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TOLERANCE OF PLANT MONOTERPENES AND DITERPENE ACIDS BY FOUR SPECIES OF LYMANTRIIDAE (LEPIDOPTERA) EXHIBITING A RANGE OF FEEDING SPECIFICITIES

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ABSTRACT

Lymantriidae (Lepidoptera) is a family of leaf-feeding insects that includes some of the most damaging forest pests worldwide. Species within this family vary widely in feeding specificity. We evaluated the ability of four species, Douglas fir tussock moth (*Orgyia pseudotsugata* McDunnough), nun moth (*Lymantria monacha* L.), rusty tussock moth (*Orgyia antiqua* (L.)), and white-marked tussock moth (*Orgyia leucostigma* (J. E. Smith)), to contend with one of the most ubiquitous and effective groups of plant defense compounds, terpenoids. We selected these species to provide a range of feeding specificities on conifer hosts, from obligate to occasional. We evaluated the effects of three monoterpenes (bornyl acetate, limonene, and myrcene) and two diterpene acids (isopimaric acid and neoabietic acid) on larval performance. Although these four species differ in their feeding ranges, utilization of conifers as hosts, and other life history processes, each shows a relatively high tolerance for conifer terpenes. The mean relative growth rates, relative consumption rates, and development times were not affected by these monoterpenes and diterpene acids when administered at concentrations present in the foliage of conifers in which they are most abundant. The most likely explanation seems to be metabolism, as a) no limonene or myrcene were recovered from frass or larvae, and b) borneol, an apparent metabolite of bornyl acetate, was recovered from frass of Douglas fir tussock moth, rusty tussock moth, and white-marked tussock moth, and from tissues of Douglas fir tussock moth and white-marked tussock moth.

Lymantriidae (Lepidoptera) is a family of folivorous insects with worldwide distribution. It contains some of the most important pests affecting trees in forest and urban settings (Schaefer 1989). In addition to native defoliators, several invasive species pose particular challenges to environmental quality and forest resources. This family of over 350 genera and 2500 species contains members ranging from monophagous to polyphagous. This diverse range of host breadths includes both angiosperm and conifer genera. Consequently, lymantriid larvae encounter a broad range of phytochemicals.

Terpenes are among the largest groups of defensive chemicals occurring in plants (Gershenzon and Croteau 1991, Langenheim 1994). They occur in both angiosperm and gymnosperm trees (Staudt et al. 2001), but are generally more prevalent in the latter, especially conifers. In particular, monoterpenes and diterpene acids common in conifers exhibit a wide degree of efficacy against a broad range of herbivores, bacteria, and fungi (Trapp and Croteau 2001). Terpenes can negatively impact herbivores through toxic and deterrent effects (Gershenzon and Croteau 1991, Langenheim 1994). Toxicity may result from several mechanisms, including inhibition of ATP formation, interference with hormone production, and binding proteins or sterols in the gut (Langenheim 1994). Mechanisms of deterrence are less well characterized but may involve interaction with sensory receptors (Gershenzon and Croteau 1991).

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Insect herbivores may metabolize phytochemicals, excrete them unchanged, passively accumulate them in body tissues, or actively sequester them for defense against predators (Blum 1981). Different species employ different mechanisms to process the same phytochemicals. Several characteristics have been proposed to explain this variation, including feeding breadth (Krieger et al. 1971, Gould 1984, Berenbaum 1991, Osborn and Jaffe 1998) and strategies of predator avoidance (Bowers and Puttick 1986). It is difficult to draw conclusions based on feeding breadth because most studies have compared taxonomically distant species, or species that feed on distantly related plants or phytochemicals. Therefore, we evaluated four species within a single family, that have overlapping host ranges, and whose behaviors range from specialist to generalist. In a more detailed study, we conducted similar evaluations of a fifth lymantriid, the highly polyphagous gypsy moth, *Lymantria dispar* (L.), (Powell and Raffa 2003).

The Douglas fir tussock moth, *Orgyia pseudotsugata* (McDunnough), is a specialist on conifers, feeding on only 2 genera. It is one of the most important pests of Douglas fir, *Pseudotsuga menziesii*, white fir, *Abies concolor,* and grand fir, *Abies grandis*, in western North America (Wallner 1989). The nun moth, *Lymantria monacha* (L.), feeds primarily on conifers, and to a lesser extent on angiosperms. Preferred conifer hosts include *Picea*, *Pinus*, *Larix*, and *Abies* (Grijpma 1989). Its native range extends from Western Europe to Siberia, and from southern Scandinavia to northern Spain, Portugal, Italy, Greece and Bulgaria (Grijpma 1989). The rusty tussock moth, *Orgyia antiqua* (L.), is found worldwide in northern regions. It feeds on all conifer genera except *Juniperus*, and on over 50 species of angiosperms (Wallner 1989). White-marked tussock moth, *Orgyia leucostigma* (J. E. Smith), feeds on over 140 tree species. Host trees are primarily angiosperms, but include some conifers. Its geographic range includes most of the central and eastern United States, and southern Canada (Wallner 1989).

The purpose of this work was to a) evaluate effects of various terpenes on lymantriid larvae displaying a range of feeding specificities, and b) explore general categories by which lymantriid larvae likely contend with these phytochemicals.

MATERIALS AND METHODS

Insect sources and rearing. Douglas fir tussock moth egg masses were field collected in Idaho and Oregon, and obtained from a laboratory colony maintained by the Canadian Forest Service in Victoria, British Columbia, Canada. Nun moth egg masses were obtained from laboratory colonies maintained by the USDA Forest Service in Ansonia, Connecticut, USA. Rusty tussock moth and white-marked tussock moth egg masses were obtained from laboratory colonies maintained by the Canadian Forest Service in Sault Sainte Marie, Ontario, Canada. All experiments were performed at the University of Wisconsin, Madison, Wisconsin, USA, except those with nun moth, which were performed at the USDA Forest Service Laboratory in Ansonia due to quarantine restrictions.

Upon receipt, egg masses were surface sterilized in a solution of 97% (v/v) deionized water, 1% (v/v) tween (Polyoxy-sorbitan monooleate) and 2% (v/v) bleach (Chlorox: 5% hyperchlorite) for 3 minutes, then rinsed 3 times in deionized water. Egg masses were allowed to air dry for 30 minutes and placed individually in large petri dishes (d = 14.0 cm, h = 3.9 cm; TriState Plastics, Dixon, KY). Upon eclosion, larvae were fed an agar- and wheat germ-based artificial (ICN gypsy moth) diet. Larvae were reared in growth chambers at 16:8 (L:D) h and 25° C. Larvae were offered fresh diet every other day until they reached the appropriate stadium. Nun moth larvae were fed agar- and wheat germ- based artificial diet amended with 3 ml of linseed oil per liter.

Bioassays. The monoterpenes bornyl acetate, limonene, and myrcene (Aldrich Chemical Company, Milwaukee, WI) were diluted individually in a 0.75% solution of Triton X 405 (triton) (Aldrich Chemical Company, Milwaukee, WI) in dH_2O before being added to artificial diet (Powell and Raffa 1999). The diterpene acids isopimaric acid and neoabietic acid (Helix Biotechnologies, Canada) were dissolved in HPLC grade methanol (MeOH; Fisher Scientific) before being added to artificial diet (Powell and Raffa 1999). Excess methanol was evaporated under a gentle stream of nitrogen. Diet was amended with 0. 75 ml treatment per mg wet weight artificial diet.

Larvae showing head capsule "slippage" just before ecdysis were isolated and placed in large petri dishes as above, without food. After 24 h, newly molted larvae were weighed and used in experiments. Individual larvae were placed in 40 ml cups (Polar Plastics, Winston-Salem, NC) and fed amended artificial diet. Newly amended diet was provided every 24 hours for the duration of the stadium. All uneaten diet was collected daily, dried, and weighed. Development time, relative consumption rates, and relative growth rates were calculated for each instar (Waldbauer 1968). Frass was collected daily and frozen until chemical analysis. Larvae and exuviae were collected after each larva molted into the next stadium, and kept frozen until chemical analysis.

Three sets of experiments were performed. In the first set of experiments, bornyl acetate, limonene, and myrcene were tested individually at 0.01%, 0.1%, and 1.0%. The controls were distilled water and triton, separately. These experiments were conducted separately with second, third, and fourth instar Douglas fir tussock moths, rusty tussock moths, and white marked tussock moths. An additional group of 10 second instar larvae were tested at 5.0% bornyl acetate. In the second set of experiments, piperonyl butoxide (PBO), a broad inhibitor of P450 enzymes (Brattsten and Metcalf 1970), was added to artificial diet (0.1%) in combination with monoterpenes to explore potential involvement of these enzymes in terpene metabolism. These experiments were performed using Douglas fir tussock moth, nun moth, rusty tussock moth, and white-marked tussock moth. Controls consisted of distilled water, triton, and PBO. The third set of experiments was conducted with the diterpenes isopimaric acid and neoabietic acid at 12.5 mg/ml, 25 mg/ml, and 125 mg/ml. The controls were distilled water and methanol. These experiments were conducted with second instar Douglas fir tussock moths. Due to uneven availability of insects of different species, the exact treatment combinations varied within experiments. Therefore the exact treatments for each insect species – geographic source - instar combination are shown at the bottom of Table 1. Likewise, sample sizes also varied depending on insect availability, with the totals being 449 Douglas fir tussock moths, 125 rusty tussock moths, 571 white marked tussock moths, and 88 nun moths. Exact sample sizes for each terpene – dose – instar - geographic source-species combination are in Powell (2002).

Chemical analyses and fate of phytochemical substances. Larvae were macerated before chemical analysis; frass and exuviae did not require grinding. Monoterpene analyses were performed as described in Powell and Raffa (2003). Briefly, monoterpenes were extracted with hexane from frass or larval tissues for 24 h. The extract was analyzed using a Shimadzu GLC 17A, fitted with an AOC 20i autosampler (Shimadzu Scientific Instruments, Inc. Columbia, MD). Separations were performed on a 25 m x 0. 25 mm bonded fused silica open tubular polyethylene glycol column (Alltech Assoc., Deerfield, IL). Oven temperature was 60°C for the first 10 minutes, and was increased 10°C per minute for 10 minutes, until 160°C. Helium, the carrier gas, was maintained at 30 cm per second. Compounds were quantified by comparing their percentage of the total with the percentage of a known amount of the internal standard para-cymene (Aldrich Chemical Company, Milwaukee, WI).

The details of our approach to evaluating the fate of terpenes are in Powell and Raffa (2003). Briefly, terpenes were considered excreted if they

Table 1. Analyses of variance of effects of terpenoids on four species of tree-feeding tussock moths (Lepidoptera: Lymantriidae) species: DFTM= Douglas Fir tussock moth; RTM= Rusty tussock moth; WMTM= White marked tussock moth; NM= Nun moth

				Relative Growth Rate (g/g/day)[a]		Stadium Duration (days to molt)		Relative Consumption Rate (g/g/day)[b]	
Exp.	**Insect (Source)**	**Instar**	**df**	***P***	***F***	***P***	***F***	***P***	***F***
1	DFTM (ID)	2 [c]	10, 40	0.43	1.08	0.019	2.51	0.1995	1.44
		3 [d]	4, 17	0.3681	1.15	0.5915	0.72	0.0222	3.79
		4 [d]	4, 16	0.4751	0.923	0.5253	0.83	0.3957	1.09
	DFTM (OR)	2 [e]	6, 35	0.8651	0.41	0.8112	0.49	0.7844	0.53
		3 [e]	6, 32	0.0097	3.45	0.207	1.51	0.1972	1.55
		4 [e]	6, 20	0.263	1.4	0.1143	2	0.9744	0.19
	DFTM (BC)	2 [f]	5, 52	0.5702	0.78	0.1808	1.59	0.1045	1.93
	RTM (CFS)	2 [c]	10, 38	0.1642	1.54	0.4034	1.08	0.1526	1.57
		3 [c]	10. 37	0.4106	1.07	0.2234	1.39	0.038	2.32
		4 [d]	4, 18	0.9527	0.17	0.978	0.11	0.4553	0.98
	WMTM (CFS)	2 [c]	10, 96	0.6418	0.79	0.4434	1.01	0.4042	1.06
		3 [c]	10, 96	0.1085	1.63	0.0166	2.33	0.8862	0.5
		4 [c]	10, 98	0.347	1.13	0.077	1.77	0.5649	0.87
2	DFTM (ID)	2 [g]	8, 44	0.2362	1.37	0.756	0.62	0.0963	1.84
		3 [g]	8, 46	0.6265	0.78	0.3161	1.21	0.289	1.27
		4 [g]	8, 20	0.4256	1.063	0.9904	0.185	0.353	1.2
	RTM (CFS)	3 [h]	4, 16	0.1647	1.87	0.0386	3.27	0.055	3.07

Table 1. Continued.

Exp.	Insect (Source)	Instar	df	Relative Growth Rate (g/g/day)[a]		Stadium Duration (days to molt)		Relative Consumption Rate (g/g/day)[b]	
				P	*F*	*P*	*F*	*P*	*F*
	WMTM (CFS)	2[g]	8, 63	0.3742	1.1	0.0001	6.74	0.0256	2.44
		3[g]	8, 72	0.3167	1.19	0.9373	0.362	0.1156	1.69
		4[g]	8, 79	0.9147	0.41	0.4464	0.1	0.5686	0.84
	NM (USDA)	4[g]	8, 79	0.0031	3.24	0.0019	3.43	0.1995	1.43
3	DFTM (BC)	2[i]	7, 47	0.4186	1.04	0.246	1.36	0.4449	1.0

a: RCR = [weight of diet consumed / weight of insect at the beginning of the stadium] / duration of the stadium
b: RGR = [weight gain over stadium / weight of larva at the beginning of the stadium] / duration of the stadium
c: Treatments = 1, 0.1, 0.01% each of bornyl acetate, limonene, myrcene, plus water and triton controls
d: Treatments = 1, 0.1, 0.01% bornyl acetate, plus water and triton controls
e: Treatments = 1, 0.1, 0.01% bornyl acetate, 1 % each of limonene and myrcene, plus water and triton controls
f: Treatments = 5, 1, 0.1, 0.01% bornyl acetate, plus water and triton controls
g: Treatments = 1% bornyl acetate, limonene, myrcene, each w/wo piperonyl butoxide (PBO), plus water, triton, and PBO controls
h: Treatments = 1% bornyl acetate, w/wo PBO, plus water, triton, and PBO controls
i: Treatments = 125, 25, 12.5 ug/ml each of isopimaric and neoabietic acid, plus methanol and water controls

were recovered from frass or exuviae, accumulated if they were recovered from larvae, or putatively metabolized if they were not recovered from frass, exuviae or larvae, or if metabolites of the parent terpenes were detected. Procedures for evaluating volatiles and other potential sources of degradation are likewise in Powell and Raffa (2003). Briefly, recovery rates after diet incorporation average 87%, 72%, and 84% for myrcene, limonene, and bornyl acetate, respectively, and recovery rates for these compounds average 66%, 59%, and 57% after 24 h under these conditions.

Statistical analysis. All insect performance and chemical data were analyzed using SAS (1988). A significance level of $P < 0.05$ was used for all analyses. All analyses were conducted separately for each insect species.

Insect performance data were analyzed using one-way ANOVA, with treatment as the independent variable. Treatments included all concentrations of terpenes and controls. Variables were tested for assumptions of normality and homogeneity of variance by graphical analysis of residuals for all variables. No insect performance data required transformation.

Chemical data were analyzed using two complementary methods: Two-Way ANOVA (Treatment, Instar, Treatment × Instar) and chi-squared analysis of binomial data. Chemical data were not normal and could not be transformed due to high numbers of zeroes. Therefore, to ensure that interpretations were correct, all chemical data were re-analyzed using binomial regression. Data were transformed into binomial data (0 = absence, 1 = presence of compound), and four possible models were tested for fit using chi-squared analysis: 1) treatment, instar, treatment × instar; 2) treatment; 3) instar; and 4) treatment × instar. Once the chi-squared model yielding the best fit was determined, it was compared with the results of the ANOVA. In all cases, the results of the binomial and ANOVA analyses agreed.

RESULTS

Insect performance. Overall, monoterpenes and diterpene acids had little to no effect on larval performance of any of the species tested. Table 1 summarizes these results. Complete statistical analyses are reported in Powell (2002). There were several instances of statistical significance, but these were not consistent. Because our data led us to the unexpected conclusion of no treatment effect for any species (Table 1), we report results of analyses using the most liberal approach possible, i.e., the most likely to counter our conclusion, evaluating each experiment independently. Even under these conditions, only 10 of 66 relationships were significant.

The relative growth rates, development times, and relative consumption rates of these four tussock moth species are shown in Table 2. Because Douglas fir tussock moth larvae obtained from different sources did not differ in growth, development time, or consumption (Powell 2002), their data are pooled. To be conservative, we removed those larvae in treatments for which there were putative treatment effects (Table 1), although this did not substantially alter any values.

Chemical analyses and fate of phytochemical substances. Neither limonene nor myrcene were recovered from frass or larval tissue of any species tested. Bornyl acetate (Retention Time (RT) = 16.7 min) was recovered in small quantities from frass of some Douglas fir tussock moth, rusty tussock moth, and white marked tussock moth larvae fed bornyl acetate, but not from any fed limonene, myrcene, or the triton or dH_2O controls. Borneol (RT = 18.4 min), a likely metabolite of bornyl acetate, also was recovered in small quantities from frass and/or tissues of some larvae of all three species fed bornyl acetate, but not from any fed the triton or dH_2O controls, limonene, or myrcene. There was some variation in quantities of borneol and bornyl acetate due to instar, species, population, and concentration, but these differences were inconsistent, and showed no overall pattern (Powell 2002).

Table 2. Relative growth rates, Development times, and Relative consumption rates of four tree-feeding tussock moth (Lepidoptera: Lymantriidae) species. DFTM = Douglas Fir tussock moth (pooled); RTM = Rusty tussock moth; WMTM = White marked tussock moth; NM = Nun moth

			Relative Growth Rate (g/g/day)[a]		Development Time (days to molt)		Relative Consumption Rate (g/g/day)[b]	
Insect	**Instar**	**N**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
DFTM	2	256	0.55	0.4-0.7	5.011	4.4-5.8	2.18	0.9-3.3
	3	137	0.352	0.2-0.6	6.42	5.3-8.5	1.23 3	0.6-2
	4	56	0.2	0.1-0.4	8.37	7.0-10.1	1.21	0.6-3.4
RTM	2	49	0.38	0.2-0.6	5.18	4.8-5.6	1.29	0.7-1.9
	3	53	0.19	0.1-0.3	9.93	7.2-9.0	2.874	0.8-3.2
	4	23	0.26	0.2-0.3	2.39	5.3-5.7	1.5	0.4-3.5
WMTM	2	179	1.01	0.9-1.1	3.65	3.3-4.3	1.16	0.5-1.7
	3	195	0.65	0.5-0.8	4.196	4.1-4.4	0.92	0.6-1.9
	4	197	0.56	0.5-0.6	4.69	4.1-5.2	0.88	0.7-1.1
NM	4	88	0.347	0.4-0.5	4.95	4.7-5.3	0.64	0.4-0.9

a: RCR = [weight of diet consumed / weight of insect at the beginning of the stadium] / duration of the stadium
b: RGR = [weight gain over stadium / weight of larva at the beginning of the stadium] / duration of the stadium
1: N = 248; 2: N = 114; 3:N = 128; 4: N = 26; 5:N = 119; 6:N = 146; 7: N = 68; 8: N = 60

In addition to the association of borneol and borneal acetate with consumption of boreal acetate, six compounds (RT = 2.6, 3.1, 3.7, 4.5, 5.0, 17.8) were detected in Idaho, three (RT = 3. 5, 5.0, 8.5) in Oregon, and five (RT = 2.6, 3.7, 4.5, 9.1, 10.2) in Goose Lake, Douglas Fir Tussock Moth treated and control larvae. Twelve compounds (RT = 2.6, 3.1, 3.7, 4.0, 4.2, 4.5, 5.0, 5.5, 6.0, 6.9, 7.5, 10.2) were found in frass from both treated and control rusty tussock moth larvae. Two compounds (RT = 9.1, 17.8) were present in frass of both treated and control white-marked tussock moth larvae. In addition to the association of borneol with boreal acetate feeding, several compounds were present in both treated and control larval tissues. Among Douglas fir tussock moths, these were RT's 2.6, 3.1, 3.7, 4.0, 4.5, and 6.0 from Idaho, RT's 3.5, 5.0, and 8.5 from Oregon, and RT's 2.6, 3.7, 4.5, and 9.1 from Goose Lake larvae. Ten compounds (RT = 2.6, 3.1, 3.7, 4.0, 4.2, 4.5, 5.0, 6.9, 10.2, 22.1) were recovered from rusty tussock moth larval, and eight (RT = 2.6, 3.1, 3.7, 4.0, 4.2, 4.5, 5.0, 6.7) were recovered from white-marked tussock moth larval tissues, in both treated and control samples. By comparison, the internal standard para-cymene had a retention time of 8.6 min.

Overall, the addition of PBO had little or inconsistent effects on larval performance and monoterpene recovery. Douglas fir tussock moth and white marked tussock moth showed no effects. In nun moth, almost twice as much bornyl acetate was recovered from the frass of larvae fed bornyl acetate + piperonyl butoxide as from larvae fed bornyl acetate alone. No borneol was recovered. In rusty tussock moth, more borneol was recovered from frass and larval tissue of larvae fed bornyl acetate + PBO than bornyl acetate alone. Complete statistical analyses are reported in Powell (2002).

No compounds were recovered exclusively from frass or larval tissues of larvae fed diterpene acids. Neither the parent diterpene acids (neoabietic RT = 8.52; isopimaric RT = 9.58) nor their potential metabolites were recovered from frass or larvae.

DISCUSSION

These four tussock moth species appear highly tolerant of monoterpenes and diterpene acids, despite the adverse effects these compounds exert on many other herbivores, including Lepidoptera. A fifth species, *L. dispar,* is likewise highly tolerant (Powell and Raffa 2003). This tolerance is independent of the wide range of feeding breadths, degrees of association with conifers, and life history strategies among these five lymantriids. The ability to contend with compounds that so strongly deter other insects may partially explain why tussock moths undergo outbreaks across such a diversity of habitats, and their ability to invade new regions.

Metabolism appears to be the most likely mechanism of tolerance in all five of these generalist and specialist lymantriids (Powell and Raffa 2003). Metabolism is likewise the most important mechanism some other lepidopterans, such as *Spodoptera litura* Fabricius (Noctuidae) and *Peridroma saucia* (Hubner) (Noctuidae) use to contend with monoterpenes (Harwood et al. 1990, Miyazawa et al. 1996). In systems where specific mechanisms of terpene metabolism have been studied, P450 enzymes were the most important group, and these enzymes were induced by terpenes (Rose 1985). Several studies show that the toxicity of phytochemicals (Wheeler et al. 1993) or insecticides (e. g. Brattsten and Metcalf 1970, Martin et al. 1997) to lepidopteran larvae increases when they are combined with PBO. However, PBO did not affect insect performance or terpene recovery in Douglas fir tussock moth, white-marked tussock moth, or gypsy moth. Further, gypsy moth performance and terpene recovery were not affected by consuming S,S,S-tri-n-butyl phosphorotrithioate (DEF), an enzyme inhibitor of esterases (Powell and Raffa 2003). We cannot rule out the possibility that P450 enzymes are involved in terpene degradation, as insects possess many isoforms, not all of which are inhibited by PBO (Feyereisen 1999).

Physical and chemical properties have been proposed as important determinants of how insect herbivores contend with host compounds (Duffey 1980, Gardner and Stermitz 1988, Shapiro 1991). In our study, the two more lipophilic monoterpenes, limonene and myrcene, appear completely metabolized. The relatively hydrophilic compound, bornyl acetate, was metabolized in part to borneol, and also was excreted in small amounts. Borneol was both accumulated in larvae and excreted. Our results, in combination with the above studies suggest that physical properties of phytochemicals may be a better predictor than feeding specificity of how an insect contends with them.

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