or a competitor's germination rate. When faced by one of these challenges, the lower the extent of herbivory, or the growth rate of bacteria, fungi or grass seeds, the higher the relative deterrent value of a given chemotype in the presence of that biotic challenge.

The feeding choices observed in the various taxa studied here illustrate the potential influences of a combination of herbivores and parasites upon the chemical polymorphism in thyme. One example is the clear and consistent preference for linalol manifested by two common herbivores: *Helix aspersa*, a generalist herbivore, which can be common in moister thyme habitats; and *Arima marginata*, a specialist feeder on Labiatae, which reaches densities high enough to create noticeable damage to natural thyme stands. Given these strong preferences, it is particularly interesting that seedling 'linalol' genotypes (up to the age of about 3 months) are C or T phenotypes, the least preferred chemotypes (Vernet *et al.*, 1986); perhaps linalol genotypes 'hide' behind the phenol chemotypes during the life-cycle stage at which they are most vulnerable to herbivore damage. This type of scenario has also been observed in the mint *Satureja douglasii*, a thyme relative. In those plants of *S. douglasii* that grow in the cooler, shadier and moister environments favoured by the slug *Ariolimax dolichocephalus*, concentrations of specific monoterpenes that deter this animal are elevated by phenotypic plasticity (Rice *et al.*, 1978).

Another complex scenario involves the carvacrol (C) and thymol (T) chemotypes, expected to be uncommon since they are only produced when four (C) or five (T) of the epistatic loci are homozygous recessive (Fig. 2). Under any scenario that posits alleles at all loci being in relatively intermediate frequencies (e.g. at all five loci $p = q = 0.5$), the C chemotypes would represent $3/(4)^5$ or 3/1024 of all possible combinations; while T, produced only by homozygous cc genotypes, would only represent $1/(4)^5$ or 1/1024 of all plants. Different values of p and q (e.g. 0.9 and 0.1) would change these fractions, but C and T chemotypes would still constitute small fractions of the total. In other words, one might expect such chemotypes to be rare in natural populations, since their expression is concealed by a dominant allele at any of four unlinked loci. However, contrary to these expectations, in some habitats C and T phenotypes are in fact by far the most common chemotypes, probably because of their relatively high deterrence against many biological challenges (Fig. 6).

We tested the relationship between average deterrent value determined under experimental conditions and abundance of the chemotypes in nature, an indirect but reasonable estimate of their fitness at the landscape scale. To determine abundance, we used a map produced by Gouyon *et al.* (1986) based on a series of 320 stations sampled in and around the basin of St. Martin de Londres. The purpose of the original sampling was to examine the spatial distribution of the six chemotypes within the basin and in the surrounding garrigue habitats. The results (Fig. 7) show there is a very strong positive association ($r^2 = 0.707$) between abundance as measured by geographic extent (km^2 of area occupied)

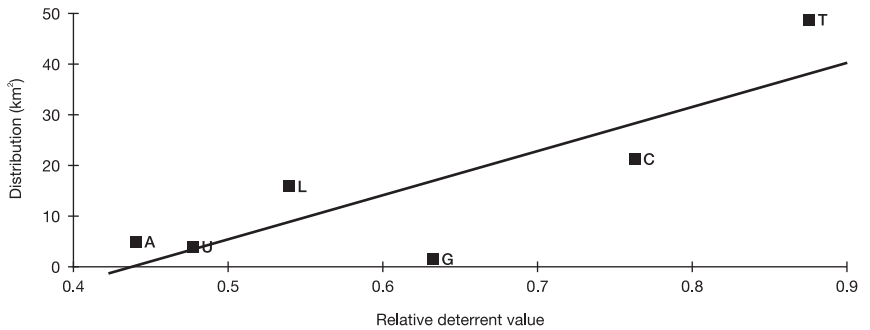


Fig. 7. Association between mean relative deterrent value of a chemotype (from Table 1) and geographic distribution of *T. vulgaris* chemotypes as determined from a map (from Gouyon *et al.*, 1986) and measured in km^2. $R^2 = 0.707$.

of the chemotypes in this landscape and the relative deterrent values calculated for the six chemotypes based on the protection afforded against the various animals, bacteria and fungi and the plant competitor. These deterrent values are highest for T and C and somewhat lower for L, whose seedlings are C or T. Conversely, A and U, which have lower deterrent values, also have more restricted distributions. The chemotype for which deterrent values (intermediate) and abundance (low to very low in this area) are most clearly uncorrelated is G. This chemotype is somewhat more common at higher elevations at cooler and moister sites. For example, in the Cévennes mountains, at elevations of 500–1500 m, *Thymus nitens*, a close relative of *T. vulgaris*, has a very high frequency of geraniol chemotypes (Granger *et al.*, 1973). However, G does not appear to be particularly common in southern France. The lower consumption of C and T, the phenolic chemotypes, is also in accord with a range of other studies which have documented the effectiveness of phenolic compounds as feeding deterrents (Levin, 1976; Rhoades and Cates, 1976; Swain, 1977; Sinclair and Smith, 1984; Greig-Smith and Willson, 1985; Gauthier and Bédard, 1990).

Analyses of the maintenance of genetic polymorphisms should address at least two separate questions. One concerns how the polymorphism may have arisen and established itself; the other is how the polymorphism is currently maintained. To examine these questions in the context of selective pressures generated by herbivory is no easy task (Endler, 1986; Fritz and Simms, 1992). Marquis (1991) has argued that the following phenomena must be demonstrated: (1) intraspecific variation in damage, which is (2) correlated with variation in a specific plant trait or traits, and (3) correlation between trait, damage and plant fitness. In addition, we believe that, for the sake of providing a precise description of the evolutionary dynamics of the system, (4) the precise genetic basis of the trait must be demonstrated; (5) selection must be shown to act directly on the phenotype produced by the locus responsible for the polymorphism (i.e. herbivore choice must be modulated primarily by the resistance trait); and (6) allele frequencies must be shown to change following episodes of herbivory. These issues were originally discussed in the context of single locus polymorphisms, but they are relevant to multilocus polymorphisms such as the thyme one as well, with the caveat that further complexities arise (e.g. from epistatic interactions among loci).

Studies of thyme genetics and herbivory have so far dealt at least to some extent with items 1, 2, 4 and 5 above. Items 3 (fitness effects) and 6 (changes in allele frequencies) have

yet to be addressed directly. Fitness effects can be inferred from observational evidence: selected thyme seedlings and young plants can be completely eaten by molluscs (Gouyon *et al.*, 1983; Linhart and Thompson, 1995; Linhart *et al.*, in prep.), insects (Linhart *et al.*, in prep.) or mammals (Y.B. Linhart and J.D. Thompson, personal observation). They can also be defoliated by mammals with remarkable precision, after which the bare branches do not regrow (Y.B. Linhart, personal observation). Finally, they can lose more than half of their above-ground biomass, both in feeding trials and in nature, an impact known to reduce significantly reproductive fitness in other plants (e.g. Crawley, 1983; Denno and McClure, 1983; Fritz and Simms, 1992). Experimental tests of one- and multi-species herbivory on phenotype and allele frequencies are planned. In addition, the strong association between overall deterrent values derived from herbivory and allelopathy and the geographic distribution of chemotypes (Fig. 7) do provide some indirect evidence for items 3 and 6. Unfortunately, for reasons discussed below, direct evidence for the impact of herbivory upon allele frequencies (item 6) is no longer testable in these ecosystems.

The possible role of mutations in the maintenance of this polymorphism should also be addressed. Epistatic interactions among loci often involve several loci that control or modify the processes associated with a single biosynthetic pathway. In thyme, this pathway is complex, as it involves terpenes being produced from branches of a single pathway (see fig. 1 in Passet, 1971).

C and T are strongly selected for, yet they are not ubiquitous in thyme. One possibility is that frequent mutations from recessive to dominant alleles may interrupt the pathway that leads to C and T, and contribute to the maintenance of the other chemotypes. However, as summarized in Fig. 6, other chemotypes besides C and T are repellent in their own right: G is most repellent to fungi, L is most repellent to *Leptophyes* and sheep, U is most repellent to goats. These factors, together with the association of various chemotypes with abiotic environmental features at the landscape scale (see below), suggest that mutations are unlikely to play a major role in the maintenance of the variability observed.

Additional factors operating at the landscape scale

The general question regarding the current maintenance of any given genetic polymorphism can only be answered precisely in a setting involving natural populations in the presence of the natural selection pressures which may influence the polymorphism; in this case, the association between native herbivores and the genetic constitution of thyme populations. This setting is no longer available for studies involving thyme, which grows in habitats that have been modified repeatedly as a result of human activities during the past 4000 years. For example, prior to large-scale human settlement, the local vegetation in many areas of Mediterranean France consisted of forests with relatively closed canopies to a height of 10–15 m. Thyme was restricted to dry, open slopes, and other sites with little or no shade. Following settlement, deforestation, fires and grazing, up to 60 cm of soil have been lost in various regions through erosion, and the garrigue landscape of open, dry scrubland, in which thyme has become much more common, has developed and been maintained by fire, erosion and heavy grazing by sheep and goats (Harant and Jerry, 1987; Lepart and Debussche, 1990).

Even this landscape is unstable. Many areas which were heavily grazed until the Second World War are currently utilized to a much lesser extent, in keeping with the accelerating exodus of the rural population towards urban areas. As a result, in the St. Martin de

Londres basin, where the initial studies of the ecology of the thyme chemical polymorphism were initiated in the 1970s (Granger and Passet, 1973; Vernet *et al.*, 1986; Gouyon *et al.*, 1981, 1986), the area of land that has changed from low herbaceous cover to shrub/tree cover has increased many-fold (Lepart and Debussche, 1990). In addition, the garrigue habitat suffers massive perturbations from heavy storms, occasional intense freezing in winter, plus informal human leisure activities. For these reasons, many thyme populations are not at equilibrium (Belhassen *et al.*, 1989), and current chemotype frequencies may change rapidly. This multiplicity of disturbances means that factors explaining the current maintenance of this polymorphism are difficult to elucidate. However, assuming that deterrent values are reasonable indices of fitness, these values may help explain the relative frequencies of chemotypes at the landscape scale, at least in the area we studied, given the high r^2 value of the relationship illustrated in Fig. 7. Deterrence does not tell the whole story, however. Theoretically, if a given allele or genotype has a higher fitness than a different allele or genotype, it should eventually become fixed, or at least reach extremely high frequencies unless, as noted earlier, mutation is very frequent. Consequently, one might expect chemotypes A and U to be uncommon, but they are relatively common where thyme is adequately sampled and, in some sites, they predominate (Mazzoni and Gouyon, 1985; Gouyon *et al.*, 1986). This patchiness may be closely linked to a differential intensity of selection by different herbivores, coupled with the epistatic effects of the loci involved, small-scale disturbances and founder effects (Gouyon *et al.*, 1986; Belhassen *et al.*, 1989).

Physical features of thyme habitats may also affect chemotype distribution (Granger and Passet, 1973; Gouyon *et al.*, 1986). For example, in the region studied, T and C chemotypes are more common on sites with dry, rocky soils, which are also more prone to fires, with C being less common in cooler zones, implying that it may be less cold-tolerant. The U chemotype is common in moister areas with deep soils in this region, but at higher elevations it can be common on shallow, rocky soils as well. In general, the non-phenolic (G, L, A, U) chemotypes are more common in zones with moister, deeper soils. Of course, these physical features, which are easy to measure, are often associated with biotic factors; for example, in the drier plateaus, *Helix* are uncommon and *Brachypodium* are common, and the reverse is true in the moister basin (Gouyon *et al.*, 1986; Y.B. Linhart and J.D. Thompson, personal observation). Informal surveys carried out on a larger scale in the south of France suggest that C and T may not be the most common phenotypes throughout the region, but L may be. At elevations above 700 m, G appears quite common (Granger and Passet, 1973; J.D. Thompson, personal observation).

In conclusion, these data appear to provide strong evidence to support our hypothesis that diverse parasites and herbivores respond to the biochemical polymorphism of *Thymus vulgaris* in a species-specific manner. In addition to the results we present here, there is further potential for complex impacts upon this polymorphism. For example: (1) the differential tolerance among chemotypes of various soil types and physical conditions (Gouyon *et al.*, 1986) noted above means that, as the climate becomes cooler and moister, non-phenolic chemotypes, whose monoterpenes volatilize at lower temperatures than the phenolic ones, become more common (Granger and Passet, 1973; Vernet *et al.*, 1986); and (2) geraniol (G) is a very strong attractant for native honeybees (*Apis mellifera*) because it is a primary component of the aggregation pheromone secreted via the Nassonoff gland (Bock and Shearer, 1962; Becker *et al.*, 1989). Bees, as important pollinators of *T. vulgaris*, may select G chemotypes for feeding and thereby increase their fitness. These results further illustrate the association between environmental heterogeneity and genetic variability that

has been documented both experimentally and theoretically (Levene, 1953; Dempster, 1955; Hedrick *et al.*, 1976; Antonovics, 1992; Linhart and Grant, 1996). These associations also indicate that evolutionary genetics is not necessarily synonymous with population genetics, but often needs to be done at the community level.

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