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Faeces' odours attract gregarious locust hoppers

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Abstract

Collective motion is one of the most impressive common features of gregarious locusts : once formed, bands and swarms get moving for long distances. It was shown that visual of neighbours plays a key role in maintaining marching behaviour at a local scale. But at a larger scale, mechanisms underlying band cohesion are less understood. It was shown in several field studies that individuals separated from the band were able to get back to the group, even after being separated since a night. In this context, faeces' odours could be a possible indicator of the recent passage of a group. In this study, we tested if nymphs are attracted by faeces' odours and if this effect is modulated by the age of the faeces. To this end, we conducted individual olfactometric behavioural assays of 3rd instar hoppers of desert locust, *Schistocerca gregaria*, exposed to odours of 1h-old and 24h-old faeces. We also used Gas Chromatography-Mass Spectrometry (GC-MS) to identify odours' volatile organic compounds from faeces. The results of behavioural assays indicated a strong attractive effect of faeces, with no preference for one of the two faecal age classes. Nymphs spent significantly more time in the side of the olfactometer where the faeces' odours came from, and 72.7% of tested individuals chose this side first. We filtered and annotated 11 volatile organic compounds present in both fresh and old faeces in GC-MS

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analyses, including guaiacol and phenol, which are known to cause an aggregative effect on desert locusts. As the attractive effect lasted over 24h, band's faeces could still have an attractive effect when individuals are separated from the band since one day. In this situation, latecomers individuals would be able to get back to the group by following the traces of their predecessors.

1. Introduction

Locusts are grasshoppers able to swarm and showing phase polyphenism, an extreme form of phenotypic plasticity allowing them to shift from a solitarious to a gregarious phase according to population density (Pener & Simpson, 2009; Cullen et al., 2017). The transition from one phase to another implies many changes, including morphological, physiological, and, above all, behavioural changes (Pener & Simpson, 2009; Cullen et al., 2017). Whereas solitarious locusts remain alone and are relatively inactive, gregarious locusts aggregate in huge active groups of nymphs or adults, called bands or swarms respectively. Those groups can be very dense, with swarms sometimes covering several hundred square meters (Uvarov, 1977).

Collective motion is one of the most impressive common features of gregarious locusts : once formed, bands and swarms get moving for long distances. For example, the desert locust *Schistocerca gregaria* can travel up to several kilometers per day (Uvarov, 1977). These massive coordinated displacements are, since antiquity, a major threat to agriculture, causing destruction of crops and starvation in countries affected by locusts outbreaks (Zhang et al., 2019). Collective motion of gregarious locusts has been extensively studied (Ariel & Ayali, 2015). It has been reported that moving bands can take different forms such as banana-shaped and column-shaped (Dkhili et al., 2017). Banana-shaped bands are characteristic of most locust species (Ellis & Ashall, 1957; Lecoq et al., 1999) and display a high density at the front, which is generally wider than the

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length of the group. Column-shaped groups (with narrow fronts) are mostly described in non-locust grasshoppers showing a low aggregating behaviour (Dkhili

25 et al., 2017). It was reported that some marching bands follow the same global direction from day to day (Ellis & Ashall, 1957) and that bands follow daily behavioural cycles, with basking, marching, and roosting phases (Ellis & Ashall, 1957; Maeno et al., 2021b; Piou et al., 2022).

However, some gaps remain in our knowledge of the mechanisms underlying
30 band cohesion. At a local scale, it was shown that perception plays a key role in initiating and maintaining marching behaviour between individuals via visual and tactile stimuli. Buhl et al. (2006) showed that directed collective motion occurs when a critical density of locusts in the group is reached. They propose that the switch from disordered to ordered marching in locust nymphs result
35 from the increase in local interactions with density. Bazazi et al. (2008) observed that groups of blind individuals had significantly lower levels of marching than control individuals in ring-shaped arena. They also showed that abdomen denervation of individuals reduce marching behaviour in groups. Ariel et al. (2014) observed in their experiments that visual detection of variation in the number
40 of walking neighbours is an important factor to walking initiation events.

At a larger scale, however, little is known about the drivers of band cohesion. Knebel et al. (2021) showed that individuals that experience a separation from the rest of the group adopt a marching behaviour that will minimise the time to rejoin them. Field studies showed that these separated individuals were able to
45 get back to the band, even when unable to perceive it visually. Ellis & Ashall (1957) followed a *S. gregaria* band and reported that individuals at the rear, out of visual contacts with their conspecifics, did not lose their way and continued to march until they found the band. The same kind of behaviour was observed in Argentina in a *Schistocerca cancellata* band, even for latecomers after spending
50 a night in vegetation (Piou et al., 2022). These observations indicate that other kind of perceptions are implicated in collective motion at an intermediate scale that allows scattered nymphs to continue to follow the group.

Olfaction could be a plausible sense involved in the mechanisms leading to

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band cohesion. Indeed, some studies about attractiveness of locusts' odours provided interesting results. Obeng-Ofori et al. (1993) observed that gregarious *S. gregaria* nymphs and adults released in an olfactometer were attracted by odours of their conspecifics. Similar results were obtained by Despland in 2001 on gregarious 2nd instar nymphs of *S. gregaria*. In 1988, Fuzeau-Braesch et al. conducted chemical analysis of the volatile organic compounds (VOCs) present in the atmosphere surrounding gregarious *S. gregaria* and *Locusta migratoria* cages and realized behavioural assays on groups of 10 gregarious nymphs in a 3-choices cross-shaped olfactometer. They detected 2 VOCs emitted by both species, guaiacol and phenol. In the presence of those VOCs, the groups of tested insects released in the arena tended to gather significantly more. Recently, Guo et al. (2020) discovered that gregarious *L. migratoria* emitted 4-vinylanisole, a VOC responsible for the attractive effect. They also conducted a field test with sticky traps releasing 4-vinylanisole and successfully cought more locusts on it than on control traps.

However, in some cases, locusts odours diffusion could be limited. Some parameters like distance from the front to the rear of the marching band or meteorologic conditions could impact odours detection. In cases where the band may be out of scent range of latecomers, faeces, which are dropped regularly along the way, may constitute efficient indicators of the path taken by the band. Indeed, some studies indicate aggregative effect of faeces odours on groups of gregarious nymphs and show that guaiacol and phenol are present in locust faeces of *S. gregaria* (Obeng-Ofori et al., 1994; Torto et al., 1996). 4-vinylanisole is also detected in faeces of *L. migratoria* (Guo et al., 2020).

To our knowledge, no study so far tested faeces' odours attractiveness by individual behavioural assays, nor took into account the age of faeces in experiments to explore if there are behavioural differences. Previous studies explored the effect of faeces' odours on the aggregative behaviour of grouped individuals (Obeng-Ofori et al., 1994; Torto et al., 1994, 1996). Other studies analysed the attraction of individuals towards groups of individuals (Fuzeau-Braesch et al., 1988; Obeng-Ofori et al., 1993; Roessingh et al., 1993; Despland, 2001). How-

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ever, previous experimental designs did not allow to differentiate the faeces'
odours from that of the insects themselves. Since only individual behavioural as-
says can test faeces attraction without confounding with aggregative effects, the
present study investigates faeces' odours attraction by individual behavioural
assays, while taking into account the age of faeces. We wanted to address the
following questions : (1) Do faeces odours attract 3rd instar nymphs ? (2) Is
there behavioural differences according to the age of faeces (1h or 24h-old), with
a preference for fresh faeces (1h-old) ? (3) Which volatile organic compounds
are present in faeces of different ages (1h, 4h and 24h-old) ? To answer those
questions, we conducted behavioural assays on 3rd instar gregarious hoppers
of *S. gregaria*, individually exposed to faeces odours according to their state
of degradation (1h or 24h). We considered 24h degradation as old faeces and
expected that the released volatile organic compounds would be reduced by this
time. We also tested whether locusts exhibited a preference between those two
types of faeces. Finally, we conducted Gas Chromatography - Mass Spectrom-
etry (GC-MS) analysis to detect and annotate VOCs emitted by 1h-old, 4h-old
and 24h-old faeces.

2. Material and methods

2.1. Insects

The insects used in this study came from a *S. gregaria* strain reared under
crowded conditions for 20 generations in the CBGP laboratory (Montferrier-sur-
Lez, France) and inherited from a strain maintained for more than 30 genera-
tions in the Research group of Molecular Developmental Physiology and Signal
Transduction (Leuven, Belgium). Gregarious locusts were reared in cages of
dimensions 40 x 40 x 50 cm at densities around 150 - 250 insects per cage. They
were fed daily with fresh wheat seedlings and bran. They were maintained at a
temperature around 32 ± 1 °C, with relative humidity of 40 - 50 % and a 12L
: 12D photoperiod.

2.2. Faeces collection

Insects used for faeces production were moved in smaller cages (20 x 20 x 35 cm), easier to use for manipulations, and placed in the same rearing room. Faeces were collected hourly from both sexes of 3rd instar locusts kept at densities of 150 insects per cage. To prevent presence of cuticular molecules on our faeces samples, a small grid (20 x 20 cm) was placed at the bottom third of every cage so that faeces fall through and thus prevent the insects from coming into contact with their faeces. We let nymphs produce faeces in cages for 1h, and then collected them. Faeces were either used directly after collection (aged no more than one hour old, hereafter referred as to 1h-old faeces) or stored in the rearing room until they reach the desired age. We used 1h-old or 24h-old faeces for behavioural assays and chemical analysis. In addition, we also used 4h-old faeces for chemical analysis in order to have a better kinetic of degradation of volatile organic compounds.

Faeces samples for chemical analysis were prepared using a piece of nalophan (20 x 20 cm), as this material does not release residual compounds (Miller & McGinley, 2008). Nalophan piece was placed at the bottom of cages, under the grid. After 1 hour collecting faeces, samples were folded as small bags closed with elastic to allow headspace sampling. Blank samples were made with empty nalophan bags of the same dimensions, closed and stored in the laboratory room to have the same conditions as faeces samples. These blank samples were used to monitor the odours coming from the room and thus control the presence of possible pollution in our chemical analysis.

Faeces samples for behavioural analyses were stored in the rearing room in little plastic boxes ($\varnothing$ 5 cm, height 3 cm) that were either closed or open. Closing boxes slow down evaporation process of volatile organic compounds but makes comparison easier with compounds detected in GC-MS.

2.3. Olfactometer

The olfactometer was a two-part transparent plexiglas device, made up of a tube with a removable central part used to introduce insects in the arena

(Figure 1b). As faeces were invisible, stored below the opaque device, nothing but odours could point nymphs in the faeces direction. Dimensions have been thought to allow individuals to easily change their direction inside the arena (a 3rd instar body length is around 20 mm). Airflow was monitored by two Laboport N811 KNF pumps at a flow rate of 11.5 L.min^{-1}. Incoming air was purified by passing through charcoal filter and was split into two tubes that connect the sources of odours to each end of the device. Outgoing flow occurred in the central part so that tested insects received odours from both sides equally (Figure 1a).

(a) Setup of the device.

(b) Blueprints of the dual-choices arena. Odours came from each side of the device.
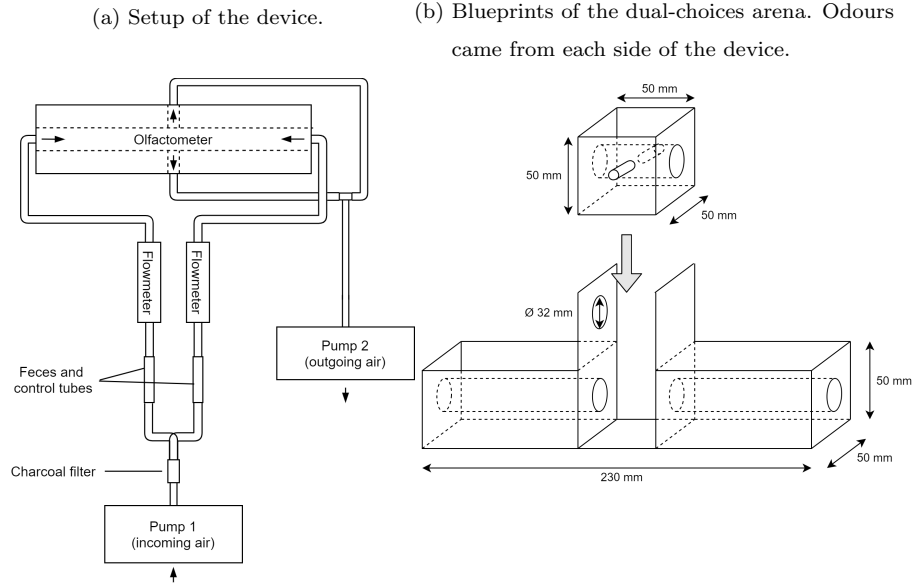
Figure 1: Olfactometer used for behavioural assays.

2.4. Behavioural assays

As nymphs are less active just after and just before a moult (Uvarov, 1977), we selected third-instar nymphs between 24h to 72h after their moult and placed them in smaller cages, as described in section 2.1. Food was removed from the cages at least one hour before the tests started to prevent lack of activity due to post-prandial period (Ellis, 1951). Prior to the assays, individuals were sexed by

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examining external genitalia (following Duranton & Lecoq, 1990) to control for a possible gender difference in behaviour (see Appendix) and placed in thermal

₁₆₀ conditions suitable for activity. The testing room was maintained at a mean temperature of $42.2 \pm 2.2°C$ by using two electric heatings, one placed in the center of the room and the other besides the arena. Furthermore, we placed a heating lamp in the cage to allow them to thermoregulate : temperature in the cage ranges approximately from 37 to $43.6°C$, with a maximum of $53.5°C$

₁₆₅ reached at the surface of the light bulb. According to the field study of Maeno et al. (2021b), the mean ground temperature recorded when locusts are marching is 43 °C. Three led spots were laid on the top and at each side of the olfactometer to ensure a uniform lighting in the arena.

Faeces were put in removable small tubes (length 20mm, ⌀ 8mm, i.e. a

₁₇₀ faeces volume of about $1cm^3$) connected to the pump and the olfactometer via connector tubes (Fig. 1b). Each sample of faeces was replaced after 1h of test. Control tubes were empty tubes of the same dimension as treatment tubes. The olfactometer was cleaned between each individual test. Twice a day, the connector tubes and flow meters were flushed with clear air to minimize contamination

₁₇₅ with volatile organic compounds of previous experiments. Connector tubes were switched after each test so that control and treatment tubes were alternately linked to the two sides of the olfactometer during experiments.

The different situations tested were (1) 1h-old faeces *vs.* neutral odours (empty) (2) 24h-old faeces stored in closed box *vs.* neutral odours (3) 24h-

₁₈₀ old faeces stored in open box *vs.* neutral odours (4) 1h-old faeces *vs.* 24h-old faeces, and (5) neutral odours *vs.* neutral odours (controls). A Logitech HD C930e webcam was fixed in front of the arena to capture movement of insects. Assays lasted 10 min as it appears to be a sufficient time to observe a stable choice over time during preliminary experiments. The 3 response variables were

₁₈₅ (1) first side chosen by the insect (2) time needed to make this first choice and (3) total time spent in each sides of the device (left, right or center). Those variables were computed by visual observation of the videos with rounding to 5 seconds.

2.5. Statistical analysis of behavioural assays

We conducted statistical analysis on behavioural assays data to test our hypothesis of an attractive effect of faeces on locusts and whether it was age-dependent.

First, to assess if faeces' odours had an attractive effect on nymphs, we conducted statistical tests on the first side of the arena chosen by individuals and the time spent in faeces' odours side. We tested if the side of the arena emitting faeces odours was chosen significantly more by comparing distribution of the first side chosen to an uniform distribution using a Pearson's Chi-Square goodness of fit test. To test if individuals spent more time on faeces side than in the other sides, we used Wilcoxon signed-rank paired test.

Secondly, we tested if nymphs exhibited behavioural differences according to the age of faeces (1h or 24h-old). We used a Chi-Square goodness of fit test, as described above, to compare distribution of the first side chosen with a uniform distribution for individuals exposed to both faeces ages simultaneously. To assess if results in term of first side chosen were significantly different according to faeces ages, we compared distributions for each class of assay (1h-old faeces vs. neutral odours, 24h-old faeces in closed boxes vs. neutral odours and 24h-old faeces in open boxes vs. neutral odours) by a Chi-Square homogeneity test. We also explored if nymphs' activity level was different according to faeces' ages. For that purpose, we compared time needed to make the first choice using a Kruskal-Wallis rank sum test. Finally, we compared time spent in faeces side between all type of assays using a Kruskal-Wallis rank sum test.

Finally, we looked at whether there might be any physiological or experimental factors that could affect the results. We conducted Chi-Square goodness-of-fit on control individuals to check if they were more attracted by one of the two sides of the arena. We also assessed if activity level and time spent in faeces odours side was different according to temperature in the room and the number of days since 3rd instar moult using Kruskal-Wallis rank sum, and between sexes using Wilcoxon rank sum test.

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Statistical analysis were made using R 3.6.1 and appropriate packages (`stats` v 4.0.5) and functions (`wilcox.test`, `kruskal.test` and `chisq.test`).

2.6. Volatile organic compounds analyses

Volatile organic compounds (VOCs) analyses were conducted at the Platform for Chemical Analyses in Ecology (PACE) located in the Center of Functional and Evolutive Ecology (CEFE, Montpellier, France), technical facilities of the LabEx CeMEB. Detection and annotation of VOCs present in hoppers faeces were made using gas chromatography–mass spectrometry (GC-MS). Extraction was made in the CBGP laboratory between 27/10/2020 and 30/10/2020. We made 6 replicates for each class of faeces (1h, 4h or 24h-old), plus at least one blank sample per day to capture potential parasitic odours from the laboratory. One of the 1h-old faeces absorbent trap was broken and could not be analyzed. In total, 24 samples were analyzed : 5 samples for 1h-old faeces, 6 samples for 4h-old, 6 for 24h-old faeces and 7 blanks. We also made controls to check the gaz chromatography system non-contamination.

Sampling was made using Gerstel PDMS Twisters® adsorbent traps. Traps were handled with a Multi Purpose Sampler (Gerstel, Mülheim, Germany). A double stage desorption system composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstel, Mülheim, Germany) was used to inject traps on the GC-MS system. VOCs were analyzed by a gas chromatograph (GC, Trace 1310, Thermo Scientific, Milan, Italy) coupled to a mass spectrometer (ISQ QD Single Quadrupole, Thermo Scientific, Milan, Italy) with an Optima 5-MS capillary column (30 m, 0.25 mm ID, 0.25 μm film thickness, Machery-Nagel, Düren, Germany). The method was similar to that of Proffit et al. (2020). To convert retention times into retention indexes, a series of n-alkanes (C8-C20 Alkane standard solution, 04070, Sigma Aldrich, Munich, Germany) was used. Analyses were processed with MZMine2 software (Pluskal et al., 2010). Annotations of VOCs were based on computer matching of mass spectra with commercial spectra databases (NIST 2011 library; Wiley, 9th edition) and by comparing the calculated retention indices with those reported in

the literature (Adams, 2007) when available. Every annotation has then been
²⁵⁰ verified by a chemist to evaluate the proposed level of annotation. Following
Blaženović et al. (2018), we classified annotations from level 0 (unambiguous
3D structures with full stereochemistry) to level 4 (unknown feature of interest,
present in the samples).

Volatile organic compounds were filtered using R software to discriminate
²⁵⁵ potential pollutants from VOCs actually originating from faeces, following these
rules : (1) one of the peak area of the faeces samples should be higher than 1e6
and (2) at least 1/3 of all the faeces samples should had a peak area 1.75 times
higher than the blank samples. Then, we applied the following conditions to
select the VOCs present both in 1h-old faeces and 24h-old faeces and not or
²⁶⁰ little present in blank samples : (1) at least 50% of replicates of each category
of faeces samples (1h-old, 4h-old and 24h-old) must contain the VOC (2) the
VOC must be present in at least 25% of its replicates of one of the faeces samples
categories (1h-old, 4h-old or 24h-old) (3) 75% of the blank samples must have a
peak area smaller than 1e5 *or* at least 75% of the replicates of one of the faeces
²⁶⁵ samples categories must have a peak area higher than 75% of the replicates of
the blank samples.

3. Results

3.1. Behavioural assays

3.1.1. Do faeces odours attract 3rd instar nymphs ?

²⁷⁰ There was a clear preference of individuals for the side from which faeces'
odours came from, regardless of their age (Table 1). When exposed to faeces
odours, a majority of nymphs (72.7%) chose the faeces's side first. This differ-
ence was significant for individuals tested with 1h-old faeces and those tested
with 24h-old faeces stored in open box, while results were not significant when
²⁷⁵ testing 24h-old faeces stored in closed boxes (Table 1). Furthermore, nymphs
spent more time in the side where the smell of faeces came from than in the
neutral part of the arena (Fig 2), with an average time spent in faeces' side

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approximately 4 times higher than the average time spent in the neutral side (353s *vs.* 92s). Those differences appeared significant for both faeces classes using a Wilcoxon signed-rank paired test ($V = 421.5$, $P = 6.692 \times 10^{-4}$ for 1h-old faeces, $V = 479.5$, $P = 5.804 \times 10^{-5}$ for 24h-old faeces stored in closed boxes, and $V = 460$, $P = 2.561 \times 10^{-4}$ for 24h-old faeces stored in open boxes).

Odour 1	Odour 2	N_{ind}	N_{odour1} (%)	N_{odour2} (%)	$N_{motionless}$ (%)	χ^2	P-value
1h-old	Neutral	33	24 (72.7%)	8 (24.2%)	1 (3%)	8	**0.0047**
24h-old (c)	Neutral	33	21 (63.6%)	11 (33.3%)	1 (3%)	3.125	0.0771
24h-old (o)	Neutral	33	27 (81.8%)	5 (15.2%)	1 (3%)	15.125	**0.0001**
1h-old	24h-old	37	22 (59.5%)	15 (40.5%)	0 (0%)	1.324	0.2498

Table 1: First side of the arena chosen by the individuals for each class of test.

1h-old: faeces of 1h of age; 24h-old (c): faeces of 24h of age stored in closed boxes; 24h-old (o): faeces of 24h of age stored in open boxes; 24h-old: faeces of 24h of age stored either in closed or open boxes; N_{ind}: number of tested individuals; N_{odour1}, N_{odour2}: number of individuals that chose first the side of the odour 1 (odour 2 respectively); $N_{motionless}$: number of individuals that did not make a choice during the 10 min of assays. Significance between the number of individuals that chose odour 1 and those that chose odour 2 is tested by Goodness-of-fit Chi-square test, compared to a uniform distribution (considering 50% of individuals that chose the side of the odour 1 and 50% that chose the side of the odour 2). P-values under $\alpha = 0.05$ are in bold.
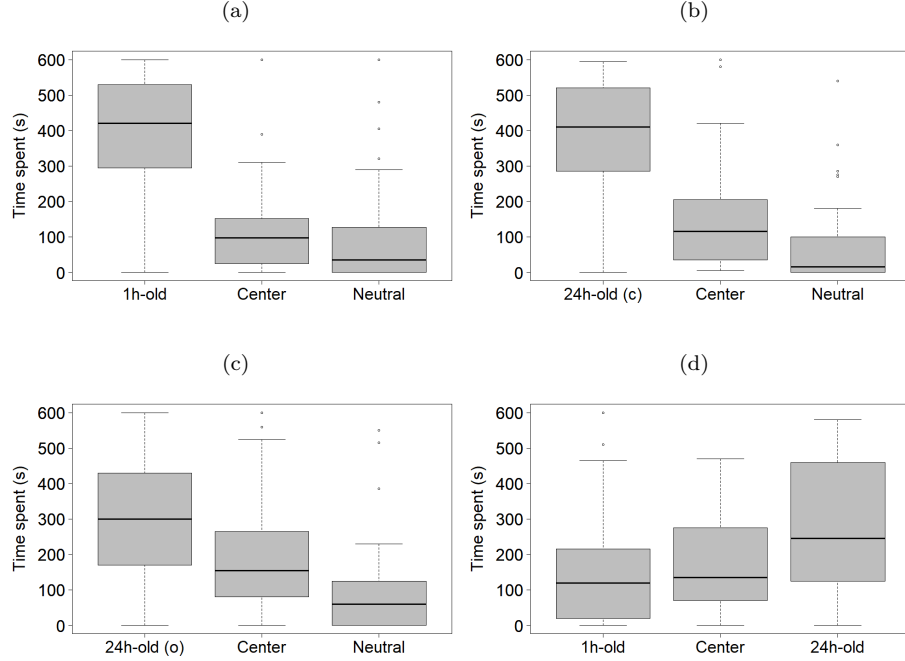
Figure 2: Time spent in each side of the arena for individuals tested with (a) 1h-old faeces, (b) 24h-old faeces in closed boxes, (c) 24h-old faeces in open boxes and (d) 1h-old and 24h-old faeces at the same time.

3.1.2. Is there behavioural differences according to the age of faeces ?

Distributions of the first side chosen by nymphs did not differ between the 3 classes of faeces age (Table 2). Hence, we considered 24h-old faeces stored in closed or open boxes together in the 1h-old vs. 24h-old tests. Locust nymphs exposed to both 1h-old and 24h-old faeces did not show any significant preference for an age class (Table 1). Furthermore, the age of faeces did not impact the time spent on faeces' odours side on our tests. Nymphs exposed to both 1h-old and 24h-old faeces did not spent significantly more time in one of the two sides of the arena (Wilcoxon signed-rank paired test; $V = 477$, $P = 0.059$). The comparison of time spent in faeces's odour side by nymphs for the 3 different age classes of faeces (1h-old, 24h-old open and 24h-old closed) was not significant on Kruskal-Wallis rank sum test ($KW = 3.263$, $P = 0.196$). Fi-

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nally, there was no difference in term of reactivity between each class of tested individuals (Fig 3). Indeed, the time needed to make the first choice was not significantly different between the classes of individuals (Kruskal-Wallis rank sum test; $KW = 3.487$, $P = 0.480$). Some individuals did not make a choice: they were motionless during the assay (8 individuals, including 5 controls). Results were identical if we considered the motionless individuals as individuals that wait 600s to make a choice (see Supplementary Material A.1). These individuals where thus discarded from further analyses.

Classes compared	χ^2	P-value
1h-old and 24h-old (c)	0.299	0.584
1h-old and 24h-old (o)	0.386	0.534
24h-old (c) and 24h-old (o)	2.083	0.149

Table 2: Chi-Squared test of homogeneity between individuals exposed to different classes of test. 1h-old: faeces of 1h of age; 24h-old (c): faeces of 24h of age stored in closed boxes; 24h-old (o): faeces of 24h of age stored in open boxes.

Figure 3: Boxplots of the time taken by individuals to make their first visit towards one side of the arena (whatever their first choice) for each type of assay (from bottom to top). Controls: individual exposed to neutral odours; 24h-old: faeces of 24h of age stored either in closed or open boxes; 1h-old: faeces of 1h of age; 24h-old (o): faeces of 24h of age stored in open boxe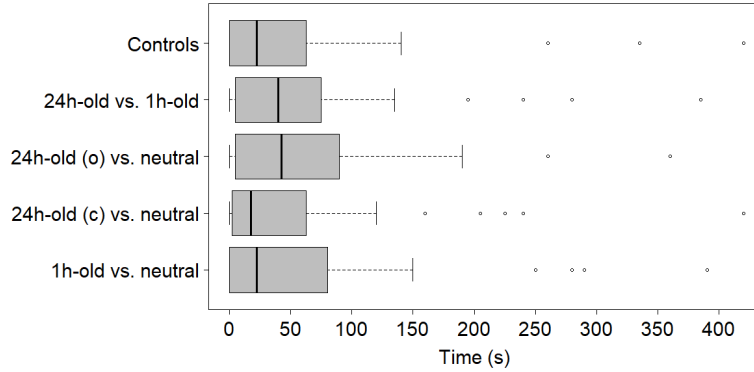s; 24h-old (c): faeces of 24h of age stored in closed boxes. The first side of the arena chosen for all tests with faeces are in Table 1.

3.1.3. Exploration of other factors of variation

The only factor that could affect result was the number of days since 3rd instar moult, that impact significantly the activity level of tested individuals ($K = 7.091$, $P = 0.029$). It is known that nymphs are less active when close to a moult (Uvarov, 1977). Nonetheless, the number of days since last moult did not affect significantly the time spent on the faeces side of individuals exposed to faeces odours (see Supplementary Material A.2), so we considered that this effect was negligible compared to faeces behavioural effect.

We did not find any significant effect of other factors (see Supplementary Material A.2). In controls, there were slightly more individuals attracted first to the left side (62.5% of the controls that made a choice) but this difference was not significant on Pearson's Chi-Square test when compared to an uniform distribution ($\chi^2=2$, $P = 0.1573$). There were no significant differences on Wilcoxon signed rank test for time spent in left and right side of the arena in controls (see Supplementary Material A.2.1), nor on Wilcoxon rank sum test between sexes, temperature, and number of days after the 3rd instar moult (see Supplementary Material A.2.2).

3.2. Volatile organic compounds analysis

In total, 300 volatile organic compounds (VOCs) were detected in faeces extracts and 75 of them remained after the filtration process. Out of these 75 remaining VOCs, 12 were present both in 1h-old and 24h-old faeces while being absent or in low quantity in blank samples, following the selection rules described in section 2.6. All but one of these VOCs could be annotated at level 2 (following Blaženović et al. (2018)), including guaiacol and phenol, already known to be present in locust faeces (see Cram representations of molecules and Mass Spectra in Supplementary Material A.3 and A.4). Temporal dynamics of a majority of VOCs were increasing or presenting a relative maximum at 4h (Fig 4), which suggests bacterial activity or chemical reactions due to other VOCs degradation. Guaiacol and phenol were the most expressed VOCs, in particular in 4h-old faeces samples (Fig 4). Further information about the different VOCs

is detailed in Table 3.
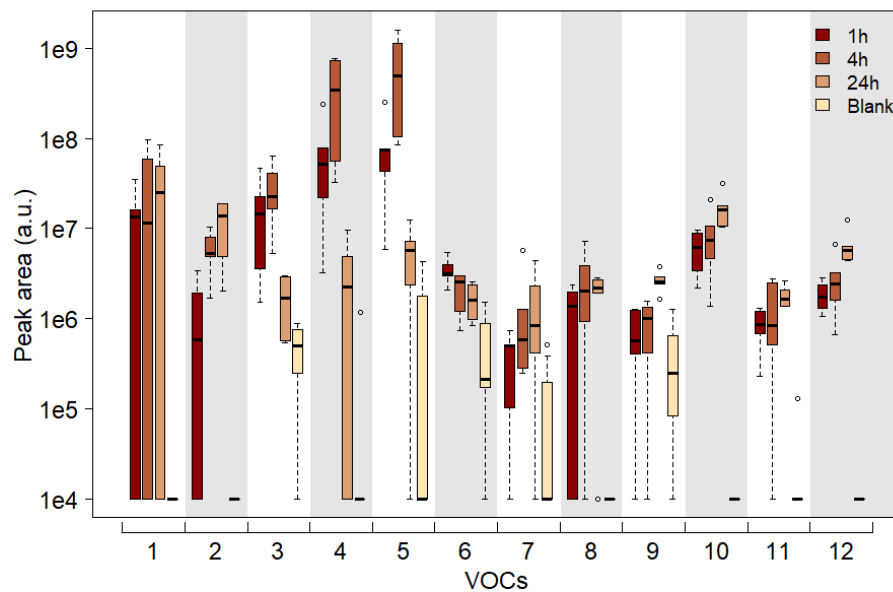
Figure 4: Abundance (in a logarithmic scale) of the 12 filtered volatile organic compounds detected in all age classes of faeces (1h-old, 4h-old and 24h-old) and mostly absent in blank samples by GC-MS analysis. 1 = Butan-2,3-diol, 2 = α-methyl-γ-butyrolactone, 3 = 3-octanone, 4 = Phenol, 5 = 2-methoxyphenol (Guaiacol), 6 = Nonan-1-ol, 7 = 2,6,6-trimethylcyclohex-1-ene-1-carbaldehyde, 8 = Unknown VOC, 9 = 4,8-dimethyl-1,7-nonadien-4-ol, 10 = Trans-α-ionone, 11 = (4ar,8as)-8a-methyl-decahydronaphthalene-1,8-dione, and 12 = (R)-dihydroactinidiolide.

N°	Name	Mass (Da)	Temporal dynamics	Detected in
1	Butan-2,3-diol	90.0680	Stable	Ubiquitous, produced by a variety of microorganisms (Syu, 2001)
2	α-methyl-γ-butyrolactone	100.0524	Increasing	Plant, like *Evolvulus alsinoides* (Kashima & Miyazawa, 2014)
3	3-octanone	128.1201	Relative maximum at 4h	Ubiquitous, present in plants, fruits and fungi
4	Phenol	94.0418	Relative maximum at 4h	In locust faeces, produced by bacteria (Dillon et al., 2000)
5	2-methoxyphenol (Guaiacol)	124.0524	Relative maximum at 4h	In locust faeces, produced by bacteria (Dillon et al., 2002), and in faeces of *Pseudoplusia includens* (Ramachandran et al., 1991)
6	Nonan-1-ol	144.1514	Decreasing	Ubiquitous, came from the acetate pathway (Syu, 2001)
7	2,6,6-trimethylcyclohex-1-ene-1-carbaldehyde	152.1201	Increasing	Plants, including wheat (Shibamoto et al., 2007; Yacout et al., 2012; Chhouk et al., 2018)
8	Unknown	/	Increasing	/
9	4,8-dimethyl-1,7-nonadien-4-ol	168.1514	Increasing	Plants, essential oils of kaffir lime *Citrus hystrix* (Loh et al., 2011)
10	Trans-α-ionone	192.1514	Increasing	Plants (teas, raspberries) (Lin et al., 2021; Aaby et al., 2019)
11	(4ar,8as)-8a-methyl-decahydronaphthalene-1,8-dione	180.1150	Increasing	Pollutant from plastic (Charpentier et al., 2012)
12	(R)-dihydroactinidiolide	180.1150	Increasing	Queen of fire red ant (*Soleneopsis invicta*) (Rocca et al., 1983; Mori & Nakazono, 1986).

Table 3: Name, mass, temporal dynamics observed in the present study and literature-survey of the possible origin of the 12 volatile organic compounds detected in 1h-old and 24h-old faeces. Except for VOC number 8 corresponding to a level 4 annotation (unknown VOC), other VOCs were annotated at level 2 (i.e. are consistent with, at least, libraries fragmentation mass spectra and theorical Retention Indexes).

4. Discussion

In this study, we assessed the individual behavioural responses of 3rd instar hoppers of *Schistocerca gregaria* to faeces odours of different ages (1h-old or 24h-old) in order to test for an attractive effect of conspecifics' faeces and for a dependency of this effect on the faeces' ages. Our results support an attractive effect of both 1h-old and 24h-old faeces on nymphs, with no preference observed between the two classes of faeces age. About 73% of the tested individuals chose faeces' side first and spent significantly more time there, regardless of faeces' age. Furthermore, we observed that this attractive effect lasted at least 24 hours, in which time 12 volatile organic compounds were emitted by the locust faeces.

In the following, we first discuss in detail the VOCs results, their relation to bacterial activity and other insects' behaviours when possible. In the second part, we come back to the attractive effect and individual responses. To finish, we discuss how these individual behaviours may play an important role in band formation and movements.

4.1. Volatile organic compounds found from locust faeces

To detect which VOCs could be implicated in the attractive behaviour, we conducted chemical analysis with GC-MS on 1h-old, 4h-old and 24h-old faeces. We found and annotated 11 VOCs that were emitted by 1h-old faeces and still released by 24h-old collected faeces. One of these, (4ar,8as)-8a-methyl-decahydronaphthalene-1,8-dione, is a pollutant from plastic. Its presence in our samples could be due to faeces that were in contact with the sides of the cage before being collected. We did not detect 4-vinylanisole, the molecule having an attractive effect on *Locusta migratoria* (Guo et al., 2020), in our nymphs' faeces samples. However, this VOC has never been detected in *S. gregaria* nymphs' faeces, although it is emitted in small quantities by adult males (from 0.02 to 0.22 ng per insect per day, Torto et al. (2021). Four of the detected VOCs seem particularly interesting because of their known effect on insect behaviour: phenol, guaiacol (2-methoxyphenol), 3-octanone and (R)-dihydroactinidiolide.

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Phenol and guaiacol have already been detected in locusts faeces in previous studies (Obeng-Ofori et al., 1994; Torto et al., 1996). These two VOCs are involved in aggregative responses on groups of *S. gregaria* and *Locusta migratoria* (Fuzeau-Braesch et al., 1988; Hassanali et al., 2005). It was shown by Dillon et al. (2002) that guaiacol production result from bacterial activity, and two of the three bacterial genera known to be involved in its production (Pantoea, Klebsiella and Enterobacter) were detected in our locusts' faeces, through a supplementary analysis based on 16S rRNA metabarcoding (see Supplementary Material A.5). Phenol production may also be linked to bacterial activity, since Dillon et al. (2000) detected it at only a reduced level in faecal pellets of germ-free locusts. In vitro inoculation of germ-free faecal pellets with *Pantoea agglomerans* resulted in the production of small but higher amounts of phenol. Furthermore, guaiacol is a relevant VOC to other insect species: it was detected in faeces of the moth *Pseudoplusia includens* and is attractive to female of the parasitoid wasp *Microplitis demolitor* (Ramachandran et al., 1991). It is also attractive to the moth *Spodoptera littoralis* (Revadi et al., 2021). Combined with the attractive response that we observed in our study, the aggregative effect of guaiacol and phenol emphasizes the hypothesis of faeces having a role in maintaining locusts bands.

The two other VOCs, 3-octanone and (R)-dihydroactinidiolide, were not yet reported in other locusts VOCs studies but are involved in behavioural responses of other insect species, including social insects. The first one, 3-octanone, is an ubiquitous VOC produced by many organisms (Table 3). It was found in faeces of the moth *Pseudoplusia includens* (Ramachandran et al., 1991) and in mandibular gland constituents of the ants *Manica mutica* and *M. bradleyi* (Fales et al., 1972). It appears to be an alarm pheromone of species in the subfamily *Myrmicinae* (Fales et al., 1972), can deter female flies of *Megaselia halterata* at high concentrations (Ramachandran et al., 1991; Pfeil & Mumma, 1993), and attracts social wasps *Vespula vulgaris* and *V. germanica* (Unelius et al., 2014). The second VOC, (R)-dihydroactinidiolide, is known to be a recognition pheromone of the queen of fire red ant (*Soleneopsis invicta*) that attracts worker-

ants (Rocca et al., 1983; Mori & Nakazono, 1986). The dynamics of emission that we observed on GC-MS revealed an increasing signal on 4h-old and 24h-old faeces samples (Fig. 4), suggesting that they could result of bacterial activity too.

Some of the remaining detected VOCs had been previously found in various plants: α-methyl-γ-butyrolactone has been found in *Evolvulus alsinoides* (Convolvulaceae) (Kashima & Miyazawa, 2014). 2,6,6-trimethylcyclohex-1-ene-1-carbaldehyde was found in *Cananga latifolia* (Annonaceae) (Chhouk et al., 2018), *Lantana camara* (Verbenaceae) (Yacout et al., 2012) and common wheat *Triticum aestivum* (Poaceae) (Shibamoto et al., 2007). 4,8-dimethyl-1,7-nonadien-4-ol is present in essential oils of *Citrus hystrix* (Rutaceae) (Loh et al., 2011) and trans-α-ionone can be found in teas (Theaceae) and raspberries (Rosaceae) (Aaby et al., 2019; Lin et al., 2021). Maybe those VOCs came from the wheat use to feed our locusts and eventually not degraded in the faeces. Finally, butan-2,3-diol and nonan-1-ol are ubiquitous VOCs produced by most living organism and does not seem to be linked to some behavioural effects on insects in the literature (Syu, 2001).

It is worth noting that three of these most interesting VOCs (3-octanone, phenol and guaiacol) shared the same temporal dynamics of emission, with highest levels of emission reached in 4-old faeces samples. This questions if a preference might be observed when testing individuals exposed to 4h-old *vs.* 1h-old or 24h-old faeces. With our results of no difference of attraction between 1h-old and 24h-old faeces, we do not know if attraction is a presence-absence response to VOCs (as illustrated recently by Nizampatnam et al., 2022) or if a gradual response to VOCs intensity could exist. Further experiments could clarify this point. It would also be informative to test from what age faeces stop being attractive to locust. For that purpose, it would be interesting to use Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) analyses to refine temporal dynamics of the VOCs. Unlike GC-MS, PTR-ToF-MS allow almost real-time quantification (Majchrzak et al., 2018). Also, it would be interesting to conduct Electroantennography analyses to explore which

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VOCs are detected by locusts, like in Torto et al. (1994). This technique record electrical potentials from insect antennae when exposed to odours to find which ones are detected by the insect and can be coupled with Gas-Chromatography (Arn et al., 1975). A further investigation about physiological functions of the VOCs is also needed. Indeed, after the perception of the VOCs, not much is known about the physiological pathways triggered by the stimulus.

4.2. Individual locust attraction to faeces' VOCs

Our experiments were realized with a strain of desert locusts reared in laboratory for several generations. It could have an effect on the outputs, especially concerning microbiota composition and chemical analysis. In the work of Torto et al. (2021), volatile emissions of *S. gregaria* were compared between individuals reared in laboratory for one and for seven generations. Results indicate that locusts from the field reared in laboratory for 1 generation produced 4 to 40 times more volatile organic compounds than locusts reared for 7 generations in the laboratory. However, the 2 strains of locusts of Torto et al. (2021) did not come from the same population, which makes it difficult to compare. The work of Lavy et al. (2019) on wild and laboratory strains differences shows that the gut-microbiota is very variable. Nonetheless, they frequently observed the presence of the *Enterobacter* genus and *Klebsiella* genus, from Enterobacteriaceae family, that is one of the dominant family of our faeces samples analyses (see Supplementary Material A.5). It was shown that *Klebsiella pneumoniae pneumoniae* and *Enterobacter cloacae* are involved in guaiacol production (Dillon et al., 2002). Although our laboratory strain may be restricted in microbiota and hence poor in VOCs compared to field desert locusts, our results are quite stable in term of presence of phenol and guaiacol, and the strong attractive response to faeces of nymphs that we observed argue in favour of faeces odours as a good candidate to contribute to orientation of locust nymphs.

Recent progress in the understanding of odour recognition of locusts show that duration, dynamics or changes in ambient conditions do not hinder the detection of important odours (Nizampatnam et al., 2022). Environnemental

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factors such as humidity, temperature and UV may modify the faeces' odours. However, the results of Nizampatnam et al. (2022) give confidence to the idea that even in natural conditions, locust nymphs may still be able to decipher faeces odours to orientate towards a feeding band and hence potential food source. This phenomenon may also explain why the attraction response in our experiments was robust to the age of faeces, in spite of changes in the chemical blend composition.

Further studies on the detected VOCs could test each of them to elucidate which ones are implicated in attractive behaviour. Because guaiacol, phenol, 3-octanone and (R)-dihydroactinidiolide are present in large amount in our faeces samples with a very clear signal on GC-MS analysis and because of their reported behavioural effect on locusts or other insects, we hypothesize that one of these VOCs is the most important in behavioural response. Eventually, it could be a combination of several VOCs that serve as trigger of attraction. Then, it would be interesting to conduct individual behavioural assays on nymphs exposed to those 4 VOCs in priority. For future works, it would also be interesting to reproduce those tests on locust from wild populations. Ultimately, trying to orientate hoppers in natural conditions with large amount of faeces or the annotated set of VOCs could verify this role of faeces odours.

4.3. Band movements and faeces odours

To our knowledge, this study is the first to consider faeces' state of degradation. Our results highlight the persistence in time of VOCs, that were still emitted 24h after faeces production. This long persistence create a trace through time of the way taken by individuals. In cases where the band may be out of scent range of latecomers, faeces may constitute more efficient indicators of the path taken by the band than individual nymphs' odours. The potential role of faeces in orienting locust nymphs is consistent with the field observation of Piou et al. (2022) on *S. cancellata* hoppers. Following a large hopper band for several days, one evening, Piou et al. observed that the band was split in two groups. The next day, the second group followed the same path that the group of the

front took the day before their separation. The faeces odours could probably be involved in this orientation.

It is known that hopper bands display a density gradient with more individuals at the front than at the rear (Uvarov, 1977). This results in an accumulation of fresh faeces at the front. Then, faeces left by the band follow an age gradient with more fresh faeces at the front than at the rear. As we observed in chemical analysis that VOCs emitted by faeces evolve over time, this age gradient could provide an additional information on the travelling direction of the band and contribute to the global cohesion of the group, with the back of the band following fresh faeces left by the front. The degradation of faeces through time, longer than 24h, may give an orientation to follow. Even if we did not observe a clear preference between 1h-old and 24h-old faeces in our experiments, there obviously should be an age when faeces are not attractive at all, or simply less attractive. Then, the direction to take would result from this difference. Further works should be conducted to identify when faeces' odours stop to be attractive and from how far faeces are detected by hoppers.

Faeces odours may also explain large band formation from small scattered hopper groups. Indeed, first and second instar nymphs of gregarious desert locusts create "hopper spots" generally without walking direction (Ellis & Ashall, 1957), as a result of synchronization of egg-laying and hatching (Uvarov, 1977; Maeno et al., 2021a). As hoppers grow, they start marching in a coordinated way (Uvarov, 1977). So far, some authors considered that the direction taken by young hopper bands is random (e.g. Lecoq et al., 1999). However, faeces odours from other groups may be a signal that influence the individuals in the front of the band, and consequently the entire band direction. It would then participate in the formation of large bands by regrouping different small hopper spots. Such convergence of several small group was observed in the field (Uvarov, 1977; Symmons & Cressman, 2001). Further studies should be conducted to test this hypothesis.

If the attractive effect of certain VOCs is strong enough, we could imagine possible application in locusts pest management. Those VOCs could be used to

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deviate marching bands from crops or to trap insects. Instead of the physical barriers used in the 1950s or afterwards in case of lack of pesticide to orientate hopper bands toward traps (Dobson, 2001; Stride et al., 2010), chemical attraction would be a way to lower necessary man power and reduce the environmental impacts of chemical pesticides. In the case of 4-vinylanisole, preliminary test with sticky traps emitting the VOC in the natural wetland reserve of North Dagang on *L. migratoria* show promising results (Guo et al., 2020).

5. Declarations of interest

Declarations of interest: none.

Data availability

The data obtained from the experiments and scripts used for analyses can be found in the data repository of CIRAD Dataverse (`https://doi.org/10.18167/DVN1/D3OP8O`).

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A Supplementary materials

A.1 Inclusion of motionless individuals

Question	Dependant variable	Independant variable(s)	Statistical Test	Statistical Value	P-value
Is there a difference of activity level between males and females ?	Time needed to make the first choice	Sex	Wilcoxon rank sum test	3371	0.502
Does the number of days since 3rd instar moult impact significantly the activity level of individuals ?	Time needed to make the first choice	Number of days since 3rd instar moult (1,2 or 3)	Kruskal-Wallis rank sum	4.824	0.09
Does temperature variation impact significantly the activity level of individuals ?	Time needed to make the first choice	Temperature	Kruskal-Wallis rank sum	12.796	0.464

TAB. A.1.1 – *Statistical test results for the time needed to make the first choice when motionless individuals are included in the database. We set the time needed to make the first choice at 600s.*

A.2 Exploration of other factors of variation

We explored physiological and experimental factors that could affect behavioural results : sex of the individuals, number of days since the 3rd instar moult and the temperature in the room.

Question	Dependant variable	Independant variable(s)	Statistical Test	Statistical Value	P-value
Is one side of the arena more attractive than the other one ?	Time spent on each side	Sides of the arena	Wilcoxon signed rank test	333	0.200
	Firt side chosen	Sides of the arena	χ^2	2	0.1573
Is activity level different between sexes ?	Time needed to make the first choice	Sex	Wilcoxon rank sum test	157	0.256
Is activity level different according to number of days since last moult ?	Time needed to make the first choice	Number of days since 3rd instar moult	Kruskal-Wallis rank sum test (df=2)	1.333	0.514
Is activity level different according to temperature variation ?	Time needed to make the first choice	Temperature	Kruskal-Wallis rank sum test (df=8)	4.863	0.772

TAB. A.2.1 – *Statisical tests made on control individuals to explore other potential factors of variation.*

Question	Dependant variable	Independant variable(s)	Statistical Test	Statistical Value	P-value
Is activity level different between sexes ?	Time needed to make the first choice	Sex	Wilcoxon rank sum test	1856	0.246
Is activity level different according to number of days since last moult ?	Time needed to make the first choice	Number of days since 3rd instar moult	Kruskal-Wallis rank sum	6.943	0.031
Is activity level different according to temperature variation ?	Time needed to make the first choice	Temperature	Kruskal-Wallis rank sum	14.748	0.256
Is one sex more attracted by faeces than the other one ?	Time spent on faeces' odour side	Sex	Wilcoxon rank sum test	1142	0.850
Is there an effect of number of days since 3rd instar moult on behavioural responses (faeces attractiveness) ?	Time spent in faeces's odours side	Number of days since 3rd instar moult	Kruskal-Wallis rank sum test (df=2)	1.139	0.566
Does temperature variation impact behavioural responses (faeces attractiveness) ?	Time spent on faeces' odour side	Temperature	Kruskal-Wallis rank sum test (df=10)	8.388	0.591

TAB. A.2.2 – *Statistical tests made on tested individuals to explore other potential factors of variation*

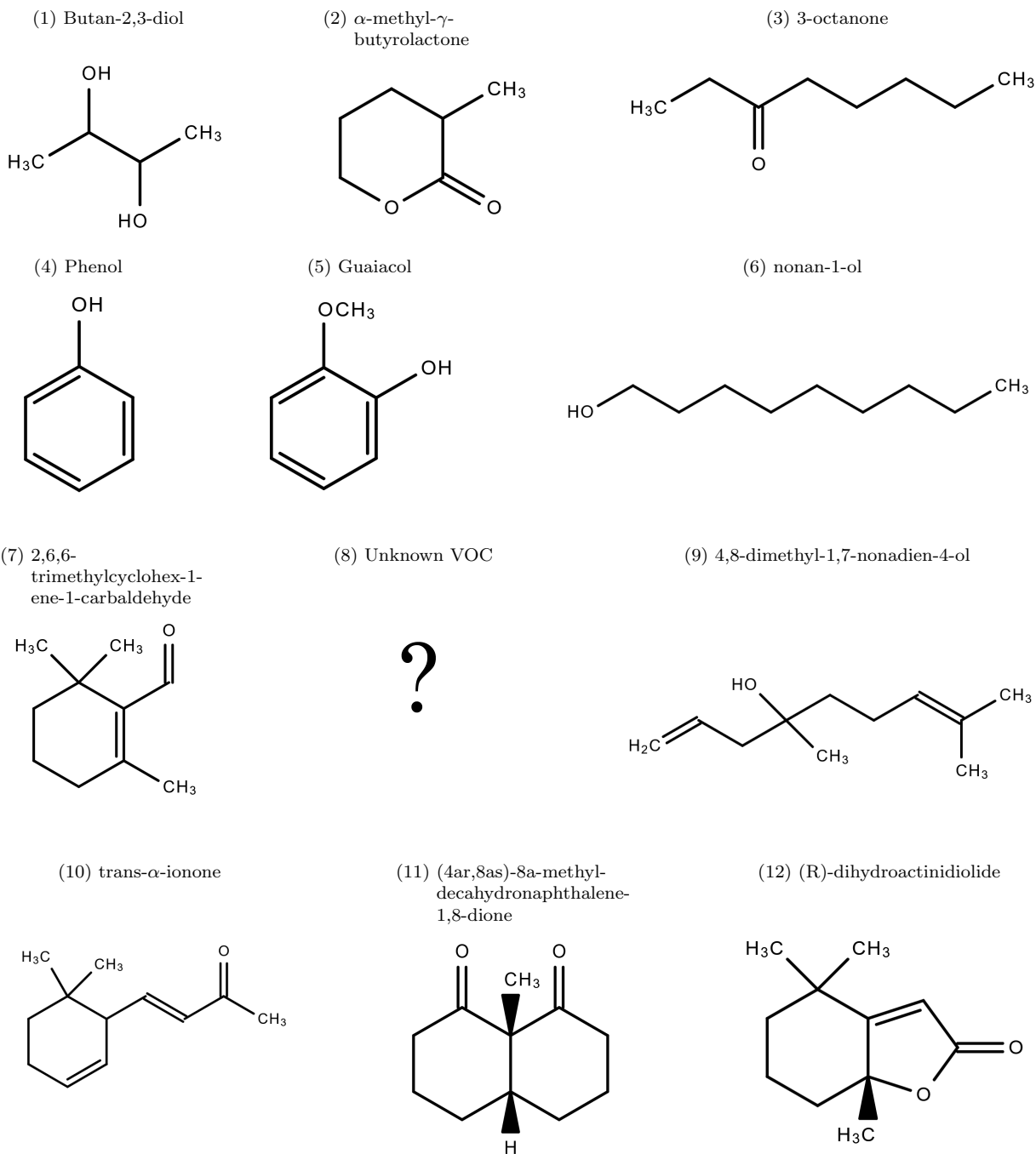## A.3 Cram representation of detected VOCs

(1) Butan-2,3-diol

(2) α-methyl-γ-butyrolactone

(3) 3-octanone

(4) Phenol

(5) Guaiacol

(6) nonan-1-ol

(7) 2,6,6-trimethylcyclohex-1-ene-1-carbaldehyde

(8) Unknown VOC

(9) 4,8-dimethyl-1,7-nonadien-4-ol

?

(10) trans-α-ionone

(11) (4ar,8as)-8a-methyl-decahydronaphthalene-1,8-dione

(12) (R)-dihydroactinidiolide

FIG. A.3.2 – *Cram representation of the 11 detected VOCs.*

4

A.4   Mass Spectra of annotated VOCs

(1) Butan-2,3-diol

(2) α-methyl-γ-butyrolactone

(3) 3-octanone

(4) Phenol

(5) Guaiacol

(6) nonan-1-ol
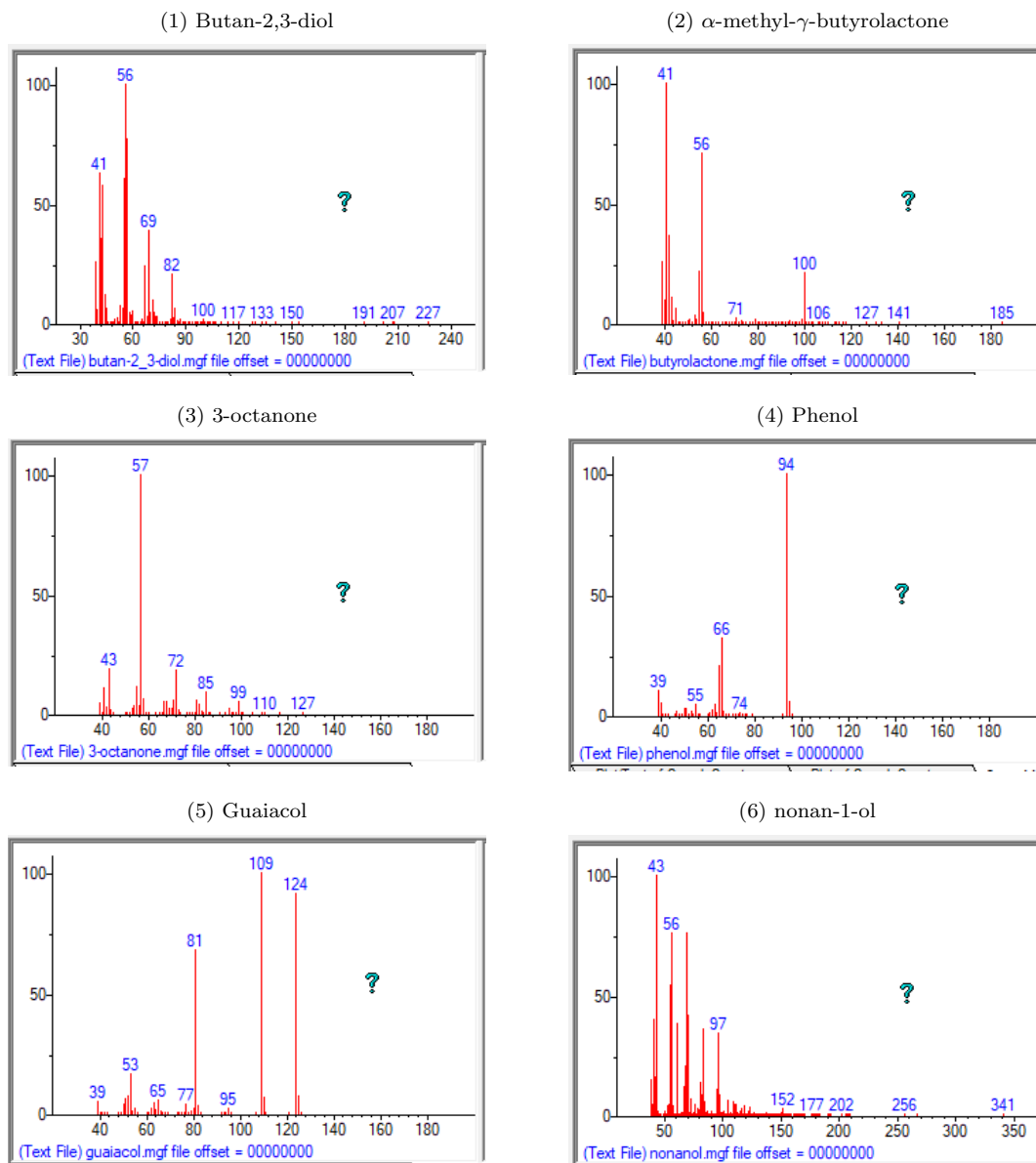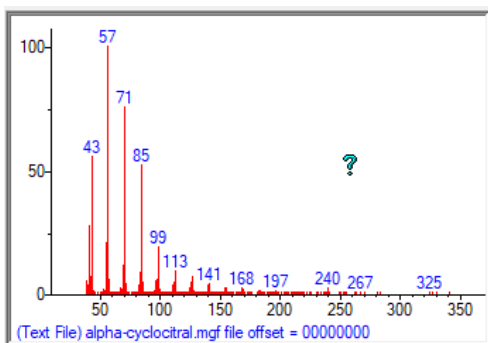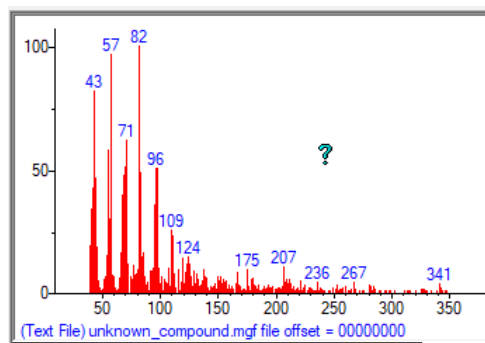
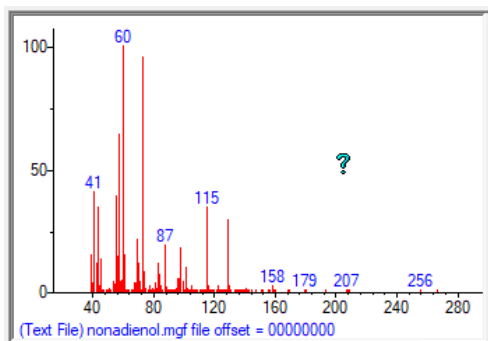

FIG. A.4.2 – *Mass Spectra of the 12 annotated VOCs.*
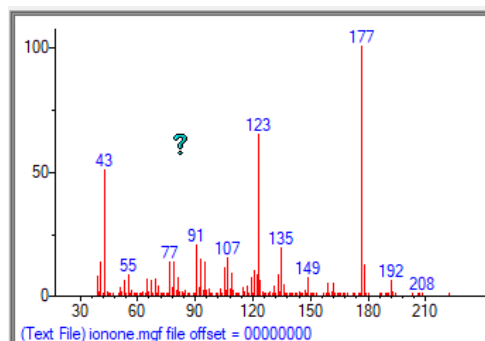
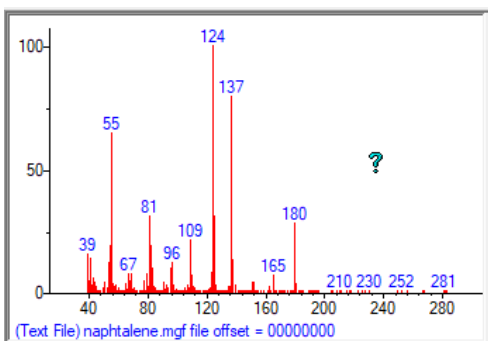(g) 2,6,6-trimethylcyclohex-1-ene-1-carbaldehyde

(h) Unknown VOC

(i) 4,8-dimethyl-1,7-nonadien-4-ol

(j) trans-α-ionone

(k) (4ar,8as)-8a-methyl-decahydronaphthalene-
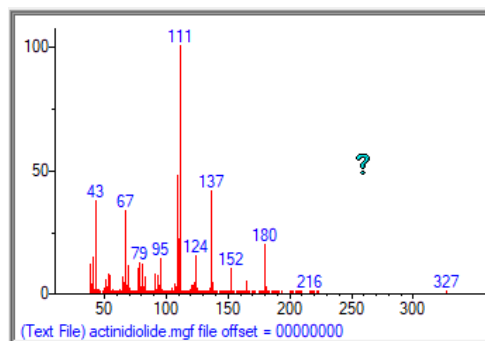1,8-dione

(l) (R)-dihydroactinidiolide

FIG. A.4.2 – *Mass Spectra of the 12 annotated VOCs (end).*

A.5 Presence of bacterial agents in faeces

Methods

Four batches of faeces produced by desert locust nymphs during the experiment (24h of age or more) were used to identify associated bacteria by high-throughput sequencing of a DNA amplicon coding for the 16S ribosome RNA gene. DNA was extracted using the DNeasy 96 Blood & Tissue Kit (Qiagen) instructions, with the addition of a mechanic cell lysis procedure to provide high quality microbial nucleic: after lysis by incubation overnight at 56°C, lysate was bead-beaten for 5 min with 500 mg of 0.45/0.55 mm zirconia beads Zirmil ®Y (Saint-Gobain), in a TissueLyser II (Qiagen) at the maximum speed setting. We used universal primers to amplify a 251-bp portion of the V4 region of the 16S rRNA gene (16S-V4F:587 GTGCCAGCMGCCGCGGTAA; 16S-V4R: GGACTACHVGGGTWTCTAATCC) and a dual-index method to multiplex our samples (Kozich et al., 2013; Galan et al., 2016). Laboratory preparation for PCRs (in triplicate and including negative controls), and library preparation was performed as in Galan et al. (2016). We performed a run of 2 x 251 bp paired-end sequencing, which yielded high-quality sequencing, through the reading of each nucleotide following the assembly of reads 1 and reads 2.

We processed the paired-end sequencing data from the Illumina MiSeq system using the following approach. We preliminarily used a Shell script that allows to prepare Illumina reads (quality control, removal of primers using cutadapt v. 1.9.1 (Martin, 2011), and contiguity filtering using FLASH v. 1.2.11 (Magoč and Salzberg, 2011). Then, in FROGS pipeline (Escudié et al., 2018), we filtered sequences by length (expected value of 251b ± 10b), dereplicated sequences, removed chimeras using the algorithm of Edgar et al. implemented in VSEARCH v. 1.1.3, clustered sequences with SWARM v. 1.3.0 using a local clustering threshold using the default d-value (d = 1) (Mahé et al., 2014) and returned taxonomic affiliation for each OTU using NCBI Blast+ on Silva SSU 132 with pintail quality 100%. Finally, we filtered for false positives or incomplete taxonomic affiliations, applied data reduction by keeping only bacterial variants with at least 0.005% of the total number of reads in the abundance table, following Escudié et al. (2018), and cleaned for wheat contamination due to homology between bacterial and chloroplast 16S (Hanshew et al., 2013).

Results

We collected *S. gregaria* faeces used in behavioural assays to identify their bacterial communities using high-throughput sequencing. Overall, PCRs generated a minimum of 4,867 reads and an average of 20,099 reads per sample after filtering, reduction, and cleaning for chloroplast variants, and all the samples reached the plateau. The 25 bacterial variants found in faeces samples represented Gammaproteobacteria and Bacteroidia classes only, 8 families and 21 genera (Table A.5.3). The Enterobacteriaceae family represent about 75 percent of reads, at least 13 genera and 13 variants.

Family	Genus	Proportion of reads	Prevalence
Enterobacteriaceae (522) or Erwiniaceae (2)	Klebsiella (343), Enterobacter (165), Buttiauxella (9), Salmonella (1), Cronobacter (1), Kluyvera (1), Escherichia-Shigella (1), Erwinia (2), Siccibacter (1)	72.1%	100%
Enterobacteriaceae	Raoultella (54), Enterobacter (2), Citrobacter (1)	10.4%	100%
Xanthomonadaceae	Stenotrophomonas	5.1%	75%
Moraxellaceae	Acinetobacter	4.0%	100%
Enterobacteriaceae (67) or Erwiniaceae (3)	Kosakonia (33), Cronobacter (15), Salmonella (17), Enterobacter (1), Escherichia-Shigella (1) Siccibacter (3)	3.1%	100%
Sphingobacteriaceae	Sphingobacterium	2.1%	50%
Xanthomonadaceae	Stenotrophomonas	1.3%	100%
Flavobacteriaceae	Flavobacterium	1.0%	100%
Pseudomonadaceae	Pseudomonas*	0.4%	100%
Enterobacteriaceae	Yersinia (405), Serratia (87), Rahnella (6)	0.3%	75%
Erwiniaceae (3) or Enterobacteriaceae (1)	Erwinia (3) Citrobacter (1)	0.1%	50%
Xanthomonadaceae	Stenotrophomonas	0.04%	50%
Comamonadaceae	Aquabacterium	0.02%	75%

TAB. A.5.3 – *Proportion of reads, prevalence among the four faeces samples and taxonomic affiliation of bacterial genera revealed by 16S metabarcoding in S. gregaria faeces. For the bacterial variants 1, 2 and 5, the 16S V4 region cannot discriminate among several genera of the Silva SSU 132 database (i.e., 'Multi-affiliation' in FROGS results). In these cases, the numbers of the different taxonomies in the best blast hits (i.e., with an equal score) were written in parentheses.*: five different bacterial variants blasted to the Pseudomonas genus (all other genera were represented by a single bacterial variant)*

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