5. Neuberger H (1950) Arago's neutral point: a neglected tool in meteorological research. Bull Am Met Soc 31:119–125
6. Van de Hulst HC (1952) Scattering in atmospheres. In: Kniper GP (ed) The atmosphere of the earth and planets. University of Chicago Press, Chicago
7. Sekera Z (1957) Light scattering in the atmosphere and the polarization of skylight. J Opt Soc Am 47:484–490
8. Holzworth GC, Rao CR (1965) Studies of skylight polarization. J Opt Soc Am 55:403–408
9. Können GP (1985) Polarized light in nature. Cambridge University Press, Cambridge
10. Bellver C (1987) Influence of particulate pollution on the positions of neutral points in the sky at Seville (Spain). Atmospheric Environment 21:699–702
11. Coulson KL (1988) Polarization and intensity of light in the atmosphere. Deepak, Hampton
12. Walraven RL (1981) Polarization imagery. Opt Eng 20:14–18
13. Wehner R (1976) Polarized-light navigation by insects. Sci Am 235(1):106–114
14. Wehner R (1997) The ant's celestial compass system: spectral and polarization channels. In: Lehrer M (ed) Orientation and communication in arthropods. Birkhäuser, Basel, pp 145–185
15. Coulson KL, Whitehead VS, Campbell C (1986) Polarized views of the earth from orbital altitude. Proc SPIE 637, Ocean Optics VIII:35–41
16. North JA, Duggin MJ (1997) Stokes vector imaging of the polarized sky-dome. Appl Opt 36:723–730
17. Prosch T, Hennings D, Raschke E (1983) Video polarimetry: a new imaging technique in atmospheric science. Appl Opt 22:1360–1363
18. Egan WG (1986) Proposed design of an imaging spectropolarimeter/photometer for remote sensing of earth resources. Opt Eng 25:1155–1159
19. Wolff LB (1993) Polarization camera technology. Proc DARPA Image Understanding Works, pp 1031–1036
20. Deschamps PY, Bréon FM, Leroy M, Podaire A, Bricaud A, Buriez J C, Séze G (1994) The Polder mission: instrument characteristics and scientific objectives. IEEE Trans Geosci Rem Sens 32:598–615
21. Cronin TW, Shashar N, Wolff LB (1994) Portable imaging polarimeters. Proc 12th IAPR Int Conf Pattern Recogn, pp 606–609
22. Shashar N, Cronin TW, Johnson G, Wolff LB (1995) Portable imaging polarized light analyzer. SPIE Proc series 2426:28–35
23. Horváth G, Zeil J (1996) Kuwait oil lakes as insect traps. Nature 379:303–304
24. Horváth G, Varjú D (1997) Polarization pattern of freshwater habitats recorded by video polarimetry in red, green and blue spectral ranges and its relevance for water detection by aquatic insects. J Exp Biol 200:1155–1163
25. Horváth G, Gál J, Wehner R (1997) Why are water-seeking insects not attracted by mirages? The polarization pattern of mirages. Naturwissenschaften 84:300–303

Naturwissenschaften 85, 339–342 (1998) Springer-Verlag 1998

Novel and Highly Specific Transport of a Volatile Sex Pheromone by Hemolymph Lipophorin in Moths

Coby Schal, Veeresh Sevala
Department of Entomology, North Carolina State University, Box 7613, Raleigh, NC 27695-7613, USA

Ring T. Cardé
Department of Entomology 041, University of California, Riverside, CA 92521, USA

Received: 22 December 1997 / Accepted in revised form: 16 April 1998

Here we report that in *Holomelina* tiger moths (Lepidoptera: Arctiidae) pheromone is synthesized by tissues associated with the abdominal integument, and that lipophorin, a multifunctional plasma lipoprotein, transports the pheromone to an abdominal gland that stores and releases the pheromone only during active calling behavior. We suggest that such transport pathways are common not only among insects that emit hydrocarbon pheromones but also among insects that sequester hydrophobic plant-derived metabolites.

Animals have evolved sophisticated chemical communication systems, including volatile sex pheromones that are used in mate recruitment, and cuticular constituents that elicit courtship and copulation upon contact (Cardé and Minks 1997). In most insects sex pheromones are produced in and emitted from specialized pheromone glands, and in female moths these glands generally constitute an epidermal layer near the female's ovipositor (Percy-Cunningham and MacDonald 1987). These glands synthesize de novo all the components of the pheromone blend (Bjostad et al. 1987; Jurenka and Roelofs 1993), and although mechanisms of pheromone export to the exterior remain unknown, these lipophilic compounds presumably require little if any interaction with an aqueous environment.

Species within the genus *Holomelina* emit pheromone blends of normal and 2-methyl-branched alkanes ranging in chain length from 16 to 19 carbons. The main pheromone component of several species is 2-methylheptadecane (2Me-17:Hy) which attracts males in field assays (Roelofs and Cardé 1971; Schal and Cardé 1985). In *H. lamae*, as in other arctiids, the pheromone emanates from paired tubular glands that vent between the 8th and 9th abdominal segments (Yin et al. 1991). Two independent observations led us to test the hypothesis that pheromone is not syn-

Correspondence to: C. Schal

thesized within this gland. First, although most lipid-synthesizing cells are rich in smooth endoplasmic reticulum (SER), ultrastructural evidence shows little SER in the epidermal cells lining the gland of *H. lamae* (Yin et al. 1991). Second, labeled leucine and acetate applied directly to the pheromone glands of *H. lamae* and *H. aurantiaca* fail to label pheromone, but when injected into the hemocoel, these substrates are effectively incorporated into 2Me-17:Hy within the gland, implying pheromone or precursor transport (Charlton and Roelofs 1991). This contrasts with the sex pheromone glands in other moths that readily incorporate topically applied precursors into pheromone, and the glands synthesize pheromone in vitro (Bjostad et al. 1987; Jurenka and Roelofs 1993). Lastly, we have recently shown that the contact sex pheromone of a cockroach is abundant on cuticular regions that do not synthesize it; lipophorin, a plasma lipoprotein, shuttles pheromone from integumental biosynthetic sites to sites that do not synthesize it (Gu et al. 1995).

To determine which tissues synthesize pheromone in *H. aurantiaca* we incubated tissues from 2-day-old virgin females in 0.5 ml Grace's medium (Sigma) fortified with 0.5 μCi sodium[1-^{14}C]acetate (51.7 mCi/mmol, Sigma). After 4 h, 1 ml methanol was added, the tissue/medium was ultrasonicated for 30 s, and lipids were extracted with *n*–hexane. Hydrocarbons were fractionated from the hexane extract on a silica gel column and analyzed by liquid scintillation spectrometry. The pheromone gland failed to incorporate radiolabel into hydrocarbon (Fig. 1a), even in 24-h assays and in the presence of hemolymph (data not shown). By contrast, the abdominal integument efficiently produced radiolabeled hydrocarbons in the absence of any pheromone gland tissue or hemolymph. Radio-gas chromatography (GC; FloOne/Beta Radiomatic, Packard) confirmed that pheromone produced in vitro was labeled (Fig. 1b,c).

A hemolymph transport pathway was thus implicated. Lipids were extracted, as above, except that 1 μg *n*–eicosane was added as an internal standard. Hydrocarbons were separated on a 25 m × 0.32 mm × 1 μm HP-1 capillary column in a GC (Hewlett Packard 5890II) interfaced with a 3365II Chemstation. Chromatographic hydrocarbon profiles showed that 2Me-17:Hy was present in the hemolymph of females (Fig. 2d) but not in that of males (Fig. 2c). In vivo radiotracer experiments and radio-GC verified that radiolabeled 2Me-17:Hy was present in the pheromone gland (Fig. 2a,b), as shown earlier (Charlton and Roelofs 1991). Moreover, our data supported the conclusion that the pheromone gland is not required for the biosynthesis of pheromone. We injected [^{14}C]acetate into intact (control) females and into females whose terminal abdominal segments, including the pheromone gland, had been ablated. The two sets of females produced equal amounts of radiolabeled hydrocarbons (intact: 12,873 ± 1150 dpm, $n = 10$; glandless: 12,974 ± 1166 dpm, $n = 10$; $t = 0.0617$, $P_{2\text{-tailed}} = 0.951$), and radio-GC confirmed that both synthesized pheromone de novo (Fig. 2e).

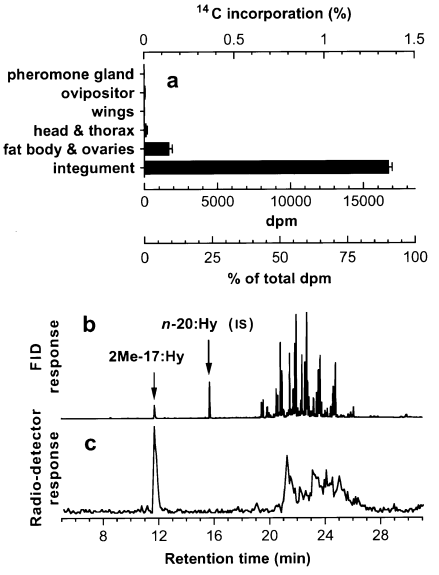

Fig. 1. a) Hydrocarbon synthesis in vitro by tissues from *Holomelina aurantiaca*. Data are presented as percentage of ^{14}C dpm from acetate incorporated into hydrocarbons, and as percentage of total dpm in newly synthesized hydrocarbons. b) Pheromone and long-chain hydrocarbons in the integument. *IS*, *n*–Eicosane internal standard; *FID*, flame ionization detector. c) Radio-GC of [^{14}C]-labeled hydrocarbons

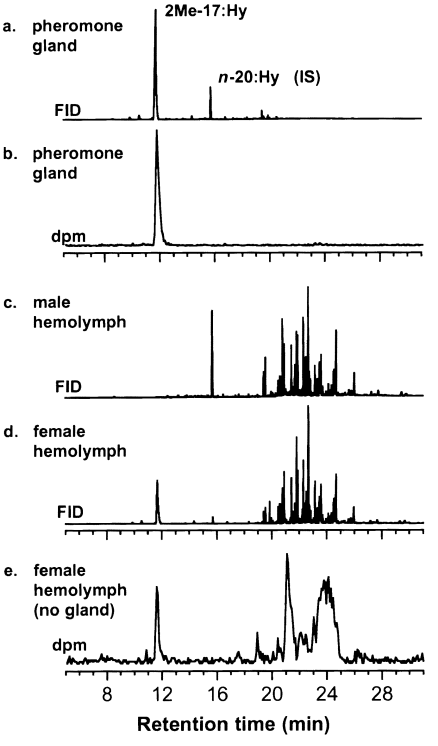

Fig. 2. a), b) Hydrocarbons from the pheromone gland of *Holomelina aurantiaca* females. 2Me-17:Hy, the sex pheromone, is a major component of the extracted hydrocarbons. For radiotracing, females were injected with [^{14}C]acetate and the pheromone glands dissected and processed 4 h later. Radio-GC shows selective sequestration of labeled 2Me-17:Hy in the gland. c), d) Hemolymph hydrocarbons of *Holomelina aurantiaca* males and females. Male hemolymph lacks 2Me-17:Hy. e) Radio-GC of hydrocarbons from the hemolymph of females from which the pheromone gland had been ablated. Females were injected with [^{14}C]acetate, and hemolymph samples were processed 4 h later for radio-GC

The chromatographic profile of the hemolymph revealed an abundance of long-chain hydrocarbons in both females and males (Fig. 2c,d), and these were radiolabeled in both in vivo and in vitro experiments. Yet, we found only trace amounts of long-chain hydrocarbons in the female's pheromone gland (Fig. 2a), and there was no pheromone anywhere on the female's exposed epicuticle. This highlights the stringent selectivity of pheromone uptake by the pheromone gland and long-chain hydrocarbon uptake by epidermal cells that then deliver them to the epicuticle. Simi-

larly, deposition of various lipophorin-bound lipids is tissue-specific: diacylglycerol is deposited for use in muscles whereas hydrocarbons are deposited primarily in the cuticle and ovaries (Chino 1985).

To identify hydrocarbon carrier protein(s) we fractionated serum proteins from 2-day-old females by KBr density-gradient ultracentrifugation (Fig. 3a,b) as described previously (Gu et al. 1995). All the hydrocarbons associated with a high-density lipoprotein (HDLp), lipophorin (density = 1.115 ± 0.007 g/ml; Fig. 3c), and coupled GC–mass spectrometry confirmed the presence of 2Me-17:Hy. Newly synthesized hydrocarbons (in females injected [^{14}C]acetate) also associated with HDLp, whereas more polar compounds that incorporated ^{14}C from acetate associated with proteins of higher density (Fig. 3d).

Neither hemolymph nor purified HDLp could stimulate pheromone synthesis in vitro by isolated pheromone glands (from [^{14}C]acetate). However, when coincubated with abdominal integument and HDLp, the pheromone gland effectively sequestered ^{14}C-labeled pheromone. Hemolymph was collected 8 h after females were injected with [^{14}C]acetate. Lipophorin, together with associated ^{14}C-labeled lipids, was purified by KBr gradient ultracentrifugation, dialyzed, and concentrated. A pheromone gland pair or an accessory reproductive gland (a tissue of approximately the same size as pheromone glands, serving as experimental control) was incubated in Grace's medium with purified HDLp (5 μg/μl). After an 8-h incubation in vitro the tissue was washed, and hydrocarbons were purified separately from the tissue and medium. Pheromone glands incubated with purified HDLp that had been pre-loaded with ^{14}C-labeled lipids accepted 2Me-17:Hy (1.90 ± 0.22% of [^{14}C]hydrocarbons in the medium, $n = 9$), while accessory reproductive glands from the same females accepted only 0.03 ± 0.03% ($n = 4$). These results underscore the importance of HDLp in pheromone transport and the selective uptake of pheromone by the gland.

We propose the following model for processing of hydrocarbons in *H. aurantiaca*: Short-chain hydrocarbon pheromone constituents and long-chain hydrocarbons are synthesized in tissues that are associated with the abdominal integument, probably in the oenocytes. Both are taken up by HDLp for transport through the hemolymph. Long-chain hydrocarbons are specifically unloaded at the epidermis for transport to the epicuticle, whereas volatile pheromone components are specifically unloaded at the pheromone gland. The pheromone gland thus serves as a reservoir, not as a biosynthetic site, while its opening serves as a nozzle, aerosolizing the pheromone into the air.

Lipophorin transport of volatile and long-chain hydrocarbons likely operates in other arctiids and geometrids, many of which possess tubular pheromone glands (Wunderer et al. 1986) and emit volatile hydrocarbons (Conner et al. 1980; Bell and Meinwald 1986; Rule and Roelofs 1989; Li et al. 1993). Recently we have shown that in the housefly, *Musca domestica*, HDLp carries (*Z*)-9-tricosene, a less volatile, medium-chain female sex pheromone (unpublished results). Long-chain cuticular hydrocarbons and pheromones that are derived from them also fit this transport scheme, as shown for locusts, beetles, cockroaches, termites, and fruit flies (Schal et al. 1998; unpublished). Because transport of epicuticular hydrocarbons by HDLp appears to be common (universal?) in insects, we suggest that pheromone transport by HDLp was evolutionarily coopted from preexisting systems that deliv-

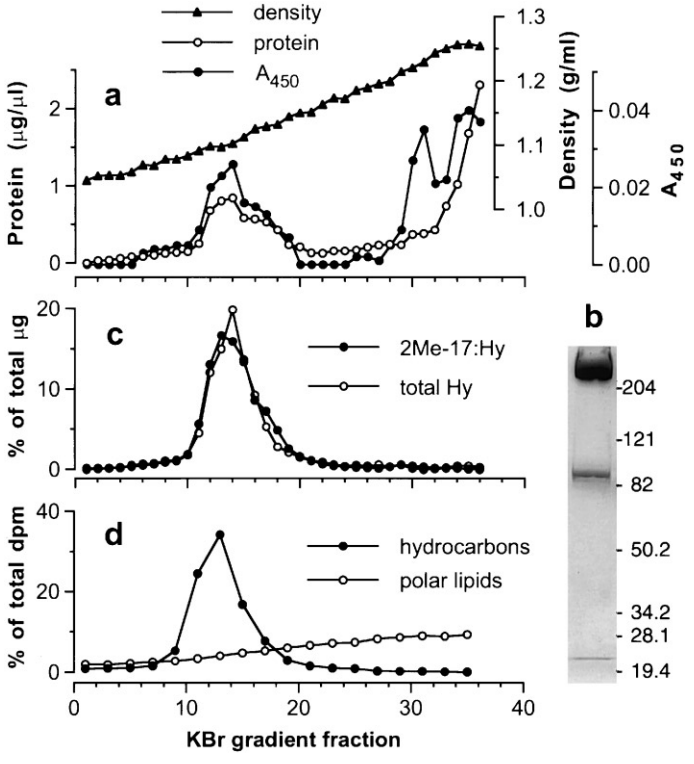

Fig. 3a–c. KBr gradient fractionation of *Holomelina aurantiaca* hemolymph proteins. a) Hemolymph fractions collected from the top to bottom of the tube. The protein content in fractions was determined using the BioRad protein assay with bovine serum albumin as a standard. The densities of fractions were determined gravimetrically, and absorbance at 450 − nm was a measure of carotenoids. b) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4–15% gradient slab gels of fraction 13, showing purity of HDLp and its three constituent apoproteins, with molecular markers (in kilodaltons) on the right. ApoLp-I, Apolipophorin-I (approx. 250 kDa); apoLp-II, apolipophorin-II (approx. 85 kDa); apoLp-III, apolipophorin-III (approx. 20 kDa). c) Distribution of 2Me-17:Hy and total hydrocarbons in fractions, as determined by GC. d) Distribution of radiolabeled hydrocarbons and polar lipids in fractions, as determined by eluting silica columns with hexane and diethyl ether, respectively, followed by LSC. Hydrocarbons, including sex pheromone, associate only with lipophorin

ered hydrophobic (and potentially cytotoxic) water-proofing lipids from internal biosynthetic sites to the developing oocytes and the epicuticle. This model can be extended to other hydrophobic compounds, including plant secondary metabolites that serve as pheromones or pheromone precursors, and ingested plant compounds that are translocated to specialized glands and to oocytes [as in beetles where females ingest male sequestered cantharidin and transfer it to oocytes (Eisner et al. 1996)]. The evolution of lipophorin as a shuttle for hydrophobic ligands probably preadapted insects to transport xenobiotics, including pesticides, through the hemolymph to sites of sequestration and catabolism.

Lipophorin serves as a reusable multifunctional shuttle of lipids in insects (Chino 1985; Law et al. 1992; Trowell 1992; Soulages and Wells 1994; Sevala et al. 1997). Its functions are analogous to those of other lipid-binding proteins, including low molecular weight pheromone-binding proteins and general odorant-binding proteins (OBPs) in antennal lymph and vertebrate nasal and vomeronasal epithelia (Pelosi 1994; Pelosi and Maida 1995; Breer 1997). All three lipid-binding proteins occur in large quantities within their respective aqueous environment and appear to have relatively low affinities for their ligands. Lipophorin's role in insects is also analogous to mammalian lipocalins, which bind and transport small hydrophobic molecules, but in most cases the identity or function of the lipid ligands (putative pheromones) is not clear. Lipophorin/pheromone interactions in insects may prove to be a tractable system in which to study lipid-protein interactions and the evolution of chemical signaling pathways. In *H. aurantiaca*, and probably in other species that use this pathway, species specificity of the emitted pheromone blend depends to a large extent on interactions between pheromone receptors in the gland and hemolymph carrier proteins. An analogous mechanism appears to operate in odor reception: odorant- and pheromone-binding proteins that deliver odorants to membrane receptors in the sensillar dendrites initiate and terminate the signal transduction pathways that mediate the response to odors. The degrees of specificity and affinity of the pheromone acceptor in the pheromone gland remain to be elucidated through structure-activity studies, as do mechanisms that regulate sex-specific pheromone production in insects that use this pathway.

We thank G.J. Blomquist, P. Estes, G.L. Holbrook, J.H. Law, W.L. Roelofs, and H.P. Young for careful reviews and critiques of earlier drafts of this paper. We gratefully acknowledge support from the Blanton J. Whitmire Endowment at North Carolina State University, NSF, USDA/NRI, and an equipment grant (radio-GC) from NCSU/NCARS.

Bell TW, Meinwald J (1986) Pheromones of two arctiid moths (*Creatonotos transiens* and *C. gangis*): chiral components from both sexes and achiral female components. J Chem Ecol 12:385–409

Bjostad LB, Wolf WA, Roelofs WL (1987) Pheromone biosynthesis in lepidopterans: desaturation and chain shortening. In: Prestwich GD, Blomquist GJ (eds) Pheromone biochemistry, Academic, Orlando

Breer H (1997) Molecular mechanisms of pheromone perception in insect antennae. In: Cardé RT, Minks AK (eds) Insect pheromone research: new directions. Chapman and Hall, New York

Cardé RT, Minks AK (eds) (1997) Insect pheromone research: new directions. Chapman and Hall, New York

Charlton RE, Roelofs WL (1991) Biosynthesis of a volatile, methyl-branched hydrocarbon sex pheromone from leucine by arctiid moths (*Holomelina* spp.). Arch Insect Biochem Physiol 18:81–97

Chino H (1985) Lipid transport: Biochemistry of hemolymph lipophorin. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology, biochemistry and pharmacology, vol. 10. Pergamon, New York

Conner WE, Eisner T, Vander Meer RK, Guerrero A, Ghiringelli D, Meinwald J (1980) Sex attraction of an arctiid moth (*Utetheisa ornatrix*): a pulsed chemical signal. Behav Ecol Sociobiol 7:55–63

Eisner T, Smedley SR, Young DK, Eisner M, Roach B, Meinwald J (1996) Chemical basis of courtship in a beetle (*Neopyrochroa flabellata*): Cantharidin as "nuptial gift." Proc Natl Acad Sci USA 93:6499–6503

Gu X, Quilici D, Juarez P, Blomquist GJ, Schal C (1995) Biosynthesis of hydrocarbons and contact sex pheromone and their transport by lipophorin in females of the German cockroach (*Blattella germanica*). J Insect Physiol 41:257–267

Jurenka RA, Roelofs WL (1993) Biosynthesis and endocrine regulation of fatty acid derived sex pheromones in moths. In: Stanley-Samuelson DW, Nelson DR (eds) Insect lipids: chemistry, biochemistry and biology. University Nebraska Press, Lincoln

Law JH, Ribeiro JM, Wells MA (1992) Biochemical insights derived from insect diversity. Annu Rev Biochem 61:87–111

Li J, Gries R, Gries G, Slessor KN, King GGS, Bowers WW, West RJ (1993) Chirality of 5,11-dimethylheptadecane, the major sex pheromone component of the hemlock looper, *Lambdina fiscellaria* (Lepidoptera: Geometridae). J Chem Ecol 19:1057–1062

Pelosi P (1994) Odorant-binding proteins. Crit Rev Biochem Mol Biol 29:199–228

Pelosi P, Maida R (1995) Odorant-binding proteins in insects. Comp Biochem Physiol 111B:503–414

Percy-Cunningham JE, MacDonald JA (1987) Biology and ultrastructure of sex pheromone-producing glands. In: Prestwich GD, Blomquist GJ (eds) Pheromone biochemistry. Academic, Orlando

Roelofs WL, Cardé RT (1971) Hydrocarbon sex pheromone in tiger moths (Arctiidae). Science 171:684–686

Rule GS, Roelofs WL (1989) Biosynthesis of sex pheromone components from linolenic acid in arctiid moths. Arch Insect Biochem Physiol 12:89–97

Schal C, Cardé RT (1985) Rhythmic extrusion of pheromone gland elevates pheromone release rate. Experientia 41:1617–1619

Schal C, Sevala VL, Young HP, Bachmann JAS (1998) Sites of synthesis and transport pathways of insect hydrocarbons: cuticle and ovary as target tissues. Am Zool 38:382–393

Sevala VL, Bachmann JAS, Schal C (1997) Lipophorin: a hemolymph juvenile hormone binding protein in the German cockroach, *Blattella germanica.* Insect Biochem. Mol Biol 27:663–670

Soulages JL, Wells MA (1994) Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. Adv Protein Chem 45:371–415

Trowell SC (1992) High affinity juvenile hormone carrier proteins in the hemolymph of insects. Comp Biochem Physiol 103B:795–808

Wunderer H, Hansen K, Bell TW, Schneider D, Meinwald J (1986) Sex pheromones of two Asian moths (*Creatonotos transiens*, *C. gangis*; Lepidoptera – Arctiidae): behavior, morphology, chemistry and electrophysiology. Exp Biol 46:11–27

Yin LRS, Schal C, Cardé RT (1991) Sex pheromone gland of the female tiger moth *Holomelina lamae* (Lepidoptera: Arctiidae). Can J Zool 69:1916–1921