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ORIGINAL ARTICLE

Composition and emission dynamics of migratory locust volatiles in response to changes in developmental stages and population density

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Abstract Chemical communication plays an important role in density-dependent phase change in locusts. However, the volatile components and emission patterns of the migratory locust, *Locusta migratoria*, are largely unknown. In this study, we identified the chemical compositions and emission dynamics of locust volatiles from the body and feces and associated them with developmental stages, sexes and phase changes. The migratory locust shares a number of volatile components with the desert locust (*Schistocerca gregaria*), but the emission dynamics of the two locust species are significantly different. The body odors of the gregarious nymphs in the migratory locust consisted of phenylacetonitrile (PAN), benzaldehyde, guaiacol, phenol, aliphatic acids and 2,3-butanediol, and PAN was the dominant volatile. Volatiles from the fecal pellets of the nymphs primarily consist of guaiacol and phenol. Principal component analysis (PCA) showed significant differences in the volatile profiles between gregarious and solitary locusts. PAN and 4-vinylanisole concentrations were significantly higher in gregarious individuals than in solitary locusts. Gregarious mature males released significantly higher amounts of PAN and 4-vinylanisole during adulthood than mature females and immature adults of both sexes. Furthermore, PAN and 4-vinylanisole were completely lost in gregarious nymphs during the solitarization process, but were obtained by solitary nymphs during gregarization. The amounts of benzaldehyde, guaiacol and phenol only unidirectionally decreased from solitary to crowded treatment. Aliphatic aldehydes (C7 to C10), which were previously reported as locust volatiles, are now identified as environmental contaminants. Therefore, our results illustrate the precise odor profiles of migratory locusts during developmental stages, sexes and phase change. However, the function and role of PAN and other aromatic compounds during phase transition need further investigation.

Key words GC–MS/MS; *Locusta migratoria*; phase change; phenylacetonitrile; volatile chemicals

Introduction

Locusts display significant phase polyphenisms in behavior, physiology and morphology in response to changes in population density (Uvarov, 1966; Pener, 1991). The biological foundation of notorious locust menaces is due to behavioral transition from the solitary to gregarious phase, which leads to local marching juvenile bands and adult migratory swarming (Lovejoy *et al.*, 2006; Guershon & Ayali, 2012). Phase change is triggered essentially by

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conspecific communication via visual, olfactory and tactile information across different individuals (Pener & Simpson, 2009). However, behavioral attraction and repulsion during the phase change of migratory locusts are mediated significantly by olfactory stimuli and related genes (Guo *et al.*, 2011; Wang & Kang, 2014).

Substantial progress has been achieved in the chemical analyses of locust-associated volatiles and in understanding their roles in the aggregation behavior of the desert locust, *Schistocerca gregaria* (Pener & Simpson, 2009). Gregarious nymphs of desert locust mainly release aliphatic acids and aldehydes (C6, C8–C10), as well as aromatic compounds (guaiacol and phenol). Gregarious juveniles respond postively to crude nymphal volatiles and synthetic blends of acids and aldehydes (Obeng-Ofori *et al.*, 1993; Torto *et al.*, 1996). The volatiles of male desert locust during adulthood primarily consist of six aromatic compounds, namely, phenylacetonitrile (PAN), benzaldehyde, veratrole, anisole, guaiacol and phenol (Torto *et al.*, 1994; Njagi *et al.*, 1996; Niassy *et al.*, 1999). The blend of these compounds has a corresponding ratio of 100 : 15 : 5 : 5 : 4 : 4 and attracts young/ mature gregarious adults but not the nymphs of the desert locust (Torto *et al.*, 1994).

Several studies have shown that solitary desert locust adults exhibited consistent preferences for male adult odors over control (Njagi *et al.*, 1996). Therefore, the volatiles have been assumed to be involved in sexual communication (Seidelmann & Ferenz, 2002; Ferenz & Seidelmann, 2003; Seidelmann *et al.*, 2005). PAN has long been considered a pivotal component of mature male desert locust in behavioral aggregation (Obeng-Ofori *et al.*, 1993; Torto *et al.*, 1994, 1996; Njagi *et al.*, 1996; Niassy *et al.*, 1999; Hassanali *et al.*, 2005) and a specific adult male sex pheromone in male–male competition (Seidelmann & Ferenz, 2002; Ferenz & Seidelmann, 2003; Seidelmann *et al.*, 2005). The migratory locust, *Locusta migratoria*, is another important pest species worldwide and displays significant density-dependent phase transition, although the styles of its phase transition evidently differ from that of the desert locust (Guo *et al.*, 2011; Ma *et al.*, 2011; Wang & Kang, 2014). However, little information is available on the locust-emitted chemicals and their roles in the aggregation of the migratory locust. Fuzeau-Braesch *et al.* (1988) reported that gregarious nymphs and adults of the migratory locust release three major compounds, namely, veratrole, guaiacol and phenol, all of which can increase the aggregation behavior in nymphs and adults. Niassy *et al.* (1999) explored the migratory locust-produced volatiles and found that PAN was produced by nymphs, whereas aliphatic aldehydes and alcohols dominated the adult volatiles. A study on the fecal volatiles of a 2-day-old adult migratory locust identified 11 electrophysiologically active compounds, namely, aromatic derivatives (benzyl alcohol, benzaldehyde, guaiacol and phenol), aliphatic aldehydes (C6–C10), cyclohexanol and 2,5-dimethyl-pyrazine. Moreover, a synthetic blend consisting of cyclohexanol, 2,5-dimethyl-pyrazine, benzaldehyde, nonanal and hexanal increase the attractiveness to young adults (Shi *et al.*, 2011). However, the complete component composition and emission patterns of the migratory locust volatiles associated with developmental stages and phase change remain ambiguous.

Recent studies on molecular and genomic levels have highlighted the important role of peripheral and central olfactory systems during the phase change of the migratory locust. Differences in the expression of chemosensory proteins, olfactory receptors and cuticle protein genes between solitary and gregarious migratory locusts on the basis of expressed sequence tags (Kang *et al.*, 2004; De Loof *et al.*, 2006), genome and transcriptomes (Wang & Kang, 2014; Wang *et al.*, 2015) are the most significant. Moreover, the oligonucleotide microarray analysis of the gene expression reveals that antenna-rich chemosensory protein (CSP) and *takeout* genes exhibit contrasting expression trends during gregarization and solitarization. The RNA interference (RNAi) of the *CSP*s and *takeout* gene significantly shifts the behavior of gregarious and solitary migratory locusts in response to the gregarious volatiles (Guo *et al.*, 2011). This study explores the important role of the peripheral olfactory system in the initiation of behavioral phase changes in the locusts (Wang & Kang, 2014). Moreover, a recent study based on locust genomic and transcriptomic data on the odorant receptors (ORs) and ionotropic receptors (IRs) of the migratory locust identified 142 ORs and 32 IRs, most of which show olfactory-specific and development-independent expression patterns (Wang *et al.*, 2015). Olfactory assays following the RNA interferences of the co-receptor genes *LmigOrco* or *LmigIR8a/IR25a* confirmed that OR-based signaling pathway and not IR-based signaling pathway regulates the preference of gregarious nymphs for gregarious locust-emitted volatiles (Wang *et al.*, 2015). These studies provide important cues for exploring the molecular mechanism of phase-dependent olfactory plasticity in locusts. Changes in the neurotransmitters and neuromodulators, such as dopamine, serotonin, octopamine and tyramine, in the central nervous system of desert locust and migratory locust modulate the olfactory preferences of gregarious and solitary locusts to gregarious locust-emitted volatiles (De Loof *et al.*, 2006; Guo *et al.*, 2013, 2015; Ma *et al.*, 2015). Furthermore, methylome and transcriptome analyses suggested phase change-related

synapse plasticity in the migratory locust (Wang *et al.*, 2014). However, insufficient information on the chemical profiles of migratory locust volatiles limits the understanding of the roles of individual components during phase change and the molecular and neural mechanisms of the locust olfactory system.

In this study, the volatile composition and emission dynamics from the solitary and gregarious individuals, two sexes and different developmental stages, were identified. Principal component analysis (PCA) was used to investigate the variations in the locust-emitted volatiles during phase transition. The results demonstrated that aromatic compounds PAN, benzaldehyde, guaiacol, 4-vinylanisole and phenol considerably changed during phase change. PAN and 4-vinylanisole were reliable volatile markers of gregarious locusts.

Materials and methods

Insects

The gregarious and solitary locusts used in the experiments were reared as reported by Guo *et al.* (2011). The gregarious locusts were cultured in cages (30 cm × 30 cm × 30 cm) at densities of 500–600 first-instar insects per cage in a well-ventilated insectary room. The solitary locusts were reared individually in a ventilating cage (10 cm × 10 cm × 25 cm) in another room. Both colonies were reared at 30 ± 2°C, 60% ± 5% relative humidity, 14 : 10 L : D photoperiod, and on a diet of fresh greenhouse-grown wheat seedlings for at least 3 years.

Absorbent trapping method for gregarious fourth-instar nymphs

A dynamic headspace collection system was used to collect the volatiles from the fourth-instar gregarious locusts of both sexes. The absorbent trapping method was performed as described previously by Wei *et al.* (2007) with slight modifications. A total of 50 males and 50 females or 5 g of feces of fourth-instar nymphs were enclosed in a 2 L round-bottomed glass flask. The fresh feces of nymphs were collected and stored at −20°C until fecal volatile collection. The compressed air (Beijing Gas Main Plant, Beijing, China) was purified and humidified through three glass jars (500 mL) individually filled with a molecular sieve (0.5 nm, Beijing Chemical Company, Beijing, China), freshly activated charcoal (Beijing Chemical Company) and distilled water. Air was pushed into the flask and then drawn from the jar via a collector (a glass tube with an internal diameter of 3 mm containing 100 mg of Porapak Q 80–100 mesh, Supelco Bellefonte, PA, USA) at the jar outlet at a rate of 400 mL/min by using a membrane pump (Beijing Institute of Labor Instruments, Beijing, China) at the end of the system for 5 h (usually from 10:00 until 15:00 hours). We ensured that no ambient air was sucked into the flask because of the positive pressure inside. Collections from each treatment were replicated five or six times. Volatile compounds were rinsed with 600 mL of high-performance liquid chromatography-grade dichloromethane (Tedia Company, Fairfield, OH, USA). Thereafter, the extracts were stored at −20°C until chemical analyses.

Static solid phase microextraction (SPME)

The absorbent trapping method is frequently applied for locust volatile collections (Heifetz *et al.*, 1996; Niassy *et al.*, 1999; Seidelmann *et al.*, 2003). However, the high density of insects and long collection duration in the collection system could interfere with the phase state of solitary locusts (Guo *et al.*, 2011). Therefore, SPME was used with a small number of locusts (10 locusts for nymphs of first and second stadia, five individuals for nymphs from third to fifth stages, and one individual for adult) and a short collection period (30 min). Gregarious locusts also underwent the same treatment to compare the volatile profiles of gregarious and solitary locusts. A fiber (PDMS/DVB 65 μm) was introduced into a glass jar (10.5 cm high × 8.5 cm internal diameter) at approximately 1 cm above a stainless steel lid (9 cm in diameter with holes of 2 mm diameter and 2 mm apart), which served as a barrier to confine the locust at the bottom of the jar to avoid direct body contact with the fiber. The headspace volatiles of nymphal (from first instar to fifth instar) and adult (immature adult refers to 5-day-old post-adult eclosion, and mature adult is 15-day-old post-adult eclosion) stages taken from solitary rearing conditions or gregarious conditions were absorbed for 30 min. The SPME volatiles collected from an empty glass jar for 30 min served as control. The fibers with absorbed odors were subjected to chemical analyses.

Volatile collections for phase change treatments

Isolation of gregarious locusts: the third-instar gregarious locusts of both sexes were moved individually to solitary rearing cages supplied with wheat seedlings. The insects were subjected to SPME system as described earlier after 2–3 days from previous molt in the fourth-instar stage.

Crowding of solitary locusts: to achieve the crowding effect, a total of five third-instar solitary locusts reared individually in a ventilating cage were introduced into a gregarious rearing cage (an optic perplex-made box, 15 cm × 15 cm × 11 cm) containing 20 gregarious nymphs of the same developmental stage as the stimulus group. Crowd-treated solitary locusts were placed into the SPME system after staying with the stimulus group for 8–10 days and reaching the middle stage in the fourth-instar stadium.

The same cohort of gregarious or solitary locusts without solitarization or gregarization served as controls in headspace volatile collections. All insects were sampled at the same time point (9:00 to 16:00 hours) for six biological replicates, and five nymphs were sampled in each biological replicate.

Chemical analysis

The structural analysis of volatile compounds was performed using an Agilent gas chromatograph (GC) (6890N) coupled with a mass spectrometry (MS) system (5973 MSD). Methods were as described by Wei *et al.* (2007) with slight modifications. The GC was equipped with a polar DB-WAX column (30 m × 0.25 mm × 0.15 μm, Agilent Technologies, Palo Alto, CA, USA) or a nonpolar DB-1MS column (30 m × 0.25 mm internal diameter [i.d.] × 0.25 μm film thickness, Agilent Technologies). The oven initial temperature on the DB-WAX column was maintained at 40°C for 4 min and then increased to 180°C at 5°C/min to 230°C at 10°C/min, and 230°C was maintained for 3 min. The GC oven temperature on the DB-1MS column was maintained at 40°C for 4 min and then increased to 120°C at a rate of 5°C/min, followed with a programmed rate at 10°C/min to 160°C to 320°C at 40°C/min. The injector temperature was maintained at 250°C with a constant flow rate of 1.0 mL/min of helium. The inlet was operated in splitless injection mode. The GC–MS electron impact source was operated in scan mode with the MS source temperature at 240°C and MS Quad at 150°C. Volatile compounds were identified by comparing their retention times with the synthetic standards on the same column. The referenced mass spectra were from the NIST11 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA).

Mixed samples consisting of standard compounds listed in Table 1 in different dosages (1, 5, 10, 50 and 100 ng/μL) were used as external standards to develop the standard curves to quantify the volatiles. The DB-WAX column and same thermal program were adopted. No quantitative and qualitative differences were detected in the volatile blends between the male and female nymphs of the fourth-instar gregarious locusts (for detailed comparison see Fig. S1). The samples were pooled and analyzed by GC–MS as body volatiles (Table 1).

A Bruker GC system (456-GC) coupled with a triple quadrupole (TQ) mass spectrometer (Scion TQ MS/MS, Bruker Daltonics, Bremen, Germany.) equipped with an DB-1MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent Technologies) was used to quantify the volatile compounds in the SPME samples. The same thermal program was adopted as described above for chemical analysis. The GC–MS/MS electron impact source was operated in multiple reaction monitoring (MRM) mode with the MS source temperature at 200°C, mainfold temperature at 40°C, transfer line temperature at 250°C, and collision-induced dissociation on argon as collision cell gas with pressure 2.0 Torr. The injector temperature was maintained at 250°C with a constant flow rate of 1.0 mL/min of helium. The fiber was injected into the inlet operated in splitless injection mode and held for 1 min. The two quantitative ions (precursor and product ions) of each compound and the corresponding collision energy are listed in Table 2. A Bruker chemical analysis MS workstation (MS Data Review, Data Process, version 8.0) was used to analyze and process the data. Mixed samples consisting of standard compounds listed in Table 2 in different dosages (1, 5, 10, 50 and 100 ng/μL) were used as external standards to develop the standard curves to quantify the volatiles. The same thermal program and MRM method were used.

Data analysis

SPSS 20.0 (IBM Inc., Chicago, IL, USA) statistical analysis software was used to process all data. The Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) and PCA of SIMCA 13.0 (Umetrics AB, Umeå, Sweden) were used to separate the volatile profiles of gregarious and solitary locusts at each developmental stage and during phase changes. Moreover, these techniques were performed to reveal the volatile compounds that are important for separating the volatile blends emitted by two phases of locusts. Furthermore, the variable importance in the projection (VIP) was calculated. Variables with VIP $>$ 1 are the most influential for the model (Wei *et al.*, 2014). The levels of volatiles released from locusts were normalized to nanogram per hour per 10 locusts in absorbent trapping and to nanogram per half-hour per locust in SPME studies. Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test were used to compare volatiles from the two sexes of immature and mature adults in gregarious or solitary locusts.

Table 1 Absolute amounts of locust-associated compounds (mean ± SE), collected from the headspace volatiles of fourth-instar gregarious locusts (ng/10 locusts/h) and their feces (ng/1 g fresh weight [FW]/h).

Chemical compound	Body volatiles[§] ng/10 locusts/h	Fecal volatiles[§] ng/1 g FW/h
Aromatic compounds		
1 Phenylacetonitrile[†]	26.3 ± 1.8	1.81 ± 0.7
2 Benzaldehyde[†]	4.28 ± 1.0	5.89 ± 1.5
3 Phenol [†]	3.62 ± 0.5	11.61 ± 2.4
4 Guaiacol[†]	1.33 ± 0.3	4.47 ± 0.8
5 Veratrole[†]	0.87 ± 0.1	0.44 ± 0.2
6 Benzeneacetaldehyde[†]	0.56 ± 0.2	0.85 ± 0.1
7 4-Vinylanisole[†]	0.52 ± 0.1	0.58 ± 0.1
8 Benzoic acid[†]	0.5 ± 0.2	1.34 ± 0.5
9 Anisole[†]	0.42 ± 0.3	0.12 ± 0.1
10 Phenethyl alcohol[†]	0.15 ± 0.1	2.5 ± 0.7
11 Indole[†]	ND[¶]	1.75 ± 0.4
Aliphatic compounds		
12 Acetic acid[†]	51.4 ± 14.3	26.6 ± 5.3
13 Hexanoic acid[†]	1.27 ± 0.1	8.30 ± 1.4
14 3-Methyl, butanoic acid[‡]	0.66 ± 0.2	6.1 ± 1.3
15 Decanoic acid[†]	0.42 ± 0.1	0.16 ± 0.1
16 Pentanoic acid[†]	0.34 ± 0.1	1.66 ± 1.1
17 Butanoic acid[†]	0.31 ± 0.1	3.34 ± 1.1
18 Nonanoic acid[†]	0.26 ± 0.1	0.24 ± 0.1
19 Octanoic acid[†]	0.14 ± 0.1	0.24 ± 0.1
20 (*E*)-3-Hexenoic acid[‡]	0.09 ± 0.1	0.91 ± 0.4
21 Propanoic acid[†]	ND	1.68 ± 0.2
22 3-Hydroxy, 2-butanone[‡]	22.7 ± 2.9	5.0 ± 0.8
23 2,3-Butanediol[†]	13.11 ± 2.7	42.1 ± 8.1
24 [R-(R*,R*)], 2,3-Butanediol[†]	1.86 ± 0.3	15.1 ± 3.2
25 2,5-Dimethyl-pyrazine[†]	0.83 ± 0.1	7.75 ± 1.5
26 1-Pentanol[†]	0.63 ± 0.2	7.54 ± 1.6
27 (Z)-2-Penten-1-ol[†]	0.22 ± 0.1	8.93 ± 1.4
28 Cyclohexanol[†]	ND	5.46 ± 0.8
29 β-Ionene[†]	ND	5.40 ± 1.5
30 β-Ionone epoxide[‡]	ND	3.11 ± 0.6
31 1-Methoxy-butane[‡]	ND	2.99 ± 0.9
32 2,6-Dimethyl-pyrazine[‡]	ND	2.58 ± 0.3
33 (*E*)-2-Hexenal[†]	ND	2.43 ± 0.3
34 Methyl-pyrazine[‡]	ND	1.44 ± 0.5
35 (*Z*)-3-Hexen-1-ol[†]	ND	0.86 ± 0.2

[†]Compounds were identified by comparison of their retention time and mass spectrometry (MS)-spectra with those of authentic compounds.

[‡]Compounds were tentatively identified by comparison of their MS-spectra with those of in the NIST11.

[§]Given that no quantitative and qualitative differences were detected in volatile blends between male and female nymphs of the fourth-instar gregarious locusts (G4) (for detailed comparison see supporting Fig. S1), the samples were pooled and analyzed by gas chromatograph (GC)-MS as G4 odors ($n = 6$). Fecal odors of G4 were repeated five times ($n = 5$). Quantification of each compound was done by comparing the peak area relative to standard curve developed on a DB-WAX column (30 m × 0.25 mm × 0.15 μm, Agilent Technologies, Palo Alto, CA, USA).

[¶]ND: not detectable (absent or peak too small to determine composition).

Table 2 Relative amounts of locust-released volatiles (mean ± SE) collected by static solid phase microextraction (SPME) method from the headspace of fourth-instar gregarious and solitary locusts.

Compounds[†]	% in G4[‡]	% in S4[‡]	Multiple reaction monitoring precursor and product ions and their collision energies (CE)[§]			
			Quantifier 1	CE1 (V)	Quantifier 2	CE2 (V)
Benzaldehyde	34.4 ± 1.2	41.8 ± 4.8	106→78	15	106→51	30
Phenylacetonitrile	33.1 ± 2.4	ND[¶]	117→90	15	90→63	25
Phenol	17.1 ± 2.2	36.8 ± 3.6	94→65	15	94→51	30
Guaiacol	6.5 ± 0.8	18.8 ± 2.0	124→109	15	124→81	20
Benzeneacetaldehyde	2.8 ± 0.7	0.3 ± 0.07	120→91	15	91→65	15
4-Vinylanisole	1.8 ± 0.4	ND	134→119	15	134→91	20
Phenethyl alcohol	1.3 ± 0.5	0.20 ± 0.1	122→91	15	91→65	15
Veratrole	0.7 ± 0.5	0.42 ± 0.1	138→123	10	138→77	15
Anisole	0.4 ± 0.2	0.3 ± 0.2	108→78	10	108→65	20
Hexanoic acid	0.8 ± 0.1	0.83 ± 0.2	73→55	10	60→42	10
2,5-Dimethyl-pyrazine	0.6 ± 0.2	0.23 ± 0.1	108→81	10	108→42	10
Pentanoic acid	0.4 ± 0.1	0.23 ± 0.1	73→55	10	60→42	5

[†]Compounds were identified by comparison of their retention time and dual mass spectrometry (MS/MS)-spectra with those of authentic compounds in the NIST11.

[‡]Volatiles of the fourth-instar gregarious or solitary locusts (G4 and S4) were collected from a glass jar containing five locusts within 30 min by a static SPME method. Totally, each phase of five individuals was repeated for six times (G4 $n = 6$ and S4 $n = 6$).

[§]Precursor and product ions used for quantification of volatile compounds. Quantification of each compound was done by comparing the peak area relative to standard curve developed on a DB-1MS column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness, Agilent Technologies, Palo Alto, CA, USA).

[¶]ND: not detectable (absent or peak too small to determine composition).

Student's *t*-test was used to compare absolute emissions of key volatiles between gregarious and isolated gregarious (IG) nymphs or solitary and crowded solitary (CS) nymphs. Absolute quantities were $\log(x + 1)$-transformed to correct for the heterogeneity of variances before data analysis.

Results

Volatiles from locusts

The headspace collection system was employed to collect the volatiles from the locusts and their feces. Twenty-five volatile compounds were identified in the fourth-instar gregarious individuals. The volatile compounds from the fecal pellets of fourth-instar nymphs included the 25 volatiles mentioned above and another 10 chemicals (Table 1). Among the 25 volatiles from gregarious individuals, the aromatic compounds (PAN, benzaldehyde, guaiacol and phenol) and aliphatic acids (C2 and C6), 3-hydroxy 2-butanone, and 2,3-butanediol dominated the volatiles of gregarious fourth-instar individuals, accounting for 70% of total volatile emissions (Table 1). In addition to the 25 nymphal volatiles, the following fecal volatiles were included: indole, propanoic acid, methyl-pyrazine, 2,6-dimethyl-pyrazine, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, cyclohexanol, β-ionene, β-ionone epoxide and 1-methoxy-butane (Table 1). Nymphal body volatiles were quantitatively characterized by a considerable amount of PAN, whereas fecal volatiles were represented by a large amount of guaiacol and phenol.

We identified the volatiles of fourth-instar gregarious and solitary locusts by SPME coupled with GC–MS/MS system and MRM analysis to investigate the difference in the compositions of volatile compounds between solitary and gregarious locusts. Contrary to the absorbent trapping method, which enriched over 20 compounds (Table 1), the SPME method during a 30 min collection frequently showed 12 volatile compounds from gregarious and solitary locusts (Table 2). PAN and benzaldehyde are the most dominant components, with which guaiacol and phenol

constituted more than 90% of the whole blend in gregarious locusts. By contrast, no PAN and 4-vinylanisole were detected in the volatiles of solitary locusts, but relatively high percentages of phenol, guaiacol and benzaldehyde were observed (Table 2).

Emission dynamics of locust volatiles across developmental stages

MRM and orthogonal projection to latent structures–discriminant analysis (OPLS-DA) were conducted to identify the emission dynamics of volatiles emitted from solitary and gregarious locusts at different developmental stages and sexes. The volatile profiles of gregarious and solitary locusts significantly differed and were categorized into two separate clusters, thus illustrating the increasing divergence from different young hoppers to adults (Fig. 1). OPLS-DA of nymphal stages showed a model with two significant principal components (OPLS-DA C1: phase and OPLS-DA C2: developmental stage; model statistics: R2X = 0.804, R2Y = 0.55 and Q2 = 0.55). The first two principal components clearly separated the data points for the two phases and developmental stages and contributed 55% of total variance (Fig. 1A). The importance of the variables (compounds) in the two clusters was determined based on the VIP. Compounds with a VIP > 1 remarkably contribute to the separation of groups. Only PAN had a VIP > 1 (VIP = 2.38). Therefore, PAN was the most important compound among the phase-related chemicals. Similarly, the OPLS-DA of adult stages of two sexes showed a model with two significant principal components (OPLS-DA C1: phase and OPLS-DA C2: sex; model statistics: R2X = 0.89, R2Y = 0.74 and Q2 = 0.64). The first two principal components contributed 64% of the total variance and clearly separated the data points for the two phases and sexes (Fig. 1B). PAN with VIP = 2.48 was the most important compound to separate adult solitary and gregarious locusts.

The chemical analyses of volatiles of adult locusts showed significant differences in the emissions of PAN and 4-vinylanisole between two sexes or immature and mature adults of gregarious locusts, thus indicating the highest releasing rate in mature adult males (ANOVA: PAN, $F_{3,23} = 23.38$, $P < 0.0001$; 4-vinylanisole, $F_{3,23} = 3.49$, $P = 0.035$; Fig. 2A). However, adult gregarious locusts emitted similar levels of guaiacol, phenol and benzaldehyde (ANOVA: guaiacol, $F_{3,23} = 0.246$, $P = 0.863$; phenol, $F_{3,23} = 0.710$, $P = 0.557$; benzaldehyde, $F_{3,23} = 0.165$, $P = 0.919$; Fig. 2A). By contrast, PAN and 4-vinylanisole were undetectable during the adult stages of the two sexes of solitary locusts. Guaiacol, phenol and benzaldehyde were released at identical rates among the two sexes and different adulthoods of adult solitary locusts (ANOVA: guaiacol, $F_{3,23} = 0.876$, $P = 0.166$; phenol, $F_{3,23} = 0.328$, $P = 0.805$; benzaldehyde, $F_{3,23} = 0.582$, $P = 0.634$; Fig. 2B).

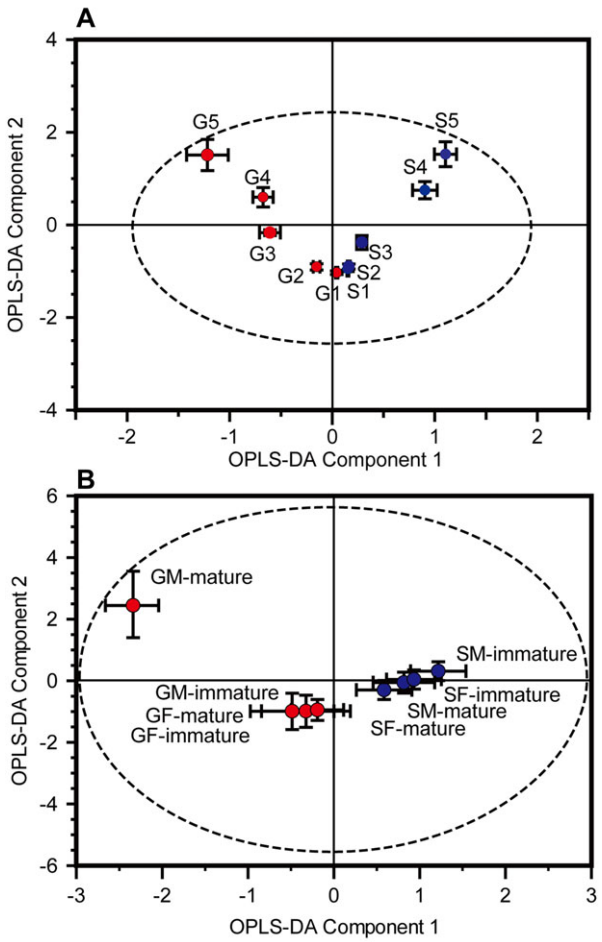

Fig. 1 Emission patterns in the headspace volatiles from the different developmental stages between gregarious and solitary locusts. (A) Two-dimensional orthogonal projection to latent structures discriminant analysis (2D OPLS-DA) score plot separating gregarious (G: red circles) and solitary (S: blue circles) samples collected from (A) first-instar (G1 and S1) to fifth-instar (G5 and S5) stages and from (B) immature adult females (5-day-old GF-immature, gregarious immature female; SF-immature, solitary immature female), mature adult females (15-day-old GF-mature and SF-mature), immature adult males (5-day-old GM-immature and SM-immature), and mature adult males (15-day-old GM-immature and SM-immature) stages of gregarious and solitary locusts. Each dot represents an averaged volatile profile of six biological replicates, and the bar lines indicate the standard error (SE) of OPLS-DA components 1 and 2. The ellipse defines the Hotelling's T2 confidence region (95%).

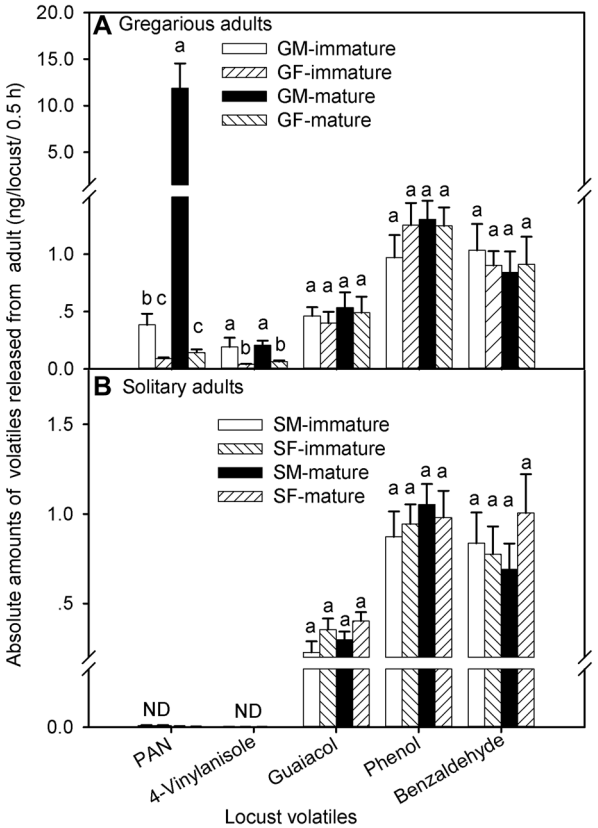

Fig. 2 Key aromatic volatile emissions from immature and mature adult locusts. Comparison of five major aromatic volatiles among two sexes of immature and mature adult (A) gregarious and (B) solitary locusts. Bars for the same compound labeled with different letters show statistically significant differences in average amounts for different treatments (analysis of variance and Tukey's Honestly Significant Difference test, $P < 0.05$). Each bar represents an averaged volatile profile of six biological replicates ($n = 6$). ND, not detectable (absent or peak too small to determine composition). Mean volatile amounts are presented with the SE; PAN, phenylacetonitrile.

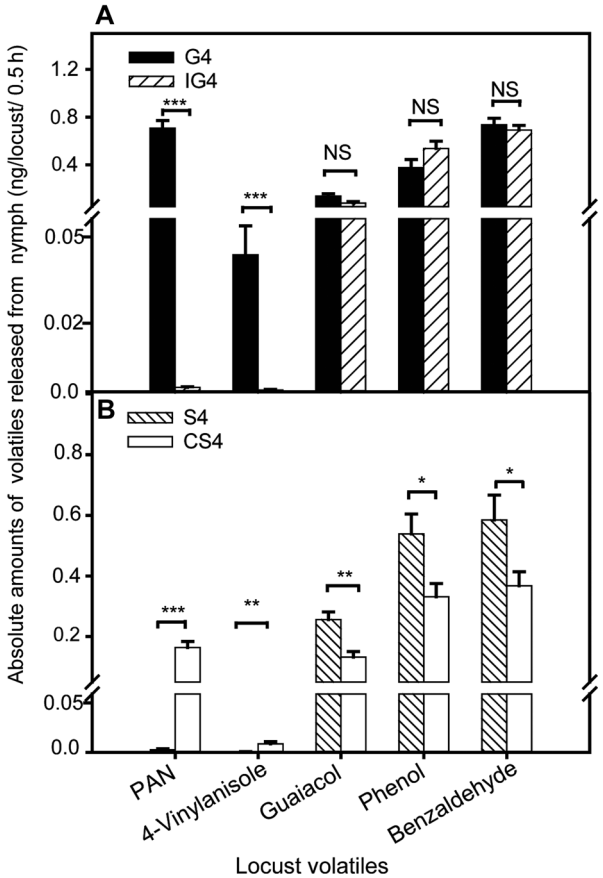

Fig. 3 Key aromatic volatile changes during solitarization and gregarization processes. Comparison of volatiles between (A) fourth-instar nymphs of gregarious (G4) and isolated gregarious (IG4) locusts and (B) fourth-instar solitary (S4) and crowded solitary (CS4) locusts. Bars for the same compound labeled with asterisks show statistically significant differences in average amounts for different treatments (two-tailed Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Each bar represents an averaged volatile profile of six biological replicates ($n = 6$). NS, not significant. The mean volatile amounts are presented with SE; PAN, phenylacetonitrile.

Therefore, PAN is the most significant compound to display the differences between solitary and gregarious locusts in nymphal stages and adulthoods.

Volatile dynamics in response to population density changes

The profiles of volatile compounds were analyzed on the basis of the time course experiments for IG locusts and CS locusts. Significant differences in the emissions of five key aromatic compounds were found during the gregarization (CS) and solitarization (IG) processes (Fig. 3). The productions of PAN and 4-vinylanisole were completely switched off in IG locusts compared with typical gregarious locusts (two-tailed *t*-test, PAN: $t = 16.99$, df $= 10$, $P < 0.0001$; 4-vinylanisole: $t = 12.14$, df $= 10$, $P < 0.0001$), whereas phenol, guaiacol or benzaldehyde were maintained in relatively steady amounts in the IG locusts and their gregarious controls (Fig. 3A). Conversely, CS treatment significantly promoted the amounts of PAN and 4-vinylanisole (two-tailed *t*-test, PAN: $t = 12.29$, df $= 10$, $P < 0.0001$; 4-vinylanisole: $t = 4.15$, df $= 10$,

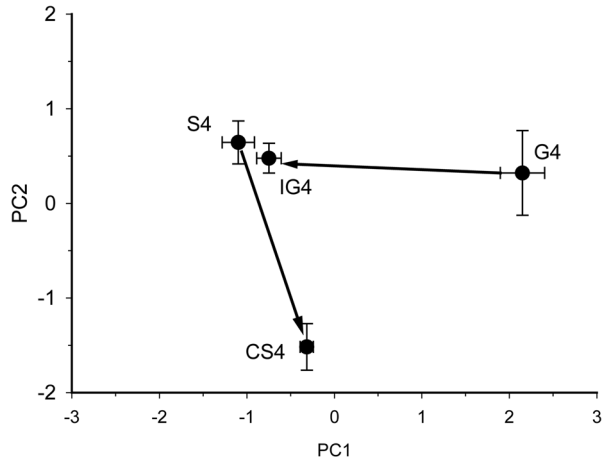

Fig. 4 Two-dimensional principal component analysis (PCA) score plot of volatile profiles in response to fourth instar isolated gregarious (IG4) and crowded solitary (CS4) treatments. Arrow lines indicate the changes in the emission patterns in fourth-instar nymphs of the treatment groups (IG and CS) and their corresponding gregarious (G4) and solitary (S4) controls after gregarization and solitarization from third-instar to fourth-instar stadia. Each dot represents an averaged volatile profile of six biological replicates, and the bar lines indicate the SE of PC1 and PC2.

$P = 0.002$), but reduced the amounts of guaiacol, phenol or benzaldehyde to approximately two-thirds compared with those of the solitary locust controls (guaiacol: $t = -3.99$, df $= 10$, $P = 0.003$; phenol: $t = -2.62$, df $= 10$, $P = 0.026$; benzaldehyde: $t = -2.316$, df $= 10$, $P = 0.043$; Fig. 3B). Similarly, the 2D PCA score trajectories of IG and CS processes clearly showed that the entire volatile profiles of the IG and CS groups changed extensively compared with their corresponding controls (Fig. 4). IG treatment was sufficient to change the volatile profile from typical gregarious to solitary (PCA model of IG and S: Q2 = 0.129, no significance), whereas CS resulted in different volatile profiles compared with that of solitary (PCA model of CS and S: Q2 = 0.526, significance R1) or gregarious phase (PCA model of CS and G: Q2 = 0.624, significance R1). PAN was the most important compound among volatiles with notable transformation from gregarious to IG locusts (OPLS-DA model with a VIP = 2.384). PAN, benzaldehyde, guaiacol and phenol were equally significant in the phase transformation from solitary to CS locusts (OPLS-DA model: VIP values, PAN = 1.37, benzaldehyde = 1.41, phenol = 1.46 and guaiacol = 1.05). Therefore, PAN and 4-vinylanisole were the most sensitive volatiles in both directions of locust phase change.

Discussion

The desert locust and migratory locust in approximately 10 locust species have been paid much attention because of their economic importance in agriculture worldwide (Pener & Simpson, 2009). The molecular regulatory mechanisms of the two locust species during the phase change obviously differed, although these species display similar behavioral phenotypes in response to variation in population density (Wang & Kang, 2014). Results from the present study show that the volatile composition and dynamic emission of migratory locust qualitatively and quantitatively differed from those of the desert locust. The migratory locusts predominantly not only produced aromatic compounds (PAN, guaiacol, benzaldehyde and phenol), aliphatic acids (C2 and C6), 3-hydroxy 2-butanone and 2,3-butanediol, but also released some minor components, such as anisole, veratrole, phenethyl alcohol, 4-vinylanisole, benzeneacetaldehyde, aliphatic acids (C3–C10), aliphatic alcohols [1-pentanol and (*Z*)-2-penten-1-ol], and 2,5-dimethyl-pyrazine. However, the gregarious nymphs of desert locust mainly released aliphatic acids and aldehydes (C6 and C8–C10), guaiacol and phenol (Torto *et al.*, 1996). PAN was evidently undetectable in the nymphal stages of desert locust (Njagi *et al.*, 1996; Torto *et al.*, 1996; Niassy *et al.*, 1999). PAN was a dominant component in the nymphal and adult stages of the migratory locust. Moreover, benzaldehyde and phenol were major volatiles in the migratory locust, but in trace amounts in the desert locust. Previous studies have shown that the fecal volatiles of both species mainly consisted of guaiacol, phenol and indole, but far less abundant than body volatiles (Fuzeau-Braesch *et al.*, 1988; Niassy *et al.*, 1999). However, our present study showed that volatile composition of fecal pellets involved almost nymphal volatiles and was more diverse than that of nymphs in the migratory locust. The gregarious adult of the migratory locust generally produced seven aromatic compounds (PAN, guaiacol, 4-vinylanisole, veratrole, benzaldehyde, benzeneacetaldehyde and phenol) and an aliphatic acid (hexanoic acid). By contrast, an adult desert locust mainly released six aromatic compounds (PAN, benzaldehyde, veratrole, anisole, guaiacol and phenol) (Torto *et al.*, 1994; Njagi *et al.*, 1996; Niassy *et al.*, 1999). Specifically, the volatile profiles of immature males and females of whole adulthood in the migratory locust were very similar, consisting of moderate amounts of guaiacol, benzaldehyde and phenol as well as trace amounts of 4-vinylanisole, veratrole, benzeneacetaldehyde and PAN. However, the volatiles of the corresponding stages of the desert locust only included trace amounts of benzaldehyde, veratrole, anisole, guaiacol and phenol (Torto *et al.*, 1994).

Interestingly, mature male adults of both species similarly released the highest amounts of PAN and small amounts of benzaldehyde, guaiacol and phenol. Previous studies indicated that the migratory locust showed limited capability in volatile emissions in terms of quantity and quality compared with the desert locust (Fuzeau-Braesch *et al.*, 1988; Niassy *et al.*, 1999). Our study clearly showed that volatile compositions and their relative amounts of migratory locust in adulthood were identical to those of desert locust. However, existing data demonstrated that the migratory locust during developmental stages and sexes significantly differed in the volatile composition and emission patterns from the desert locust.

The two locust species have common volatiles, especially the aromatic compounds (e.g., PAN, benzaldehyde, veratrole, guaiacol and phenol) and aliphatic acids (e.g., from C5 to C10). This characteristic may suggest that the volatile compound produced by two locust species involves the same biosynthetic pathway *in vivo* and plays similar roles in chemical communication between conspecific individuals. Although phenylalanine has been proven to be a precursor for the biosynthesis of PAN in the desert locust (Seidelmann *et al.*, 2003), this biosynthetic pathway is conserved from microbe to plants and to animals (Asano & Kato, 1998; Irmisch *et al.*, 2014; Vavricka *et al.*, 2014). This compound has been reported as a mature male-specific component in the desert locust, serving as an aggregation pheromone of adults, but not nymphs (Torto *et al.*, 1994; Hassanali *et al.*, 2005; Rono *et al.*, 2008), as a courtship inhibition pheromone in male–male competition and homosexual attempt (Seidelmann *et al.*, 2005), or as a maturation-accelerating pheromone (Mahamat *et al.*, 2011). The different opinions toward the behavioral role of PAN have led to the so-called "PAN paradox" and these debates still need further clarification (Pener & Simpson, 2009). However, PAN was a dominant volatile in both the gregarious nymphs and mature males of migratory locust. Notably, the roles of PAN during the nymphal stage and adulthood of the migratory locust needs further exploration. Therefore, the ultimate resolution of volatile similarities or differences between the two locust species should be determined when they are measured and compared in the same laboratory.

Further chemical analysis showed that nymphs and adults of the migratory locust released different volatiles between solitary and gregarious individuals with a remarkable trend of a major component (PAN) and a minor component (4-vinylanisole) in gregarious locusts and inhibition in solitary counterparts. Moreover, changes in the concentrations of the two volatiles showed extremely sensitive response to changes of population densities, because these volatiles were lost completely during solitarization and were gained to a certain level during gregarization. The emission patterns of PAN and 4-vinylanisole during phase change were consistent with the rate of behavioral change in our previous reports, in which the migratory locust displayed quick solitarization and slow gregarization in behavioral phase change (Guo *et al.*, 2011; Ma *et al.*, 2011). Meanwhile, we also reported that solitary locusts avoided the gregarious locust-emitted volatiles, while they become attracted to the gregarious volatiles during crowding and *vice versa* for gregarious locusts to volatiles of each other during isolation (Ma *et al.*, 2015). We speculate that PAN would have more complex roles in the migratory locust combined with the dual roles of PAN in the desert locust.

Phase-related volatile studies on the desert locust exclusively focused on adult males and showed quantitative and qualitative differences in male adult-produced aromatic compounds between two phase individuals (Njagi *et al.*, 1996). The study further showed that aromatic compounds (PAN, benzaldehyde, veratrole, anisole, guaiacol and phenol) tested singly or in combination had the same behavioral effects on the preferences of two phases and sexes. For example, PAN alone or a synthetic blend consisting of four aromatic compounds (PAN, benzaldehyde, guaiacol and phenol) showed the highest responses of gregarious and solitary adult locusts (Njagi *et al.*, 1996). The emissions of PAN by crowded- and solitary-reared adult males shifting from different developmental stages indicated that PAN was a more sensitive measure than morphometrics to associate with rearing condition and time course of phase change in the desert locust (Deng *et al.*, 1996). However, the behavioral responses of solitary-reared nymphs to nymphal volatiles of gregarious locusts in the desert locust were rarely studied, and emission patterns of volatile profiles during phase change at nymphal stages were also largely unknown (Pener & Simpson, 2009). Therefore, we can conclude that PAN is a gregarious-phase specific component in the two locust species, and no solitary-phase specific volatiles have been reported yet.

Surprisingly, we also found that PAN was a major component of volatiles in each nymphal stage (10%–40% of whole blends) and feces of gregarious nymphs. Incomplete records of PAN in these samples in previous studies may have been caused by nonpolar columns for GC–MS analysis (a CpSil 5 CB Chrompack column in Fuzeau-Braesch *et al.* [1988] and a HP-5 column in Shi *et al.* [2011]), because PAN is likely to co-elute with containment peaks. The samples were particularly collected at a special time before or after molting (Seidelmann *et al.*, 2003). This speculation was supported by our study and that of Niassy *et al.* (1999), in which the polar DB-wax and carbowax 20 M columns were used to successfully

separate PAN with other compounds in volatile emissions from migratory locust nymphs. However, Niassy *et al.* (1999) failed to detect PAN in volatiles from mature adult migratory locust.

The other two major aromatic compounds, namely, guaiacol and phenol, emitted from the desert locust fecal pellets were verified to have been produced by gut bacteria but not locusts themselves (Dillon *et al.*, 2000, 2002). These compounds have been reported as important components of aggregation or cohesion pheromones in both locust species (Fuzeau-Braesch *et al.*, 1988; Torto *et al.*, 1994, 1996; Niassy *et al.*, 1999; Shi *et al.*, 2011). Furthermore, although the origins of veratrole and benzaldehyde in locusts are still being investigated, the roles of these compounds in behavioral aggregation of adult desert locust have been proven to be less important than those of PAN or guaiacol (Torto *et al.*, 1994). Aliphatic acids, from pentanoic acid to decanoic acid, present during the nymphal stages of the desert locust, were assumed to have been degraded from fatty acids in plants and animals, but the exact biosynthetic pathway in desert locusts was unclear (Seidelmann *et al.*, 2003). Nevertheless, a blend consisting of C6–C10 acids elicited strong aggregation response from gregarious nymphs of the desert locust, and each of these locusts alone showed moderate activity (Torto *et al.*, 1996). Interestingly, pentanoic acid was extracted from the Comstock-Kellog gland of sexually mature female desert locust, and this acid was suggested as a sex pheromone to evoke sexual behavior of mature males (Njagi & Torto, 2002). Therefore, the origins and behavioral roles of these aromatic and aliphatic compounds in the migratory locust should be fully explored in the future to better understand the evolution of pheromone systems and the application for pest management of swarming locusts.

Volatile extracts are also likely to be contaminated by environmental chemicals because of a relatively longer collection duration, although the absorbent trapping method is advantageous to enrich a large number of insect-associated headspace volatiles. Previous studies reported aliphatic aldehydes (C6–C10) as pheromones in the desert and migratory locusts (Torto *et al.*, 1996; Shi *et al.*, 2011). However, we confirmed that the four aliphatic aldehydes (C7–C10) were environmental contaminants, because their amounts in the control empty jars were not significantly different from the amounts collected from insects and their feces (Fig. S2). These aldehydes were also reported as environmental contaminants in other studies (Svensson *et al.*, 2010; Knauer & Schiestl, 2015). Furthermore, individual aldehydes were reported to have no significant behavioral activity in the desert locust (Toulemonde & Richard, 1983; Torto *et al.*, 1996). Compared with other modules of GC–MS, MRM analysis can detect as low as 1 pg for a compound that facilitates detectability and quantification of some minor components in the blends. Therefore, we accurately detected and quantified the compounds in the headspace of the locust body and eliminated the contaminants in the samples from the environments using the powerful tool of GC–MS/MS MRM analysis on samples collected in short duration (30 min).

Our study on the locust-emitted volatiles is the first report of 4-vinylanisole as a marker component for gregarious-phase locusts. This compound was only reported as a volatile of dry elder (*Sambucus nigra* L.) flowers (Toulemonde & Richard, 1983) or essential oil of *Ephedra sinica* plant (Tellez *et al.*, 2004). The biological activity of this compound to other organisms has not been investigated.

In summary, our present study contributes important information for exploring the roles of individual components in behavioral phase changes, although the roles of PAN and 4-vinylanisole need further investigation. In this study, we clearly showed the difference in the volatiles between the desert locust and migratory locust, thus indicating the diverse biological traits and behavioral mechanism in the two locust species. Moreover, this study provides important chemical cues to understand the molecular and neural mechanisms of olfactory coding in the migratory locust. The achievements in locust genome (Wang *et al.*, 2014), transcriptomes (Wang & Kang, 2014) and RNAi techniques (Ma *et al.*, 2011; Guo *et al.*, 2011; Wang & Kang, 2014) provide powerful tools to determine the molecular mechanisms of locust pheromone productions. Moreover, their functions were established by manipulating the expression levels of related genes in the biosynthetic pathways (Li *et al.*, 2016; Song *et al.*, 2016). Related discoveries will be useful in developing environment-friendly behavioral regulators for locust management.

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Disclosure

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. The major volatile components in the headspace of gregarious males and females.

Figure S2. A comparison of the TICs (Total ion chromatograms) of volatile profiles of control empty jar (blue trace) with either fecal volatiles of gregarious 4th-instar nymphs (inverted black trace) (A), or body volatiles of gregarious 4th-instar nymphs (inverted black trace) (B).