(Z)-3-Dodecenoic Acid Is the Main Component of Full-Body *n*-Hexane Extracts from Two *Acacia* Gall-Inducing Thrips (Thysanoptera) and May Function as an Alarm Pheromone

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A major interest in the gall-inducing thrips of Australia began with the discovery that some species have eusocial colonies. The origin of social castes remains one of the outstanding questions in evolutionary biology. The inference of the ancestral stage from study of solitary species is important to understanding the evolutionary history of semiochemicals in social species. Here we investigated two solitary species, *Kladothrips nicolsoni* and *K. rugosus*. Whole body extracts revealed that (Z)-3-dodecenoic acid, here reported for the first time in a thrips species, is the main component. (Z)-3-Dodecenoic acid and (E)-3-dodecenoic acid were synthesized in high stereoisomeric purity ($> 99.8\%$) and exposed to *K. nicolsoni* 2nd-instar larvae in a contact chemoreception bioassay to test for potential bioactivity. Both isomers decreased the average time spent in the treated area per entry suggesting repellence at the tested dose. (Z)-3-Dodecenoic acid may function as alarm pheromone. (E)-3-Dodecenoic acid increased also the absolute change in direction of larvae compared to an *n*-hexane control and could potentially function as a repellent.

Key words: Kladothrips, Semiochemicals, (E)-3-Dodecenoic Acid

Introduction

Thrips (order Thysanoptera) are tiny insects, 1 – 3 mm long, spread worldwide, and mainly studied as crop pests. However, the discovery of eusociality among the *Acacia* gall-inducing thrips living in Australia (genus *Kladothrips*) and its recent evolutionary origin revealed a good candidate for the study of the evolution of sociality in this genus (“model clade” approach) (Chapman *et al*., 2008). Indeed, the division of labour (reproductives and sterile helpers) is not complete, *i. e.* the helping caste (soldiers in this clade) is still able to reproduce, especially in the basal social species. Comparing solitary and social *Kladothrips* species is an appropriate way to discover the evolutionary novelties acquired during the evolution of sociality.

Our study focused on two species of gall-inducing thrips of Australia: *K. nicolsoni* and *K. rugosus*. Initially, both thrips species were thought to fall within a large grouping of populations, the *rugosus* complex that makes galls on a dozen *Acacia* species, but large differences in gall morphology coupled with genetic data supported the description of *K. nicolsoni* as a distinct taxon (McLeish *et al*., 2006). *K. nicolsoni* induces elongated galls with a smooth surface or fine ridges on its host species, *A. papyrocarpa*. Being a rather isolated host species compared to others, it offered better opportunities for speciation (McLeish *et al*., 2011). *K. nicolsoni* and *K. rugosus* are both solitary. Their life-cycle starts with the gall foundress inducing a gall which ultimately encapsulates it and creates a suitable environment (food and shelter) for its brood. A mature gall of solitary species can house hundreds of individuals; in the case of *K. nicolsoni* and *K. rugosus*, 2nd-instar larvae which will pupate in the soil once the gall dries out (Crespi *et al*., 2004). Large brood size coupled to a short life-cycle is thought to be a selective adaptation to counteract parasitism (Chapman *et al*., 2006).

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The comparison between solitary and social species to investigate the evolution of sociality can be extended also to semiochemicals. A recent study showed that soldiers of the social *K. intermedius* might produce an alarm pheromone contained in their defensive anal droplets (De Facci *et al*., 2013), but it is still unknown which of the compounds present in the droplet (De Facci *et al*., 2014) are responsible for the observed behaviour. The droplets, from larvae as well as adults (reproductives and helpers), of this species contain mainly a series of short-chain ($C_8 - C_{10}$), saturated or monounsaturated, fatty acids, but also other potentially bioactive compounds (De Facci *et al*., 2014). The secretion of anal droplets under threat is most probably symplesiomorphic among Thysanoptera, so that all thrips are expected to show this behaviour, including *Kladothrips* solitary species. A variety of functions (alarm/aggregation pheromones, repellents) have been described for the several chemical components of these secretions (Moritz, 2006), but the knowledge of semiochemicals in the genus *Kladothrips* is still in its infancy. Unsaturated fatty acids are common in full-body extracts and defensive anal secretions of thrips (Moritz, 2006; MacDonald, 2002). For the majority of these insects the bioactivity of these compounds is largely unknown. Haga and colleagues (1989) found two unsaturated fatty acids in the anal secretions of *Hoplothrips japonicus* (Phlaeothripidae): (*E*)-3-dodecenoic acid (*E*3-12:COOH) and (*Z*)-5-dodecenoic acid. The cryptic behaviour of this species and their low propensity to attack did not permit a proper behavioural assay. Instead, the authors speculated that the secreted compounds could act as an alarm/aggregation pheromone similarly to what was observed on a previously studied gall-inducing species, *Varshneyia pasaniae* (= *Leeuwenia pasanii*) (Suzuki *et al.*, 1988). *E*3-12:COOH was also found in a few other species belonging to the subfamilies Idolothripinae and Phlaeothripinae (family Phlaeothripidae) (Suzuki *et al.*, 2000, 2004). Since the chemical identification of these compounds was based on the analysis of derivatives (Suzuki *et al.*, 2000, 2004), the mass spectra of monounsaturated fatty acids are often still unavailable in the literature (El-Sayed, 2013).

In this study, full-body extracts of *K. nicolsoni* and *K. rugosus* 2nd-instar larvae were subjected to different chemical analyses, and two substances, (*Z*)-3-dodecenoic acid (*Z*3-12:COOH) and *E*3-12:COOH, were synthesized in high stereoisomeric purity for comparisons with the extracts and for a behavioural assay to assess the compounds' function using a recently developed bioassay (De Facci *et al.*, 2013).

Results and Discussion

Chemical analyses and identification of extracted compounds

Extracts of individuals from fresh galls were relatively clean and consisted mainly of an unknown compound; the chromatogram and mass spectrum are shown in Fig. 1. GC-EIMS and GC-CIMS (positive mode) indicated a molecular weight of 198. In some cases, *i. e.* for methyl esters, the methods allow to obtain information on the position of double bonds (Oldham and Svatos, 1999; Kroiss *et al.*, 2011). By utilizing acetonitrile (ACN) as reagent gas for CI and by lowering the temperature of the mass spectrometer, a base peak of m/z 199 ($[M+H]^+$) was obtained, and typical adduct peaks were m/z 233 (23% of base peak, $[M+34]^+$) and m/z 252 (2% of base peak,

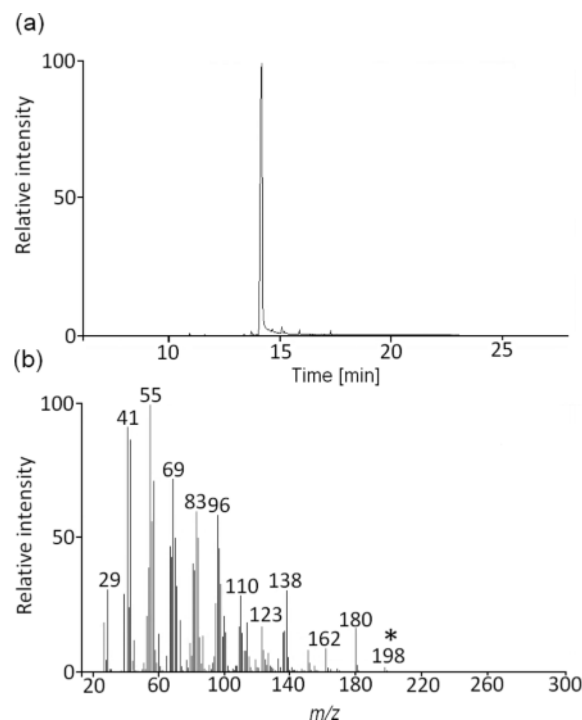

Fig. 1. (a) GC-MS total ion current (TIC) chromatogram of the *n*-hexane full-body extract of *K. rugosus*, illustrating the purity of extracts when analysed on a polar Varian FactorFOUR VF-23ms column. (b) Mass spectrum of the unknown compound.

$[M + 54]^+$). The lowered temperature of the mass spectrometer enhanced the stability of the $[M + 54]^+$-adduct, which is diagnostically important since this adduct upon fragmentation forms two fragments that reveal the position of the double bond (Oldham and Svatos, 1999). Using this method for unsaturated acids (results not shown here) the $[M + 54]^+$-adduct was successfully identified giving that the double bond is in position 3. But, the intensity of the $[M + 54]^+$-adduct was quite low, and the method probably needs to be optimized to be suitable also for double bond diagnostics of acids. Due to the low intensity of the $[M + 54]^+$-adduct, no CIMSMS was performed, which may give an even stronger indication of the double bond position (Oldham and Svatos, 1999). The compound lost m/z 18 (H_2O) in both EI and CI analyses, which is indicative of an oxygen-containing functional group.

The high-resolution (HR) EI mass spectrum in the positive mode of the *K. nicolsoni* extract provided a molecular formula of $C_{12}H_{23}O_2$ for $[M + H]^+$ (observed, m/z 199.1694; calculated, m/z 199.1693). This resulted in the molecular formula $C_{12}H_{22}O_2$ (86% certainty and no other molecular formula was suggested) of a structure, which has two double bond equivalents

Table I. Summary of NMR shifts for natural and synthetic compounds.

Position number and peak description	*K. nicolsoni*	*K. rugosus*	*Z*3-12:COOH	*E*3-12:COOH
1 COOH (1H, br)	11.3 – 10.9	–	10.4 – 10.0	–
2 (2H, d)	3.15	3.15	3.14	3.07
3 (1H, dtt)	5.55	5.55	5.54	5.51
4 (1H, dtt)	5.63	5.63	5.63	5.60
5 (2H, q)	2.04	2.04	2.04	2.03
6 – 11 (10H, m)	1.39 – 1.22	1.39 – 1.22	1.39 – 1.22	1.39 – 1.22
12 (3H, t)	0.88	0.88	0.88	0.88

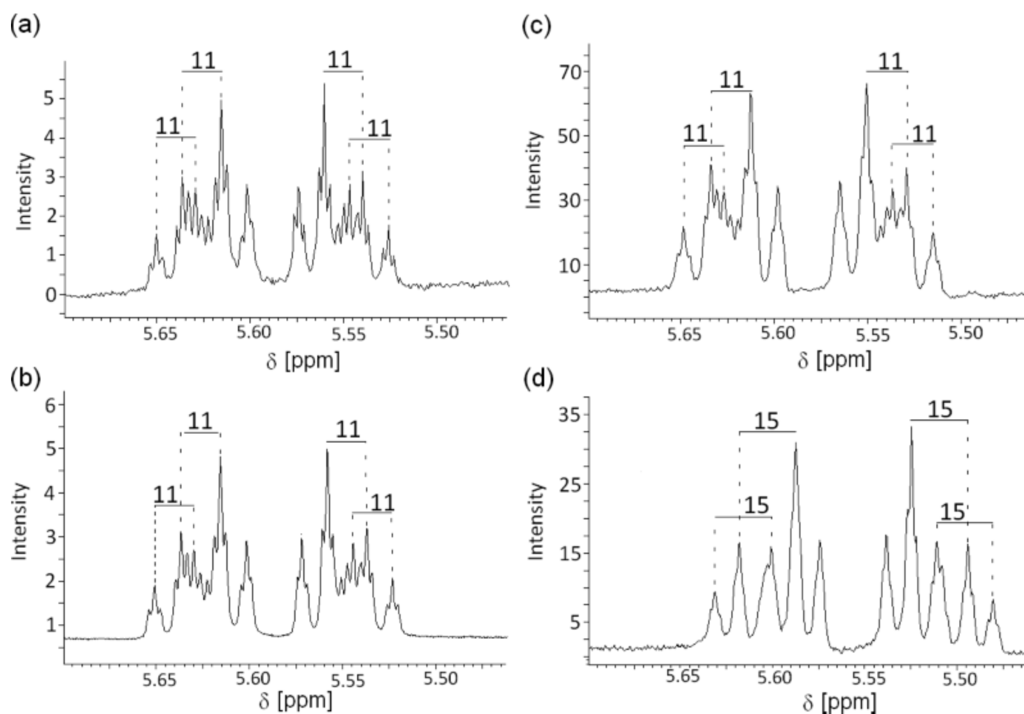

Fig. 2. ^{1}H NMR spectra of the alkene areas for (a) extract of *K. nicolsoni*, (b) extract of *K. rugosus*, (c) synthetic *Z*3-12:COOH, and (d) synthetic *E*3-12:COOH. The spectra show that the coupling constants and the chemical shifts are the same for the synthetic *Z*3-12:COOH and the two extracts.

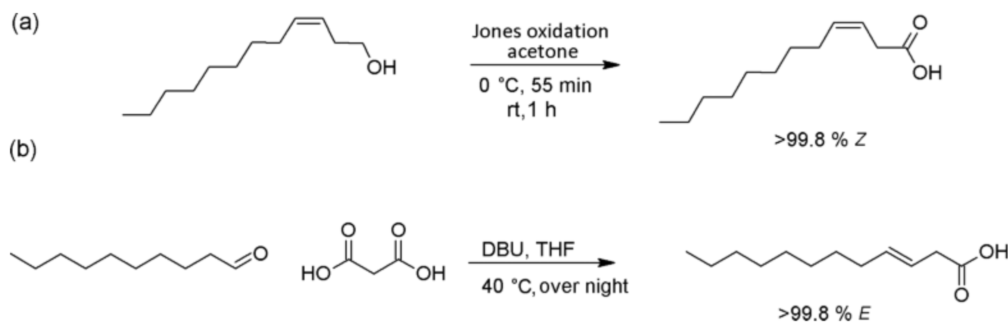

Fig. 3. Reaction scheme for the synthesis of the two reference compounds: (a) *Z*3-12:COOH, (b) *E*3-12:COOH; rt, room temperature; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; THF, tetrahydrofuran.

(DBE) giving the degree of unsaturation. HRMS was not performed on the *K. rugosus* extracts since all other analyses were identical for the two extracts.

In order to compensate for the differences in the amounts of the investigated compound in the two extracts, the ^{1}H NMR spectrum of *K. nicolsoni* was run for 4 days, while that of *K. rugosus* was run overnight. Here, we also show a diagnostically important chemical shift in the ^{1}H NMR spectrum for the determination of the double bond position in acids as an alternative to the traditional dimethyl disulfide (DMDS) method (Dunkelblum *et al.*, 1985). A doublet at δ_H 3.15 ppm in the ^{1}H NMR experiments confirmed that the double bond is in position 3; the rather high chemical shift can be explained by the fact that the protons are close to and between two electron-withdrawing groups (acid and alkene) (Frost and Gunstone, 1975). The chemical shifts for olefinic protons are slightly different for *Z*- and *E*-alkenes, as can be seen in Table I and Fig. 2, with *Z*-alkenes having a slightly higher shift than *E*-alkenes. The doublet at δ_H 3.15 ppm and the apparent quartet at δ_H 2.04 ppm correspond to hydrogen atoms adjacent to the alkene group, and these were irradiated in order to receive more information on the coupling constants and the splitting pattern. The results from this experiment support a coupling constant of 11 Hz and hence *Z*-double bond. To further strengthen the conclusion that the double bond is of *Z*-stereochemistry, the alkene area of the extract was compared with the alkene area of synthetic references and the *Z*-stereochemistry was confirmed (Fig. 2). All NMR data are summarized in Table I.

*Z*3-12:COOH was synthesized, for the first time, via Jones oxidation from (*Z*)-3-dodecen-1-ol in 89.9% yield and a stereoisomeric purity of > 99.8% (Fig. 3a). By comparing ^{1}H and ^{13}C NMR data for (*Z*)-3-dodecen-1-ol and *Z*3-12:COOH, it was deduced that no isomerization had occurred during oxidation. *E*3-12:COOH was synthesized via a decarboxylative Knoevenagel reaction from malonic acid and decanal in 40.2% yield and a stereoisomeric purity of > 99.8% (Fig. 3b).

GC-FTIR confirmed the NMR results since no peak was present in the 970−960 cm^{-1} region (Fig. 4), whereas a peak was found at 3031 cm^{-1} (Attygalle *et al.*, 1994) for the extracts and *Z*3-12:COOH, which strongly indicates *Z*-geometry of the double bond. The results are summarized in Table II.

Comparison of retention times on an INNOWax column confirmed that the unknown compound present in *K. nicolsoni* and *K. rugosus* 2nd-instar larvae full-body extracts eluted at the same retention time (29.810 min) as *Z*3-12:COOH, confirming the presence of this isomer while the *E*-isomer eluted at 29.413 min.

The amount of *Z*3-12:COOH was determined from an external standard (henicosane). A *K. nicolsoni* larvae produced (59 ± 11) ng per individual (based on 5 extracts, each with 10 individuals) and a *K. rugosus* larvae produced (84 ± 49) ng per individual (based on 4 extracts, each with 10 individuals). It is indeed interesting to note that the dominating compound in the two species studied here was not detected in the closely related but eusocial *K. intermedius* (De Facci *et al.*, 2014), which mainly contained shorter fatty acids. The reason behind this divergence remains to be elucidated in future investigations of the biosynthesis and behavioural role of the compounds.

Contact chemoreception bioassay

The entry duration (Table III) differed significantly for *K. nicolsoni* 2nd-instar larvae exposed to the treatments *vs.* *n*-hexane control (ANOVA on log-transformed data: N_{group} = 32, $F_{2,93}$ = 7.333, P =

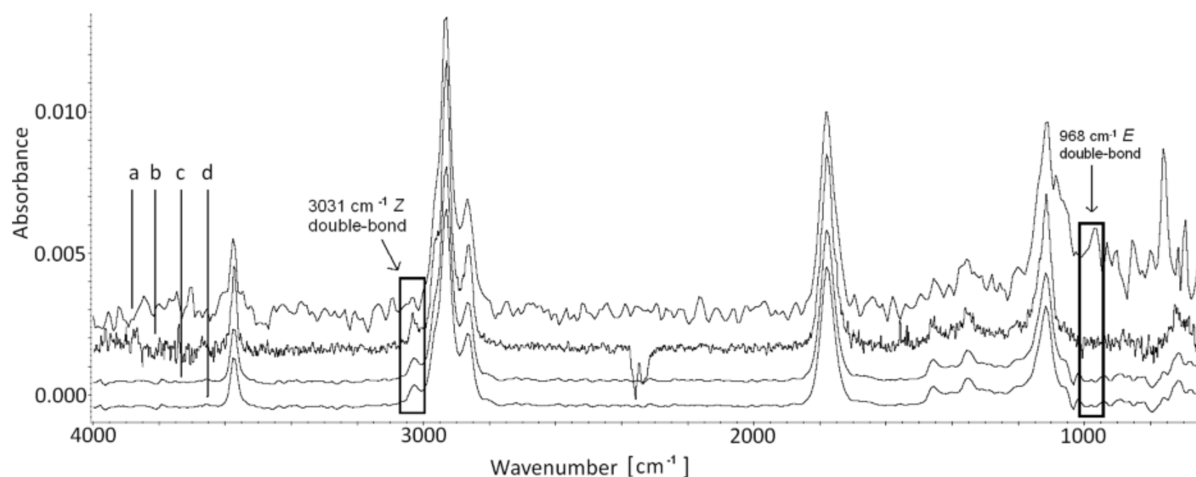

Fig. 4. FTIR spectra of (a) synthetic *E*3-12:COOH, (b) synthetic *Z*3-12:COOH, (c) extract of *K. nicolsoni*, and (d) extract of *K. rugosus*. The peak at 761 cm^{-1}, present in the spectrum of *E*3-12:COOH, is missing in the spectra of *Z*3-12:COOH and of both extracts.

Table II. Wavenumbers (in cm^{-1}) from GC-FTIR spectra of natural and synthetic compounds.

Functional group and peak origin	*K. nicolsoni*	*K. rugosus*	*Z*3-12:COOH	*E*3-12:COOH
O-H str	3576	3576	3577	3577
H-C=C-H *Z*	3031	3031	3031	–
C-H str	2934	2934	2934	2935
C=O str	1780	1780	1782	1781
O-H bend	1353	1353	1353	1356
C-O str	1117	1117	1117	1114
H-C=C-H *E*	–	–	–	968
H-C=C-H *Z*	696	696	696	–

0.001; Fig. 5a). Both isomers induced the same behaviour (Tukey's HSD post-hoc test: $P = 0.733$). However, the absolute angular velocity (Table III) showed a dissimilar pattern (ANOVA: $N_{\text{group}} = 32$, $F_{2,93} = 3.29$, $P = 0.042$; Fig. 5b), with only the *E*3-12:COOH treatment significantly differing from the *n*-hexane control (Tukey's HSD post-hoc test: $P = 0.032$). Again, isomers did not differ significantly when compared (Tukey's HSD post-hoc test: $P = 0.313$).

Track features can give insights regarding the stimuli animals encounter while exploring the environment and how these stimuli affect their movement (Bell, 1991). With our contact chemoreception bioassay we were able to assess that both 3-12:COOH isomers appear to act as repellents at the high dose used (1 $\mu g/\mu l$), but not always in the same way when compared with the control. The isomers affected significantly two behavioural parameters when compared

Table III. Behavioural parameters considered in the analysis.

Parameter	Definition
Entry duration [s]	Average time spent in the zone at each complete entry (in/out), therefore excluding the starting period and eventual incomplete entrances at the end of the recording
Absolute angular velocity [°/s]	Absolute (unsigned) rate of change in direction of movement of the insect body-centre between two consecutive samples over the recording period of 15 min. It is also known as absolute turning rate (Bell, 1991)
Distance from centre [cm]	Average of all distances of the insect body-centre from the arena-centre computed per each second over the recording period of 15 min

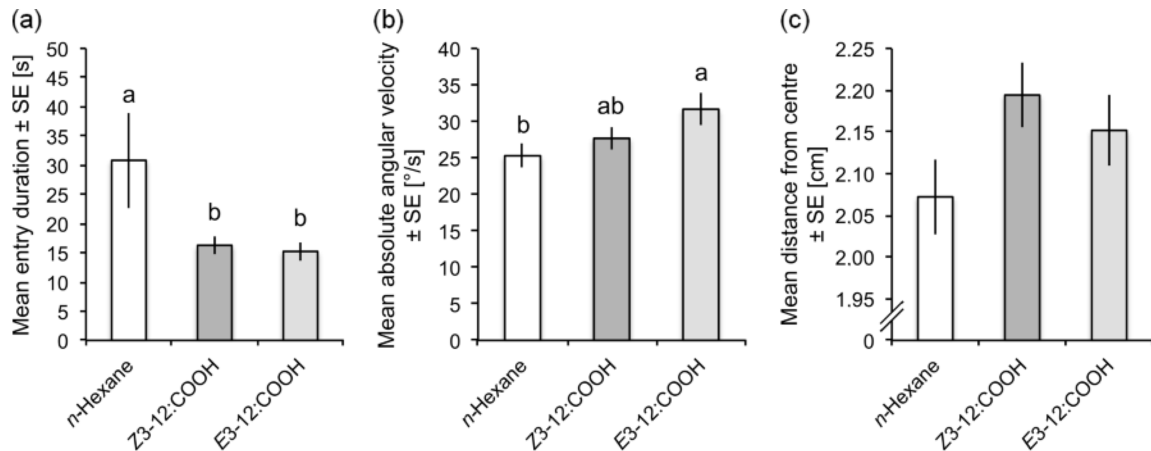

Fig. 5. Variables (means ± SE) describing *K. nicolsoni* 2nd-instar larvae behaviour in response to the two different 3-12:COOH isomers: (a) entry duration in the treated zone (in/out); (b) absolute angular velocity measured during the entire recording duration; (c) distance from the arena centre maintained during the whole recording. Letters indicate treatments with different significance level at $\alpha = 0.05$ according to the Tukey's HSD post-hoc test applied to transformed data. $n = 32$ for each group.

to the *n*-hexane control. In detail, *K. nicolsoni* 2nd-instar larvae spent on average less time in the treated zone at each entry suggesting repellence at the selected dose. However, the similar effect induced by both isomers on thrips denotes that 3-12:COOH itself acts as a repellent, independent of the structure, at the tested dose. Although not significant, the isomers seemed to affect the other behavioural parameters somewhat differently, *i. e.* the absolute angular velocity and the distance from centre (Table III, Figs. 5b, c). *K. nicolsoni* exposed to *E*3-12:COOH showed a higher absolute angular velocity compared to the *n*-hexane control. This can be seen again as an effect of a repellent, since insects encountering an undesired stimulus should change direction more frequently in an attempt to avoid it: frequent changes of direction over time translate into high absolute angular velocity. This was not noticed for *Z*3-12:COOH. Gregarious insects, like gall-inducing thrips, are also expected to respond stronger to alien stimuli not shared by their group mates, *i. e.* *E*3-12:COOH in this case (Fig. 5), according to the nest mate recognition theory (Wyatt, 2003). *E*3-12:COOH was previously identified from a few idolothripine and phlaeothripine species (Haga *et al.*, 1989; Suzuki *et al.*, 2000, 2004), and (*Z*)-3-decenoic acid (*Z*3-10:COOH) from the idolothripine *Holurothrips morikawai* (Suzuki *et al.*, 2000), family Phlaeothripidae. Haga and colleagues (1989) inferred *E*3-12:COOH to be an alarm and aggregation pheromone in *Varshneyia pasaniae* (= *Leeuwenia pasanii*), and the concept was extended to other species in which it was identified (Moritz, 2006; Suzuki *et al.*, 2000, 2004). However, there are no behavioural data available to support this function so far. Thus, this compound could be rather common in thrips and cover several functions. In addition, to our knowledge this is the first report of *Z*3-12:COOH in a thrips species. This compound was previously found in the defensive anal gland secretions of the ant *Bothriomyrmex syrius* (Lloyd *et al.*, 1986). Tracks showed the tendency of insects to seek for refuge close to the arena border as indicated by the higher distance from centre maintained by the larvae when exposed to *Z*3-12:COOH (Fig. 5c). Therefore, the compound could either act as an alarm pheromone or as a cue to avoid conspecifics, *e. g.* during latency in the soil. Unfortunately, given the recent discovery (McLeish *et al.*, 2006), our knowledge of *K. nicolsoni* ecology is extremely limited. If *Z*3-12:COOH acts as an alarm pheromone, the "model clade" genus *Kladothrips* would help us to understand the evolution of semiochemicals taking place during the evolution of sociality. Recent work has focused attention on the anal droplets produced by a congeneric social species, *K. intermedius* (De Facci *et al.*, 2013, 2014). In this species, only soldiers seem to produce an alarm pheromone. Since in social thrips caste dimorphism evolutionarily preceded the division of labour (Chapman *et al.*, 2008), investigating the presence of an alarm pheromone especially in the basal social species, where soldiers still reproduce at high rate,

would give insights into the transition from a phenotype shared by all group members to a caste phenotype.

In conclusion, *E*3-12:COOH and *Z*3-12:COOH have been synthesized and, based on different chemical analyses, it was determined that extracts from two thrips species, *K. nicolsoni* and *K. rugosus*, contain the *Z*3-12:COOH isomer. No significant difference regarding induced behavioural effects on the thrips *K. nicolsoni* was detected between the isomers, but trends suggested that they are not perceived exactly as the same compound. It is plausible that the *K. nicolsoni* 2nd-instar larvae are actually able to distinguish the compounds better by olfaction rather than by "taste" given their volatility and the higher specificity of insect olfactory receptors (Wyatt, 2003). A categorizing semiochemical activity still needs to be properly assessed and therefore other behavioural assays are suggested, preferably testing also olfaction and other species belonging to the genus *Kladothrips* for comparison.

Experimental

Handling of galls and insects

Galls induced by *K. nicolsoni* on *A. papyrocarpa* trees and shrubs were collected in South Australia (Middleback, S 32°56.759′, E 137°23.403′) while *K. rugosus* galls on *A. melvillei* were collected in Victoria (Merbein South, S 34°12.953′, E 142°01.322′) in January 2012. Galls contained mainly living larvae (2nd-instar) that were used for chemical analyses. Galls on *A. melvillei* were pouched with a smooth surface, having been induced by a physogastric foundress, and were different from the galls that are more commonly found on this host species, *i. e.* the flat, pouched galls with spikes of *K. rugosus*. Therefore, the few adults found were used for taxonomical confirmation (L. Mound, personal communications). Vouchers are stored at the Australian National Insect Collection, Canberra. A second collection of *K. nicolsoni* for use in behavioural tests was made in January 2013. Behavioural tests were attempted also for *K. rugosus*, but, given the early stage of galls on *A. pendula* in October 2012 in southern Queensland (close to Dirranbandi, S 28°35.146′, E 148°13.565′), the low number of individuals found limited a proper analysis. Therefore, results are not shown here. For preservation prior to bisection, galls were stored in a refrigerator at ~ 9 °C. Healthy thrips can survive up to four months under these conditions. Insects were processed immediately after bisection of galls.

The stickiness of the larval surface made the larvae difficult to handle according to the method described by De Facci *et al.* (2013). Therefore, as commonly found in the literature (MacDonald, 2002), full-body extracts were preferred, which were prepared by immerging ten or twenty-five 2nd-instar larvae in 250 μl *n*-hexane for 30 min. Extracts were transferred to new vials, concentrated to ~ 30 μl, and stored in a freezer until needed. Five extracts of *K. nicolsoni* from ten individuals each and four extracts of *K. rugosus* from ten individuals each were prepared in order to estimate the amount of unknown in the larvae. An external calibration curve prepared with henicosane was used for the estimation.

Chemical analyses and syntheses

All chemicals were used without further purification. (*Z*)-3-Dodecen-1-ol was bought from U-Chemo (Shanghai, China). Preparative liquid chromatography was performed on straight-phase silica gel (Merck 60, 230 – 400 mesh, 0.040 – 0.063 mm, 10 – 50 g/g of product mixture; Merck, Darmstadt, Germany) employing a gradient technique with an increasing content (0 – 100 %) of distilled ethyl acetate in distilled cyclohexane. Thin-layer chromatography (TLC) was performed to monitor the progress of the reaction on silica gel plates (Merck 60, precoated aluminium foil) using ethyl acetate in cyclohexane (40:60, v/v) as an eluent; plates were developed by means of ultraviolet irradiation and/or by spraying with vanillin in sulfuric acid and heating at 120 °C. Gas chromatography-mass spectrometry (GC-MS) was performed on two different instruments. Extracts and synthetic reference substances were analysed with an HP 6890N gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a polar FactorFOUR VF-23ms column (30 m $\times$ 0.25 mm i. d., 0.25 μm film thickness; Varian, Palo Alto, CA, USA) coupled to an HP 5973 mass spectrometer in full scan mode. The injector was operated in splitless mode at 250 °C, and a helium flow rate of 1 ml/min and a transfer line temperature of 280 °C were used. The source temperature was 250 °C with a filament bias voltage of -70 eV. The mass range used was m/z 20 – 500. For each injection 1 μl of sample was used. An Agilent 7890 gas chromatograph (Santa Clara, CA, USA) equipped with a non-polar FactorFOUR vf-5ms column (30 m $\times$ 0.25 mm i. d., 0.25 μm film thickness; Agilent) coupled to an Agilent 240 ion-trap mass spectrometer was used for both EI and CI experiments. The injector

was operated in splitless mode at 275 °C, and a helium flow rate of 1 ml/min and a transfer line temperature of 280 °C were used. The analyses were performed in external ionization configuration. EI spectra were recorded in the mass range of m/z 50 – 300 at fast scan rate. CI spectra were recorded using acetonitrile (ACN) as a reagent gas in the mass range of m/z 50 – 500 at normal scan rate. A modified temperature regimen in the mass spectrometer was used for the CI experiments: ion-trap temperature of 90 °C, manifold temperature of 40 °C, and ion-source temperature of 160 °C (Kroiss *et al.*, 2011). The same oven temperature program was used for all GC-MS analyses; the temperature was increased from 50 °C by 10 °C/min up to 230 °C, and the temperature was held isothermally for 10 min.

Gas chromatography coupled to Fourier transform infrared spectroscopy (GC-FTIR) analysis of the natural and synthetic compounds was carried out on a Thermo Scientific Nicolet 6700 FTIR spectrophotometer (Waltham, MA, USA), coupled via a GC-FTIR interface to an Agilent 7890A gas chromatograph with an HP-5 column (30 m × 0.25 mm i. d., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The carrier gas (1 ml/min) was helium. One μl of the sample was injected splitless, the injector temperature was 250 °C, and the transfer line and flow cell temperature were both set at 280 °C. The column temperature was increased from 50 °C by 10 °C/min up to 260 °C, and kept at 260 °C for 8 min.

^{1}H NMR experiments of extracts and ^{1}H and ^{13}C NMR spectra on synthetic references were run on a Bruker Avance 500 (^{1}H, 500 MHz; ^{13}C, 125.8 MHz) spectrometer (Karlsruhe, Germany) using deuterated chloroform ($CDCl_3$) as solvent and tetramethylsilane (TMS) as internal standard. Irradiation experiments were performed to deduce the splitting pattern and coupling constants, at pl24 = 45 db or 50 db, depending on the extract. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hz. Data are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet.

High-resolution mass spectrometry (HRMS) was performed on an Agilent 6520 – QTOF ESI-mass spectrometer (positive mode) with ACN/H_2O (70:30) and 1 % formic acid as eluent, and 3 μl of sample were injected. An isotope calculator in the software was used to determine the molecular formula.

To test the conformation of the compound present in *K. nicolsoni* and *K. rugosus* 2nd-instar larvae full-body extracts, the latter were analysed together with the synthesized 3-12:COOH geometrical isomers in an HP 5890-5972 GC-MS instrument equipped with an HP-INNOWax column (30 m × 0.25 mm i. d., 0.25 μm film thickness; J & W Scientific). The oven temperature was increased from 80 °C at 5 °C/min to 195 °C, and held for 5 min.

Preparation of (Z)-3-dodecenoic acid

According to Harding *et al.* (1975), Jones reagent (0.54 ml) was added to (*Z*)-3-dodecen-1-ol (0.54 mmol, 0.1 g) in acetone (5 ml) at 0 °C. The mixture was allowed to reach room temperature after 55 min and subsequently stirred for 1 h. The reaction was quenched by addition of Et_2O and H_2O (5 ml). The aqueous phase was extracted with Et_2O (2 × 5 ml), and the combined organic layers were washed with H_2O (2 × 5 ml) and brine (1 × 5 ml), then dried over $MgSO_4$. Evaporation of the solvent resulted in 0.096 g (89.9 % crude yield) of a clear oil, which was purified by Kugelrohr distillation to remove impurities: B.p. 150 °C (1.3 mbar). – ^{1}H NMR: δ = 0.88 (t, 3H, J = 7 Hz), 1.38 – 1.23 (m, 14H), 2.04 (m, 2H), 3.14 (d, 2H, J = 7 Hz), 5.58 (ddt, 2H, J = 11, 8.5, 1.5 Hz), 10.45 (br, 1H). – ^{13}C NMR: δ = 14.1, 22.7, 27.4, 29.3, 29.5, 31.9, 32.4, 119.9, 134.2, 176.9. – GC-FTIR: ν = 3577 (O–H), 3031 (C=C *cis*), 2934 (C–H), 1782 (C=O), 1353 (O–H), 1117 (C–O), 696 (C=C *cis*) cm^{-1}.

Preparation of (E)-3-dodecenoic acid

(*E*)-3-Dodecenoic acid was prepared according to the procedure of Kemme *et al*. (2010). The NMR data corresponded to those given by Ragoussis and Ragoussis (1998). – GC-FTIR: ν = 3577 (O–H), 2935 (C–H), 1781 (C=O), 1356 (O–H), 1114 (C–O), 968 (C=C *trans*) cm^{-1}.

Contact chemoreception bioassay

To test the bioactivity of the two isomers, we used a walking bioassay based mainly on contact chemoreception (“taste”), performed in still air. Both *K. nicolsoni* 2nd-instar larvae and *K. rugosus* adults were tested. The setup was readjusted from De Facci *et al.* (2013). A filter paper (grade 1001; Munktell, Falun, Sweden) disk, 5.5 cm in diameter, represented the arena and was housed in a 9-cm-diameter glass Petri dish. Covering the sector between the edge of the arena and the wall of the Petri dish, a layer of Chesebrough clover vaseline (Lilleborg AS, Oslo, Norway) contain-

Table IV. Pearson's correlation coefficients r (2-tailed) between several behavioural parameters.

Parameter	Entry duration [s][a]	Absolute angular velocity [°/s][b]	Distance from centre [cm][b]	Distance moved [cm][b]	Time spent in zone [s][b]	$N^{a}_{entries}$	Absolute min. distance from centre [cm][a]
Entry duration [s][a]	–						
Absolute angular velocity [°/s][b]	0.040	–					
Distance from centre [cm][b]	−0.548***	−0.063	–				
Distance moved [cm][b]	−0.391***	−0.412***	−0.046	–			
Time spent in zone [s][b]	0.555***	−0.001	−0.878***	0.175	–		
$N^{a}_{entries}$	−0.379***	−0.130	−0.270**	0.777***	0.463***	–	
Absolute min. distance from centre [cm][a]	−0.147	0.247*	0.211*	−0.609***	−0.400***	−0.473***	–

[a] Computed per entry.
[b] Computed for the entire recording.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ing 20% saponin (ICN Biomedicals, Irvine, CA, USA) constrained the thrips within the arena. Insects able to exit the arena ($n = 10$), thereby taking the risk to get stuck on the vaseline, were excluded from further analyses.

Behaviour under two treatments was analysed: exposure to 75 μg of the synthesized *Z*3-12:COOH or, respectively, *E*3-12:COOH, in 75 μl *n*-hexane. As control, we used 75 μl *n*-hexane. The solvent created a treatment/control zone comprising half of the surface area of the Petri dish arena, and was allowed to evaporate for 5 min under a sucking vent. The filter paper was changed and the stimulus newly applied for each replicate. We ensured the immediate acquaintance with the stimulus from the start by gently positioning each individual at the arena centre using a paintbrush. Each insect was recorded for 15 min with an Ikegami CCD video camera (Ikegami Electronics Europe, Neuss, Germany) and simultaneously video-tracked live using the EthoVision® XT 8.5 (Noldus Information Technology, Wageningen, Netherlands) (Noldus Information Technology, 2011). Experiments were conducted in the dark, and the arena was lighted from above with infrared lights at the sides of the camera, as performed by De Facci *et al*. (2013), and from below with a third infrared light. The latter was placed under the paper sheet holding the Petri dish and held by a cardboard box. In this way the yellowish *K. nicolsoni* 2nd-instar larvae could contrast better the bright background offered by the filter paper matching the detection settings of EthoVision® XT 8.5. Trials were performed between 10:00 – 20:00 at (20.6 ± 0.3) °C and (32.9 ± 3.4) % relative humidity.

The sample rate was set at 1 sample/s (video frame/s). Such fast sample rate and the tiny size of thrips were occasionally problematic for the video-tracking system. Tracks were edited using the interpolation function to correct eventual errors (Noldus Information Technology, 2011). As default, measurements were applied on the body centre. To test the attraction/repellence of single compounds, three main variables were considered: entry duration, absolute angular velocity, and distance from centre (cf. Table III). Since the entry duration is calculated on complete entries, one insect was disregarded from further analysis. Other variables were ignored given the strong correlation with these three variables (Table IV).

Data analysis was performed using parametric statistics with SPSS v.19 and Excel for Mac v.14. Exploratory tests (Shapiro-Wilk's and Levene's) were used to evaluate the assumptions of normality and homoscedasticity among groups. Normality was improved by data transformation $[\log_{10}(x+1)]$ before applying one-way ANOVA to determine equality of means. For unplanned multiple range comparisons, Tukey's HSD post-hoc test followed ANOVA (Sokal and Rohlf, 2013). Pearson's correlation coefficient r was applied to test the linear correlation between two variables.

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