Sex Pheromone Components in the New Zealand Greenheaded Leafroller *Planotortrix excessana* (Lepidoptera: Tortricidae)

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Planotortrix excessana was found to include moths of two distinct pheromone-types which were not mutually attractive. Tetradecyl acetate and (*Z*)-8-tetradecenyl acetate were identified as pheromone components in one, and two other tetradecenyl acetates, probably (*Z*)-5- and (*Z*)-7-tetradecenyl acetate, in the other. By contrast with other pheromones reported from the tribe Archipini, Δ11-tetradecenyl compounds were not found in either pheromone-type.

Introduction

The greenheaded leafroller, *Planotortrix excessana* (Walker), along with the brownheaded leafroller *Ctenopseustis obliquana* (Walker) and the light-brown apple moth *Epiphyas postvittana* (Walker) are predominant among the complex of leafroller pests of horticulture in New Zealand. All three moths are classified in the Tortricidae, subfamily Tortricinae, tribe Archipini [1]. Sex pheromone components have been identified in the Australian *E. postvittana* [2]. We have examined the other two species, which are endemic to New Zealand, and here describe the identification of pheromone components in *P. excessana* and give evidence for a second pheromone-type within the species as currently defined.

Materials and Methods

A colony of *P. excessana* was established from moths collected at Auckland. They were reared individually on a diet similar to that of Roelofs and Feng [3], substituting dried and powdered leaf of a locally available host plant, *Acmena smithii* (Poiret), in place of alfalfa leaf meal. Pupae were removed, sexed, and separated accordingly. Pheromone extract was collected from virgin female moths as previously described [4]. Male moths were maintained separately under a natural light cycle until required for bioassay.

Electroantennogram (EAG) responses of antennae of male moths were determined by the method of Roelofs [5] with standards presented as previously described [4]. A field cage bioassay, following that of Tamaki *et al.* [6] was also used. In this bioassay the pheromonal activity of fractions was determined by captures of released male moths in traps containing the fractions. Test fractions in pentane solution were spotted on filter paper strips which were placed in sticky traps (Pherocon IC; Zoecon Corp., USA) hung 1 m above the ground in a $7 \times 10 \times 2$ m field cage. The amount of extract or fraction per trap was 20 female-equivalents. Male moths were released in the cage each evening shortly before dusk, just prior to their normal period of activity. Counts of moths trapped were made the next morning. Synthetic materials were tested either on filter paper in the same way as extracts, or applied in pentane solution to 5 mm sleeve-type rubber caps

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(1780-B10, Arthur H. Thomas Co., USA), which were used for the duration of the test.

Materials on filter paper were replaced nightly. Field tests of synthetic materials used rubber caps in sticky traps hung 1.5 m above the ground and 15 m or more apart in areas infested with *P. excessana*. The trap positions were rotated weekly when the catches were recorded. For statistical comparison trap catches were transformed to $\sqrt{(\chi + 1/2)}$ and differences between means tested by analysis of variance (with fractions, replicates and successive night's catches treated as separate factors) and Duncan's new multiple range test. In the tables, the means listed are of untransformed catches.

Gas chromatography and mass spectrometry instrumentation and procedures were as previously described [4]. Female tip extract was fractionated by preparative GC using OV-1 (3% on Chromosorb W-HMDS 100/120 mesh in a 2 m × 5.3 mm ID stainless steel column), with 10% of the effluent split to the detector and 90% to the collector port [4]. Under the operating conditions used the retention times (R_T) of dodecyl, tetradecyl, and hexadecyl acetates were 4.7 min, 7 min, and 9.5 min respectively. Collections were made from 3 to 6 min (A), 6 to 8.5 min (B) and 8.5 to 11 min (C).

Female abdominal tip extract was also fractionated by column chromatography on Florisil [7]. Some fractions were saponified by 10% KOH in 80% ethanol/water overnight, or acetylated by acetic anhydride and pyridine. Preparative thin layer chromatography (TLC) was carried out on silica gel G impregnated with silver nitrate (30% w/w) and the plates developed with benzene. Zones corresponding to the alkyl, *E*-alkenyl, *Z*-alkenyl and alkadienyl acetates were located by running reference substances in the margins of the plate and locating these with a fluorescein spray.

Double bond location was determined by a procedure based on that of Francis and Veland [8] as modified by Buser *et al.* [9], involving bis-thiomethylation of the alkenyl acetates with dimethyl disulphide in the presence of iodine. Female tip-extract was first fractionated by preparative GC on OV-1 as described above. Derivatization was then carried out in a 100 µl conical reaction vial. A pentane solution of the relevant fraction was evaporated to about 20 µl and then treated with dimethyl disulphide (1 µl) and a solution of iodine in diethyl ether (60 mg ml^{-1}, 3 µl). After this reaction mixture had been kept at 40 °C overnight it was washed with aqueous $Na_2S_2O_3$ (50 µl), centrifuged, and the aqueous layer carefully removed. The pentane solution so obtained was evaporated to dryness under a gentle stream of nitrogen. Hexane (2 µl) was added to give a solution that was analyzed by GC-MS using a DB-1 bonded phase WCOT column (30 m × 0.3 mm fused silica, J & W Scientific Inc., USA). The MS was scanned continuously and the spectra stored on disc and later searched for the molecular and major fragment ions expected for derivatized alkenyl acetates.

Results

In initial tests, traps containing either virgin female moths or abdominal tip extracts both caught male *P. excessana*. The abdominal tip extract was then subjected to preparative GC on OV-1, collecting successive 1 min fractions over a time-span encompassing R_Ts of simple alkyl compounds up to about C20 chain length. These fractions were then screened using the EAG response of a male antenna. Maximal response was obtained for the fraction corresponding to the R_Ts of tetradecyl/tetradecenyl acetates (14 : Ac, U 14 : Ac).

When female abdominal tip extract was fractionated by column chromatography on Florisil only the "esters" fraction, eluted with 5% diethyl ether/pentane, was active in the field-cage bioassay (Table I (a)). Furthermore, saponification of the "esters" fraction destroyed its activity, which was then restored by acetylation (Table I (b)), in accord with the principal pheromone components being acetates. When the "esters" fraction was further fractionated by silica gel/silver nitrate TLC, only the *Z*-alkenyl acetate fraction was active in the field cage bioassay (Table I (c)), and this activity was not significantly increased by recombining it with the saturated, *E*-alkenyl and alkadienyl acetate fractions (Table I (d)).

When tip extract was fractionated by preparative GC on OV-1 a major signal was observed with R_T matching tetradecyl/tetradecenyl acetates, and upon field-cage bioassay only fraction B, incorporating this region, was active (Table I (e)). This activity was not significantly increased by any recombination of fractions (Tables I (e) and (f)). On the other hand, fraction B or combinations with it were significantly more active than crude tip extract,

Table I. Field cage bioassays: catches of male *P. excessana* in traps containing fractions of female abdominal tip extract (20 female equivalents per trap): (a) fractionation by Florisil chromatography (2 replicates; 130 moths released, 100 trapped); (b) saponification and acetylation of active fraction from (a) (2 replicates; 130 moths released, 93 trapped); (c) fractionation by silica gel/silver nitrate TLC (2 replicates; 100 moths released, 73 trapped); (d) recombinations of silver nitrate TLC fractions (4 replicates; 160 moths released, 56 trapped); (e) fractionation by GLC on OV-1 (2 replicates; 150 moths released, 137 trapped); (f) recombinations of GLC fractions (2 replicates, 350 moths released, 316 trapped).

Test fraction	Mean catch [males/trap][a]
(a) 0% ether in pentane	0.5 b
5%	26.0 a
10%	0.5 b
20%	0.0 b
50%	0.5 b
crude extract	22.0 a
blank	0.5 b
(b) 5% ("esters") fraction from (a)	21.0 a
saponified	0.5 b
saponified and acetylated	24.5 a
blank	0.5 b
(c) saturated	0.0 b
E	0.5 b
Z	15.5 a
diene	0.0 b
control (5% fraction from (a))	20.5 a
blank	0.0 b
(d) *Z*	8.25 a
saturated + *E* + diene	0.5 b
Z + saturated + *E* + diene	5.25 a
blank	0.0 b
(e) A (12C region)	0.5 b
B (14C region)	35.0 a
C (16C region)	0.0 b
A + B + C	33.0 a
blank	0.0 b
(f) A + B	80.5 a
A + C	1.5 c
B + C	56.5 a
crude extract	19.0 b
blank	0.5 c

[a] Means within the same treatment followed by the same letter are not significantly different at the 5% level.

which may therefore contain some material inhibiting pheromone activity.

Since both the EAG and field cage bioassay results indicated one or more tetradecenyl acetates as principal components of the *P. excessana* pheromone, confirmation of their presence in female tip-extract was sought by GC-MS analysis using selected ion monitoring (SIM). Ions monitored were *m/z* 61 (corresponding to $CH_3CO_2H_2^+$ species formed from acetates), and *m/z* 196 and 194 (corresponding to M-60 fragment ions from 14:Ac and U14:Ac respectively). Tip extract was fractionated by preparative GC on OV-1 as above and the combined fractions A, B and C subjected to GC-MS analysis on a Carbowax 20M SCOT column. Strong signals were detected with R_T's corresponding to 14:Ac (*m/z* 61 and 196 channels) and a U14:Ac (*m/z* 61 and 194 channels). The R_T of the latter relative to 14:Ac (R_T^{rel}) matched that of (*Z*)-8-tetradecenyl acetate (8Z14:Ac) within experimental error. In addition there was a very weak signal in the same region on both *m/z* 61 and 194 channels with an R_T^{rel} which could have corresponded to 10Z14:Ac or perhaps 11E14:Ac. No 8E14:Ac was detected. Other signals detected on the *m/z* 61 channel corresponded to 12:Ac, 16:Ac, and a U16:Ac with R_T^{rel} matching that of 10Z16:Ac within experimental error.

Further support for 8Z14:Ac as a major pheromone component in *P. excessana* was obtained when series of U12:Ac, U14:Ac and U16:Ac standards were screened using the EAG response of a male antenna: the maximum response was to 8*Z*14:Ac.

The presence of this compound in female moths was further substantiated when fraction B of the tip-extract from about 50 moths was subjected to bis-thiomethylation. Searches of stored spectral scans from GC-MS analysis of the derivatization products revealed spectra at the correct retention time, and with the molecular ion (*m/z* 348) and three major fragment ions (*m/z* 217, 157 and 131) expected from bis-thiomethylated Δ8-tetradecenyl acetate. Taken together with the silica gel/silver nitrate TLC and other results this provided strong evidence for the presence of 8*Z*14:Ac in the female tip extract. No fragment ion sets were found corresponding to Δ10- or Δ11-tetradecenyl acetates (*m/z* 245, 185, and 103; and 259, 199 and 89 respectively): if any such compound was present it was below our detection limit.

Traps baited with 8*Z*14:Ac caught male *P. excessana* both in the cage (Table II) and in the field (Table III), where the optimum loading on a rubber stopper was about 200 µg. Other possible pheromone components were tested in combination with 8*Z*14:Ac at ratios suggested by GC-MS analysis of tip extract. Addition of 10*Z*14:Ac had no signif-

Table II. Field cage bioassays: catches of male *P. excessana* in traps containing synthetic mixtures. Means followed by the same letter are not significantly different at the 5% level: (a) comparison of 8*Z*14:Ac and female extract (2 replicates, 4 nights; 600 moths released, 436 trapped); (b) test of added 10*Z*14:Ac (2 replicates, 3 nights; 325 moths released, 246 trapped); (c) test of added 12:Ac, 14:Ac, 16:Ac (2 replicates, 4 nights; 425 moths released, 227 trapped); (d) test of added 14:Ac (2 replicates, 6 nights; 260 moths released, 144 trapped).

Test mixture	Mean catch [males/trap/night][a]
(a) 100 µg 8*Z*14:Ac on rubber stopper	16.1 b
female extract (20 *FE* on paper, replaced nightly)	37.9 a
blank	0.5 c
(b) 5 µg 8*Z*14:Ac on paper	11.7 a
plus 0.5% 10*Z*14:Ac	11.5 a
plus 2% 10*Z*14:Ac	7.8 a
plus 5% 10*Z*14:Ac	9.5 a
blank	0.5 b
(c) 100 µg 8*Z*14:Ac on rubber stopper	5.6 b
plus 2% 12:Ac	4.9 b
plus 30% 14:Ac	10.0 a
plus 2% 16:Ac	7.3 a b
blank	0.6 c
(d) 100 µg 8*Z*14:Ac on rubber stopper	4.1 b
plus 30% 14:Ac	7.1 a
blank	0.8 c

[a] Means within the same treatment followed by the same letter are not significantly different at the 5% level.

Table III. Field catches of male *P. excessana* in traps containing synthetic mixtures on rubber stoppers. Havelock North, 20 May to 4 July 1983. 2 replicates; catches followed by the same letter are not significantly different at the 5% level.

Bait composition in µg		Catch/trap/week
8*Z*14:Ac	11*E*14:Ac	
50	0	10.9 a b
100	0	17.0 a
200	0	17.7 a
500	0	6.0 b c
50	1	3.3 c d
0	0	0.0 d

Table IV. Field cage catches of male Lincoln and Tokoroa *P. excessana* in traps containing female abdominal tip extract (2 replicates; 225 Lincoln and 25 Tokoroa moths released).

	Female extract in traps		
	Lincoln	Tokoroa	Blank
Lincoln males trapped:	214	1	1
Tokoroa males trapped:	0	14	0

icant effect on trap catch (Table II (b)), while addition of 11*E*14:Ac lowered trap catch (Table III). Addition of 12:Ac or 16:Ac had no effect on trap catch but addition of 14:Ac increased it significantly (Table II (c)) despite the previous negative results with the equivalent combination of fractions from silica gel/silver nitrate TLC (Table I (d)). In a further test, addition of 14:Ac to 8*Z*14:Ac again significantly increased trap catch (Table II (d)). We conclude that both 8*Z*14:Ac and 14:Ac are pheromone components in *P. excessana*. A more sensitive bioassay might elucidate their precise roles.

Traps baited with 8*Z*14:Ac alone or with added 14:Ac caught male *P. excessana* in several areas of New Zealand: Auckland and Hawkes Bay (North Island), and Canterbury and Otago (South Island). However in one North Island area (Tauranga), 8*Z*14:Ac failed to attract *P. excessana*, although other trapping methods confirmed that moths of this species were present (D. Steven, pers. comm.). A possible reason for this disparity became apparent as a result of a fortuitous finding.

When rearing problems led to the loss of our original colony of *P. excessana* (derived from Auckland moths) in winter 1982, we had difficulty obtaining insects to start a new colony. Eventually *P. excessana* was obtained from two sources: a laboratory colony from Lincoln (Canterbury), and eggs from a single gravid female collected at Tokoroa (central North Island). The latter were kept separately and reared to adult moths but did not become established as a continuous culture.

We were surprised to find that abdominal tip extract from Tokoroa female moths did not attract Lincoln males. A test was therefore made of cross attraction between the two populations. Traps were baited with extract from female moths from each colony and suspended in the field cage. Males from both colonies were then released; as we could not otherwise distinguish them, Tokoroa males were marked by dusting with fluorescent powder ("Day-glo Saturn Yellow"). The results (Table IV) demonstrated strong attraction of male moths to extract from females of the same colony, but no cross attraction.

We therefore re-examined both the identification and the pheromones of these moths. As to the former, the current taxonomic description of *P. excessana* is that of Dugdale [10]. He examined moths from our Auckland, Lincoln and Tokoroa colonies,

confirmed the identification of all three as *P. excessana* and found no consistent morphological differences between them (Dugdale, pers. comm.). We checked their pheromones by the EAG responses of male antennae to U12:Ac, U14:Ac and U16:Ac standards and by GC-MS analysis of female abdominal tip extract. For Lincoln moths the results were no different to those from Auckland. However for Tokoroa moths, the maximum EAG response was not to 8*Z*14:Ac but to 5*Z*14:Ac. GC-MS examination of Tokoroa female abdominal tip extract (using the Carbowax 20M SCOT column and SIM at *m/z* 61, 194 and 196) revealed 14:Ac, no detectable 8*Z*14:Ac but two other signals on both the *m/z* 61 and 194 channels with R_T^{rel}'s which could have corresponded to any of several U14:Acs with double bond position 5, 6 or 7. When the remaining tip extract (from about 15 females) was subjected to bis-thiomethylation and GC-MS analysis, searches of stored spectral scans for the molecular ion of (derivatized) tetradecenyl acetate (*m/z* 348) again indicated two such compounds. Searches also revealed two of the three major fragment ions expected from Δ5-tetradecenyl acetate (*m/z* 173 and 175, but not 115, for which there was a high background level) in spectra corresponding to the derivatized tetradecenyl acetate of shorter R_T, and all three major fragment ions expected from Δ7-tetradecenyl acetate (*m/z* 143, 145 and 203) in spectra corresponding to that of longer R_T. No such fragment ions were detected corresponding to Δ8-tetradecenyl acetate, or any other tetradecenyl acetate.

Field tests were made of all combinations of 5*Z*-, 5*E*-, 6*Z*- or 6*E*14:Ac with 7*Z*- or 7*E*14:Ac in orchards at Tauranga where 8*Z*14:Ac had failed to attract *P. excessana*. Traps were baited with rubber caps loaded with 50 µg of mixtures of each of the 8 combinations at ratios (3:2 and 4:1) close to that observed in the GC-MS analysis of Tokoroa female tip extract. The whole series was replicated twice. Catches after 2 weeks were low, but of the 8 combinations, male *P. excessana* were attracted only to those traps baited with 5*Z*14:Ac plus 7*Z*14:Ac (3 and 11 in the 3:2 replicates, 0 and 1 in the 4:1 replicates) and to no others. At a site (Havelock North, Hawkes Bay) where traps baited with 8*Z*14:Ac continued to catch *P. excessana*, traps baited with the above mixtures of 5*Z*14:Ac and 7*Z*14:Ac caught none.

Discussion

Our data show that *P. excessana* as currently defined is pheromonally heterogeneous. The sex pheromone of moths from Auckland and Lincoln contains 8*Z*14:Ac and 14:Ac, while moths from Tokoroa, which we were unable to distinguish morphologically, employ a distinctly different pheromone incorporating two other tetradecenyl acetates, probably 5*Z*14:Ac and 7*Z*14:Ac. We have no information on whether they differ in any other respect such as behaviour or host range; further work is required to determine these, as well as the full pheromone composition, geographic distribution and taxonomic status of the different types.

These distinct pheromone-types of *P. excessana* which do not cross-attract and are therefore unlikely to interbreed, may represent morphologically similar species within *P. excessana* as currently defined – *i.e.* sibling species in the sense of Mayr [11]. Several instances of sibling species distinguished by their pheromones have been previously reported, including a pair of species from Archipini [12–14].

An alternative explanation could be that *P. excessana* exhibits pheromonal polymorphism without reproductive isolation between the different forms, as in the pyralid *Ostrinia nubilalis* [15]. This seems less likely given the lack of cross-attraction demonstrated for the *P. excessana* pheromone-types and their lack of the aggregation behaviour which in *O. nubilalis* is thought to enable cross-mating without longer range cross-attraction [15].

On the basis of their identified or likely pheromone components both types of *P. excessana* differ from those Tortricinae whose pheromones are known. Neither 8*Z*14:Ac nor 5*Z*14:Ac nor 7*Z*14:Ac has been previously reported from this group, though 8*Z*14:Ac has been reported as a sex attractant for two species from the Olethreutinae, another subfamily of Tortricidae [16, 17].

Pheromones have been previously reported from over 30 species of Archipini [17–21] and thus far all have included Δ11-tetradecenyl compounds. However we did not find such compounds in either pheromone-type of *P. excessana*. This unconformity suggests that either those species whose pheromones have so far been reported are not representative of the whole tribe (and certainly they include few species from outside North America, Europe or Japan [2, 18, 20, 22]), or the present tribal classifica-

tion based on morphology does not entirely reflect the pattern of pheromone constituents in the Tortricinae. The current situation in tortricine taxonomy has been described as "confused" by Horak [23] who also concluded that Archipini in particular "clearly are a polyphyletic group". Information on pheromones may assist in the revision of their classification. Finally, it should also be noted that at least one if not both of the pheromone-types of *P. excessana* are serious pests in New Zealand and hence the resolution of their status and the differences between them will be of economic as well as scientific interest.

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