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IDENTIFICATION OF THE MAJOR COMPONENTS IN THE SECRETION FROM THE RECTAL PHEROMONE GLANDS OF THE QUEENSLAND FRUIT FLIES *Dacus tryoni* AND *Dacus neohumeralis* (DIPTERA:TEPHRITIDAE)

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Abstract—The secretion from the rectal pheromone glands of male *Dacus tryoni* and *Dacus neohumeralis* is largely a mixture of six aliphatic amides. In order of decreasing quantity these are *N*-3-methylbutylpropanamide, *N*-3-methylbutylacetamide, *N*-(3-methylbutyl-2-methylpropanamide, *N*-2-methylbutylpropanamide, *N*-2-methylbutylacetamide, and *N*-(2-methylbutyl)-2-methylpropanamide. The proportions of the various amides in the two species are similar.

Key Words—Queensland fruit fly, *Dacus tryoni*, *D. neohumeralis*, Diptera, Tiphritidae, rectal pheromone gland, aliphatic amides, sex pheromone.

INTRODUCTION

The common name, Queensland fruit fly, is used for two very closely related species of *Dacus*, *D. tryoni* (Froggatt) and *D. neohumeralis* (Perkins), which breed in a wide range of native and introduced fruits in the eastern part of Australia (May, 1963). In the northern part of their range they occur sympatrically and infest a similar range of hosts. However *D. neohumeralis* only occurs as far south as northern New South Wales whereas the range of *D. tryoni* extends to East Gippsland in northern Victoria.

In the laboratory the two species produce fertile hybrids, but in the wild they seem to be reproductively isolated (Vogt, 1977); the main barrier to cross mating appears to be a difference in mating times (Gibbs, 1965). *D.*

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neohumeralis normally mates at high light intensities during the middle of the day whereas *D. tryoni* will only mate under a low light intensity at dusk (Tychsen and Fletcher, 1971). In both species sexually excited males emit an oily secretion with a sweet pungent odor which acts as a sex pheromone. The source of this secretion is a glandular complex with an associated reservoir which opens into the rectum (Fletcher, 1969). Similar gland structures are known to occur in some other species of this economically important genus (Fletcher, 1969; Schultz and Boush, 1971).

We examined the composition of the rectal pheromone gland secretion of *D. tryoni* and *D. neohumeralis* to determine whether they are the same or different. Any differences might act as a further barrier to mating between the two species. In addition, it is possible that pheromone components might be of use in control programs for the two species, particularly in view of the successful use of the synthetic attractants methyl eugenol and 4-*p*-acetyloxyphenylbutan-2-one in monitoring and suppression programs for fruit flies (Steiner et al., 1965; Bateman et al., 1966).

METHODS AND MATERIALS

The infrared spectra were measured in CCl_4 solution with a Perkin Elmer model 221 spectrophotometer. The ultraviolet spectra were determined in ethanol solution on a Perkin Elmer model 402 spectrophotometer. Mass spectra were measured with a GEC-AEI MS-902 mass spectrometer to which was connected a Philips Research model PV4000 gas chromatograph. For high-resolution measurements the output from the mass spectrometer was fed through an A/D converter to an online Raytheon 706 computer.

Preparative gas chromatography was conducted on a Varian 1200 instrument which had been modified to incorporate an annular splitter (Brownlee and Silverstein, 1968). Samples were collected in glass capillaries with the collection zone cooled with dry ice.

Kováts retention indices were measured on a Varian 2100 gas chromatograph. Retention times were measured with a Hewlett-Packard model 3370A integrator. Calculations were made on a CDC 3600 computer with use of the program KLINDEX (Bellas, 1975). Unless otherwise indicated the gas chromatography support was Gas-chrom Z of 80/100 mesh, the phase loading was 5%, columns were made of stainless steel 2 m × 2.3 mm and the carrier gas was helium at a flow rate of about 20 cm^3/min.

Collection of Pheromone Gland Secretion and Separation of Components for Spectrophotometry. The secretion was collected from the pheromone glands of mature male specimens of *D. tryoni* and *D. neohumeralis* which had been reared on standard artificial diet (Bateman, 1968)

and fed as adults on protein hydrolysate, sugar, and water. Some samples were also obtained from field-collected mature male *D. tryoni* for comparison with the laboratory stocks. After dissection of the reservoir, the pheromone gland secretions were collected in micropipettes and were stored as solutions in ether. The ether phase was withdrawn from the small quantity of water also present and filtered through anhydrous $MgSO_4$. The dried solutions were concentrated by slow distillation of the solvent through a short fractionating column.

Samples for spectrophotometry were collected by preparative gas chromatography. Injections of an ether solution containing about 8 male equivalents of the secretion from *D. tryoni* onto a column containing OV-225 at 140° afforded the major components as two peaks with retention times of 12.0 and 14.4 min. These components were collected in one tube and were washed from the tube with the appropriate solvent. UV (ethanol), end absorption; IR ν_{max} (CCl_4 solution, 5× scale expansion) 3460, 2960, 2930, 2875, 1680 cm^{-1}.

The columns used for GC-MS were made of stainless steel and were 2 m or 4.2 m × 2.3 mm. The stationary phases were OV-1 (on Gas-chrom Q) and Carbowax 20M. Conditions were chosen so that each peak took at least one minute to elute.

Determination of Ratios of Components in Individual Flies. The gland content of each fly was collected in a micropipette and dissolved in about 100 mm^3 of acetone. Each sample was injected onto two columns: OV-225 at 140° which resolved the amides into two peaks, and terephthalic acid-terminated Carbowax 20M at 130° which resolved them into three peaks. The carrier gas was helium at a flow rate of 20 cm^3/min in each case. For each peak a relationship between the peak height and the area under the curve was derived using solutions of mixtures of the synthetic amides. The areas were measured with a planimeter on eightfold time-expanded traces. The relative areas for the fly material were then determined by measuring the peak heights on the two traces for the secretion from each animal.

Synthesis of Amides. The amides were prepared by one of two methods. Propanamides were prepared by heating the amine with propanoic anhydride. Acetamides and 2-methylpropanamides were prepared from the amines and the acid chlorides. The amides were purified by gas chromatography before measuring the mass spectra. The mass spectra of the synthesized amides are listed in Table 1.

Measurement of Retention Indices. The columns and conditions used for determining the retention indices were A: 2 m × 4 mm glass, Carbowax 20M at 125°, B: same column at 140°, C: 2 m × 3 mm glass, EGSS-X at 125°, D: 3.7 m × 3 mm glass, Carbowax 20M at 130°, E: same column at 150°. The carrier gas was nitrogen at a flow rate of 25 cm^3/min.

TABLE 1. MASS SPECTRA OF SYNTHESIZED AMIDES[a]

N-3-Methylbutylacetamide: $M^{+\cdot}$ 129.11574, calculated for $C_7H_{15}NO$, 129.11537: *m/z* 129(11%), 114(4), 86(11), 73(36), 72(31), 60(17), 44(25), 43(25), 30(100).

N-3-Methylbutylpropanamide: $M^{+\cdot}$ 143.13107, calculated for $C_8H_{17}NO$, 143.13102: *m/z* 143(13%), 128(8), 114(4), 100(10), 87(44), 86(31), 74(20), 57(44), 44(41), 43(23), 30(100).

N-(3-Methylbutyl)-2-methylpropanamide: $M^{+\cdot}$ 157.14669, calculated for $C_9H_{19}NO$, 157.14667: *m/z* 157(18%), 142(13), 114(13), 101(37), 100(11), 88(17), 72(25), 71(67), 44(47), 43(100), 41(18), 30(34).

N-Pentylacetamide($C_7H_{15}NO$): $M^{+\cdot}$ 129.11573: *m/z* 129(13%), 114(2), 100(7), 73(21), 72(29), 60(10), 44(16), 43(24), 30(100).

N-Pentylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13108: *m/z* 143(22%), 128(1), 114(22), 100(7), 87(35), 86(33), 74(11), 57(49), 44(32), 43(25), 30(100), 29(14).

N-Pentyl-2-methylpropanamide($C_9H_{19}NO$): $M^{+\cdot}$ 157.14689: *m/z* 157(23%), 142(3), 128(21), 114(16), 101(24), 100(12), 88(12), 72(29), 71(75), 44(34), 43(100), 30(40).

N-2-Methylbutylacetamide($C_7H_{15}NO$): M^{+} 129.11550: *m/z* 129(10%), 114(<1), 100(10), 73(20), 72(35), 60(24), 43(20), 30(100).

N-2-Methylbutylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13111: *m/z* 143(9%), 128(<1), 114(9), 87(18), 86(32), 74(31), 58(12), 57(36), 44(8), 43(10), 30(100), 29(29).

N-(2-Methylbutyl)-2-methylpropanamide($C_9H_{19}NO$): $M^{+\cdot}$ 157.14645: *m/z* 157(29%), 142(2), 128(9), 114(5), 101(19), 100(23), 88(67), 72(49), 71(88), 58(20), 44(10), 43(100), 41(21), 30(56).

N-1-Methylbutylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13089; *m/z* 143(6%), 114(5), 101(10), 100(23), 74(10), 72(14), 57(15), 45(28), 44(100), 43(11), 29(17).

N-1-Ethylpropylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13105: *m/z* 143(7%), 114(19), 74(7), 58(100), 57(11), 29(12).

[a] The high-resolution spectra were measured in the Mass Spectrometry Unit of the Division of Entomology. Only the major and diagnostic ions are given for the low-resolution spectra but high-resolution data are available for most ions above *m/z* 38 of relative abundance greater than 1% of the base peak. The complete low resolution spectra have been submitted to the Mass Spectrometry Centre, Aldermaston, U.K.

RESULTS

Gas chromatography on four phases (OV-225 at 140°, OV-1 at 110°, PEGA at 160°, and Carbowax 20M at 120°) indicated the presence in the secretion collected from *D. tryoni* of at least four components which had similar retention characteristics. The mixture of these components collected from the gas chromatograph showed only end absorption in the UV. Mass spectrometry-gas chromatography showed molecular ions at *m/z* 143 and 129 for the major components of the two large peaks that were resolved on the OV-1 column used. A high-resolution spectrum of the major component gave the molecular ion as *m/z* 143.13029. $C_8H_{17}NO$ requires 143.13102 and the majority of the fragment ions contained both N and O. These results and the presence in the infrared spectrum of absorptions at 1680 cm^{-1} and 3460 cm^{-1} suggested that the compound was an *N*-substituted primary amide.

One freshly prepared Carbowax 20M 4.2m column resolved the four

major components in the rectal pheromone gland secretion of *D. tryoni* and these four were estimated to form more than 95% of the total volatile material from the glands (Figure 1). The separation of the amides obtained on Carbowax columns deteriorated rapidly with age and use. Within several days of use the major propanamide and acetamide (3P and 3A in Figure 1) became coincident. It was on an aged column that the ratios of components in individual flies were measured. The retention times at 140° for these four peaks were 21.2, 23.0, 24.4, and 27.2 min. The molecular ions were at m/z 157, 143, 129, and 143. The spectra of these four components suggested that they were all amides, and a fragment ion attributable to the protonated primary amide ($RCONH_3^+$) appeared at m/z 88, 74, 60, and 74, respectively. The amine moiety in each component thus contained five carbon atoms.

The fragmentation patterns for the three components of greatest amount were similar and included a substantial loss of methyl with subsequent loss of C_3H_6. The lack, in the spectrum of the component of highest

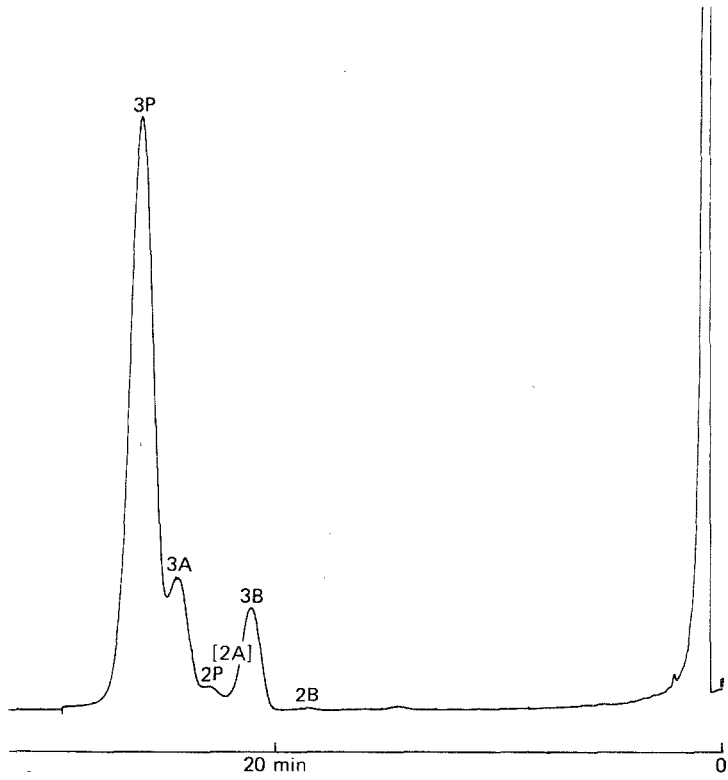

FIG. 1. Gas-chromatograph trace of volatiles from the rectal gland secretion of *Dacus tryoni*. Conditions: 4.2-m column containing 5% Carbowax 20M at 120°.

molecular weight, of a peak corresponding to a McLafferty rearrangement to give an ion at M-28 showed that the 4-carbon acyl fragment was derived from 2-methylpropanoic acid. These three components were therefore in order of abundance *N*-3-methylbutylpropanamide (3P), *N*-3-methylbutylacetamide (3A), and *N*-(3-methylbutyl)-2-methylpropanamide (3B).

The smallest peak of the four, some 3% of the total, with a molecular ion at m/z 143, was a propanamide. The mass spectrum lacked the prominent loss of CH_3 shown by the *N*-3-methylbutyl series of amides but did show a loss of C_2H_5. This indicated either a straight chain or a 2-methylbutyl substituent. The spectrum of this component corresponded to that of *N*-2-methylbutylpropanamide (2P) but not to those of the propanamides derived from 1-, 2-, or 3-pentanamine. The presence of this propanamide prompted a search for the corresponding *N*-2-methylbutylacetamide (2A) and *N*-(2-methylbutyl)-2-methylpropanamide (2B) which were found to be present in even smaller amounts.

Coinjection of synthetic 2A and 2B with the rectal gland secretion showed that 2B was coincident with a small component which in Figure 1 is at 18.4 min, while 2A appeared between 2P and 3B. The mass spectrum of this small component was very similar to that of synthetic 2B. The first peak (retention time 12.0 min) to emerge from an OV-225 column at 140° was collected and reinjected for GC-MS onto a 4.2 m Carbowax 20M column at 150°. Two peaks, in a ratio of about 1:15, emerged at retention times of 30.3 and 34.3 min. The mass spectrum of the first component was very similar to that of 2A. The second component was 3A.

The secretion from *D. neohumeralis* was analyzed by gas chromatography and mass spectrometry and the same six amides were identified. In this species too they formed the major part of the volatiles of the rectal gland secretion.

The identity of the six amides was confirmed by a comparison of the retention indices (Wehrli and Kováts, 1959) and mass spectra with those of authentic materials. With the exception of the minor 2-methylpropanamide (2B), the retention index of which was measured using the fly secretions directly, the amides from the secretions were first isolated using gas chromatography by first separating the acetamides from the other four amides on OV-225 at 140° and then separating these two fractions into their components on Carbowax 20M at 110° before determining the retention indices.

Retention indices (compound, conditions, index of natural compound, index of synthetic compound) were as follows:

Dacus tryoni: (3A) A, 1875,1874; (3P) A, 1884,1884; (3B) A, 1839,1838; (2P) A, 1859,1857; (2A) E, 1831,1831; (2B) E, 1797,1796.

Dacus neohumeralis: (3A) B, 1878,1880, C, 2192,2188; (3P) B, 1890,1891, C, 2192,2191; (3B) B, 1843,1841, C, 2131,2131; (2P) B, 1862,1862, C, 2174,2172; (2A) D, 1896,1896; (2B) D, 1852,1853.

Determination of Ratios of the Six Amides in Individual Males. The OV-225 column separated the amides into two peaks: the two acetamides (2A+3A) with a retention time of 6.5 min and the other four amides (3P+2P+2B+3B), which eluted as a single peak, with a retention time of 7.8 min. The Carbowax 20M column separated the amides into three peaks; (2B+3B) with a retention time of 10.8 min, (2A+2P) 11.9 min, and (3A+3P) 13.5 min.

The percentages of acetamides and 2-methylpropanamides were obtained directly by measurements of their peak size, and the figure for the propanamides was then obtained by subtraction of these from the total. These data are given in Table 2.

The secretion obtained from mature *D. tryoni* males caught in the wild contained the same compounds in about the same proportions as the laboratory-reared males.

DISCUSSION

Simple amides have not previously been reported from insects. *N*-3-Methylbutylacetamide has been identified in a Californian sherry (Webb et al. 1966) and in tobacco (Demole and Berthet, 1972). Both of the acetamides have been isolated from wines (Schreier et al., 1975).

The presence of 3-methylbutyl and of 2-methylbutyl residues parallels the presence of the corresponding amyl alcohols in fermentation products, where it has been shown, for example, in brewer's yeast (Ayrapaa, 1967), that the alcohols are derived from the amino acids leucine and isoleucine. Tracer experiments also demonstrated that the 3-methylbutanamine found in the ergot fungus *Claviceps purpurea* is formed from the leucine in the substrate, although 2-methylbutanamine was not found (Hartmann, 1965). It, therefore, appears likely that the ultimate sources of the two amine residues in *D. tryoni* and *D. neohumeralis* are also leucine and isoleucine, although

TABLE 2. PROPORTIONS OF 2-METHYLPROPANAMIDES (B), ACETAMIDES (A), AND PROPANAMIDES (P) IN INDIVIDUAL MALE FLIES OF *D. tryoni* AND *D. neohumeralis*

	3B + 2B[a]	3A + 2A	3P + 2P
D. tryoni (10 flies)	5.2 ± 2.2[b]	20.8 ± 3.5	74.0 ± 3.9
D. neohumeralis (14 flies)	3.4 ± 1.4[b]	19.4 ± 6.0	77.1 ± 6.7

[a]3 indicates a 3-methylbutyl and 2 indicates a 2-methylbutyl substituent.
[b]Mean percentage with standard deviation for the amide pair.

it is not known whether the decarboxylation is accomplished by the fly or whether the amines are derived from the food. The aliphatic acyl residues are well known from biological systems (Kahn, 1969). The source of the food appears to be unimportant, at least for the amide components, since both the laboratory-reared and the wild males of *D. tryoni* have very similar secretions.

Since both species have the same amides in about the same proportions, it would appear that these compounds are unimportant in the reproductive isolation of the two species. Behavioral studies (Fletcher and Bellas, unpublished) indicate that the mixture of amides may act as a short-range pheromone which increases the sexual excitement of the female once she has arrived in the vicinity of the males but does not act as an attractant acting over a distance. It is known, however, that other volatile compounds are present in the male pheromone gland secretion, since none of the amides has the characteristic sweet odor of the male flies, and that the total extract acts as an attractant over several meters (Fletcher, 1977). It is not yet possible to say, therefore, whether the pheromones from the male rectal gland play any role in the isolation of the two species. However, because of the differences in mating times and the associated rhythmic response of the females to the pheromone (Fletcher and Giannakakis, 1973), it is quite possible that the males of the two species do use exactly the same blend of chemicals in their pheromone. A preliminary gas chromatographic survey of the secretions of the rectal glands of *D. musae* (Tryon), *D. cucumis* French, *D. jarvisi* (Tryon), and *D. absonifacies* May, which all live in the same area and mate at about the same time as *D. tryoni* (i.e., around dusk), has shown that each species has a quite different pattern of components in its secretion.

REFERENCES

AYRAPAA, T. 1967. Formation of higher alcohols from ^{14}C-labeled valine and leucine. *J. Inst. Brew. London* 73:17–30.

BATEMAN, M.A. 1967. Adaptations to temperature in geographic races of the Queensland fruit fly, *Dacus* (*Strumeta*) *tryoni*. *Aust. J. Zool.* 15:1141–1161.

BATEMAN, M.A., FRIEND, A.H., and HAMPSHIRE, F. 1966. Population suppression in the Queensland fruit fly, *Dacus* (*Strumeta*) *tryoni*. *Aust. J. Agric. Res.* 17:699–718.

BELLAS, T.E. 1975. A computer program for calculating linear and logarithmic retention indices in gas chromatography. *Chromatographia* 8:38–40.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

DEMOLE, E., and BERTHET, D. 1972. A chemical study of *Burley* tobacco flavour (*Nicotiana tabacum* L.) I. Volatile to medium-volatile constituents (b.p. $\leqslant$84°/0.001 Torr). *Helv. Chim. Acta* 55:1866–1882.

FLETCHER, B.S. 1969. The structure and function of the sex pheromone glands of the male Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol.* 15:1309–1322.

FLETCHER, B.S. 1977. Behavioral response of Diptera to pheromones, allomones and kairomones, pp. 129-148, *in* H.H. Shorey and J.J. McKelvey, Jr. (eds). Chemical Control of Insect Behavior: Theory and Application, John Wiley and Sons, New York.

FLETCHER, B.S., and GIANNAKAKIS, A. 1973. Factors limiting the response of females of the Queensland fruit fly, *Dacus tryoni*, to the sex pheromone of the male. *J. Insect Physiol.* 19:1147-1155.

GIBBS, G.W. 1968. The frequency of interbreeding between two sibling species of *Dacus* (Diptera) in wild populations. *Evolution* 22:667-683.

HARTMANN, T. 1965. Zur Biogenese flüchtiger Amine beim Mutterkorn-pilz *Claviceps purpurea*. *Planta* 66:191-206.

KAHN, J.H. 1969. Compounds identified in whisky, wine, and beer: A tabulation. *J. Assoc. Off. Anal. Chem.* 52:1166-1178.

MAY, A.W.S. 1963. An investigation of fruit flies (fam. Trypetidae) in Queensland. 1. Introduction, species, pest status and distribution. *Queensl. J. Agric. Sci.* 20:1-82.

SCHREIER, P., DRAWERT, F., and JUNKER, A. 1975. Gaschromatographisch-massenspektrometrische Untersuchung flüchtiger Inhaltsstoffe des Weines IV. Nachweis sekundärer Amide in Wein. *Z. Lebensm.-Unters. Forsch.* 157:34-37.

SCHULTZ, G.A., and BOUSH, G.M. 1971. Suspected sex pheromone glands in three economically important species of *Dacus*. *J. Econ. Entomol.* 64:347-349.

STEINER, L.F., ROHWER, G.G., AYERS, E.L., and CHRISTENSON, L.D. 1961. The role of attractants in the recent Mediterranean fruit fly eradication program in Florida. *J. Econ. Entomol.* 54:30-35.

TYCHSEN, P.H., and FLETCHER, B.S. 1971. Studies on the rhythm of mating in the Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol.* 17:2139-2156.

VOGT, W.G. 1977. A re-evaluation of introgression between *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). *Aust. J. Zool.* 25:59-69.

WEBB, A.D., KEPNER, R.E., and GALETTO, W.G. 1966. Volatile components of sherry wine. II. Isolation and identification of *N*-(2-phenethyl)acetamide and *N*-isoamylacetamide. *Am. J. Enol. Vitic.* 115:1-10.

WEHRLI, A., and KOVÁTS, E. 1959. Gas-chromatographische Charakterisierung organischer Verbindungen Teil 3: Berechnung der Retentionsindices aliphatischer, alicyclischer und aromatischer Verbindungen. *Helv. Chim. Acta* 42:2709-2736.