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SHORT COMMUNICATION

Antennally mediated negative feedback regulation of pheromone production in the pine engraver beetle, *Ips pini*

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Abstract Bark beetles use monoterpenoid aggregation pheromones to coordinate host colonization and mating. These chemical signals are produced de novo in midgut cells via the mevalonate pathway, and pheromone production may be regulated by a negative feedback system mediated through the antennae. In this study, we explored the effect of antennectomy on pheromone production and transcript levels of key mevalonate pathway genes in juvenile hormone III-treated male pine engraver beetles, *Ips pini* (Say). Antennectomized males produced significantly greater amounts of pheromone than podectomized males and those with intact antennae. Likewise, mRNA levels of three mevalonate pathway genes important in pheromone biosynthesis were measured by quantitative real-time PCR and found to be induced to a greater extent with antennectomy, suggesting a transcriptional regulation of pheromone production.

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Introduction

The pine engraver *Ips pini* (Say) and a number of other bark beetles produce monoterpenoid pheromone components that coordinate host colonization and mating. These chemical signals are derived de novo via the mevalonate pathway, and pheromone production is induced when an adult beetle feeds on host phloem (Seybold et al. 1995; Tillman et al. 1998). These pioneer beetles, however, are faced with selection pressure to maximize the benefits associated with chemical communication while minimizing the risks (Raffa 2001). For example, pioneer beetles must attract sufficient numbers of conspecifics for mating and overcoming host defense, while minimizing intraspecific competition and undue chemically mediated predation and parasitism of their progeny. Bark beetles may optimize this cost/benefit of chemical communication through a tightly regulated negative feedback system, which is likely mediated through chemoreceptors on the antennae. In fact, there is evidence for such a negative feedback system in the Colorado potato beetle and the cotton boll weevil (Dickens et al. 1988, 2002). In both cases, antennectomized males produced significantly greater amounts of pheromone over those with intact antennae (Dickens et al. 1988, 2002). Although treatment with a juvenile hormone (JH) analog (methoprene) in the boll weevil (Dickens et al. 1988) and JH III alone in the Colorado potato beetle (Dickens et al. 2002) increased pheromone production suggesting these systems are also

under hormonal control, there was a synergistic effect of hormone treatment and antennectomy.

In *I. pini*, the male-produced aggregation pheromone is a blend of ipsdienol and lanierone (Vité et al. 1972; Birch et al. 1980; Seybold et al. 1995). Moreover, pheromone production is positively influenced by a developmental program that primes males for rapid synthesis and by a direct induction of mevalonate pathway genes by JH III (Bearfield et al. 2006; Gilg et al. 2005; Keeling et al. 2004, 2006). It appears that in *I. pini*, feeding on host phloem stimulates JH III synthesis by the *corpora allata*, which, in turn, increases transcript levels of all mevalonate pathway genes, resulting in increased pheromone production (Tillman et al. 2004; Keeling et al. 2004).

In this study, we tested the hypothesis that pheromone production in *I. pini* is modulated by a negative feedback system mediated through the antennae and that antennectomy will increase pheromone production and transcription of key mevalonate pathway genes. Accordingly, we quantified the amount of pheromone produced by intact antennectomized and podectomized males, and then further examined the effect of antennectomy on the relative expression of three mevalonate pathway genes.

Materials and methods

Source of beetles Bolts of Jeffrey pine (*Pinus jeffreyi* Grev. and Balf.) infested with immature *I. pini* were collected from the University of Nevada Whittell Forest, NV and from within the Lake Tahoe Basin Management Unit, South Lake Tahoe, CA and reared according to Browne (1972). Emerged beetles were typed, sexed, and stored at 4°C until used in experiments.

Influence of antennectomy on pheromone production To determine the influence of antennectomy on pheromone production, 25 male *I. pini* were antennectomized under a stereo microscope using sharpened no. 5 forceps. The hind right tarsal segment was also removed from another 25 males to control for any effects due to stress from extirpation, and 25 males were left intact (control). After 1 h, we applied 10 μg of racemic JH III (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 μl of acetone to the abdominal venter of all males in each treatment group as per Seybold et al. (1995). The beetles were then grouped by treatment, placed in 60 ml plastic cups (Dixie, Georgia-Pacific, Atlanta, GA), five beetles per container, and kept in the dark at room temperature for 16 h. We then quantified the amount of pheromone (ipsdienol) produced by males of each treatment group. Each group of five beetles was homogenized with a Teflon pestle for ~30 s in 500 μl of pentane spiked with 20 ng/μl of octanol as an internal standard. The extracts were analyzed by gas chromatography using an HP 5890 Series II gas chromatograph, with an HP-WAX (cross-linked polyethylene glycol) capillary column (60 m×0.25 mm×0.25 μm film thickness) in splitless mode (30 s) with helium as the carrier gas. Oven temperature was ramped from 50 to 150°C at 5°C/min then 150 to 240°C at 10°C/min with a 10 min hold at 240°C. Injection port temperature was 240°C, and the flame ionization detector was kept at 250°C. The amount of pheromone produced by beetles in each treatment group was compared by one-way ANOVA, followed by Fisher's least significant difference (LSD) test (Sokal and Rohlf 1995).

Analysis of gene expression by quantitative real-time PCR Male beetles with or without antennae were treated with JH III (10 μg in 0.5 μl of acetone) or acetone (0.5 μl) and stored in the dark in 60 ml plastic cups for 16 h (Dixie), with five beetles per cup. After incubation, anterior midguts were excised in water under a stereo microscope, purged of their contents, frozen immediately in liquid nitrogen, and stored at −80°C. Each treatment was replicated six times with each replicate containing five midguts.

Total RNA was prepared from midgut tissue using the RNeasy Mini plant kit (Qiagen, Valencia, CA). First strand cDNA was prepared from ~20% fractions of each preparation using Superscript III RNase H-reverse transcriptase and random hexamer primers (Invitrogen, Carlsbad, CA) and used as template in quantitative real-time (qRT) PCR reactions using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). *I. pini cytoplasmic actin*, which is unresponsive to JH III treatment in midguts (Keeling et al. 2006) was used as an endogenous control gene. Real-time PCR primers were designed for actin (IPG005E02) using a single EST sequence (Eigenheer et al. 2003). Primers for selected mevalonate pathway genes were designed based on sequence information as follows: *HMG-R* (Hall et al. 2002), *HMG-S* (Bearfield et al. 2006), and *GPPS* (Gilg et al. 2005; for primer sequences see Table 1). PCR amplifications were performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Each reaction was prepared in a separate well containing cDNA template, 300 nM of both forward and reverse primers, and SYBR Green PCR Master Mix (Applied Biosystems).

Results

Influence of antennectomy on pheromone production Among JH III-treated males, antennectomized beetles produced significantly greater amounts of ipsdienol than intact males or those missing tarsal segments (one-way ANOVA; $F_{2,\ 14}=23.0$, $P<0.001$; Fig. 1). Interestingly,

Table 1 Primer sequences for real-time PCR

Gene	Forward primer	Reverse primer
Cyto. actin	5′-GCCGTCTTTCCATCAATCGT-3′	5′-TTTTGCTCTGGGCTTCATCAC-3′
HMG-R	5′-CCCTGGGAAGTAATCGTTGCT-3′	5′-TAGGTTTGGTTGGTGGGATTG-3′
HMG-S	5′-TGCAATGACCGGGAAGACA-3′	5′-TTCCTGAGGTTTGATGTTGTAACG-3′
GPPS	5′-CGTGTGCCTCATGGAAATGTT-3′	5′-GGAAGAGAAGGTTGCGGAGAA-3′

there was no difference in the amount of pheromone produced by intact males treated with JH III and those with hind tarsal segments removed and JH III treatment (Fig. 1); providing no evidence that physical stress from extirpation affects pheromone production.

Influence of antennectomy on mRNA expression of mevalonate pathway genes All three mevalonate pathway genes in our analysis were JH-responsive. When compared to hormone-treated males with intact antennae, the relative expression of each significantly increased with antennectomy (*t* test: *HMG-R*, $t=2.72$, $df=5$, $P=0.021$; *HMG-S*, $t=2.10$, $df=5$, $P=0.044$; *GPPS*, $t=2.98$, $df=5$, $P=0.015$) (Fig. 2).

Discussion

In antennectomized male Colorado potato beetles, decreased antennal input resulting from extirpation correlated with increased pheromone production (Dickens et al. 2002). This reduction in antennal input may deprive beetles of information necessary to regulate pheromone production or release (Dickens et al. 2002). Interestingly, there appears to be similar negative feedback regulation of pheromone production in male *I. pini.* Antennectomized males that were JH III-treated produced greater amounts of pheromone than intact males treated with JH III and extirpation of the hind tarsi had no effect. As antennae of *I. pini* contain specialized ipsdienol receptors cells (Mustaparta et al. 1979), feedback regulation is mediated through the antennae and likely occurs via central processing to alter pheromone production. Although the mechanism by which antennal input may affect pheromone production is unclear, it has been suggested that olfactory glomeruli in the antennae are connected via output interneurons to portions of the brain, where neurosecretory cells may control juvenile hormone production by the *corpora allata* (see Dickens et al. 1988). In our study, however, the topical addition of JH may overshadow changes in hormone biosynthesis induced by antennectomy.

JH III treatment after antennectomy also increased mRNA levels of three mevalonate pathway genes important in pheromone production. In antennectomized males, *HMG-R* was the most induced of the genes tested, consistent with it being the most highly regulated step in the mevalonate pathway. Antennectomy also increased transcript levels of *GPPS*, which is an important branch point in the formation of monoterpenoid pheromones. The response of these genes to antennectomy suggests regula-

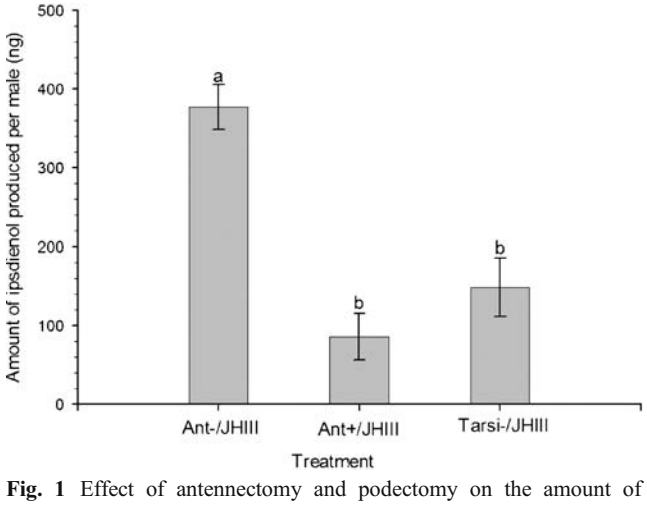

Fig. 1 Effect of antennectomy and podectomy on the amount of ipsdienol extracted per male, 16 h after treatment with 10 μg of JH III. *Bars* marked with *different letters* are significantly different (LSD, $P<0.05$). See text for details of statistical analyses

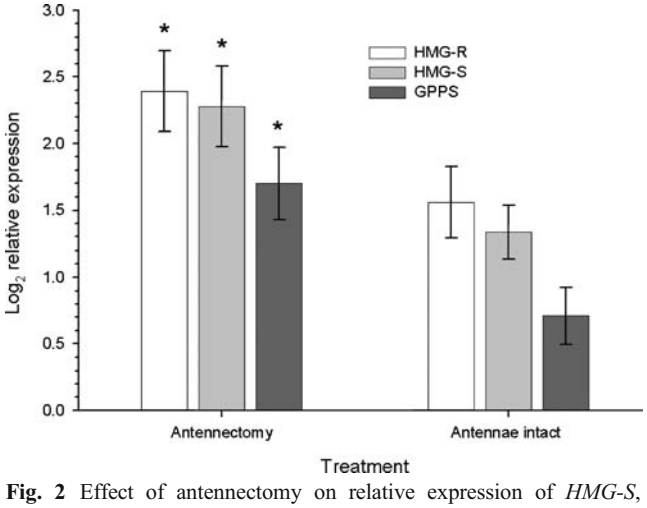

Fig. 2 Effect of antennectomy on relative expression of *HMG-S*, *HMG-R*, and *GPPS* in male midguts as measured by qRT-PCR 16 h after JH III treatment. Expression ($\log_2$) is relative to acetone-treated males for each treatment. An *asterisk* denotes a significant difference in expression in antennectomized beetles relative to those with intact antennae. See text for details of statistical analyses

tion of pheromone production by a negative feedback system occurring at a transcriptional level. In fact, a JH-induced non-mevalonate pathway gene showed no change in mRNA transcript levels with antennectomy (data not shown). We cannot, however, rule out the possibility of further post-transcriptional regulation of pheromone production.

This work provides evidence for negative feedback regulation of pheromone production in bark beetles functioning in addition to developmental and endocrine regulatory schemes. Furthermore, a negative feedback between population density and pheromone production has broad implications on host selection strategies. Although pheromone-mediated aggregation greatly enhances the likelihood of individuals colonizing a host and producing brood, a pioneer may incur fitness costs associated with heightened predation of progeny and increased competition (Raffa 2001). By tightly regulating pheromone production via a negative feedback system, colonizing beetles may more adequately balance selection pressures imposed by such factors as competition and host defense. Larval host condition may also influence the degree to which this system is regulated. For instance, far fewer beetles are required to colonize nutrient-deficient dead hosts than to overcome vigorous living trees (see Raffa 2001). In fact, an aggregation of more than a few conspecifics on a dead host can be detrimental to a pioneer (Raffa 2001); consequently, an even more tightly regulated negative feedback system may be expected.

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