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Calling Behavior of the Cerambycid Beetle *Neoclytus acuminatus acuminatus* (F.)

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Males of the cerambycid beetle Neoclytus acuminatus acuminatus *(F.) assume a body posture, never displayed by females, that appears to be associated with release of an aggregation pheromone: they periodically stop walking and fully extend their front legs, elevating their head and thorax above the substrate. In this article, we demonstrate that this body posture, the "pushup stance," coincides with release of pheromone and that it serves to elevate pheromone glands above the substrate. We also use a pheromone proxy system (sublimation of naphthalene) to demonstrate that the pushup stance increases rates of pheromone dissemination. The pushup stance provides a convenient indictor for studying the role of pheromones in reproductive behavior and facilitating collection of pheromone in the laboratory.*

KEY WORDS: reproductive behavior; pheromone; semiochemical; longhorned beetle.

INTRODUCTION

Insects that communicate through volatile semiochemicals rely on air currents to transport signals between sender and receiver. Viscous and turbulent air boundary layers can hinder dissemination of such volatile compounds (Moore and Crimaldi, 2004). Insects may maximize dissemination of pheromones by assuming body postures that expose pheromone-releasing surfaces to convective air currents above the substrate (Connor

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and Best, 1988). Such "calling" postures have been described for species in many insect orders (see Hardie and Minks, 1999; Greenfield, 2002), but rarely for beetles (but see Leal *et al.*, 1996).

Males of the cerambycid beetle *Neoclytus acuminatus acuminatus* (F.) periodically assume a body posture, never displayed by females, that suggests a calling behavior: they periodically stop walking and fully extend their front legs, elevating their head and thorax (Lacey *et al.*, 2004). In fact, this stereotypical behavior gave impetus to identification of the aggregation pheromone of this species (Lacey *et al.*, 2004). In this article, we demonstrate that the body posture assumed by male *N. a. acuminatus*, hence referred to as the "pushup stance," coincides with release of pheromone and serves to elevate pheromone glands above the substrate. We also demonstrate the functional significance of the pushup stance by testing the hypothesis that it increases dissemination rates of a pheromone proxy.

MATERIALS AND METHODS

Natural History

Neoclytus a. acuminatus, the redheaded ash borer, is endemic to North America. The larvae develop in woody tissues of stressed or dying trees (Linsley, 1964; Solomon, 1995). Adults are present from spring through late summer in the area of our study, apparently do not feed, and live fewer than 16 d (ESL, pers. obs.). Both sexes aggregate on larval hosts and are most active between ~1100 and 1700 h (ESL, pers. obs.). Adult males produce the aggregation pheromone (2*S*,3*S*)-hexanediol, and both sexes are attracted to field traps baited with the synthetic compound (Lacey *et al.*, 2004).

Source of Beetles

Adult *N. a. acuminatus* were reared from larval hosts, green ash (*Fraxinus pennsylvanica* Marshall), that had been felled in July 2004 on the grounds of the University of Illinois at Urbana-Champaign and naturally colonized by beetles. Logs cut from the trees were moved to a $3 \times 2 \times 1$ m rearing cage of window screen in a laboratory room (fluorescent lighting, ~12:12 L:D, ~20°C, ~50% RH) in December 2004. Adults began to emerge in late January 2005 and were caged individually in 0.3 m^3 cylindrical cages of aluminum window screen with plastic Petri dishes at top and bottom. All work with live beetles was conducted in February 2005 under ambient laboratory conditions (see above).

Characterizing the Pushup Stance

During photophase, males alternately walked and rested. Resting males displayed two discrete body positions: body parallel to, and in contact with the substrate (hence referred to as the "resting stance"), or forelegs extended such that head and prothorax were elevated above the substrate and only the tip of the abdomen was in contact with the substrate (the pushup stance). Males usually assumed the pushup stance between 1350 and 1600 h.

We characterized the pushup stance by photographing from the side six males in screen cages that initially were in resting stance, then in pushup stance. We used a protractor to estimate the angle of the long axis of the body relative to the substrate in digital images. We measured the dorsoventral thickness of the prothorax of each male with dial calipers (mean: 1.8 ± 0.28 mm, SD) which provided a reference for estimating the distance between the prosternum and substrate in the digital images. Differences in body angle relative to the substrate, and height of the prothoracic sternum above the substrate were tested with the nonparametric Kruskal-Wallis test (PROC NPAR1WAY; SAS Institute, 2001).

Association between Pushup Stance and Pheromone Release

We evaluated the association between the pushup stance and release of pheromone by sampling individual males by solid-phase microextraction (SPME) analyzed by gas chromatography/mass spectrometry (GC/MS; Millar and Haynes, 1998). We assumed that pheromone glands would be located on the prothorax based on previous research with other cerambycid species (Iwabuchi, 1986; Nakamuta *et al*., 1994; Noldt *et al*., 1995). We sampled the same ten males three times: during resting stance (1100–1350 h photophase), during pushup stance (1350–1600 h), and at least 15 min after resuming resting stance (1615–1800 h). We grasped the male by the abdomen and wiped the dorsal then ventral surfaces of the prothorax five times each with a SPME fiber (100 μm polydimethylsiloxane, #57300-U, Supelco®, Bellefonte, PA). Pressure was exerted on the fiber such that it was in contact with the cuticular surface but was not deflected. The fiber was desorbed in the injection port of a GC-MS (Hewlett-Packard GC model 6890, MSD 5973), with a HP-5MS column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \times \mu$m film) in splitless mode with helium carrier gas. Injection port temperature was 250°C and oven temperature was 50°C for 1 min, ramped at 10°C/min to 110°C, and held for 1 min. We identified pheromone in samples by comparing retention times and mass spectra with those of a synthetic standard of the natural pheromone. Quantification of absolute pheromone

abundance was not possible with these sampling methods, so we compared abundances between samples by integrating peaks in total ion chromatograms and comparing areas under the pheromone peak (calculated with MSD ChemStation, Version B.02.05; Hewlett-Packard, Palo Alto, CA). Areas under pheromone peaks were reproducible between samples and provided an unbiased assessment of relative abundance. Differences between samples in mean pheromone peak area were tested with the Kruskal-Wallis test.

Location of Pheromone Glands

To confirm that the prothorax was the only site of pheromone release, we sampled five body regions of four males that were in pushup stance using SPME analyzed by GC-MS (as above). Body regions were sampled sequentially in the following order: anterior surface of the head, prothoracic tergum and sternum, combined meso- and metathoracic sterna, dorsal surface of elytra, and combined ventral, lateral, and terminal surfaces of the abdomen. Beetles were held by the abdomen when sampling head and thorax, then held by the thorax when sampling elytra and abdomen. After sampling each body region, subject males invariably resumed the pushup stance. Abundances of pheromone were quantified and statistically compared as described above.

We characterized the external morphology of pheromone pores by examining the surface of the prothorax, the only body region consistently associated with production of pheromone (see Results), of both sexes of *N. a. acuminatus* by scanning electron microscopy (SEM). We first soaked one specimen of each sex overnight in a 4% Triton X100 solution (Sigma-Aldrich, St. Louis, MO) to remove dirt and lipids. They were then dehydrated by submerging in 70% and 95% EtOH for 5 min each. Residues of lipids were stripped by soaking specimens in mixed hexanes (Fisher Scientific, Fairlawn, NJ) for at least 2 h and sonicating in hexane for 30 sec. Specimens were allowed to air dry. We attached specimens to aluminum mounts (25.4 mm diameter) with double-sided adhesive carbon discs (25 mm) and secured them with Flash-Dry™ silver paint (all materials from Structure Probe, Inc., West Chester, PA). Specimens were coated with $\sim$6 μm of gold-palladium (Desk II TSC turbo sputter coater, Denton Vacuum, Moorestown, NJ). Mounts were stored in a desiccator. We imaged lateral, ventral, and dorsal surfaces of the prothorax with a Phillips XL30 environmental scanning electron microscope equipped with a field-emission electron gun (FEI Company, Hillsboro, OR) at 5.0 kV.

To confirm that pores in the prothorax of males (see Results) were associated with pheromone glands, we examined cross-sections of

prothoraces of both sexes under light microscopy. A male and female *N. a. acuminatus* were anesthetized with carbon dioxide and their entire prothoraces were excised. Prothoraces were immediately transferred to cold fixative (0.5% paraformaldehyde and 2.5% gluteraldehyde in a buffer of 0.1 M cacodylate, 0.18 mM $CaCl_2$, and 0.58 mM sucrose, pH 7.3). Tissues were refrigerated for three hours at 5°C, rinsed in buffer, transferred to a 2% solution of osmium tetroxide in buffer, and refrigerated overnight. Fixed tissues were rinsed with buffer and dehydrated with serial dilutions of ethanol (50–100% in 10% steps, 15 min each) then subjected to three changes of propylene oxide followed by a 1:1 solution of propylene oxide and Medcast resin (Ted Pella Inc., Redding, CA), then in pure Medcast resin. After agitating overnight, tissues were placed in molds. Cross sections of the prothoraces (anterior to prothoracic coxae; 1 μm thick) were cut from Medcast blocks with an ultramicrotome (0MU2, Reichert, Vienna, Austria), mounted on glass slides, and then stained with 1% toluidine blue.

Influence of Pushup Stance on Rate of Pheromone Dissemination

We tested the hypothesis that the pushup stance maximizes the rate of pheromone dissemination using a pheromone proxy system, sublimation of naphthalene. Naphthalene was the ideal pheromone proxy because we could evenly coat a small surface area with a measurable amount, it sublimed at room temperature, and sublimation rate was slow enough to compare experimental treatments (see Souza Mendes, 1991). We used 16 dead and dried male *N. a. acuminatus* as dummies. They were first relaxed by sonicating in distilled water for ~5 min, then each was mounted on an insect pin through the abdomen, their appendages were removed, and they were dried to constant weight at 150°C. We melted naphthalene moth balls (99.9% pure, Willert Home Products, St. Louis, Mo., USA) at ~90°C, coated the head and prothorax of each dummy by dipping three times in the liquid naphthalene, allowed the naphthalene to solidify, and reweighed them. The amount of naphthalene applied to dummies averaged 5.7 ± 1.9 mg (SD). Naphthalene-coated specimens then were mounted 5 cm apart on a sheet of cardboard, half positioned to approximate the resting stance (body angle ~0° above horizontal, prothoracic sternum ~0.5 mm above substrate; see Results), and half positioned with the body approximating the pushup stance (body angle ~32°, prothoracic sternum ~4.3 mm above substrate), with treatments alternating across specimens. Specimens then were immediately placed in an air stream (electric fan, airspeed ~2.5 m/s) and were reweighed after 30 min to estimate rate of naphthalene sublimation. The experiment was replicated three times. We

calculated the percent of naphthalene lost, and tested differences between treatments by 2-way ANOVA (PROC GLM, SAS institute, 2001) with main effects being treatment and trial.

RESULTS

Characterizing the Pushup Stance

While males were in resting stance (Fig. 1A), the angle between femur and tibia of each foreleg was variable but always less than 90° and the body was parallel to the substrate (0° above horizontal) and nearly in contact with it (0.7 ± 0.4 mm between substrate and prothoracic sternum). While in pushup stance (Fig. 1B), the angle between femur and tibia of forelegs was always greater than 90°, the angle between body and substrate averaged $32 \pm 1.1°$ (mean significantly different from resting stance, Kruskal-Wallis $\chi^2_{1,12} = 9.47$, $P < 0.003$), and the prothoracic sternum was elevated 4.3 ± 0.4 mm above the substrate (mean significantly different from resting stance, Kruskal-Wallis $\chi^2_{1,12} = 9.47$, $P < 0.003$).

Association between Pushup Stance and Pheromone Release

Abundance of pheromone on the prothoraces of male *N. a. acuminatus* varied dramatically before, during, and after they were in the pushup stance (Fig. 2; means significantly different, Kruskal-Wallis $\chi^2_{2,\ 30} = 21.0$, $P < 0.0001$). Pheromone was absent or present only in trace quantities while beetles were in resting stance, present in high amounts during pushup stance, and returned to low abundance after resuming resting stance (Fig. 2). Minute quantities of pheromone present after resuming resting stance (Fig. 2) apparently were the residue of that produced during pushup stance.

Location of Pheromone Glands

Pheromone was in high abundance on the prothoraces of male *N. a. acuminatus* that were sampled while in pushup stance (mean peak area ± 1 SE: $1.89 \times 10^6 \pm 3.3 \times 10^5$), but was undetectable, or present in only minute quantities (mean < 0.2% of abundance on prothorax) when we sampled their heads, meso- and metathoraces, elytra, and abdomens (means significantly different, Kruskal-Wallis $\chi^3_{4,\ 20} = 12.5$, $P < 0.05$). Small amounts of pheromone on body regions other than the prothorax may be due to contamination of SPME fibers by volatilized pheromone during sampling; these

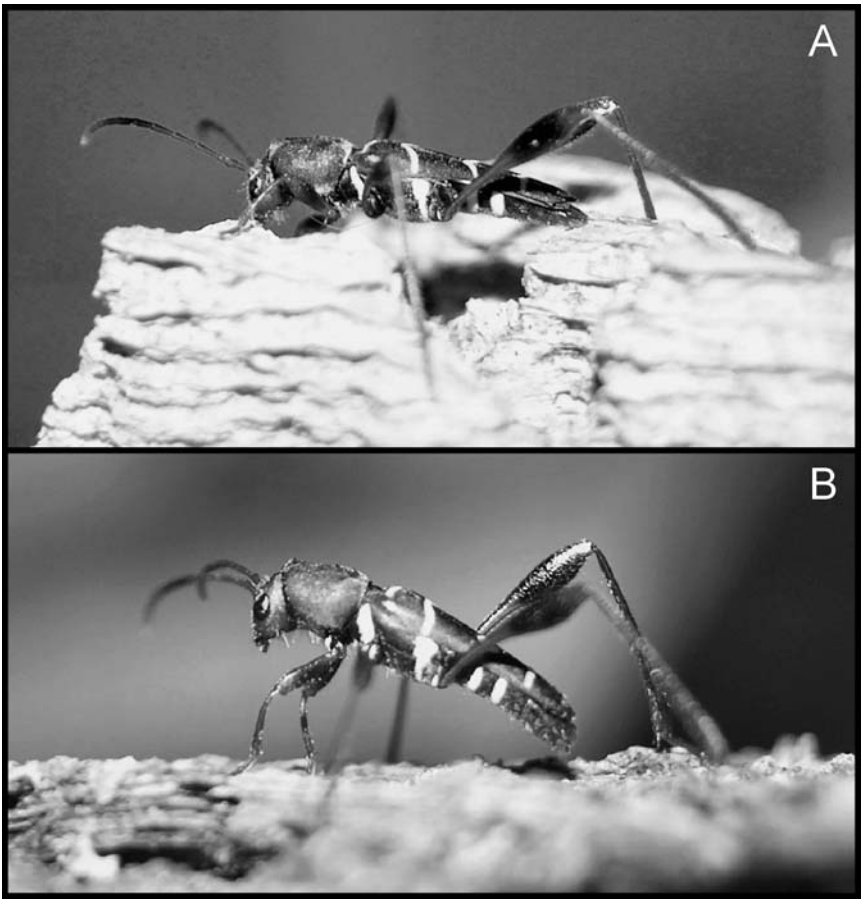

Fig. 1. Characteristic body postures of adult male *N. a. acuminatus*: A) resting stance; B) pushup stance.

fibers can capture detectable amounts of pheromone even when exposed several centimeters from calling male *N. a. acuminatus* (unpub. data).

SEM images of the prothorax of male *N. a. acuminatus* revealed many shallow ovoid indentations on the cuticular surface of males (Fig. 3A) that were absent in females (Fig. 3B). Indentations were 5–10 μm in diameter, separated by 10–50 μm, and were evenly distributed over the entire prothoracic surface with the exception of a thin collar around the anterior and posterior edges, the pronotal carina, the prosternal process, and an anterior patch along the midline of the prosternum. Distributed within each pit were 5–20 circular pores ($\sim$1 μm diameter; Fig. 3A, inset).

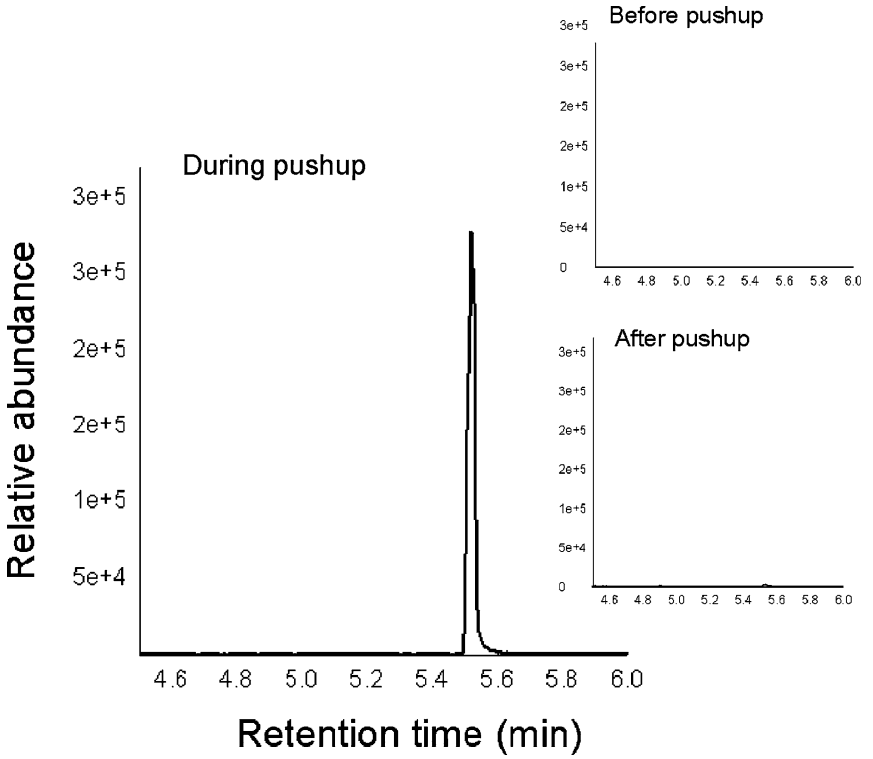

Fig. 2. Representative chromatograms for SPME samples of the prothoraces of adult male *N. a. acuminatus* before adopting the pushup stance (i.e., in resting stance), during pushup stance, and after resuming resting stance. Peak at ~5.5 min during pushup stance, and small peak after pushup stance, is the aggregation pheromone (2*S*, 3*S*)-hexanediol.

Light microscopy of sectioned prothoraces revealed a layer of epidermal cells under the cuticle of males (Fig. 4A) that was absent in females (Fig. 4C). This layer apparently contains many type III gland cells connected to the pores by conducting canals (Fig. 4B, inset; see Noldt *et al.*, 1995; Quennedey, 1998).

Influence of Pushup Stance on Rate of Pheromone Dissemination

Sublimation rate of naphthalene from dummy beetles in an air stream was significantly influenced by the body posture treatment (overall ANOVA $F_{5,35} = 7.73$; $P < 0.0001$), being ~45% faster from dummies that were positioned in the pushup stance compared to those in the resting stance (means 29.1 ± 2.0 and $20.1 \pm 1.8\%$ lost, respectively; treatment

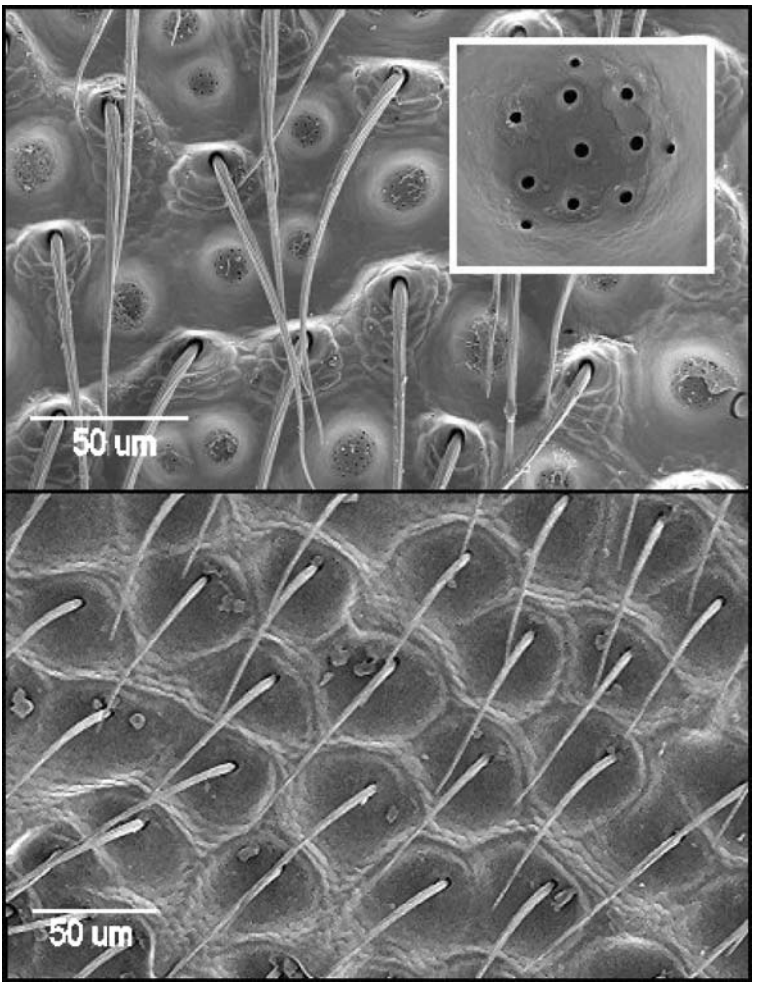

Fig. 3. SEM of the dorsal surface of the prothorax of adult *N. a. acuminatus*. A) Male (inset, close-up of pore cluster); B) Female.

effect $F_{1,35} = 16.9$; $P = 0.0003$; trial effect $F_{2,35} = 10.4$; $P = 0.0004$; interaction not significant, $P > 0.05$).

DISCUSSION

Our findings support the hypothesis that the pushup stance displayed by male *N. a. acuminatus* coincides with release of pheromone, and that

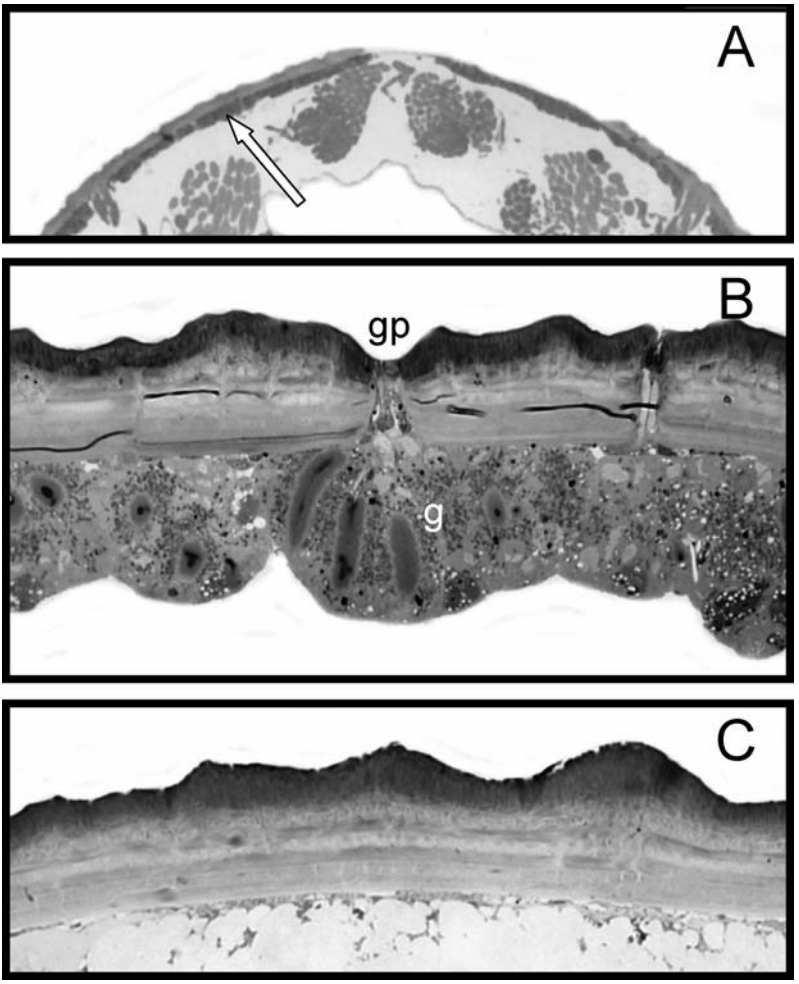

Fig. 4. Transverse histological sections through prothorax of adult *N. a. acuminatus*; A) Dorsum of male at 100X (arrow indicates cell layer underlying cuticle) B) Dorsum of male at 400X (gp = gland pore; *g* = gland); C) Dorsum of female 400X.

the prothorax is the source of pheromone. Crawshay (1907) described a similar behavior in the cerambycid *Tetropium gabrieli* Weise (subfamily Aseminae) that seems likely to be a calling behavior, but did not specify which sex was displaying the behavior: "They have also a peculiar habit of standing motionless, almost on tiptoe, with the body well away from

the bark. . .". Pheromone pores and glands of *N. a. acuminatus* are similar to those identified in three other species of the subfamily Cerambycinae (Iwabuchi, 1986; Nakamuta *et al*., 1994; Noldt *et al*., 1995). We have found similar male-specific gland pores on the prothoraces of another 48 species of the Cerambycinae (Ray *et al*., 2006), and have observed males of four of these species displaying postures similar to the pushup stance of *N. a. acuminatus* (*Enaphalodes rufulus* [Haldeman], *Megacyllene robiniae* [Förster], *Neoclytus mucronatus mucronatus* [F.], *Xylotrechus colonus* [F.]; unpublished data). These findings suggest that the mechanism of pheromone release is consistent within this specious subfamily, and that other species are likely to display the pushup stance when calling.

Rates of naphthalene sublimation from dummies of *N. a. acuminatus* supported the hypothesis that the pushup stance serves to increase dissemination of pheromones from the prothorax. The body posture effect probably is due to the elevation of the pheromone-releasing surface above the air boundary layer for dummies that were in the pushup position. Maximization of pheromone dissemination may be critical for *N. a. acuminatus* because relatively high release rates apparently are necessary to elicit a response from congeners (see Lacey *et al*., 2004).

The pushup stance of *N. a. acuminatus* is a subtle posture, but is easily recognized. It not only is evidence that a male is producing pheromone, but presumably also would indicate the period when conspecifics are responsive to pheromone. The stance therefore provides a convenient means of studying the role of pheromones in reproductive behavior of cerambycid beetles in the field and facilitates collection of pheromone in the laboratory.

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REFERENCES

Connor, W. E., and Best, B. A. (1988). Biomechanics of the release of sex pheromone in moths: effects of body posture on local airflow. *Physiol. Entomol.* **13:** 15–20.

Crawshay, G. A. (1907). The life history of *Tetropium gabrieli*. *Trans. Entomol. Soc. London* **13:** 183–212.

Greenfield, M. D. (2002). *Signalers and Receivers: Mechanisms and Evolution of Arthropod Communication*, Oxford University Press, NY.

Hardie, J., and Minks, A. K. (eds.). (1999). *Pheromones of Non-Lepidopterous Insects Associated with Agricultural Plants*, CABI Publishing, Wallingford, United Kingdom.

Iwabuchi, K. (1986). Mating behavior of *Xylotrechus pyrrhoderus* Bates (Coleoptera: Cerambycidae). III. pheromone secretion by male. *Appl. Entomol. Zool.* **21:** 606–612.

Lacey, E. S., Ginzel, M. D., Millar, J. G., and Hanks, L. M. (2004). Male-produced aggregation pheromone of the cerambycid beetle *Neoclytus acuminatus acuminatus*. *J. Chem. Ecol.* **30:** 1493–1507.

Leal, W. S., Yadava, C. P. S., and Vijayvergia, J. N. (1996). Aggregation of the scarab beetle *Holotrichia consanguinea* in response to female-released pheromone suggests a secondary function hypothesis for semiochemical. *J. Chem. Ecol.* **22:** 1557–1566.

Linsley, E. G. (1964). The Cerambycidae of North America, Part V. Taxonomy and classification of the subfamily Cerambycinae, tribes Callichromini through Ancylocerini. *Univ. Calif. Publ. Entomol.* **22:** 1–197.

Millar, J. G., and Haynes, K. F. (1998). *Methods in Chemical Ecology: Volume 1*, Kluwer, NY.

Moore, P., and Crimaldi, J. (2004). Odor landscapes and animal behavior: Tracking odor plumes in different physical worlds. *J. Marine Systems* **49:** 55–64.

Nakamuta, K., Sato, H., and Nakashima, T. (1994). Behavioral and morphological evidence for a male-produced sex pheromone in the cryptomeria twig borer, *Anaglyptus subfasciatus* Pic (Coleoptera: Cerambycidae). *Jpn. J. Entomol.* **62:** 371–376.

Noldt, U., Fettköther, R., and Dettner, K. (1995). Structure of the sex pheromone-producing prothoracic glands of the male old house borer, *Hylotrupes bajulus* (L.) (Coleoptera: Cerambycidae). *Intl. J. Insect Morphol. Embryol.* **24:** 223–234.

Quennedey, A. (1998). Insect epidermal gland cells: Ultrastructure and morphogenesis. In Harrison, F. W., and Ruppert, E. E. (eds.), *Microscopic Anatomy of Invertebrates Vol. 11A: Insecta*, Wiley-Liss, NY, pp. 177–207.

Ray, A. M., Lacey, E. S., and Hanks, L. M. (2006). Predicted taxonomic patterns in pheromone production by cerambycid beetles. *Naturwissenschaften* **93:** 543–550.

SAS Institute. (2001). *SAS/STAT User's Guide for Personal Computers, Release 8.01*, SAS Institute, Cary, NC.

Solomon, J. D. (1995). Guide to insect borers in North American broadleaf trees and shrubs. *USDA For. Serv. Agric. Handbook* **AH-706:** 747.

Souza Mendes, P. R. (1991). The naphthalene sublimation technique. *Experimental Thermal Fluid Sci.* **4:** 510–523.