Supplementary material to:

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Pine emperor moths from KwaZulu-Natal use the same pheromone component previously isolated from *Nudaurelia cytherea* (Lepidoptera: Saturniidae) from the Western Cape

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Electronic Supplementary Material 1:

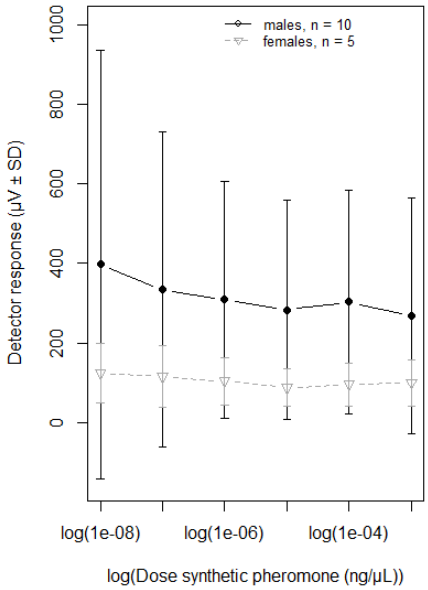
EAG Dose Response

METHODS AND MATERIALS

A serial dilution of (*Z*)-dec-5-en-1-yl-3-methylbutanoate, was prepared in dichloromethane. Concentrations ranged from 10^{-8} ng/µl to 10^{-3} ng/µl. A volume of 1 µl of each concentration was pipetted onto filter paper strips (Whatman 1, 0.5 x 2.2 cm) and inserted into Pasteur pipettes. Puffs (1 second) were generated with the help of a Syntech stimulus controller (CS-55) at a flow rate of 159.8 ± 0.6 l/hour. All concentrations were puffed over the same antenna in increasing dose order, with thirty second intervals between stimuli. Electroantennographic responses were recorded from male (n = 10) and female (n = 2) antennae. The response size for each puff was measured from raw direct current data (GcEad32 V4.3, Syntech, Hilversum, The Netherlands). Kruskal-Wallis non-parametric significance tests were performed with blank-subtracted antennal response data in R version 3.5.2.

RESULTS

Electrophysiological responses (315.7 ± 45.8 µV, mean ± SD, n = 10) from male antennae were on average larger than those of female antennae (104.7 ± 13.3 µV, mean ± SD, n = 5), but not significantly different (P = 0.416) (Supplementary Fig. 1 and Supplementary Table S1.1). A larger variance in response size was observed for male antennae when compared to female antennae. Subsequent recordings on the GC-EAD confirmed that the antennae can detect the dichloromethane (DCM) solvent that was used to dilute the pheromone standard. Electrophysiological responses measured with this technique were thus confounded by the response to the DCM solvent. The response to the solvent was removed when the same experiment was done on the GC-EAD.



Supplementary Fig. 1. A comparison of male (black, n = 10) and female (grey, n = 5) antenna response sizes (mean ± SD µV) toward the log transformed results of puffs in increasing order of concentrations (10^{-8} to 10^{-3} ng/µl) of the synthesised pheromone in an EAG dose response experiment.

Table S1.1. Statistical results for EAG puffing results. The chi-squared critical value was 10.36 in multiple Kruskal-Wallis tests at 5% confidence level (n = 5, df = 1 for each test).

Concentration (ng/µl)	Treatment 1	Treatment 2	p-value	Kruskal Wallis chi-squared
1.00E-03	Male	Female	0.255	1.2968
1.00E-04	Male	Female	0.193	1.6938
1.00E-05	Male	Female	0.143	2.1438
1.00E-06	Male	Female	0.166	1.9189
1.00E-07	Male	Female	0.329	0.95278
1.00E-08	Male	Female	0.514	0.42645

Electronic Supplementary Material 2:

DMDS-derivatisation analysis

INTRODUCTION

The synthesised pheromone of the pine emperor moth (*Nudaurelia clarki* (Lepidoptera: Saturniidae)), (*Z*)-dec-5-en-1-yl-3-methylbutanoate, was treated with DMDS similar to literature (Buser *et al.* 1983). The purpose of the experiment was to verify the location of double bonds and also the similarity of the identities of the components in the synthesised pheromone standard that elicited four repeatable electroantennographic responses during GC-EAD (gas chromatography electroantennographic detection) screenings using male and female *N. clarki* antennae.

The expected mass-to-charge ratios of *(Z)*-dec-x-en-1-yl 3-methylbutanoate, were determined theoretically (Fig. S6.1). It is expected that the correct molecule was synthesised, thus the expected prominent mass-to-charge fragments of the major component in the synthesised pheromone are A^+-SCH_3:117 m/z and B^+-SCH_3: 217 m/z, indicating the double bond at C5 from the ether functional moiety (Fig. S6.1). The pheromone has previously been shown to have (*Z*)-conformation (Henderson *et al.* 1973), and is expected to show threo-addition of DMDS to the double bond (Buser *et al.* 1983).

METHODS AND MATERIALS

The same methods were used as reported previously (Buser *et al.* 1983), except that incubation proceeded for 90 hours at a temperature of 60°C instead of 15 hours at 40°C.

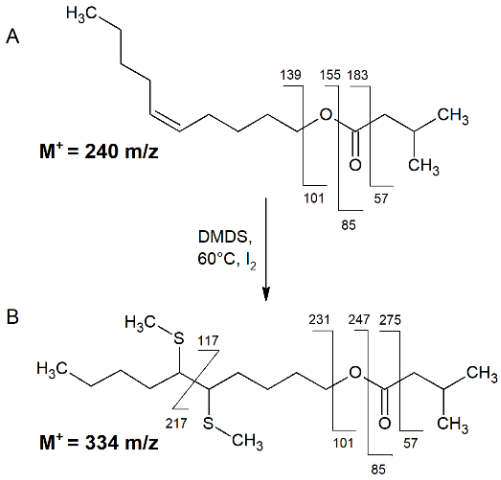
DMDS-treated or untreated synthetic (*Z*)-dec-5-en-1-yl 3-methylbutanoate (50 ng/µl) or double distilled n-hexane (blank) or DMDS was injected (1 µl) in splitless mode into the GC-MS (purge vent at 2 minutes, 20 ml/min). The oven was held at 50°C for 1 minute, and ramped (20°C/min) to 250°C where it was held for 7 minutes in the ZBWax column (30 m x 0.25 mm ID, 0.25 µm, 7HG-G007-11, Zebron™).

RESULTS

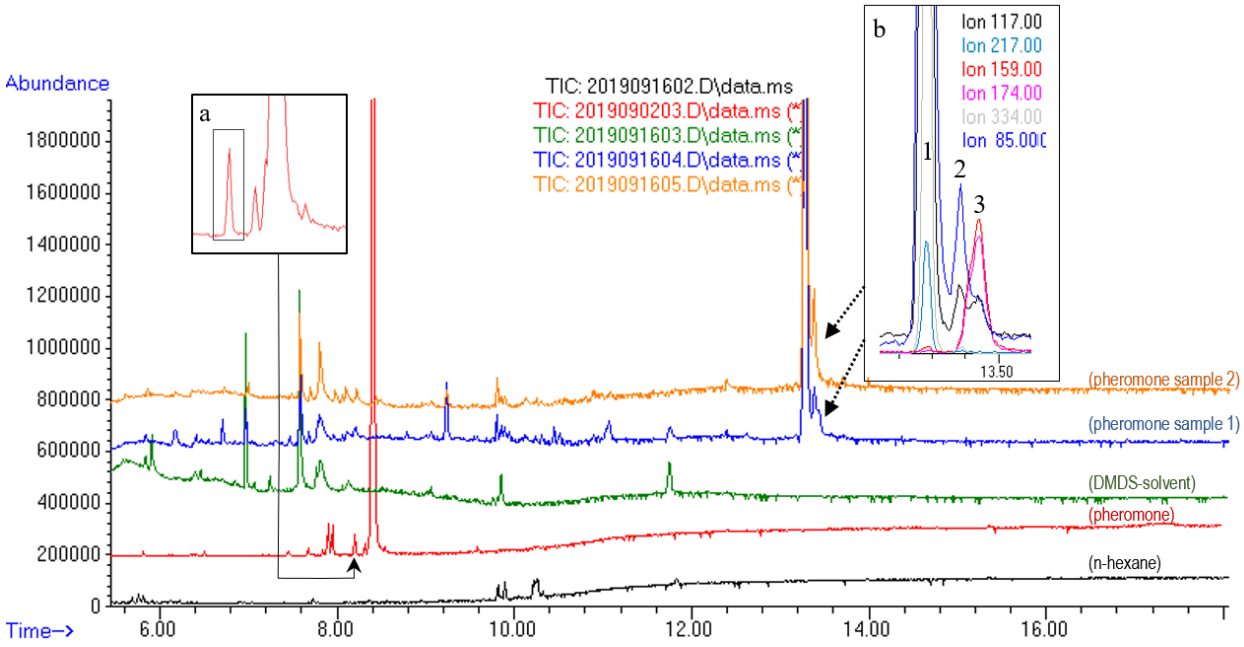
Our results confirmed that the synthetic pheromone mixture of four components was derivatised successfully and to completion. No detectable peaks were observed at the original elution time for unreacted unsaturated components and the chromatographic peaks of derivatised pheromone components eluted later than the untreated components on the ZBWax column (Fig. S6.2). One of the four components in the synthesised standard was expected to be the saturated derivative of the synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate based on its mass fragmentation results (peak 4, Supplementary Fig. 1, not shown here) and was expected not to react with the DMDS reactant (Fig. S6.2a). This expected result was confirmed upon identification of only three distinguishable peaks, and not four from treated samples (Fig. S6.2, n = 2). The saturated component was below detection limits of the GC-MS due to the addition of reagents.

The peaks of the DMDS-derivatised compounds eluted at (1) 13.26 min, (2) 13.39 min and (3) 13.44 min (Fig. S6.2b). Their respective Kovats retention Indexes (I_K) are I_K (1) = 2739, I_K (2) = 2756 and I_K (3) = 2762 on the ZBWax column. The untreated pheromone components eluted at Rt (1): 8.204 min; Rt (2): 8.314 min; Rt (3): 8.366 min and Rt (4): 8.389 min (major) when the same GC-parameters were used.

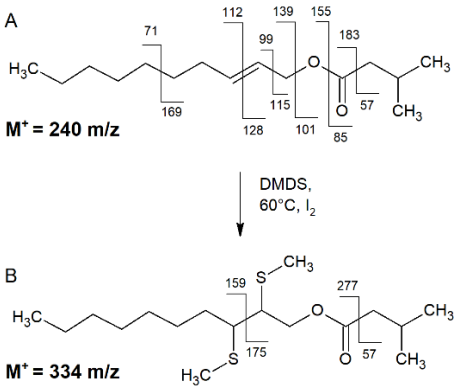
Mass fragmentation results of peak 1 (major, Fig. S6.2b) confirmed the double bond position at C5 in (*Z*)-dec-5-en-1-yl-3-methylbutanoate, based on the expected fragment ions 117 and 217 m/z and parent ion, 334 m/z (Fig. S6.1). Results were only able to show that peak 2 has similar mass fragments to peak 1. The mass fragmentation pattern of peak 3 showed the presence of prominent 158 and 174 m/z ions after treatment with DMDS, suggesting that the original compound was dec-2-en-1-yl-3-methylbutanoate (Fig. S6.3).



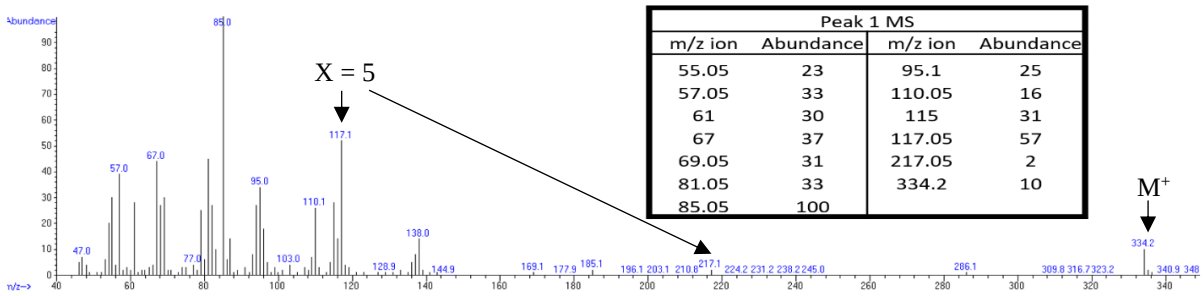
S6.1: The expected reaction scheme from the structure of the previously identified pheromone, (Z)-dec-5-en-1-yl-3-methylbutanoate (A, double bond on C5), to its derivatised form after treatment with DMDS (B). Expected mass-to-charge ratios are 117 and 217 m/z, as shown on B. This reaction scheme applies to peaks 1 and 2 in Fig. S6.2b.



S6.2: The overlaid total ion chromatographic (TIC) elution profiles of the untreated n-hexane solvent (black), 50 ng/µl synthesised pheromone (red), DMDS-treated n-hexane solvent (green), and DMDS-treated 50 ng/µl synthetic pheromone samples 1 (blue) and 2 (orange). Note the distinct difference in retention times between reagents (a) and treated products (b) as described in Buser *et al.* 1983. (a) The peak indicated in a box, eluting at 8.21 min, did not react in the DMDS derivatisation due to its saturated structure, as expected. (b) Product peaks of the treated pheromone components are indicated with dotted arrows, and their deconvoluted characteristic ions are enlarged to show three distinguishable peaks, with overlapping distinct ions of peak 3 over peak 2.

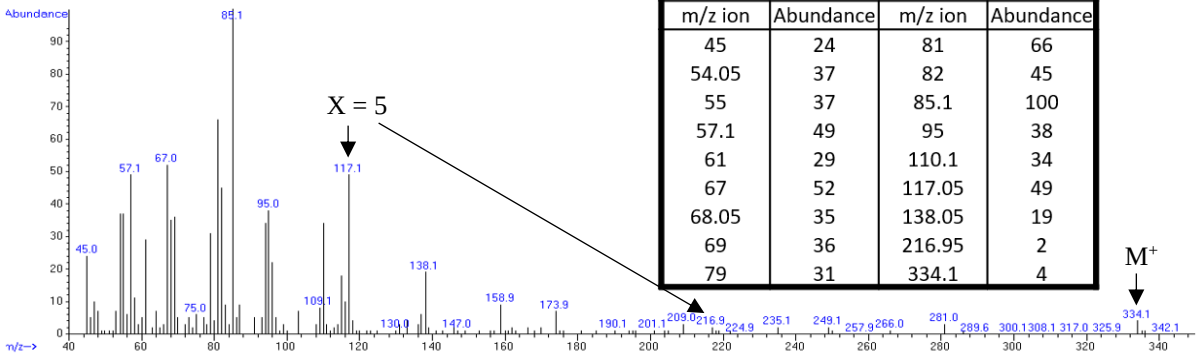


S6.3: A hypothesised reaction scheme of peak 3 (Fig. S6.2), proposed to be 2-decen-1-yl-3-methylbutanoate (A, double bond on C2) after derivatisation with DMSD (B).



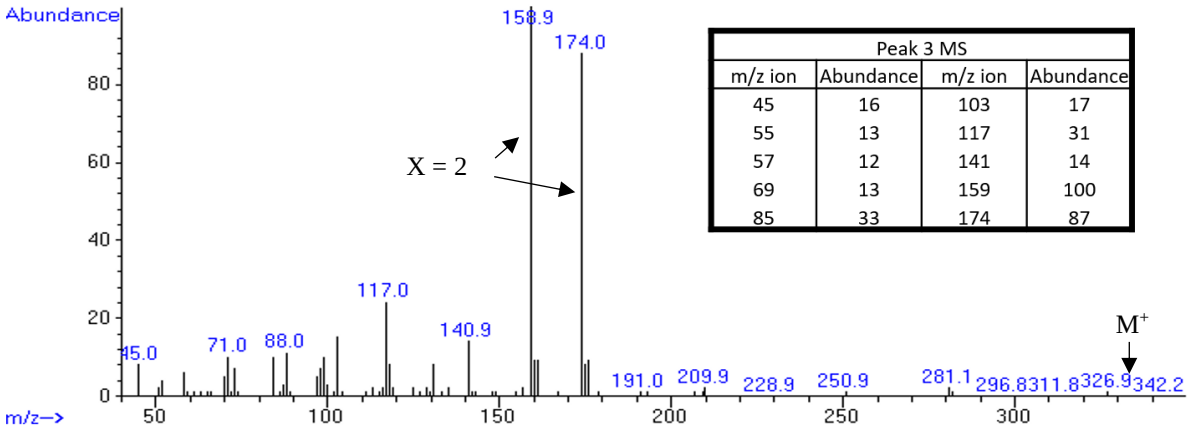
Peak 1 MS			
m/z ion	Abundance	m/z ion	Abundance
55.05	23	95.1	25
57.05	33	110.05	16
61	30	115	31
67	37	117.05	57
69.05	31	217.05	2
81.05	33	334.2	10
85.05	100		

S6.4: The MS fragmentation pattern of peak 1 (Fig. S6.2b, I_K (1) = 2739) after treatment with DMDS. The characteristic ions, 117 and 217 m/z are shown with arrows. The molecular ion at 334 m/z is also indicated.



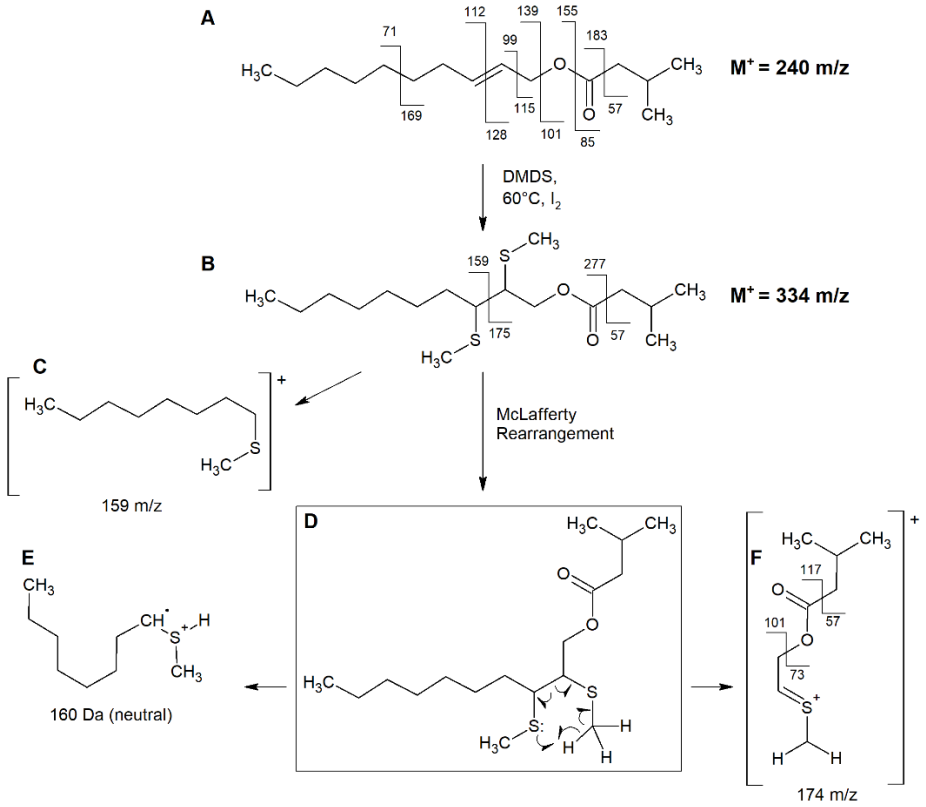
Peak 2 MS			
m/z ion	Abundance	m/z ion	Abundance
45	24	81	66
54.05	37	82	45
55	37	85.1	100
57.1	49	95	38
61	29	110.1	34
67	52	117.05	49
68.05	35	138.05	19
69	36	216.95	2
79	31	334.1	4

S6.5: MS fragmentation pattern of peak 2 (Fig. S6.2b, I_K (2) = 2756) after treatment with DMDS. Characteristic ions, 117 and 217 m/z are present and pointed out with an arrow. Other ions, including 159 and 174 m/z are also present, but these are due to a coelution with peak 3 (Fig. S6.2b). The molecular ion, 334 m/z is also indicated with an arrow.



Peak 3 MS			
m/z ion	Abundance	m/z ion	Abundance
45	16	103	17
55	13	117	31
57	12	141	14
69	13	159	100
85	33	174	87

S6.6: MS fragmentation pattern of peak 3 (Fig. S6.3/2b, I_K (3) = 2762) after treatment with DMDS. The 159 and 174 m/z fragments are shown with arrows, and are indicative of sulfur-containing fragments due to characteristic m/z + 1 and m/z + 2 isotopic ions in similar abundances for both fragments. The molecular ion, 334 m/z, is present in very low abundance (0.25%) in this fragmentation pattern.



S6.7: A hypothesised reaction scheme of dec-2-en-1-yl-3-methylbutanoate (A) after derivatisation with DMSD (B). The expected characteristic ions, C and F, are shown. We propose a mechanism of a McLafferty rearrangement of B to result in the formation of a neutral fragment (E) and the prominent 174 m/z-ion (F) seen in Fig. S6.6.

DISCUSSION

The expected molecular ion of the reacted pheromone (334 m/z, Fig. S6.1) is present in the mass fragmentation profile of the major component, peak 1 (Fig. S6.2b). This indicates that the DMDS reaction has indeed taken place. Both indicative fragments, 117 and 217 m/z are present, indicating the position of the double bond on C5, thus the correct

structure (Fig. S6.1). The base peak is 85 m/z, indicating the presence from the isovalerate moiety of the structure (B, Fig. S6.1). The major pheromone component is thus confirmed as (*Z*)-dec-5-en-1-yl-3-methylbutanoate.

Peak 1 and peak 2's fragmentation patterns are similar (Fig. S6.4 and S6.5), and have the presence of the same molecular ion (334 m/z, Fig. S6.1). The only difference is the presence of ions 159 m/z and 174 m/z, but these ions are due to a coelution of peak 3 (Fig. S6.2b). Peak 2 was distinguishable from peak 3 due to the fact that fragments including 57, 67, 81, 95 and 110 m/z were present only in peak 2 (Fig. S6.2b). The tentative identity of peak 2 is expected to be the trans-isomer of dec-5-en-1-yl-3-methylbutanoate. The presence of the same characteristic ions, 117 and 217 m/z-in this peak's elution do not contradict the expected results, even though co-elution hinders a clear result. The hypothesis that this compound identity is E-dec-5-en-1-yl-3-methylbutanoate, is not rejected. All other possible double bond positions were excluded based on the absence of other expected indicative fragments. Analysis of the fragmentation pattern and retention index of a pure form of this compound is necessary to confirm this tentative identification.

Peak 3 has a different fragmentation pattern than the other peaks, although the expected molecular ion is present in very low abundance in the mass spectral profile (334 m/z, Fig. S6.6). The presence of the m/z+1 and m/z+2 isotopic peaks in similar abundances in both 159 m/z and 174 m/z suggest that both these fragments contain a sulfur-atom, as expected for DMDS-treated fragments. These ions are also the two most abundant ions and correspond to an isomer of the major pheromone component with its double bond on C2 (Fig. S6.3). A 175 m/z fragment was expected (Fig. S6.1), but the results showed only the fragment of 174 m/z. We propose that this fragment may have been formed in a McLafferty rearrangement, where a single hydrogen is lost to a nucleophilic sulfur after the DMDS reaction (D, E and F, Fig. S6.7). Thus, 159 m/z and 174 m/z-ions (C, F, Fig. S6.7) indicate that the parent component could be dec-2-en-1-yl-3-methylbutanoate (A, Fig. S6.3).

Ultimately, we were able to confirm the double bond location of the synthetic pheromone components that are unsaturated, and the identity of the major pheromone component, (*Z*)- dec-5-en-1-yl-3-methylbutanoate. Our results showed three components in the synthetic pheromone standard are structural isomers of one another and the major pheromone component, resulting in complex mass fragmentation rearrangements. The prediction of where double bonds are in these component structures are not absolute and need further investigation. This work is part of the investigation of the pheromone of the pine emperor moth, that can aid in the sustainable management of this pine plantation pest.

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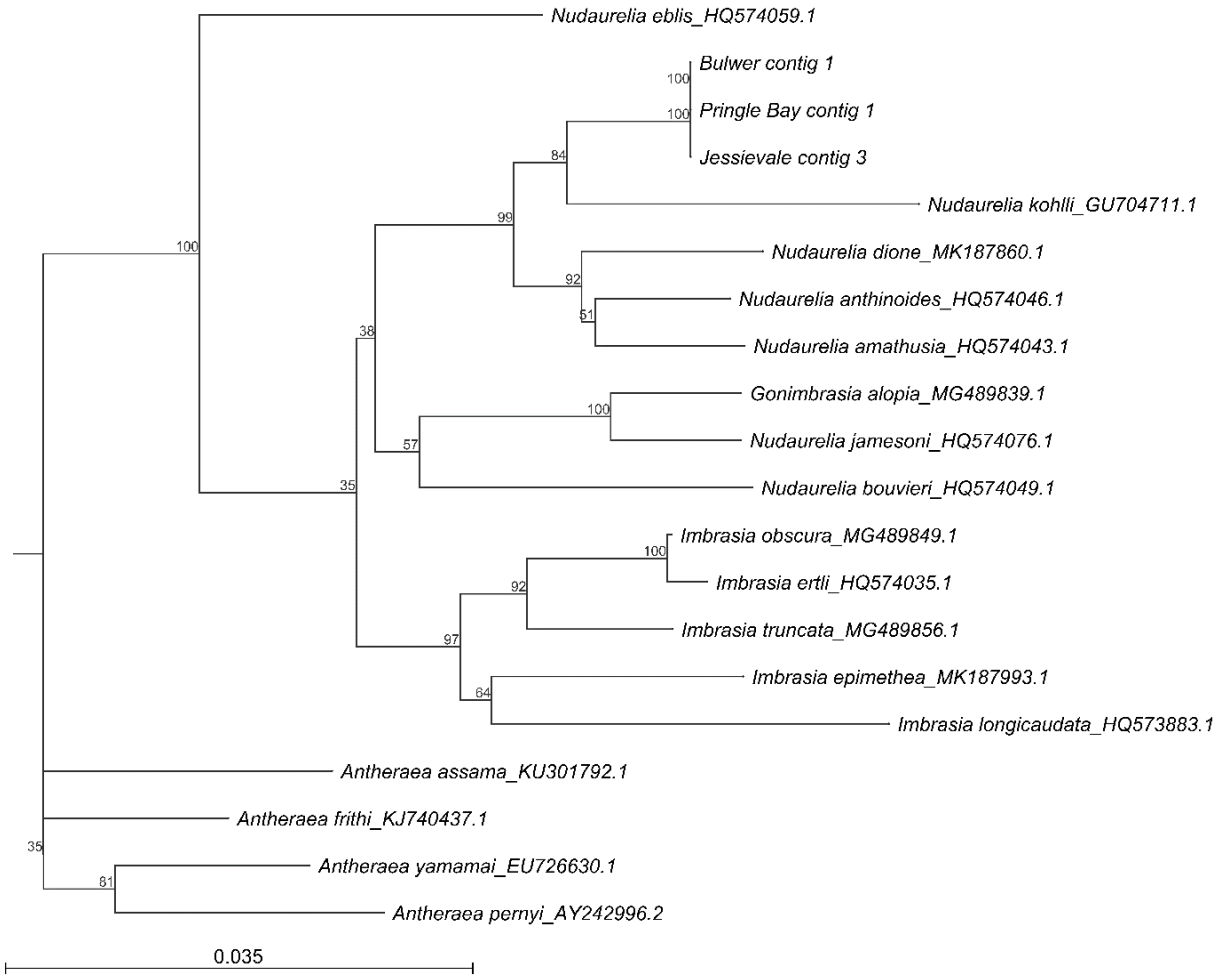

Figure S1. A neighbor-joining tree of the COI gene consensus sequence of representative samples obtained from *N. clarki* from Bulwer (KZN), Jessievale (Limpopo) and Pringle Bay (Western Cape) in South Africa. All consensus sequences were identical between moths. The position of *N. clarki* is shown in relation to other Saturniidae moth species from *Nudaurelia, Imbrasia, Gonimbrasia* and *Antherea* genera, from available sequences on GenBank. Species of the *Antherea* genus was chosen as the outgroup. The tree was constructed with a bootstrap value of 1000.

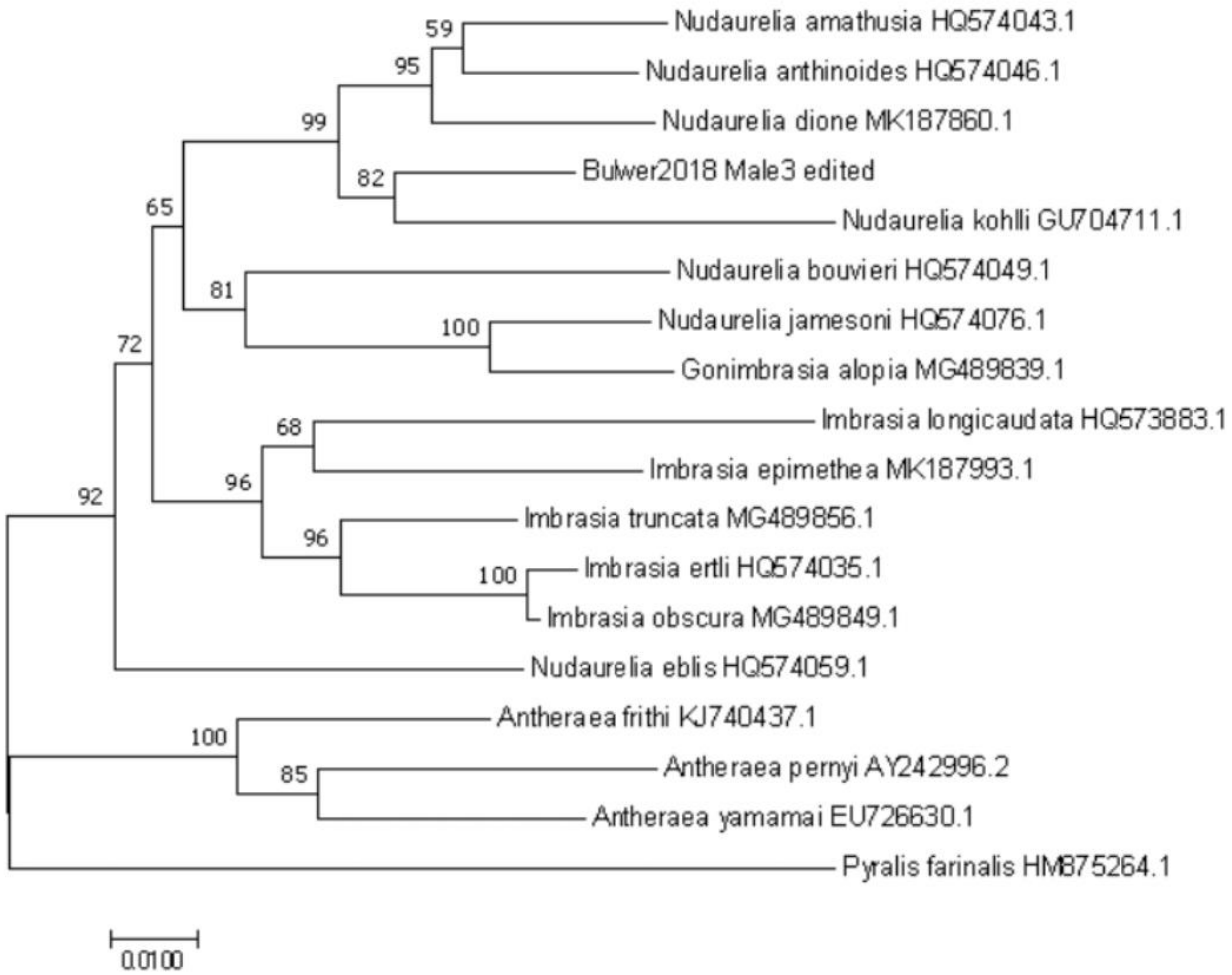

Figure S2. A neighbor-joining tree of the *COI* gene regions of the closest relatives of the Pine Emperor moth, *N. clarki,* (represented here as Bulwer2018 Male3 edited) in the Saturniidae family. Species in the *Antherea* genus group sister to a previously established outgroup for Saturniidae, *Pyralis farinalis* (Lepidoptera: Pyralidae). The support for branches in the tree was calculated from a 1000 bootstrap replicates.

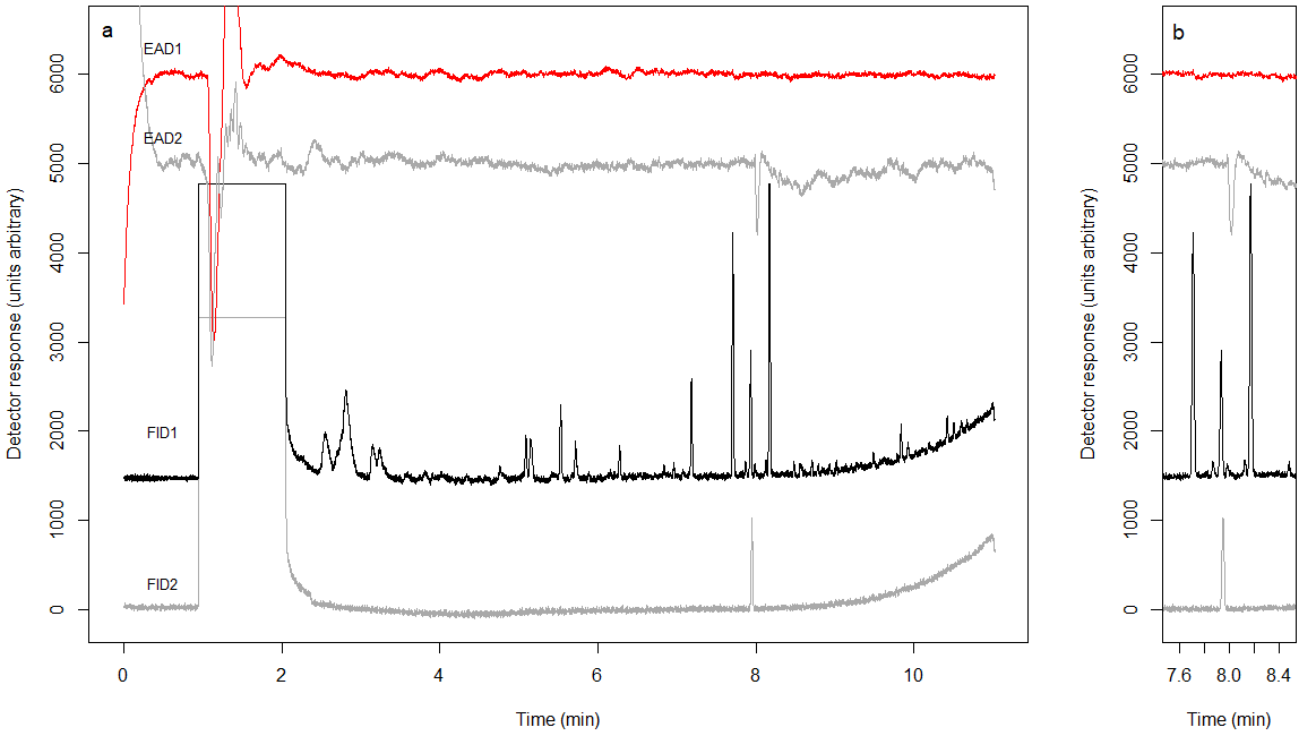

Figure S3. A comparison between a representative electroantennographic result of a male *N. clarki* antenna in response to a dynamically sampled virgin or field collected *N. clarki* female (EAD 1 and FID 1) and the synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate pheromone standard (EAD 2 and FID 2, 1 µl 1 ng/µl).

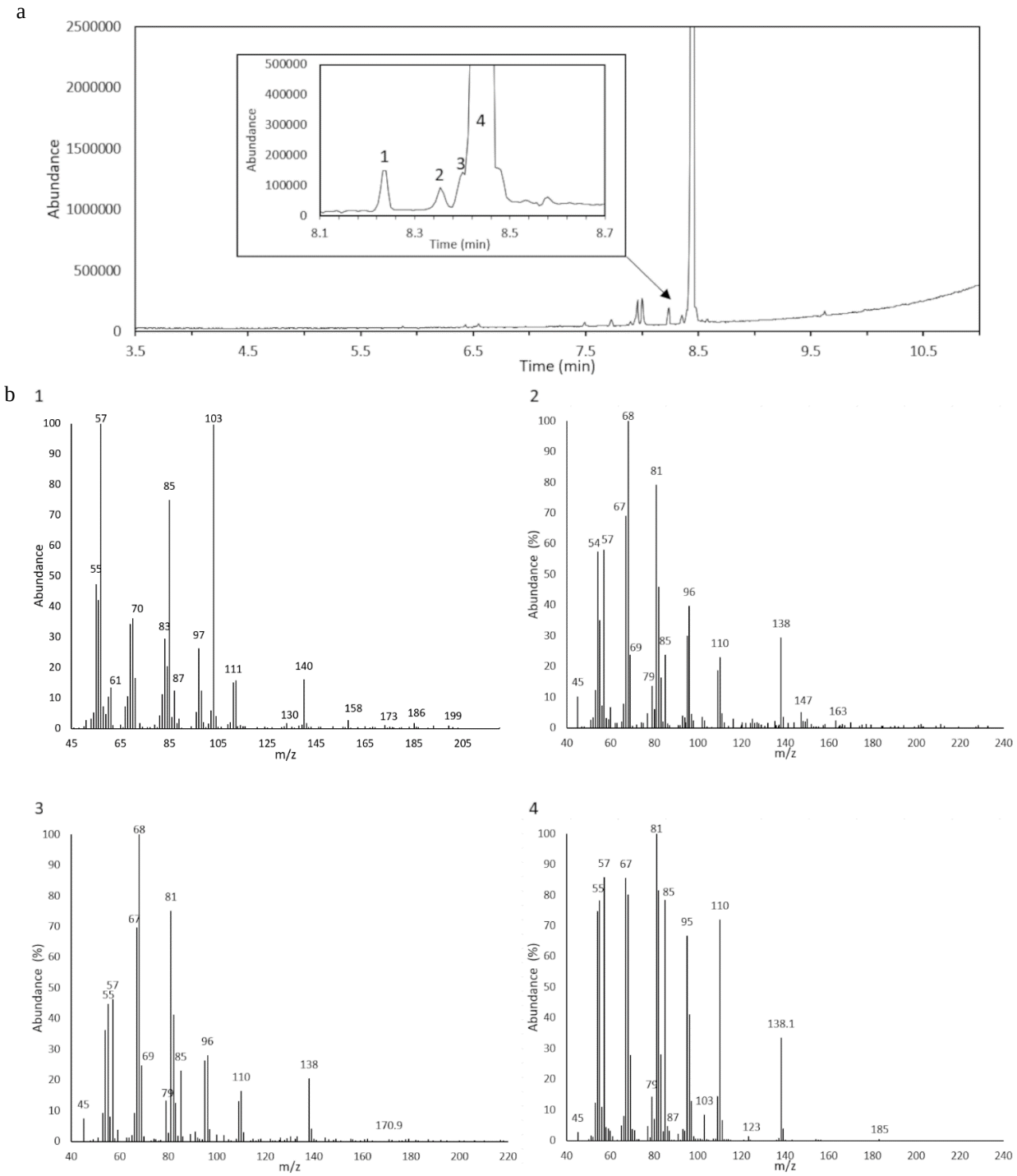

Figure S4. (a) A total ion chromatogram (TIC) elution profile of the synthesised pheromone, (*Z*)-dec-5-en-1-yl-3-methylbutanoate after injection on the GC-MS using a ZBWax column. The Kovats retention indices of the peaks from left to right are (1) = 1852 ± 0, (2) = 1873 ± 0, (3) = 1882 ± 0 and (4) = 1887 ± 1 (*n* = 3). (b) The MS fragmentation patterns for the respective peaks are shown. The major component, (*Z*)-dec-5-en-1-yl-3-methylbutanoate corresponds to peak 4. Note that the molecular ion could not be detected in any of the four mass spectra.

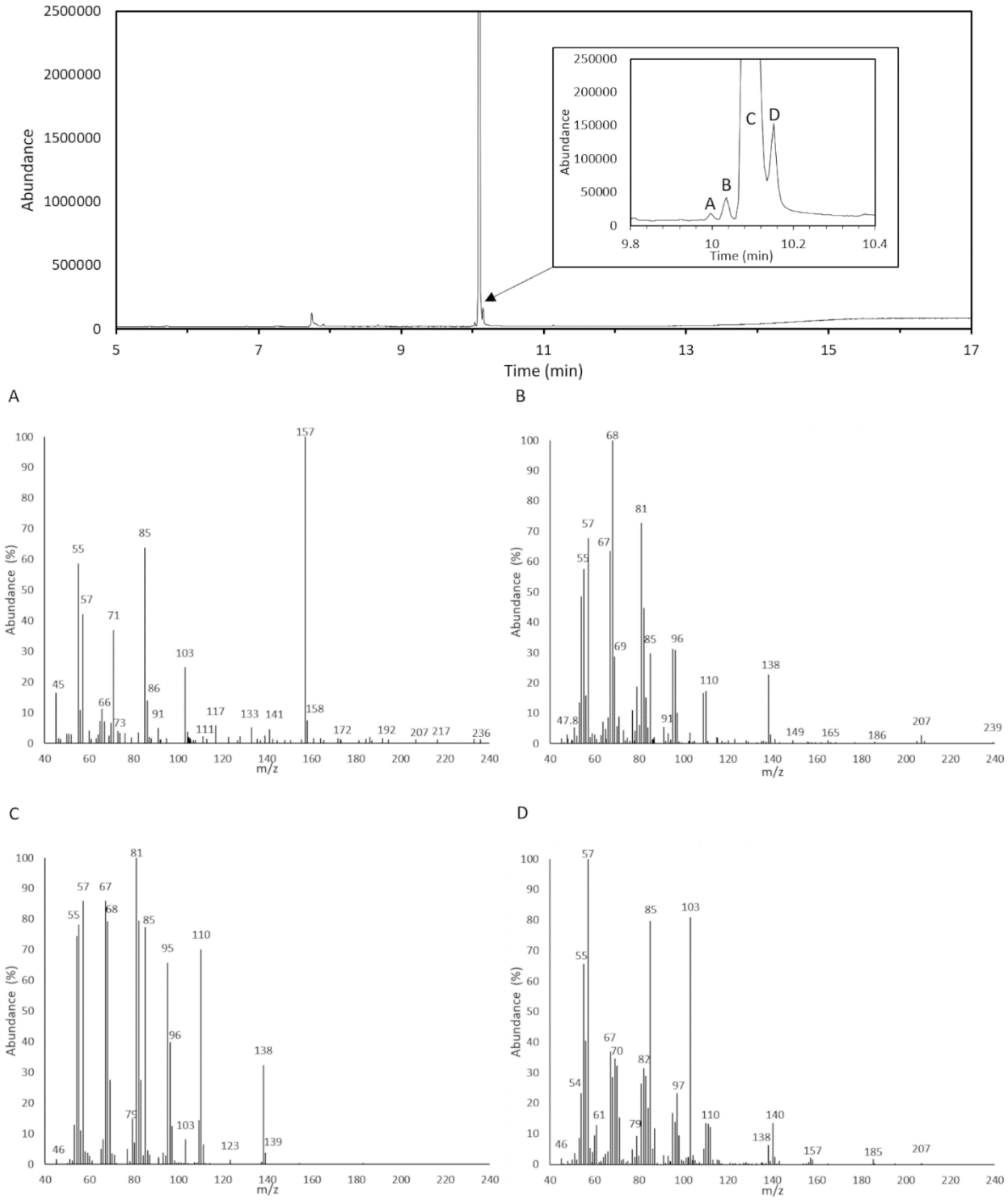

Figure S5. The total ion chromatogram (TIC) elution profile of the four chromatographic peaks in the synthetic pheromone after separation on the GC-MS system using an HP5 column and each peak's associated mass spectrum. These peaks elute at Kovats retention indices (A) = 1610 ± 1, (B) = 1617 ± 1, (C) = 1624 ± 1 and (D) = 1636 ± 0.