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RESEARCH ARTICLE

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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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Nudaurelia spp. (Saturniidae) are sporadic pests of pine plantations in South Africa. Taxonomic uncertainty exists in South Africa with this group and represents an impediment to the development and implementation of pheromone-based management tactics. Populations from the Western Cape have been described as *Nudaurelia cytherea* while those from KwaZulu-Natal and Mpumalanga have been described as *Nudaurelia clarki*. We compared *Cytochrome oxidase subunit I (COI)* gene sequences from moths from the Western Cape, KwaZulu-Natal (KZN) and Mpumalanga regions. We also analysed female pheromone gland extracts with gas-chromatography electroantennographic detection (GC-EAD) and gas-chromatography-mass spectrometry (GC-MS) for moths from KwaZulu-Natal and Mpumalanga. *COI* gene barcoding sequences were identical for moths from the Western Cape, KwaZulu-Natal (KZN) and Mpumalanga regions providing preliminary evidence to suggest that these populations may be the same species. Based on the comparison to available literature, the morphology and origin of our samples suggests that pheromone analyses in this study were done on *N. clarki*. Male *N. clarki* antennae respond to two compounds in female extracts. One of these compounds was confirmed with a synthetic standard to be (*Z*)-dec-5-en-1-yl-3-methylbutanoate, the sex pheromone previously identified from *N. cytherea*. The identity of the second compound could not be confirmed. Both male and female antennae responded to four structurally related compounds in the synthetic pheromone standard. Field trials with custom-made traps confirmed attraction of *N. clarki* males to polydimethylsiloxane and polyisoprene lures loaded with synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate.

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INTRODUCTION

Moths from the Saturniidae (Lepidoptera: Saturniidae) are among the largest known insects in the world. The subfamily Saturniinae are commonly known as emperor moths. The larvae of some emperor moths include well known edible species in southern Africa (Amadi et al. 2005; Greyling et al. 2001; Kachapulula et al. 2018). Many of these species are also polyphagous and some are pests in forestry (Staude et al. 2016; Van den Berg 1973, 1974, 1990). For example, the poplar emperor moth, *Pseudobunaea irius*, has four indigenous and 13 exotic plant species that it feeds on (Hepburn et al. 1966). This species is known to defoliate *Pinus*, *Eucalyptus* and *Acacia* trees in South Africa (Van den Berg 1973, 1979a; Hurley et al. 2017). *Nudaurelia cytherea* (Fabricius, 1775) has also been reported from several native plants, including *Rhus angustifolia*, *Rapanea melanophloeos*, *Euclea schimperi*, *Protea repens* and *Watsonia* spp. (Geertsema 1970; Pinhey 1956). *Nudaurelia* species have also undergone host range expansions onto exotic plantation trees where they have become pests, in particular on *Pinus radiata* and *P. patula* (Hurley et al. 2017; Sims 1903; Tooke 1935; Tooke and Hubbard 1941; Van den Berg 1973; Van den Berg and Van den Berg 1973).

There is uncertainty regarding the taxonomy of *Nudaurelia* species feeding on pine. Synonyms used in literature for these moths include *Antherea cytherea* (Sims 1903), *Imbrasia cytherea* (Van den Berg 1979a, b), *Bombyx cytherea*, *Gonimbrasia cytherea* and *Nudaurelia cytherea* (Geertsema 1970). Two prominent *Nudaurelia* variations were described as subspecies, *N. cytherea cytherea* and *N. cytherea clarki* (Geertsema 1971), and later as species, *N. cytherea* and *N. clarki* (Staude et al. 2016). *Nudaurelia cytherea* is reported to occur along the coastline of the Western Cape through to the Eastern Cape and in southern KwaZulu-Natal (KZN) (Staude *et al.* 2016; Van den Berg 1973) (Figure 1a). *Nudaurelia clarki* is reported to occur in Zimbabwe, the South African provinces of Limpopo, North West, KZN and the northern parts of the Eastern Cape, as well as eSwatini, previously known as Swaziland (Van den Berg 1973) (Figure 1a). The *N. cytherea* moths are reported to be brown in colour and smaller than the *N. clarki* moths that are predominantly yellow (Geertsema 1970, 1971). The colour of the third ring around the wing eye-spot is reported as white for *N. cytherea* and pinkish or maroon for *N. clarki* (Geertsema 1971; Staude et al. 2016) (Figure 2a).

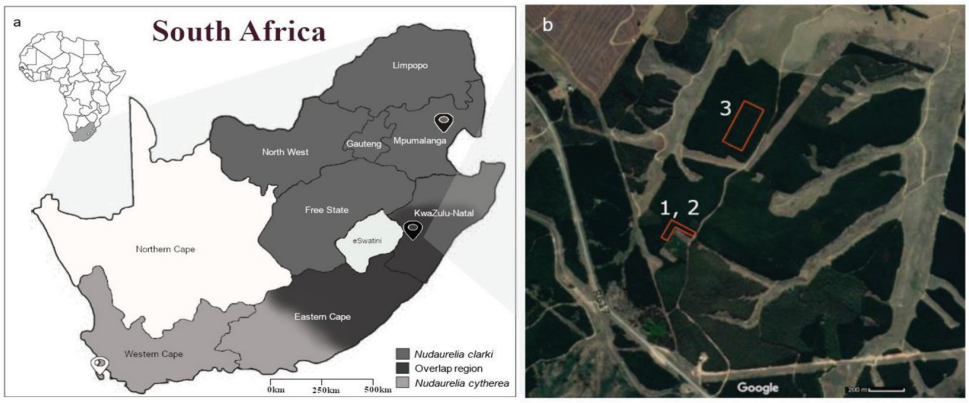

Figure 1. (a) The described distribution of *N. cytherea* (light grey) and *N. clarki* (grey) in South Africa, shown as highlighted provinces, with the proposed overlapping region where interbreeding has been reported previously (dark grey) (Geertsema 1971; Staude et al. 2016). Black pins: sampling locations in 2018 and 2019 of live moths for pheromone studies and mitochondrial gene comparisons. White pin: sampling location in 2019 of the Cape syntype for mitochondrial gene comparisons. **(b)** A map showing the layout of two *Pinus patula* compartments where trial 1, 2 (2019), and 3 (2020) took place in Bulwer, KZN, to test the attraction efficacy of synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate to *N. clarki*.

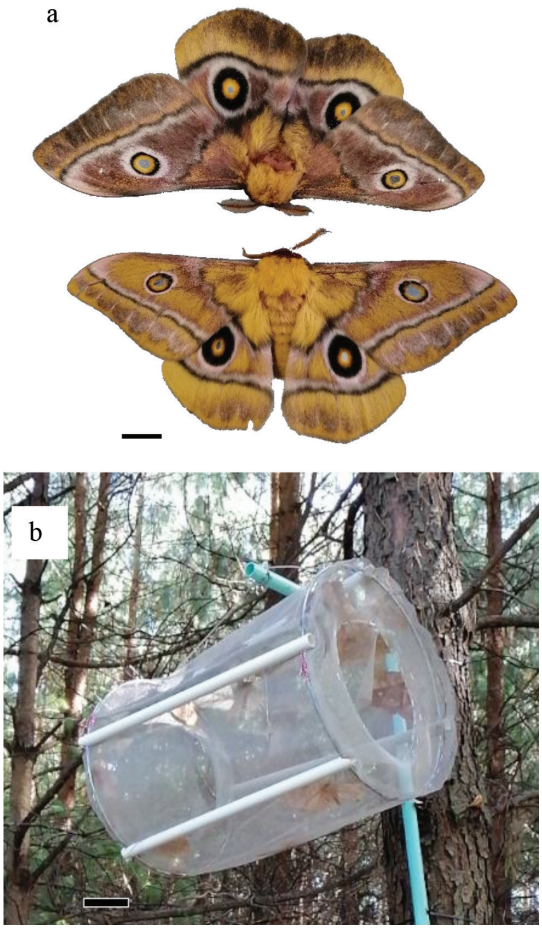

Figure 2. (a) A visual comparison between two male moths respectively collected from Jessievale (Limpopo), *N. clarki* (bottom), and Pringle Bay (Western Cape), *N. cytherea* (top). Scale bar = 1 cm. The morphological colour variations of adults and larvae, when available, were compared to those described in Supplementary material from Staude et al. (2016) to assign the respective species. **(b)** An up-close view of the suspension of a custom-built trap in a *P. patula* tree during field trials in Bulwer, KZN. Scale bar = 10 cm.

The first report of *N. cytherea* as a pest was an outbreak at the Fort Cunynghame plantation in 1896 on *Pinus insignis* that lasted for seven years (Sims 1903). These moths were rarely seen in subsequent years and damage to pine plantations now occurs sporadically (Govender 2011; Tooke 1935). Defoliation, especially at the crown of pine trees, can cause stunted growth and subsequent economic loss (Van den Berg 1973). It has been calculated that an average of 126 *N. cytherea* larvae can totally defoliate a large *Pinus patula* tree with crown depth of 7 m (Van den Berg 1973). Although historically infrequent, when infestation of pines occurs these moths can cause serious economic loss if populations are not managed (Van den Berg 1979a).

Methods that have previously been used for control of pine emperor moths in plantations include cultural control, biological control and insecticide application. Cultural control, such as ploughing and counting moth debris (Geertsema 1980; Van den Berg and Van den Berg 1974) can guide action thresholds of other control methods. Natural control agents include pigs and other predators (Geertsema and Van den Berg 1973) or larval viruses (Hendry et al. 1985; Munshi et al. 1998; Tripconey 1970). Pyrethroid and organochlorine pesticides are the preferred fast-acting control to prevent damage in pine plantations (Donald 1963; Govender 2011; Kirsten et al. 2000; Van den Berg 1979a). Even though pesticides are effective against young larvae (Donald 1963), they are known to have many non-target effects (Quinn et al. 2011).

Attractant sex pheromones could facilitate monitoring adult pine emperor moth population levels to determine the need for control and direct the timing of insecticide treatments to optimise larval mortality and minimise damage. The pheromone of *N. cytherea* was identified in the early 1970s as (*Z*)-dec-5-en-1-yl-3-methylbutanoate and used experimentally to trap *Nudaurelia* spp. in the Cape region (Henderson 1972; Henderson et al. 1972, 1973). No subsequent pheromone field trials or commercial use have been reported for this moth in South Africa. It is unknown if (*Z*)-dec-5-en-1-yl 3-methylbutanoate is a pheromone attractant for *N. clarki*. The objectives of this study were: firstly, to determine if (*Z*)-dec-5-en-1-yl 3-methylbutanoate is a constituent of the sex pheromone of *N. clarki* moths from pine plantations in northeastern South Africa (Figure 1a); secondly, to determine if (*Z*)-dec-5-en-1-yl 3-methylbutanoate attracts *N. clarki* in field tests; and thirdly, to provide *Cytochrome oxidase subunit I* (*COI*) gene sequences from moths in various regions where they occur as reference for future studies.

METHODS and MATERIALS

Insects

Adult moths were collected from Jessievale (–26.221804 S, 30.468118 E), Bulwer (–29.8049195 S, 29.7553241 E) and Pringle Bay (–34.3390330 S, 18.8309181 E), South Africa. Moth legs were used for DNA extraction and were obtained from individuals collected in April–May, 2018 from Bulwer (n = 5) and Jessievale (n = 10) and April–May, 2019 from Bulwer (n = 5) and Pringle Bay (n = 5) (Figure 1a). Field-collected adults (n = 5) and pupae

(n = 7) were obtained from Bulwer and Jessievale April–May, 2018 and females were used for dynamic headspace sampling. Pupae were reared by keeping them undisturbed in soil from the collection site in an unsealed plastic container. The container was kept in a fine-mesh cage in the insectarium at the FABI Biocontrol Centre, University of Pretoria. Rearing conditions were kept at 22 ± 2 °C, 12L:12D cycle, and 65 ± 3% relative humidity until moths emerged. The soil was kept damp by spraying with distilled water as necessary. Emerged moths did not require feeding.

DNA barcoding

In order to provide a preliminary comparison between moths from the different locations, and with previous sequences in GenBank, the *COI* barcoding region was amplified and sequenced. The legs of the collected moths (25 in total) were removed after return to the laboratory. These were stored in 95% ethanol and freeze dried for 24 h prior to DNA extraction. DNA was extracted with the phenol-chloroform method (Sambrook et al. 1989). Polymerase chain reaction (PCR) amplifications were performed in a total volume of 21 µl that consisted of 2.5 µl of 10× PCR Buffer, 2.5 µl (0.25 mM) of dNTPs, 3 µl (3 mM) of MgCl2, 1 µl (10 µM) of each primer (LepF1 5′-ATTCAACCAATCATAAAGATATTGG-3′ and LepR1 5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) (Hebert et al. 2004), 0.2 µl (5 U/µl) FastStart Taq Polymerase, 10.8 µl sterile PCR grade SABAX water and 1 µl (50 ng) DNA. PCR amplifications were done with a ProFlex PCR instrument with an initial denaturation temperature at 95 °C for 7 min. This was followed by 94 °C for 30 sec; 50,52 of 56 °C for 30–60 sec; 72 °C for 30 sec, and these steps were repeated for 36 cycles. The final extension temperature was 72 °C for 7 min. Polyacrylamide gel electrophoresis was used to visualise and compare amplified fragment lengths. PCR products were purified and sequenced in both directions using an ABI PRISM® BigDye® Terminator Cycle Sequencing Kit (version 3.1; Applied Biosystems) and the same forward and reverse primers (LepF1 and LepR1) (Hebert et al. 2004).

Sequencing was done on an ABI Prism 3130 XL capillary sequencer (Applied Biosystems), and data collected with 3500 Series data collection software. Sequences were inspected and trimmed in ClustalX 2.1 (Larkin et al. 2007) and overlapping sequences were aligned with default parameters using Mega 7.0.26. Sample consensus sequences were compared to one another, and because there was no variation between sequences, a representative sequence from each region was compared to *COI* sequences in GenBank through a BLAST search. GenBank sequences representing 17 different species were selected based on their close taxonomic relation to *N. cytherea* or *N. clarki*, including species in the *Nudaurelia*, *Imbrasia*, *Gonimbrasia* and *Antherea* genera. A previously established outgroup for Saturniidae, *Pyralis farinalis* (Lepidoptera: Pyralidae) (Kachapulula et al. 2018) was used for phylogeny construction. A neighbour-joining tree was constructed via Mega 7.0.26 with the default parameters, using representative samples from each sampled region and the selected *COI* gene sequences from GenBank.

Synthesis general procedures

Starting materials and solvents, if commercially available, were purchased from Sigma-Aldrich (Burlington, MA, USA), Fisher Scientific (Pittsburg, PA, USA), or GFS Chemicals (Powell, OH, USA) and used without further purification. All solvents used were Optima grade (Fisher Scientific, Pittsburgh, PA, USA). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE NEO 400 (400 and 100.5 MHz, respectively) spectrometer (Bellarica, MA, USA), as CDCl3 solutions. ^{1}H NMR chemical shifts are expressed in ppm relative to residual CHCl3 (7.27 ppm) and ^{13}C NMR chemical shifts are reported relative to CDCl3 (77.16 ppm). All reactions were performed in oven-dried glassware with magnetic stirring under an argon (Ar) atmosphere. Thin layer chromatography was performed on Polygram® SIL G/UV254 plates (Macherey-Nagel) with detection by UV (254 nm) or by immersion in a 10% w/v solution of phosphomolybdic acid in absolute ethanol, followed by heating. Crude products were purified by vacuum flash chromatography or column flash chromatography on silica gel (230–400 mesh; Fisher Scientific). Yields refer to isolated yields of chromatographically pure products. Mass spectra were obtained with a Hewlett–Packard (HP) 5890 GC (Avondale, PA, USA) interfaced to an HP5970 mass selective detector, in EI mode (70 eV) with helium carrier gas. The GC was equipped with a DB17-MS column (30 m, 0.25 mm ID 0.25 µm film, J and W Scientific, Folsom CA, USA).

5-decen-1-ol (2)

To a solution of 5-decyn-1-ol (1.5 g, 9.72 mmol, 1 equiv, 1 in Figure 3) in hexane (40 ml) was added Lindlar catalyst (150 mg, 10% wt) and dry pyridine (30 µl, 20% vol/wt catalyst). The resulting suspension was then stirred at r.t. under an atmosphere of H2 for 2 h. The reaction was monitored by measuring the H2 volume uptake to prevent over reduction. The mixture was then filtered through celite and washed with 1M HCl (2 × 50 ml), sat. NaHCO3 (1 × 50 ml), and brine (1 × 50 ml). The organic layer was then dried over anhydrous Na2SO4 and purified by vacuum flash chromatography (1:9 EtOAc/Hexane) to afford 1.37 g (90%, 8.75 mmol) of 5-decen-1-ol as a colourless oil.

^{1}H NMR (400 MHz, CDCl3, TMS): δ [ppm] = 5.43–5.25 (m, 2H), 3.65 (td, J = 6.7, 5.4 Hz, 2H), 2.14 (m, 2H), 2.01 (m, 2H), 1.61 (quint, J = 6.7 Hz, 2H), 1.38–1.16 (m, 6H), 0.87 (t, J = 7.0 Hz, 3H) ^{13}C NMR (100 MHz, CDCl3, TMS): δ [ppm] = 131.0, 129.2, 62.1, 33.2, 31.5, 29.2, 27.7, 23.8, 22.8, 14.3.

EI-MS (70 eV): m/z (%) = 156 (M^{+}, 1), 138 (8), 110 (16), 95 (27), 81 (41), 67 (100), 55 (90), 41 (79).

(Z)-dec-5-en-1-yl-3-methylbutanoate (3). To an ice/NaCl bath cooled (–10 °C) solution of 5-decen-1-ol (1.0 g, 6.4 mmol, 1 equiv), pyridine (575 µl, 7.2 mmol 1.13 equiv) and dimethylaminopyridine (DMAP, 100 mg) in 25 ml of CH2Cl2 was added isovaleryl chloride (878 µl, 7.2 mmol, 1.13 equiv) via syringe pump over 10 min. The reaction was allowed to stir at –10 °C for 30 min and warmed to r.t. for another 3 h. The reaction was quenched by the addition of brine (10 ml), and the reaction mixture was partitioned between Et2O (75 ml) and brine. The organic layer was separated and washed with a solution of 1M HCl (2 × 50 ml), sat. NaHCO3 (2 × 50 ml), brine (2 × 50 ml) dried over anhydrous Na2SO4, and then concentrated *in vacuo*. The crude residue was purified via vacuum flash chromatography (1:9 EtOAc/Hexane) to afford 1.41 g (92%, 6.62 mmol) of the pure product as a colourless oil.

^{1}H NMR (400 MHz, CDCl3, TMS): δ [ppm] = 5.39–5.23 (m, 2H), 4.05 (t, J =7.8 Hz, 2H), 2.28 (d, J =8.3 Hz, 2H), 2.21–2.15 (m, 1H), 2.06–1.98 (m, 4H), 1.57-1.45 (m, 4H), 1.28–1.21 (m, 4H), 0.98 (d, J =8.9 Hz, 6H), 0.88 (t, J=7.2 Hz, 3H).

^{13}C NMR (100 MHz, CDCl3, TMS): δ [ppm] = 172.8, 130.9, 129.7, 64.8, 44.1, 32.1, 29.3, 26.7, 26.4, 25.9, 25.3, 22.4, 21.9,14.1

EI-MS (70 eV): m/z (%) = 240 (M^{+}, 1), 138 (59), 123 (2), 110 (100), 95 (72), 81 (97), 68 (71), 57 (66), 41 (29).

Electrophysiology experiments

An Agilent 6890N gas chromatography system (Chemetrix, Midrand, South Africa) was coupled to an electroantennographic detection system (Syntech, Hilversum, The Netherlands) for GC-EAD recordings. The GC oven temperature for the ZBWax column (30 m × 0.32 mm ID, 0.25 µm, 7HG-G007-11, Zebron™) was held at 50 °C for 1 minute and ramped at a rate of 20°C/min to 250 °C and held for 10 minutes. The method on the non-polar

ZB5 column (30 m × 0.32 mm ID, 0.25 µm, 7HM-G002-11, Zebron™) was held at 50 °C for 2 minutes and ramped at a rate of 20 °C/min to 300 °C and held for 5 minutes. A volume of 1 µl of each extract was injected in splitless mode into the GC-EAD system (injector temperature at 250 °C) to screen for repeatable electrophysiological responses from male and a limited number of female antennae. The effluent was split with a Y-quartz splitter (Agilent, PN:5181-3398) at the end of the GC-column to the FID detector (300 °C) and antennal preparation in a 1:1 split ratio. The transfer line was kept at 250 °C. Samples that gave responses were combined, concentrated and rescreened in an attempt to find previously undetected peaks with the FID. Screening was performed on two column polarities and Kovats retention indices (IK) were calculated for all chromatographic peaks.

Antennae were removed with dissecting scissors at the pedicel. The terminal antennal nerve endings were exposed by cutting off the antennal tip. A few antennal branches on the male bipectinate antennae were removed at the terminal ends to allow for the antenna to fit into the glass capillary electrodes. This procedure minimised bubbles that can introduce noise in the EAD recordings. Each antenna was oriented with the tip connected to the recording electrode of the EAD. The antennal preparation was moved to within 5 mm of the stimulus delivery system of the EAD. Microelectrodes were made from pulled glass capillaries (Hirschmann, 120 mm) and Ag/AgCl wire electrodes. The capillaries were filled with Beadle-Ephrussi Ringer electrolyte solution (129 millimolar NaCl, 4.7 millimolar KCl, and 1.9 millimolar CaCl) and connected to a manually adjustable micro-manipulator at each electrode. All direct current recordings were made with a GcEad32 V4.3 system (Syntech, Hilversum, The Netherlands), and amplified ten times with external amplification. Baseline drift was removed by plotting the derivative of the EAD data as described in Slone and Sullivan (2007).

A serial dilution of between 0.001 ng/µl to 10 ng/µl of synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate, was prepared in double distilled n-hexane. A volume of 1 µl of the respective five doses was injected individually in splitless mode into the GC-EAD system, and the same separation method was used as for GC-EAD extract analyses (ZBWax). Response sizes of male antennae ($n = 4$) were measured for each dilution. The experiment was repeated for both increasing and decreasing concentration increments in time. Dose responses were also tested using the EAG puffing method but were not informative (Supplementary Material 1). The antennal response sizes were measured from the direct current data with GcEad32 V4.3 software (Syntech, Hilversum, The Netherlands). The response data was analysed in R version 3.5.2.

Gland extracts

Glands were extracted by removing ovipositors from selected field collected female moths from Bulwer and Jessievale and placing them individually in 500 µl n-hexane (n = 26) upon arrival at the lab. Females were selected if their abdomen size was large and filled with eggs (based on visual and touch inspection).

Dynamic headspace Ssampling

Dynamic headspace sampling was performed by aerating individual field collected (n = 5) or reared virgin female moths (n = 7) in modified 1-litre CONSOL® jars with bottled air. All jars were cleaned with soap and water and dried overnight in a drying oven (110 °C) before use. Air passed through a hydrocarbon trap (Supelco Superpure HC, #2-2445-U) for purification and distilled water for humidification via Teflon tubing (1.8" OD, Supelco, #20532). The air then moved through ¼ inch bulkhead union fittings, attached to the custom modified lids of CONSOL® jars. The airflow was adjusted with a needle valve and measured at 6.2 ± 2.1 ml/min (mean ± SD) with a glass bubble flow meter. Volatiles were trapped onto PorapakQ adsorbent cartridges (Supelco ORBO 1103, 50/80, 150/75 mg) for 24 hours. This procedure was repeated for the same reared females (7 in total) for three consecutive nights after the females emerged, onto different adsorbent cartridges. Field collected females were sampled only once. Each PorapakQ adsorbent was desorbed three times with double distilled n-hexane (500 µl, 30 min). Desorbed fractions were combined and the solvent was transferred to new vials. Extracted samples were stored at 4 °C in a fridge before analysis.

GC-MS screenings

An Agilent 7890B gas chromatography system was coupled to a 5877B MSD mass spectrometer detector. Female ovipositor extracts (1 µl) were injected in splitless mode into the GC-MS (purge vent at 2 minutes, 50 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 6 minutes for the ZBWax column (30 m x 0.25 mm ID, 0.25 µm, 7HG-G007-11, Zebron™). The temperature programme for the HP5 column (30 m × 0.25 mm ID, 0.25 µm, 19091S-433UI, Agilent™) was 50 °C for 2 minutes and ramped (20 °C/min) to 300 °C and held for 5 minutes (mass scan range of 30–550 m/z). The synthetic pheromone (50 ng/µl) was injected (1 µl) in splitless mode into the instrument.

DMDS microderivatisation

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

Field Trials

Collapsible traps were designed with openings large enough for pine emperor moths to enter (Figure 2b). Fiberglass mesh (5 mm) was wrapped around a cylindrical shape made from two (37 cm, OD) wire (4 mm) rings and four PVC pipes (50 cm × 25 mm ID) as a support for the structure (Figure 2b). Two fiberglass mesh funnels were knitted into the ends of the cylindrical trap with staples. Lure dispensers were hung in the middle of the traps on a 4 mm wire hook that was suspended from the top PVC pipe through a drilled hole (4.5 mm).

Field trials were set up near Bulwer, KZN in *P. patula* plantations. Trial 1 ran from 7–12 March 2019 and trial 2 between 12 March and 8 April 2019 in the same compartment (29°51′28.7″S; 29°42′39.1″E) and trial 3 between 2–26 March 2020 in another compartment (29°51′10.9″S; 29°42′51.0″ E). Traps were suspended with cable ties and PVC angle brackets from pine trees at a height of between 2 to 2.5 m (Figure 2b). For trial 1 and 2, 18 traps were set out in a linear transect roughly 5 m from the compartment edge (Figure 1b). Lures were loaded with 1 µl of undiluted synthesised pheromone. Trial 1 compared PDMS ring lures (n = 6) and field collected females (n = 6) to blank traps (n = 6). Moth counts were taken after 5

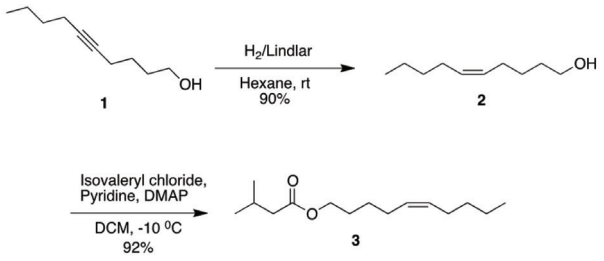

Figure 3. The reaction scheme of synthesis of the previously described pheromone from *N. cytherea* (Henderson, 1972). The compound was synthesised from 5-decyn-1-ol (1) via 5-decen-1-ol (2) to afford (*Z*)-dec-5-en-1-yl-3-methylbutanoate (3).

days. In trial 2, PDMS ring (n = 6) and red rubber septum lures (n = 6) were compared to blank (unloaded PDMS lures) traps (n = 6). Captured moths were counted and discarded weekly until a period of 27 days had passed. Trial 3 was set up in a larger compartment of *P. patula* with 40 traps. Each trap was fitted with a PDMS ring lure, which was loaded with 1 µl on-site with doses of 0.01 ng/µl, 0.1 ng/µl, 1 ng/µl or 10 ng/µl in double distilled n-hexane (n = 8). The PMDS lures were chosen based on results from Trials 1 and 2 and higher, consistent release rates in laboratory tests under regulated temperature conditions (data not shown). Blank traps (n = 8) were loaded with n-hexane only. Traps were deployed in four parallel transects that were spaced 20 m apart. The first trap in each transect was 5 m from the compartment edge. The first transect ran parallel to a road and each trap was 5 m from the road. There were 5 treatments per block, 2 replicates per transect and 8 replicate blocks in total (Figure 1b). Results were analysed with the Kruskal–Wallis test in R version 3.5.2 to determine significance between treatments for each trial. Post-hoc pairwise Wilcoxon Rank sum tests, with Bonferroni corrections, were used to show differences between treatments.

RESULTS

COI DNA barcoding

COI-gene sequences (GenBank nr: MT740714, MT740715 and MT740716) (581 base pairs after alignment and trimming) were identical for moths collected from Bulwer, Jessievale and Pringle Bay (Supplement, Figure S1). The sequences from our collected samples grouped with those known for other moths in the Saturniidae in GenBank, but no previous sequences for *N. clarki* or *N. cytherea* were available in this database and no sequences were identical to ours. The closest matching sequence differed by 7%, namely *Nudaurelia kohli*. The *Antherea* genus grouped sister to the previously established outgroup, *Pyralis farinalis* (Kachapulula et al. 2018) (Supplement, Figure S2) and was subsequently chosen as the outgroup of our dataset. The choice of outgroup was further supported by the relation of *Antherea* in a sister clade to *Nudaurelia* in a nuclear protein sequence Saturniid phylogeny (Regier et al. 2008). Species that grouped closest to *N. clarki* were *Nudaurelia kohli*, *N. dione*, *N. amathusia*, and *N. anthinoides* (Supplement, Figures S1 and S2).

Electrophysiology experiments

One large and one small male antennal response were present in the GC-EAD recordings of female pheromone gland extracts (n = 26 extracts, Figure 4a, b). The large response occurred at IK = 1887 ± 1 on the ZBWax column and the male antenna response size was 135.56 ± 42 µV (n = 9) (Figure 4b). The same large response occurred at IK = 1627 ± 3 on the ZB5 column and in this case the male response size was 104.62 ± 91 µV (n = 13). The small response occurred at IK = 1879 ± 1 on the ZBWax column, and the male response size was 23.33 ± 15 µV (n = 6). On the ZB5 column, the small male response, 22.22 ± 16 µV (n = 7), occurred at IK = 1621 ± 2. The concentration of both active compounds was below our FID detection limits. We observed no male antennal responses to dynamic headspace samples from field collected (n = 5) or reared virgin females (n = 7, Supplement, Figure S3).

We observed four chromatographic peaks in the reference standard at the 50 ng/µl concentration level (Table 1). Both male and female antennae detect all four of these chromatographic peaks. Even though female antenna responses were observed, the depolarisations were often too low to reliably measure using the EAD software. Male antennae (842.9 ± 446.2 µV, mean ± SD, n = 7) gave significantly larger (t-test p = 0.0179) antennal responses to the largest chromatographic peak (at 50 ng/µl) when compared to measured responses from female antennae (50.0 ± 42.4 µV, mean ± SD, n = 3). Kovats retention index values of the large antennal response observed from gland extract analysis (ZBWax) matched with the largest peak in the pheromone standard solution (Figure 5 and Table 1).

Table 1. A tabulation of the Kovats retention index values of the electroantennography responses of male and female *N. clarki* antennae to the synthetic pheromone and the female ovipositor extracts that were analysed on the GC-EAD (ZBWax and ZB5 column) or GC-MS (HP5 column)

ZBWax column			ZB5 column			EAD Response size[a]	
Peak[b]	Elution time (min)	I_K	Peak[c]	Elution time (min)	I_K	Male (µV) n = 7	Female (µV) n = 2
Synthetic pheromone							
1	7.75 ± 0.005	1853 ± 1	D	10.15 ± 0.000	1636 ± 0[d]	171.43 ± 133.59	[e]
2	7.88 ± 0.004	1874 ± 1	A	9.99 ± 0.000	1611 ± 1[d]	57.14 ± 31.47	[e]
3	7.93 ± 0.003	1883 ± 1	B	10.04 ± 0.000	1617 ± 1[d]	131.43 ± 107.08	[e]
4	7.96 ± 0.003[f]	1888 ± 1	C	10.09 ± 0.003[f]	1624 ± 1[d]	842.86 ± 460.83	50 ± 42.43
Female ovipositor extracts							
3	7.88 ± 0.02	1879 ± 1	B	9.88 ± 0.01	1621 ± 2	23.33 ± 15	NA
4	7.93 ± 0.02[f]	1887 ± 1	C	9.91 ± 0.02[f]	1627 ± 3	135.56 ± 42	NA

All values are mean ± SD

I_K: Kovats retention index

[a]: EAD responses determined on ZBWax column

[b]: Chromatographic peak elution order as in Supplementary Figure 1

[c]: Chromatographic peak elution order as in Supplementary Figure S7

[d]: Elution time and retention index determined on HP5 by GC-MS

[e]: Responses below EAD detection threshold (20 µV)

[f]: Major chromatographic peak intensity

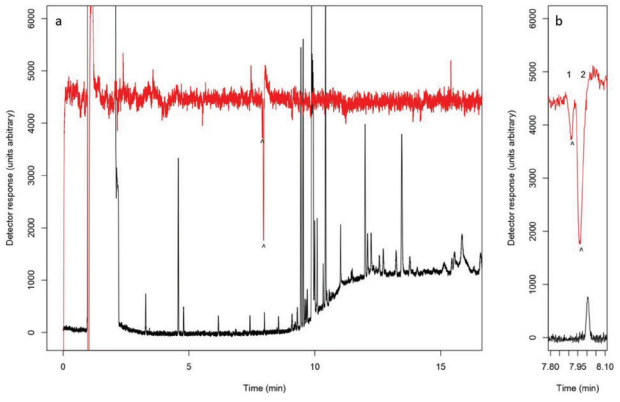

Figure 4. (a) Electroantennographic response (top, red) of a *N. clarki* male antenna to a female ovipositor extract in n-hexane (n = 8) (FID trace bottom, black, ZBWax column). **(b)** Close-up of the repeatable minor (1) and major (2) responses. Antenna response sizes were 1 = 135.56 ± 42 µV and 2 = 23.33 ± 15 µV (mean ± SD, n = 10) and occurred at Kovats index values of (1) = 1879 ± 1 and (2) = 1887 ± 1

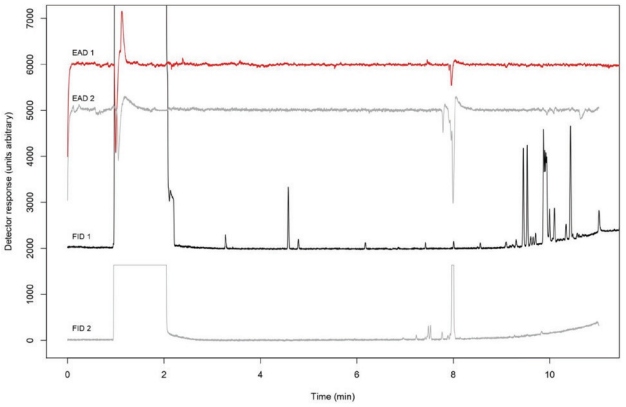

Figure 5. A comparison between the electroantennographic responses of a male *N. clarki* antenna to a female pheromone gland extract (EAD 1 and FID 1) and the synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate pheromone standard (EAD 2 and FID 2, 1 µl 50 ng/µl)

Electrophysiological responses measured with the EAG puffing technique were confounded by the response to the DCM solvent (Supplementary Material 1). The response to the solvent was removed when the same experiment was done on the GC-EAD. Dose response data confirmed a log linear regression ($F1,2 = 45.59$, MSE = 0.365, $p = 0.021$) from male antennae on the GC-EAD system ($R^2 = 0.958$, data not shown). Response sizes increased from 20 ± 40 µV to 540 ± 143.29 µV (mean ± SD) over a 0.01 to 10 ng/µl concentration range (Figure 6).

GC-MS extracts and standards

The concentration of the electrophysiologically active compounds in female *N. clarki* pheromone gland extracts were below detection limits of the GC-MS system ($n = 24$). This was also true when selected extracts were combined, concentrated and re-analysed. However, the retention indices of two of the four peaks found in the synthesised pheromone standard were almost identical when compared to retention indices calculated for the electrophysiological responses seen to pheromone gland extracts on both the polar (ZBWax) and non-polar (ZB5) columns (Table 1).

Analysis of the (*Z*)-dec-5-en-1-yl-3-methylbutanoate standard revealed four peaks that were separated on the ZBWax column (IK (peak **1**) = 1852 ± 0, IK (peak **2**) = 1873 ± 0, IK (peak **3**) = 1882 ± 0 and IK (peak **4**) =1887 ± 1) ($n = 3$)) (Supplement, Figure S4). The mass spectrum of peak one had a 140 m/z ion, in contrast to peaks two, three and four, that showed mass spectra with the 138 m/z ion (Supplement, Figure S4). The 140 m/z ion was assigned to the same fragment as the 138 m/z ion plus two hydrogen atoms. The identity of peak one was subsequently assigned to the saturated decenyl-3-methylbutanoate, and was confirmed by the lack of structural changes upon DMDS-derivatisation (Supplementary Material 2).

The identities of peak two, three and four (Supplement, Figure S4) were tentatively identified as dec-2-en-1-yl-methylbutanoate, an isomer of (*Z*)-dec-5-en-1-yl-3-methylbutanoate and (*Z*)-dec-5-en-1-yl-3-methylbutanoate. This was based on a detailed comparison of the resultant fragmentation patterns from the synthetic pheromone standard before and after derivatisation with DMDS (see Supplementary Material 2 for details).

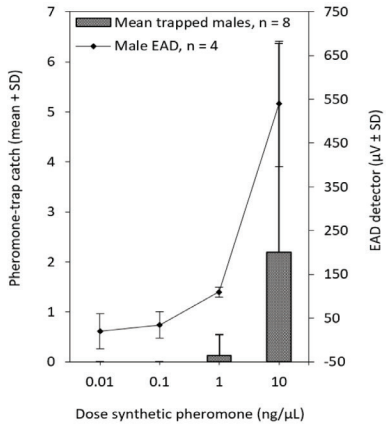

Figure 6. Electroantennographic and behavioural responses to concentrations of 0.01–10 ng/µl of the synthesised standard of the major pheromone component, (*Z*)-dec-5-en-1-yl-3-methylbutanoate. GC-EAD electroantennographic response sizes (mean ± SD µV, $n = 4$) of *N. clarki* male antennae to 1 µl of the synthesised pheromone is shown as a line graph. The mean number of male moths (+ SD) caught in traps baited with doses of synthetic (*Z*)-dec-5-en-1-yl-3-methylbutanoate ($n = 8$) in PDMS pheromone dispensers, is shown as a histogram. Significantly more moths were captured in traps baited with 10 ng/µl pheromone ($n = 70$) than traps baited with less than 1 ng/µl, and the total of four moths captured in 1 ng/µl-baited traps did not differ significantly from blank traps. No moths were captured in traps baited with concentrations lower than 1 ng/µl pheromone. A similar trend was seen in the electroantennographic responses, where larger response sizes were observed to 10 ng/µl than 1 ng/µl, and no response is seen to doses of lower than 1 ng/µl pheromone

Analysis of the (*Z*)-dec-5-en-1-yl-3-methylbutanoate standard on the HP5 column showed four distinguishable peaks, including IK (**A**) = 1611 ± 1, IK (**B**) = 1617 ± 1, IK (**C**) = 1624 ± 1, and IK (**D**) = 1636 ± 0 (Supplement, Figure S5 and Table 1). The mass spectra of peaks B, C and D on the HP5 column were identical to the mass spectra of peak three, four and one on the ZBWax column (Table 1).

Electrophysiological retention data showed (*Z*)-dec-5-en-1-yl-3-methylbutanoate (Supplement, Figure S4, peak 4, ZBWax column) had a retention index that matched well with the major antennal response found when gland extracts were analysed on the GC-EAD system (IK = 1887 ± 1, see Figure 5 and Table 1). This compound had fragmentation masses that included m/z = 240 (0.02%, M^+), 138 (33%), 110 (72%), 95 (67%), 81 (100%, base peak), 67 (86%) and 57 (86%) (Supplement, Figure S4, peak 4).

There were consistent differences between retention indices from both peak 3 (ZBWax column) and peak B (HP5 column) in the (*Z*)-dec-5-en-1-yl-3-methylbutanoate standard in comparison with the retention indices of the antennal responses to the ovipositor extracts. The third peak in the synthesised pheromone standard (Supplement, Figure S4) is thus not the same compound to which a minor response was seen from male antennae in the female gland extracts (Figure 4). In contrast, the retention index of peak C was similar to the retention index of the GC-EAD major response from male antennae toward female ovipositor extracts (IK = 1627 ± 3, ZB5 column, Table 1). The same was true for peak four (IK = 1887 ± 1, ZBWax column, Supplement, Figure S4 and Table 1). Peak four (Supplement, Figure S4) and peak C (Supplement, Figure S5) represent (*Z*)-dec-5-en-1-yl-3-methylbutanoate, the major pheromone component present in the female ovipositor extracts of the pine emperor moth (Figure 4). The compound responsible for the small antennal response in the pheromone gland extract analysis remains unknown.

Field trials

A total of 364 male moths were captured in trial 1 (Figure 7a). There were significant differences between treatments (Kruskal $p = 0.001$, $n = 6$, df = 2, $\chi^2 = 13.361$). Traps baited with the loaded PDMS pheromone dispensers captured significantly (53.5 ± 39.5, median ± interquartile range (IQR), range = 11 to 137) more moths than those baited with live female moths (0, median ± IQR, range = 0 to 28) (pairwise Wilcoxon $p = 0.030$) and unloaded PDMS blank dispensers (0, median ± IQR, range = 0) (pairwise Wilcoxon $p = 0.008$). A single female captured 28 males. The other females did not capture any males suggesting that they did not call.

A total of 517 male moths were captured in trial 2 (Figure 7b). There were significant differences between treatments (Kruskal $p = 0.001$, $n = 6$, df = 2, $\chi^2 = 13.414$). The number of male moths captured in traps baited with the loaded PDMS pheromone dispensers (48.5 ± 33.5, median ± IQR, range = 26 to 121) and RR pheromone dispensers (20 ± 34.5, median ± IQR, range = 8 to 58) was significantly (pairwise Wilcoxon $p = 0.008$) more than for unloaded PDMS dispensers (0, median ± IQR, range = 0). The number of captured moths did not differ significantly between PDMS pheromone dispensers and RR pheromone dispensers (pairwise Wilcoxon $p = 0.233$).

A total of 74 male moths were trapped in trial 3 (Figure 6). The number of moths captured with different doses differed significantly (Kruskal $p < 0.001$, $n = 8$, df = 4, $\chi^2 = 25.859$). Only four moths were captured in 1 ng/µl-baited traps (0.5 ± 0.76,

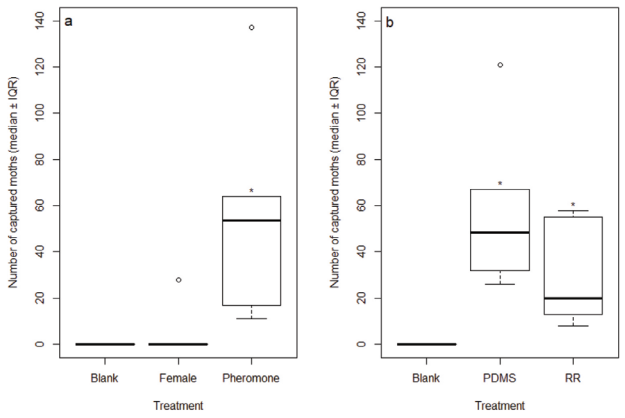

Figure 7. Numbers of male *N. clarki* moths that were captured in two field trials in a *Pinus patula* plot, represented as a box-and-whisker plot (median ± interquartile range (IQR), $n = 6$). Outliers are shown as circles. (a) The loaded PDMS pheromone dispenser treatment captured significantly more moths than the unloaded blank PDMS dispenser in trial 1. A single female moth captured 28 males in one of the traps. (b) In trial 2 the PDMS and red rubber (RR) pheromone dispensers captured significantly more moths than traps baited with unloaded blank PDMS dispensers. No significant difference was observed in the numbers of moths captured between the PDMS and red rubber isoprene pheromone dispensers

mean ± SD), and the count per trap did not differ significantly (pairwise Wilcoxon $p = 0.530$) from blank traps. No moths were caught in blank traps or those loaded with concentrations lower than 1 ng/µl. All other moths were captured in traps loaded with 10 ng/µl and the count per 10 ng/µl-baited trap (8.75 ± 7.57, mean ± SD) differed significantly from the number of moths caught in blank traps (pairwise Wilcoxon $p = 0.010$), but not to the number of moths caught in 1 ng/µl-baited traps (pairwise Wilcoxon $p = 0.12$).

Free roaming and trapped moths observed before, during and after trapping periods in the trial sites had the pink/maroon colouration of the third ring in the wing eye spots and were similar in size and colouration for *N. clarki* (Geertsema 1971; Staude et al. 2016). This suggests the prevalence of only *N. clarki* in the sampling region, despite previous indications that populations of *N. cytherea* and *N. clarki* overlap in this geographic region (Henderson 1972) (Figure 1a).

DISCUSSION

This study suggests that populations in the Western Cape and northeastern South Africa (KZN), previously described as *N. cytherea* and *N. clarki*, respectively, both use (*Z*)-dec-5-en-1-yl-3-methylbutanoate as at least their primary pheromone component and are indistinguishable by preliminary comparison of *COI* gene regions. These pine emperor moths are periodic pests on plantation pines in the Eastern and Western Cape, Gauteng, KZN, Limpopo and North West provinces of South Africa, as well as eSwatini and Zimbabwe (Staude et al. 2016; Van den Berg 1973). There is taxonomic uncertainty as to whether the two species reported in the different regions are in fact distinct (Geertsema 1971; Pinhey 1956; Staude et al. 2016). Because the same mechanisms that mediate mate location also mediate reproductive isolation in moths, studies of pheromone biology have the potential to help resolve issues of species identity (Allison and Cardé 2016; Lassance et al. 2019).

Historical chromatographic retention data allowed the successful verification of pheromone presence and identity despite operating below instrumental detection limits. Previously identified compounds were elucidated using combined, fractionated solvent extracts from more than 12 000 *N. cytherea* female ovipositors (Henderson 1972; Henderson et al. 1973). Samples used in this study were obtained from individual *N. clarki* females. Electrophysiology response data from male *N. clarki* antennae was consistent with the chromatographic data from Henderson (1972) and Henderson et al. (1973). Thus, the GC-EAD data from this study, screened on various GC column polarities, confirmed the presence of the same compound in ovipositor extracts from *N. clarki* previously identified in *N. cytherea* and that this compound stimulated antennal response. Detection of pheromone constituents by male antennae and not GC-FID instruments is a common phenomenon for Saturniidae given the small pheromone titres produced (Gago et al. 2013; Millar et al. 2010, 2016; Symonds et al. 2012).

Along with the major pheromone component, previous analyses (Henderson 1972; Henderson et al. 1973) also reported the elution of a small shoulder peak in the chromatographic elution profile of female extracts from *Nudaurelia cytherea*. Electrophysiological responses from male *N. clarki* in this study showed similar results, as there was a repeatable response with consistently smaller magnitude than the repeatable major response. This smaller response corresponds to an undetected closely eluting chromatographic shoulder peak to the major pheromone component when female *N. clarki* gland extracts were screened with GC-EAD. We were not able to identify the second component. It is possible that future work may determine that the unknown compound is part of the pheromone.

Comparison of *COI* DNA barcoding sequence data from moths collected from the Cape, Limpopo and KZN in this study found no differences. This finding is in contrast to observed morphological differences between moths collected from the different regions in South Africa for our study. Cape-collected moths were smaller, had brown wing colouration and a white third ring in the eyespots of their wings, consistent with the characteristic colours of *N. cytherea* (Geertsema 1971; Staude et al. 2016). The KZN moth type was larger with mostly yellow colouration and a pink/maroon third eye spot ring on their wings, characteristic of *N. clarki* (Geertsema 1971; Staude et al. 2016). It is possible that our methodology cannot separate the two *Nudaurelia* species. The *COI* gene region is usually able to detect variation between species (Lassance et al. 2019), but not in all species. It has been shown to be insufficient for some Saturniidae species (Janzen et al. 2012). In this scenario, quantitative or qualitative differences in the pheromone blend not measured in this study (i.e., the identity or amount of minor pheromone components) could confer specificity (Allison and Cardé 2016, Lassance et al. 2019, Tolasch et al. 2013).

Moth populations in KZN experience summer rainfall while those in the Cape experience winter rainfall. This difference in pattern of rainfall and associated biotic and abiotic effects is generally thought to promote divergence (Scriber and Ording 2005). If speciation had taken place between emperor moth populations in KZN and the Cape, in the absence of behavioural differences that isolate the two populations, the pheromone chemistry and *COI* DNA sequences would be expected to differ. While *COI* sequences clearly do not differ between moths from the two regions, it is not clear yet if qualitative differences exist in the pheromone blends of moths from the two regions. Definitive resolution of this question would require determination of whether additional minor components exist in either population.

COI DNA barcoding sequence data from moths collected in this study was considered to represent *N. clarki*, and grouped sister to *N. kohlli*, a related Saturniid from Tanzania. Other species that grouped with *N. clarki* included *N. dione*, from tropical African countries and South Africa (Pinhey 1956; Staude et al. 2016), *N. amathusia* from Uganda, Cameroon and Gabon (Pinhey 1956) and *N. anthinoides* from Gabon. These moths all have similar morphological appearance with only slight variations in colour. Despite confirmation that mitochondrial sequences from moths from our study grouped

sister to other Saturniidae, no further interpretation was possible due to big gaps in meta-morphological and molecular data for all abovementioned moths.

We confirm that the sex pheromone previously identified for *N. cytherea*, (*Z*)-dec-5-en-1-yl-3-methylbutanoate (Henderson 1972; Henderson et al. 1973), is also part of the pheromone of *N. clarki*. The similar pheromone chemistry and identical *COI* gene regions from samples representing these moth variants suggest that *N. cytherea* and *N. clarki* might be the same species. We propose that the same pheromone blend will be effective for all populations of these two putative species. Future studies will attempt to develop indirect (e.g., monitoring to time insecticide application) and direct (e.g., mass trapping, mating-disruption) applications of the sex pheromone to manage outbreaks. The use of such pheromone control-strategies in plantations could be cheaper and more environmentally friendly than pesticides (Larsson 2016; Nadel *et al*. 2012).

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICS APPROVAL

This article does not contain experiments with human participants or animals performed by any of the authors.

AVAILABILITY OF DATA AND MATERIAL

DNA sequences were deposited into GenBank (accession MT740714-MT740716).

AUTHORS' CONTRIBUTIONS

MB, JA, BS and LS conceived the research, MB and JA designed the research. LS conducted most experiments, and PM contributed partially to performing experiments. JB contributed reagents. LS and MB analysed data. LS, MB, JA, BS wrote the manuscript. All authors read and approved the manuscript.

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