

High individual variation in pheromone production by tree-killing bark beetles (Coleoptera: Curculionidae: Scolytinae)

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Abstract Aggregation via pheromone signalling is essential for tree-killing bark beetles to overcome tree defenses and reproduce within hosts. Pheromone production is a trait that is linked to fitness, so high individual variation is paradoxical. One explanation is that the technique of measuring static pheromone pools overestimates true variation among individuals. An alternative hypothesis is that aggregation behaviour dilutes the contribution of individuals to the trait under selection and reduces the efficacy of natural selection on pheromone production by individuals. We compared pheromone measurements from traditional hindgut extractions of female southern pine beetles with those obtained by aerating individuals till they died. Aerations showed greater total pheromone production than hindgut extractions, but coefficients of variation (CV) remained high (60–182%) regardless of collection technique. This leaves the puzzle of high variation unresolved. A novel but simple explanation emerges from considering bark beetle aggregation behaviour. The phenotype visible to

natural selection is the collective pheromone plume from hundreds of colonisers. The influence of a single beetle on this plume is enhanced by high variation among individuals but constrained by large group sizes. We estimated the average contribution of an individual to the pheromone plume across a range of aggregation sizes and showed that large aggregation sizes typical in mass attacks limit the potential of natural selection because each individual has so little effect on the overall plume. Genetic variation in pheromone production could accumulate via mutation and recombination, despite strong effects of the pheromone plume on the fitness of individuals within the aggregation. Thus, aggregation behaviour, by limiting the efficacy of natural selection, can allow the persistence of extreme phenotypes in nature.

Keywords Aggregation behaviour · *Dendroctonus* · Fisher's fundamental theorem of natural selection · Group selection · Individual variation · Mutation–selection balance · Pheromones

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Introduction

The importance of variation among individuals within populations has long been recognised in behavioural ecology in general and animal communication in particular (e.g., Maynard Smith 1982). However, technological limitations have largely prevented the empirical study of individual variation in chemical communication. Extraction and analyses of pheromones in bark beetles (Coleoptera: Curculionidae: Scolytinae) have been performed ever since researchers first realised the existence of an “attractive

principle" (Vité and Gara 1962; Pitman and Vité 1963) behind the phenomenon of aggregation. The sophistication of pheromone-mediated communication became evident when pheromones were first identified using packed-column gas chromatography (GC). Soon it was deduced that the site of pheromone production, accumulation and release in *Ips confusus* (Leconte) and *Dendroctonus* spp. was the posterior alimentary canal (Pitman et al. 1965, 1968). In the 1960s and 1970s, up to 20,000 bark beetle hindguts were dissected and extracted to obtain enough material for reliable identification of pheromones (Renwick 1967; Pitman et al. 1968). When pheromones from live beetles were required, emitted volatiles were collected by aerating beetles in groups (Rudinsky et al. 1973; Gries et al. 1992; Pureswaran et al. 2000).

With the development of capillary column GC and coupled GC-mass spectrometry (MS), it became possible to quantify pheromones in the hindguts of individual beetles. In the first such published study, Birgersson et al. (1984) found significant variation in the amount of pheromone within attacking groups of spruce bark beetles, *Ips typographus* L. A few beetles produced large amounts of pheromone, while most produced relatively little. Similarly skewed distributions have since been reported in the mountain pine beetle *Dendroctonus ponderosae* Hopkins (Borden et al. 1986; Pureswaran et al. 2000; Pureswaran and Borden 2003), the pine engraver *Ips pini* (Say) (Miller et al. 1989; Pureswaran 1999; Pureswaran et al. 2000) and the southern pine beetle *Dendroctonus frontalis* Zimmermann (Grosman et al. 1997; Pureswaran et al. 2006).

Pheromones are generally assumed to have strong fitness effects on tree-killing bark beetles because they potentiate the aggregation behaviour that is necessary to overcome tree defenses and permit beetle reproduction (Raffa and Berryman 1987; Raffa 2001; Aukema and Raffa 2004). The attractiveness of a tree to dispersing beetles increases as the amount of pheromone emanating from it increases (Coster and Gara 1968; Miller et al. 2005a, b). There is evidence that the isomeric composition of pheromone production in bark beetles is under strong genetic control (Hager and Teale 1996; Keeling et al. 2004) and that quantitative variation among individual beetles is not greatly influenced by the rearing environment or the dietary availability of chemical precursors to pheromones (e.g., Birgersson et al. 1988). Thus, pheromone production in tree-killing beetles is apparently heritable, linked to fitness and highly variable among individuals. This is a surprising combination because natural selection tends to shrink the population variance in heritable traits linked to fitness (Fisher 1930; Crow and Kimura 1970).

Spatiotemporal variation in the fitness surface is one general mechanism that can maintain heritable variation in fitness. For example, it has been hypothesised that

populations of *Dendroctonus* spp. are made of alternative pheromone phenotypes that are specialised for being early colonisers of trees (pioneers) or later colonisers of trees (joiners and scroungers), but Pureswaran et al. (2006) tested this for *D. frontalis* and found no relationship between timing of attack and pheromone production phenotype.

Here, we examine two previously untested explanations for the apparent paradox of high variation in pheromone production. We hypothesise that variation among individuals could be (hypothesis 1) an artifact of temporal dynamics in pheromone production or (hypothesis 2) an evolutionary consequence of aggregation behaviour.

Hypothesis 1 questions whether high individual variation is real and not an artifact of extraction procedures. Pheromone release by beetles might be based on fixed pools of previously synthesised pheromones in their hindgut, or pheromones might be released from a dynamic pool of pheromones that continues to be replenished from new biosynthesis within the hindgut. Previous studies of pheromone production by individuals have typically employed hindgut extraction techniques (Birgersson et al. 1984, 1988; Miller et al. 1989), which measure pool size at one point in time and may therefore underestimate the cumulative pheromone production by a beetle in nature and overestimate the variation among individuals. Sullivan (2005) developed a static headspace technique in which individual beetles could be aerated in vials over many days to capture all volatiles being released, thus providing a temporally integrated measurement of pheromone emission. Here, we studied the dynamics of pheromone release by individual beetles and tested whether the variation among beetles was reduced in measurements of lifetime production. Specifically, we compared the mean and variance of pheromone production by female *D. frontalis* as measured by (1) aerating beetles until they died, (2) dissecting and extracting their hindguts and (3) combining the two techniques by aerating beetles for 18 h and then extracting their hindgut.

Hypothesis 2 follows from observing that the pheromone phenotype of aggregating beetles is a group property. All of the beetles colonising a section of host tree can influence the olfactory plume that drifts and diffuses away from the tree to function as pheromones for conspecifics and kairomones for heterospecifics (Wood 1982). Attractiveness of the plume to conspecifics influences the fitness of beetles already present because conspecifics represent potential mates, cooperators in overcoming tree defenses and/or competitors for phloem resources (Reeve et al. 1998). Attractiveness to heterospecifics influences fitness because heterospecifics can be cooperators in tree attack, competitors for phloem resources or natural enemies (Ayres et al. 2001; Raffa 2001). We used the empirical measurements of pheromone production till death by individual beetles to

estimate the effects of an individual on the overall olfactory plume produced by aggregations of different sizes. The premise of hypothesis 2 is that individuals within beetle aggregations have so little effect on the ecologically meaningful phenotype (the olfactory plume emanating from a tree) that there is effectively no heritability, even if the olfactory plume influences the fitness of beetles within the group and pheromone production is under high genetic control within individuals. This follows from the principle that the rate of evolutionary change via natural selection cannot exceed phenotypic variance among individuals in the trait under selection (Fisher 1930).

Materials and methods

Study system

The southern pine beetle, *D. frontalis*, is a major killer of pines in the southern USA (Drooz 1985; Price et al. 1998). Five main pheromones mediate aggregation and antiaggregation behaviour. When a female lands on a tree, she initiates a gallery within the phloem and begins to emit the aggregation pheromone frontalin, which is attractive to both sexes, and *trans*-verbenol, which synergises the attraction of frontalin (Payne et al. 1978). The male joins his eventual mate within the gallery (probably within hours of the female's arrival) and begins to release *endo*-brevicomin (Pitman et al. 1969), which further facilitates aggregation (Vité et al. 1985) but might also promote the initiation of attacks on adjacent trees (Sullivan, unpublished data). A few days later in the attack progression on a tree, both sexes release the antiaggregation pheromones verbenone (predominantly males) and myrtenol, which tend to reduce the final density of beetle attacks (and intraspecific competition within the phloem; Payne et al. 1978).

Treatment of beetles

Four loblolly pine trees, *Pinus taeda* L. were felled in the Homochitto National Forest in Mississippi. Trees were ~20 cm DBH; two were infested with *D. frontalis* and two were not. Bolts were transported to the US Forest Service Research Station, Pineville, LA, and stored at 4°C until used in experiments (1–7 days). Infested bolts were placed in cages and beetles were used in experiments as they emerged. Females that emerged in the past 24 h were either used immediately or allowed to bore into an uninfested bolt for 24 h. These beetles were either (1) aerated in vials until they died (up to 5 days), (2) subjected to hindgut extractions or (3) aerated for 18 h before hindgut extractions (a combination of the above two techniques). Fifteen females were used for each treatment.

Aerations of individual beetles in vials

The tips of 100 µl conical glass vials were filled with 2 mm (approximately 0.3 mg) of adsorbent Super Q® (80–100 mesh) (Sullivan 2005). Individual beetles were inserted abdomen-first and confined to the bottom of the vials using perfluoroalkoxy (PFA) tubing so that the tip of the abdomen was 1–2 mm from the adsorbent. The mouths of the vials were loosely closed with a PTFE-lined cap to allow respiration. Volatiles released from the beetles were passively collected on the adsorbent Super Q® at room temperature until they died (~5 days). A few beetles that died in less than 5 days were excluded from further analysis. Vials were maintained in a stream of purified, humidified air. Once aerations were complete, beetles and PFA tubing were removed. Fifty microliters of redistilled pentane spiked with 3.5 ng/µl heptyl acetate was added to the adsorbent and allowed to sit for 15 min at room temperature. The supernatant was pipetted out, transferred to another vial, and stored at –80°C.

Hindgut extractions

Hindguts of beetles were dissected (Sullivan 2005) using sharp forceps and placed in 50 µl of hexane containing 3.5 ng/µl (175 ng) heptyl acetate as an internal standard. The samples were incubated for 30 min and the supernatant was transferred to new vials. Samples were frozen at –80°C until processed.

Determination of experimental variation due to conical vial method

One hundred microliters of a pheromone solution (1/100 by volume of frontalin, *endo*-brevicomin, *exo*-brevicomin, *trans*-verbenol, verbenone, and myrtenol in pharmacy-grade mineral oil) was placed in the bottom of a conical-base vial (5 ml capacity, 5.5 cm long, bottom 1.5 cm tapered, id 12 mm). A platform of Teflon screening was secured inside the mouth of the vial with a ring of PFA pipe. Pieces of silicone tubing (3 mm long, 2.16 mm od, 1.02 mm id, 0.58 wall, VWRbrand “Select Silicone”) were placed in a vertical orientation (axis = up/down) on the screening such that they touched one another but did not press. The vial was capped and held at room temperature for 7 days. At that time, the vial was opened and a single piece of tubing was placed into each of 13 freshly prepared conical vials (as per Sullivan 2005). A piece of 1/16-in Teflon pipe was placed above each piece of silicone tubing, and then each vial was capped loosely with a Teflon-lined septa-top and placed into a continuous stream of charcoal-filtered, humidified air. After 24 h, the vials were extracted with 50 µl pentane spiked with 3.5 ng/µl heptyl acetate. To

each vial was added ~50 µl of hexane to slow evaporation, and then each sample was analyzed by GC-MS.

Gas chromatography-mass spectrometry

Samples were analysed on an Agilent 6890-5973 coupled GC-MS detector using an HP-INNOWax (Agilent Technologies; 60 m×0.25 mm×0.25 µm film) column. The temperature program was 40°C for 1 min, 16°C/min to 80°C, then 7°C per min to 230°C and held for 10 min. Carrier gas (helium) flow was constant at 1.0 ml/min (26 cm/s), and the injector and detector ports were held at 200 and 240°C, respectively. Pheromone amounts were quantified by abundance of diagnostic ions relative to the internal standard, using MSD ChemStation Standalone software (G1701DA version D.00.00.38, Agilent Technologies© 1989–2001). Identities of compounds were confirmed by comparing retention times and mass spectra with known standards. A standard solution containing 1 ng/µl of each of the five pheromones of *D. frontalis* was run 18 times on the GC-MS to estimate measurement error of the instrument. The amounts of four major pheromones of female *D. frontalis*, frontalin, *trans*-verbenol, verbenone and myrtenol (Payne et al. 1978) were quantified with a standard curve calculated from injections of variable, known concentrations of pheromone standards.

Statistical analyses

Pheromone amounts were transformed by $\log_{10}(x+0.01)$ nanograms to improve normality and homoscedasticity. We used multivariate analysis of variance to test for differences in pheromone amounts related to (1) stage of attack (emerged vs in log), (2) extraction technique (aeration vs hindgut extraction vs the total of a combination of the two techniques) and (3) interactions between stage of attack and techniques. We used a one-way ANOVA followed by Ryan-Einot-Gabriel-Welsch multiple comparisons (Day and Quinn 1989; Reeve and Strom 2004) to evaluate possible differences in pheromone amounts among extraction techniques within each stage.

We used Student's *t* tests to compare pheromone amounts for recently emerged beetles vs mining beetles in the combination treatment (treatment 3) and for 18-h aerations vs hindgut extractions within a stage of attack. The CV (CV=100 SD/mean) of untransformed quantities of the four pheromones in standard runs and samples were calculated. We tested for patterns in the CV with an ANOVA model that included stage of attack, extraction technique, and pheromone. All analyses were performed using SAS Institute Inc.© version 8.2 (SAS 1999–2001) statistical software, and $\alpha=0.05$.

Simulations

We used measurements of pheromone production by individual beetles (Pureswaran et al. 2006; this study) to characterise the expected quantitative properties of pheromone plumes produced by beetle aggregations of different sizes. A pheromone plume produced by *D. frontalis* could be characterised in terms of the amount of each of the five pheromones, but productions of different pheromones were correlated, so 66% of the total variation in pheromone production by pairs of beetles was captured in the first two components of a principal components analysis (Table 1). The first component, which was largely driven by female contributions, reflected a tendency for pairs that produced more *trans*-verbenol to also produce more myrtenol but less frontalin (see loadings on PC1; Table 1). The second component, which was largely driven by male contributions, reflected positive covariation in the production of verbenone and *endo*-brevicomin.

We simulated the effects of individual beetles on the composite pheromone plume of aggregations of variable size by drawing replicated random samples of *n* individuals (with replacement) from the pool of beetles for which we measured pheromone production (Table 1). Following the addition of each male or female beetle in each simulated aggregation, we calculated the total amount of each pheromone that would be produced by the hypothetical group and estimated the composite quantitative properties of the plume in units of PC1 and PC2 (using the means, standard deviations, and coefficients from beetles pairs in Table 1). Effects of each individual on the plume were quantified as the Euclidian distance that the plume moved in PC1–PC2 space (Fig. 1) following the addition of the individual. We then estimated the average relative effect of one beetle individual in each replicate composite pheromone plume as

$$\Delta P = \left[\sum_{i=2}^n \frac{d_i}{S_{i-1}} \right] / (n-1) \quad (1)$$

where per capita change in the plume, ΔP , represents the average change (d_i = Euclidian distance) in the pheromone plume of a growing aggregation before and after the addition of the next individual *i*, standardised by the previous plume state (S_{i-1} = Euclidian distance to origin), up to a total aggregation size of *n*. Each replicate aggregation yielded one estimate of the average ΔP given *n*. We conducted 10,000 simulations for each aggregation size and calculated the average ΔP (which was stable to <2%). We solved Eq. 1 for both males and females (assuming that pairings are random with respect to

Table 1 Principal components analysis of pheromone production by *D. frontalis* females, males, and randomly assembled pairs

Pheromone	Female <i>D. frontalis</i> (n=28)			Male <i>D. frontalis</i> (n=59)			Pairs of <i>D. frontalis</i> (n=1,652)		
	Coefficient		ng/female (mean±SD)	Coefficient		ng/male (mean±SD)	Coefficient		ng/pair (mean±SD)
	PC1	PC2		PC1	PC2		PC1	PC2	
Frontalin	-0.34	0.65	134±205			0.11±0.31	-0.39	0.06	134±201
<i>trans</i> -Verbenol	0.64	-0.07	2,769±3,232	0.20	0.82	17.7±46.7	0.66	-0.05	2,786±3,175
Verbenone	0.27	0.75	3.90±2.50	0.64	-0.03	293±763	0.05	0.71	297±757
Myrtenol	0.63	0.11	430±616	0.64	0.11	33.5±53.6	0.64	0.01	464±607
<i>endo</i> -Brevicomin			0±0	0.38	-0.56	1.62±4.86	0.03	0.70	1.62±4.82
Eigenvalue	2.09	1.09		1.89	1.07		2.01	1.31	
% Variance	52	27		47	27		40	26	

Female data from this study (Fig. 2, combination). Male data from Pureswaran et al. (2006)

pheromone phenotype and that groups grow by the additions of pairs).

To evaluate ΔP at natural group sizes for *D. frontalis*, we applied measurements of adult attack densities in 182 infested trees (from two to five trees within each of five sites within each of three national forests in each of one to two summers; Hofstetter et al. 2006); average tree-specific attack density ($\pm$ SD) = 5.9 ± 2.0 attacking adult pairs/decimeter squared. *Dendroctonus frontalis* typically attack

trees quite synchronously (e.g., 7–10 days; Coulson 1978) over the mid-bole of host trees ($\approx 6 \text{ m}^2$ of surface area in a tree that is 25 cm DBH and 22 m tall with a height to live crown of 11 m). Therefore, we conservatively estimated that the effective aggregation size that contributes to a pheromone plume comes from 1 m^2 of bole area, yielding an average group size of about 600 attacking adult pairs/ m^2 . We weighted the frequencies of plots in the forest by the number of beetle pairs within these plots to yield the frequency distribution of densities experienced by individuals (total of $n=2,219$ attacks).

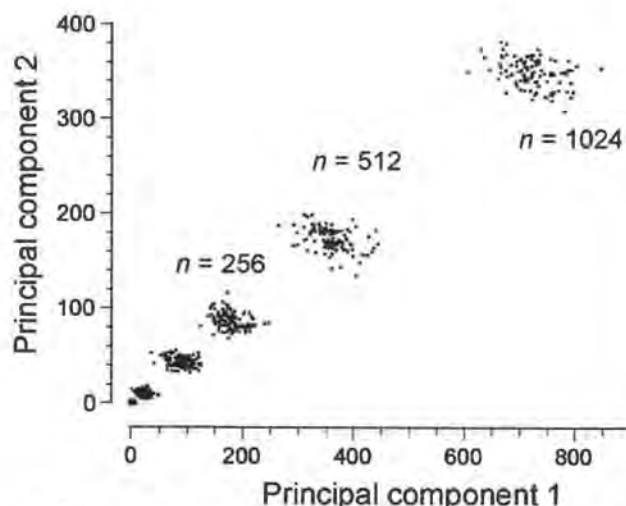


Fig. 1 Composite properties of pheromone plumes (as defined by components 1 and 2 of PCA) that would be produced by different-sized aggregations of *D. frontalis*. Each point represents one simulated aggregation (200 random replicate points/aggregation size). Clusters from left to right correspond to aggregation sizes of 2, 32, 64, 128, 256, 512, and 1,024 beetle pairs

Results

Effect of technique on total pheromone extraction, pheromone dynamics and individual variation

There were significant effects of stage of attack and extraction technique on the amount of pheromone we detected (Table 2, Fig. 2). As hypothesised, hindgut extraction alone yielded significantly lower amounts of pheromone than aerations in some cases (Fig. 2a,b). When the techniques were combined, and beetles were aerated for 18 h followed by immediate hindgut extraction, more pheromone was obtained from the hindgut than the short-term 18-h aeration (Fig. 2e–h). The amount of pheromone detected by aerating beetles until death was not usually different from the total pheromone measured by combining hindgut extraction with an 18-h aeration (Fig. 2a,b,d).

Frontalin, the key aggregation pheromone of females (Payne et al. 1978) was produced in lower amounts in freshly emerged beetles than in beetles that were boring in the log (Fig. 2a,c), while *trans*-verbenol, which synergises

Table 2 Effects of stage of attack and pheromone extraction technique on the amount of pheromone detected in female *D. frontalis*

	F-statistics				
	Frontalin	<i>trans</i> -Verbenol	Verbenone	Myrtenol	MANOVA
Stage					
Emergence vs in log	9.00**	68.78***	5.82*	49.73***	14.74***
Technique					
Aeration vs hindgut extraction vs combination	12.03***	1.83	23.59***	1.74	8.72***
Interaction					
Stage $\times$ technique	1.37	0.42	2.17	0.89	4.19***

Results of multivariate analysis of variance (df=5, 81)

MANOVA multivariate analysis of variance

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

the attraction of frontalin (Payne et al. 1978), exhibited the reverse trend (Fig. 2b,f). Freshly emerged females emitted more frontalin in their hindguts than they contained but were already actively emitting as much *trans*-verbenol as they contained in their hindgut (Fig. 2a,b). Once they started feeding on phloem, the amount of emitted frontalin increased over three-fold, while the amount of *trans*-verbenol that was emitted decreased to <10% of that from emerged beetles (Fig. 2a,b). Myrtenol, like *trans*-verbenol, declined sharply following 1 day of feeding (Fig. 2d,h). Myrtenol and *trans*-verbenol were found in quantities that were ~5–15 times higher than frontalin and verbenone (Fig. 2).

Pheromone variation among individual beetles was very high and generally unrelated to pheromone amounts, chemistry, or function: average coefficient of variation $\pm$ SD = $120 \pm 40\%$ (Table 3). Although the absolute amounts varied greatly among pheromones, the CV were indistinguishable (Table 3, $F_{3,17}=0.92$, $P=0.45$). CVs averaged somewhat higher with the hindgut extraction technique compared to aerations or the combination (mean $\pm$ SE=153 $\pm$ 10 vs 100–106 $\pm$ 10%; $F_{2,17}=7.56$, $P=0.004$). Thus, there was a signal of reduced variation from the temporal integration of pheromone emission in aerations (as hypothesised), but the variation among individuals was still 50–100 fold greater than the analytical variation among replicate analyses of synthetic standards with the same instruments and ~10-fold greater than the conical vial aeration technique on beetle-sized silicone tubing that emitted adsorbed pheromone (Table 3). CVs were somewhat higher among beetles that had been feeding for 1 day within a log compared to those that had not yet entered a log (mean $\pm$ SE=136 $\pm$ 8 vs 103 $\pm$ 8%; $F_{1,17}=7.20$, $P=0.02$).

Beetle individuals producing relatively more of one pheromone also tended to produce more of the other pheromones: average correlation coefficient (mean $r \pm$ SE) =

0.42 ± 0.07 ($n=36$; six experimental combinations $\times$ six pairwise combinations of pheromones). However, the correlation structure differed depending upon the attack stage and the pheromone combination. The correlation between myrtenol and *trans*-verbenol was always strongly positive ($r=0.77$ to 0.94 across six experiments with $n = 13 - 16$ females per experiment), as was the correlation between myrtenol and verbenone (mean $r \pm$ SE = 0.51 ± 0.08) and verbenone and *trans*-verbenol (mean $r \pm$ SE = 0.47 ± 0.10). The correlations involving frontalin were also strongly positive among beetles that had just emerged (mean $r \pm$ SE = 0.73 ± 0.07 , 0.66 ± 0.14 , and 0.57 ± 0.17 for frontalin vs myrtenol, *trans*-verbenol, and verbenone, respectively), but declined or even became weakly negative among females that had been in a log for 1 day: mean $r \pm$ SE = -0.22 ± 0.02 , -0.27 ± 0.21 , and 0.33 ± 0.12 , respectively. There was therefore no effect of extraction technique on the correlation structure.

Simulations

When characterised by the first two components of a principal components analysis, the average properties of a pheromone plume change linearly as the size of the group contributing to the plume increases (Fig. 1); this reflects the increase in plume size with increasing group size. However, when individual beetles are as variable as *D. frontalis*, there is still considerable variance among replicate assemblages drawn randomly from the same population; for group sizes of eight pairs or more, the variance/mean was constant at ≈ 2.5 for PC1 and PC2. Consequently, the CV among possible random groupings (100 SD/mean) decreased from 130 to 50 to 25 to 12 as group size increased from 4 pairs to 16, 64, or 256 pairs.

The average effect of one beetle on a pheromone plume (ΔP from Eq. (1)) decreased greatly as the group size

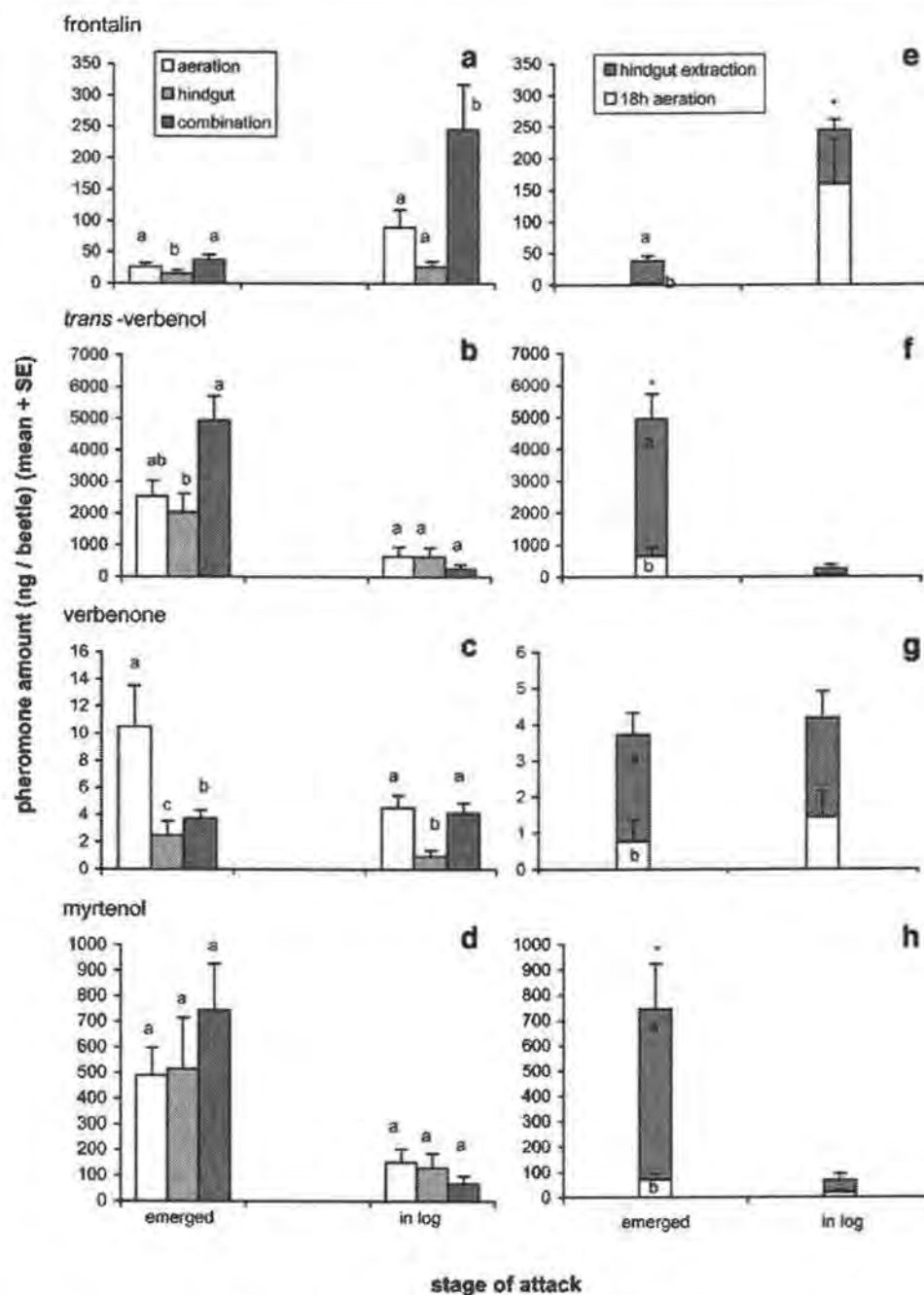


Fig. 2 Amount of pheromone detected in female *D. frontalis* from two stages of attack, using three pheromone collection techniques. Graphs **a**, **b**, **c** and **d** compare three techniques within each stage of attack. Bars with the same letter within a stage are not significantly different, ANOVA followed by Ryan-Einot-Gabriel-Welsch multiple comparisons test, $P < 0.001$. Graphs **e**, **f**, **g** and **h** compare two stages of attack

using a combination of two treatments. Stacked bars represent relative amounts captured by each technique within a stage. Asterisks indicate significant difference in total combined amount of the two techniques between stages. Letters within bars indicate difference in amounts between 18-h aeration and hindgut extraction within a stage. Bars with the same letter are not significantly different, Student's *t* test, $P < 0.001$

increased ($\approx$ one order of magnitude decrease as n increased from 2 to 64 pairs, and nearly another order of magnitude decrease from $n=64$ to 1,000 pairs) (Fig. 3, lower). At the modal group size of 600 pairs per meter squared, the

average effect of one beetle on the growing plume was only 1.8% for females and 0.8% for males (Fig. 3, upper). Even with a low group size of 300 pairs, the average effect was only 4.6 or 2.1% for males and females, respectively.

Table 3 CV (standard deviation/mean) in pheromone amounts due to measurement error of synthetic standards, experimental variation due to conical vial technique and in female *D. frontalis* from two stages of attack and three collection techniques analysed by GC-MS

Pheromone	Technique	Coefficient of variation (%)			
		Frontalin	<i>trans</i> -Verbenol	Verbenone	Myrtenol
Stage of attack	Analysis of synthetic standards on GC-MS	2	2	1	1
	Conical vial aeration of adsorbed synthetic standards emitted from silicone tubing ^a	11	10	11	12
Emerged	Aeration	89	70	109	83
	Hindgut extraction	158	115	161	152
	Combination	83	61	66	95
In Log	Aeration	114	174	82	128
	Hindgut extraction	127	181	159	168
	Combination	107	170	64	156

^a *exo*- and *endo*-brevicomin CV=10%

Discussion

Effect of technique on total pheromone extraction, pheromone dynamics and individual variation

Aerating beetles till they die seems to provide the best estimates of lifetime pheromone production in the laboratory. The increase in release of frontalin by feeding (Fig. 2a,e) may explain the apparent anomaly reported by Coster and Vité (1972), who found that 48 h of feeding by females reduced the amount of frontalin in their hindguts, despite a corresponding increase of attractiveness of such attacks. In feeding beetles compared to freshly emerged beetles, preformed frontalin in the hindgut represented a much smaller portion of total frontalin production (i.e. hindgut content plus amount released). Thus, data from hindgut extractions alone would have failed to detect the increase in frontalin in response to feeding.

While frontalin production by adult females increased following 1 day of feeding, *trans*-verbenol production declined to <10% of that by freshly emerged females (Fig. 2a,b,e,f). This is in accord with Pureswaran et al. (2006), who found ~100 times more *trans*-verbenol than frontalin in hindgut extracts of females that were landing on trees compared to those that had entered their tree the day before (see also Coster and Vité 1972). Previous studies have indicated that *trans*-verbenol and the host monoterpene α -pinene both function as attractive synergists for *D. frontalis*, and that *trans*-verbenol may function as a surrogate for host odours during the initial stages of attack when resin exudation from the host is still low or absent (Renwick and Vité 1969, 1970; Payne et al. 1978). It appears that beetles down-regulate the production of *trans*-verbenol once host tissues are damaged and α -pinene starts emanating from the host.

The decline of myrtenol following feeding (Fig. 2d,h) was surprising because myrtenol has been described as an antiaggregation pheromone (Rudinsky et al. 1974) and would therefore be expected to increase during the attack progression. It could be that myrtenol and *trans*-verbenol have similar production dynamics because of overlap in the biochemical pathways. Both myrtenol and *trans*-verbenol are direct oxygenated and detoxified derivatives of the host precursor α -pinene (Hughes 1973, 1975; Renwick et al. 1976). Frontalin is synthesised de novo by *Dendroctonus* spp. (Barkawi et al. 2003) and might therefore be more expensive to produce than pheromones that are directly derived from host precursors (Pureswaran et al. 2006).

The tendency for positive correlations suggests common metabolic or genetic controls on the production of multiple pheromones and argues against the alternative hypothesis of trade-offs between production of different pheromones (Pureswaran 1999; Pureswaran et al. 2006). We also established that there was only minimal experimental variation due to the conical vial aeration technique and measurement error of the instrument (<12%) (Table 3). The above results confirm the existence of high individual variation in pheromone production and leave unresolved how and why it persists in populations of tree-killing bark beetles.

Reduced efficacy of natural selection due to aggregation behaviour?

Pheromone production by individuals could be nearly invisible to selection if group sizes are large enough such that each individual beetle has little effect on the plume produced by the group. Thus, at natural group sizes, individual beetles have quite little effect on the phenotype (pheromone plume) that is assumed to influence the fitness

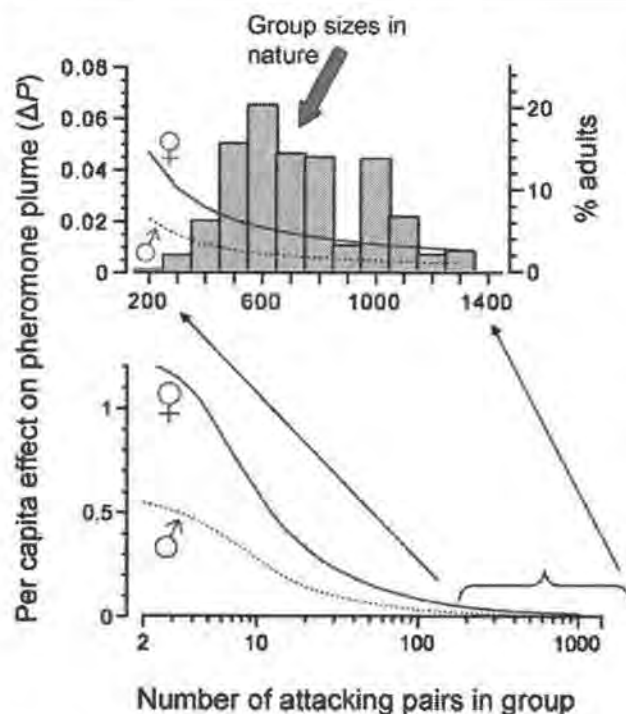


Fig. 3 Lower: Average proportional effect of one male or female beetle on the composite pheromone plume from groups of increasing size (Eq. 1). Upper: Closeup of lower figure for the range of aggregation sizes in natural attacks of pine trees by *D. frontalis* (represented by histogram)

of beetles within the group. Following Fisher's fundamental theorem of natural selection (Fisher 1930; Crow 1958; Arnold and Wade 1984), the strength of natural selection decreases as the genetic variance in fitness decreases. Therefore, even with perfect genetic control of pheromone production, the potential for natural selection on pheromone production quantity is greatly reduced in this system because individual beetles have little effect on the group property that influences fitness. As a result, the balance between selection and mutation would be expected to shift away from the variance-reducing effects of stabilizing natural selection and towards the variance-enhancing effects of mutation. That is, the high variance among individuals in pheromone production could be the result of aggregation behaviour that limits the potential for selection on attributes of individuals (supports hypothesis 2).

For two reasons, the actual potential for selection to operate on individual contributions to the pheromone plume must be even less than that indicated in Fig. 3. Equation 1 assumes that the group grows one pair of beetles at a time, which means that early-arriving beetles, which join a smaller group, can have a relatively large effect on the composite phenotype (by influencing the average pheromone phenotype of beetles that subsequently join the

aggregation and contribute to the growing pheromone plume). In fact, many beetles join attacks at about the same time, which reduces the potential for their pheromone phenotype to influence the plume of the full eventual group. If Eq. 1 is adjusted to represent synchronous arrival of beetles (S_{i-1} becomes S_n), maximum ΔP becomes vanishingly small for natural group sizes (<0.003). Finally, the genetic control of pheromone production cannot be perfect, although it may be high (e.g., Hager and Teale 1996 estimated heritability $h^2=0.82$ for the enantiomeric composition of ipsdienol in male *I. pini*). In our study, environmental (nongenetic) effects on pheromone production were deliberately minimised by drawing beetles from the same host trees.

Females influence the pheromone plume more than males. Regardless of group size, the average effect of one female was 2.2 times greater than that of one male (Fig. 3). This was by virtue of females producing about twice as much total pheromone as males (Table 1). The prediction under hypothesis 2 is that males would therefore be more variable in their pheromone production, and indeed this was conspicuously true: mean $CV \pm SE = 256 \pm 11$ vs $119 \pm 10\%$ for males and females, respectively, $t_7=4.10$, $P=0.005$.

The above results do not diminish the importance of pheromone plumes as a determinant of fitness for *D. frontalis*. In fact, surveys of natural attacks have revealed striking variation in fitness among beetles that attack adjacent trees and suggest that this variation is at least partly a function of tree-specific pheromone plumes. Across 189 trees (from 45 local infestations within three national forests during 3 years), 39% of the variance in per capita fitness, $\ln$ (progeny/adult), was attributable to trees within local infestations (Hofstetter 2004). Tree-specific fitness ranged from 0.70 progeny/adult at the lower decile to 5.8 progeny/adult at the upper decile. Furthermore, tree-specific fitness was related to tree-specific attack densities by *D. frontalis* adults ($r=-0.48$), which are influenced by tree-specific pheromone plumes.

Alternative hypotheses to explain high variability in pheromone production

The most attractive alternative hypothesis to explain high variance among individuals is that there are variable costs and benefits of pheromone production that yield variable optima: e.g., selection might favor a mixed strategy in which a fraction of the population persists as "cheaters" that produce little or no pheromone themselves and depend upon the pheromones of conspecifics for their reproductive success. In this case, variance would be a product of natural selection rather than a consequence of reduced efficacy of natural selection. The difficulty for this hypothesis is justifying the assumption that there are meaningful costs to pheromone production in *D. frontalis* because (1) larger

beetles do not produce more pheromones (Pureswaran et al. 2006), as would be expected if there are energetic constraints; (2) the production of different pheromones is correlated positively, not negatively, as expected if different pheromone pathways compete for limited resources; and (3) the amounts of pheromone per animal are so small that they seem unlikely to incur energetic costs (≈ 0.02 or 0.18% of body mass in males and females, respectively; given that adult fresh mass = 1.84 mg, Ayres et al. 2000). If pheromones are toxic, they would be costly in the sense that moose antlers are costly, but the handicap principle (Zahavi and Zahavi 1997) seems an unlikely explanation here because pheromones are probably not as toxic as host monoterpenes (White et al. 1980; Seybold et al. 2006), and beetle larvae consume ≈ 230 -fold more monoterpenes than the total pheromones they emit as adults (assuming consumption of 5.7 mg of phloem that is 1% monoterpenes; Ayres et al. 2000; Hofstetter et al. 2005). Furthermore, beetle adults commonly spend hours to days of their limited lifespan moiling within pools of oleoresin that flow from the vertical resin ducts within host trees.

Another adaptive model for the maintenance of variation invokes temporal variability in the relationship between fitness and pheromone production. *Dendroctonus frontalis* populations fluctuate between outbreaks and crashes (Turchin 1991; Reeve 1997). Almost all research has been devoted to outbreak populations. Presumably, group sizes tend to be smaller when populations are at endemic levels following crashes, which would enhance the potential for selection on individual pheromone production (Fig. 3), and presumably select for higher pheromone production because small numbers of attacking beetles are at greater peril from tree defenses. Thus, selection for high pheromone production may be episodically associated with periods of low population size. Still, this mechanism does not readily explain the maintenance of variation unless there is a cost to pheromone production that selects for lower pheromone production during periods of relatively high abundance.

The absence of appreciable costs to pheromone production precludes a large class of possible adaptive explanations for the maintenance of variation. However, not all possible costs have yet been evaluated. For example, there could be costs to individuals of relatively high pheromone production if clerid predators preferentially hunt or oviposit on a scale of centimeters within trees based on the pheromones being emitted from the galleries of individual pairs of beetles. Also, high pheromone production might be favoured in early-arriving beetles (when there is a premium on overcoming tree defenses) and disfavoured in later-arriving beetles (when the more likely problem is attracting too many competitors or predators); this potential explanation for the maintenance of variation is weakened but not eliminated by the result that there is no difference in

pheromone production between beetles that arrive early vs late on a given host tree (Pureswaran et al. 2006). Further research is required to evaluate the relationship between pheromone quantity and actual attraction across relevant emitted concentrations, and whether beetle pairs within trees experience predictable differences in fitness based on their pheromone production.

Conclusions

The maintenance of high variation in pheromone production by *D. frontalis* can be parsimoniously understood as due to the accumulation of genetic variation by mutation and recombination in the absence of efficient natural selection on individuals. That is, the pheromone produced by an individual has such a small effect on the phenotype that is expressed in nature (i.e. the aggregate plume) that pheromone production is essentially a neutral trait, even if pheromone production has high genetic variance and the properties of the aggregate plume have a strong effect on the fitness of group members. If this is true, it becomes more difficult to understand the evolution of what appear to be complex and highly functional systems of pheromone-mediated aggregation in nature.

If there is high variance in *D. frontalis* pheromone production because large aggregation sizes limit the efficacy of selection, then similar species that aggregate less should have lower variation in pheromone production among individuals. This could be tested by comparisons with species that also produce aggregation pheromones, but typically occur in smaller groups than *D. frontalis*, for example spruce beetles (*D. rufipennis*; Wallin and Raffa 2004) or pine engraver beetles (*Ips* spp.; Steed and Wagner 2004). Schlyter and Birgersson (1989) provide evidence that solitary callers like moths vary less (meanCV $\pm$ SD = $69\% \pm 20$, $n = 15$) compared to bark beetle systems (meanCV $\pm$ SD = $119\% \pm 32$, $n = 9$). Figure 3 suggests quantitative, falsifiable predictions for how the variance in pheromone production should decrease as typical aggregation sizes become smaller.

This paper presents a novel example of how aggregation behaviour can influence the evolution of ecologically important traits. Traits such as pheromone production, which yield a composite phenotype for the group, apparently permit the survival of genotypes that might otherwise be eliminated by natural selection (Dugatkin et al. 2005), thereby increasing diversity and contributing to polymorphisms in nature. It should be generally true that the potential efficacy of natural selection is diminished whenever the effective phenotype is an emergent property of the group. This theory resembles some earlier conceptions of

how selection acts on groups (e.g., “group selection” sensu Wilson 1975) in that it emphasises shared fitness within groups based on emergent properties of groups. However, group selection scenarios have frequently highlighted the enhanced potential for natural selection when populations are aggregated into demes with linked fitness, while our model argues for a diminished role of natural selection with increasing aggregation.

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