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Drosophila melanogaster Stress Odorant (dSO) Displays the Characteristics of an Interspecific Alarm Cue

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Abstract

Organisms depend on visual, auditory, and olfactory cues to signal the presence of danger that could impact survival and reproduction. *Drosophila melanogaster* emits an olfactory alarm signal, termed the *Drosophila* stress odorant (dSO), in response to mechanical agitation or electric shock. While it has been shown that conspecifics avoid areas previously occupied by stressed individuals, the contextual underpinnings of the emission of, and response to dSO, have received little attention. Using a binary choice assay, we determined that neither age and sex of emitters, nor the time of the day, affected the emission or avoidance of dSO. However, both sex and mating status affected the response to dSO. We also demonstrated that while *D. melanogaster, D. simulans, and D. suzukii*, have different dSO profiles, its avoidance was not species-specific. Thus, dSO should not be considered a pheromone but a general alarm signal for *Drosophila*. However, the response levels to both intra- and inter-specific cues differed between *Drosophila* species and possible reasons for these differences are discussed.

Keywords *Drosophila melanogaster* · *D. simulans* · *D. suzukii* · *Drosophila* stress odorant (dSO) · Alarm cue · Volatile Organic Compounds

Introduction

Social interactions using visual, auditory, olfactory, and/or tactile cues are crucial for the successful development, survival, and reproduction of organisms (Dahanukar and Ray 2011; Sokolowski 2010). These include reactions to danger, that can elicit a range of different behavioural responses (Yew and Chung 2015) and vary depending on the species and the ecological context (Verheggen et al. 2010). Olfactory alarm signals are typically made up of highly volatile, non-persistent molecules, which rapidly inform conspecifics of potential danger without generating a persistent state of alert (Verheggen et al. 2010). Alarm pheromones, which by definition modulate interactions between conspecifics, have been reported in a wide range of animals, including nematodes (Zhou et al. 2017), aphids (Vandermoten et al. 2012), bees (Hunt 2007), zebrafish (Mathuru et al. 2012), mice (Chao et al. 2018) and humans (Mujica-Parodi et al. 2009). It has been postulated that alarm pheromones evolved from chemicals initially serving other functions such as defence (Napper and Pickett 2008; Verheggen et al. 2010), and in some cases, these cues elicit responses in closely related sympatric species that share common natural enemies (Napper and Pickett 2008).

In *Drosophila melanogaster*, stressed flies emit an olfactory alarm cue, the *Drosophila* stress odorant (dSO), and individuals avoid areas previously occupied by stressed conspecifics (Suh et al. 2004). One component of dSO is CO_2 (Suh et al. 2004), and different aspects of the neural pathways involved in CO_2 detection have been identified (Dubnau et al. 2019; Kwon et al. 2007; Siju et al. 2014; Suh et al. 2007; Suh et al. 2004; van Breugel et al. 2018). However, CO_2 is ubiquitous in nature and the degree to which

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Drosophila adults are attracted or repelled varies depending on the food source (Faucher et al. 2006; Turner and Ray 2009) and the ecological context (van Breugel et al. 2018; Muria et al. 2021).

It is clear that other chemicals are present in the mixture emitted by stressed flies, as their avoidance response is weaker to CO_2 alone than to dSO (Suh et al. 2004). However, the presence of CO_2 is essential, as flies do not respond to dSO if their ability to detect CO_2 is inhibited (Turner and Ray 2009).

Both sexes respond to dSO, although the levels of response vary as a function of the genetic background (Fernandez et al. 2014) and age (Brenman-Suttner et al. 2018), as well as the density of emitters (Fernandez et al. 2014). In this study, we examined the importance of sex and mating status, as well as the time of day on the emission and perception of dSO in *D. melanogaster.* As the response to CO_2 differs between *Drosophila* species (Krause Pham and Ray 2015) we tested the responses of *D. melanogaster, D. simulans,* and *D. suzukii* to the odour cues from stressed conspecific and heterospecific adults and compared the profiles of dSO emitted by adults of each species.

Methods and Materials

Experimental Animals

Drosophila melanogaster (Canton-S) and *D. simulans* (FC) adults used in the different assays were obtained from laboratory colonies maintained at Western University. *Drosophila suzukii* were a gift from Dr Ian Dworkin, McMaster University. *Drosophila melanogaster* and *D. simulans* were reared on Drosophila Jazz-Mix™ diet (Fisher Scientific, ON, Canada) at 25 °C, 50% RH under a 12L:12D light cycle, with lights on at 08:00 and off at 20:00. *Drosophila suzukii* was maintained on a banana-cornmeal-agar medium (Jakobs et al. 2017) at 21 °C, 65% RH under a 14L:10hD light cycle, with lights on at 7:00 and off at 21:00.

We tested flies that were 3–7 days old since under laboratory conditions, behavioural performances of *D. melanogaster* have been reported to peak during those ages (Simon et al. 2006).

To obtain mated individuals, newly emerged flies (20 male and 20 females) were held together for 3 to 7 days before testing and sexed under cold anesthesia the day before the experiment, as previous experiments showed that under these conditions > 90% of all adults would have mated at least once (Yost et al. 2021). Virgins were obtained by sexing flies at emergence under cold anaesthesia and adults held at a density of approximately 40 flies in same sex containers until needed.

All flies used in this study were naïve with respect to the behavioural assays conducted. The morning of the experiment, all flies were transferred to new vials at lights on and allowed to habituate for approximately 3 h to the test conditions of 25 °C and 50% RH. All assays, except those investigating temporal patterns of emission/reception, were carried out between the 4^{th} to 8^{th} hr of the photophase to reduce variability associated with diel periodicity (Dubruille and Emery 2008). The time assays were carried out is expressed as Zeitgeber time (ZT), which is the time (in hrs) from the start of the photophase, so the majority of assays were run at ZT 4–8.

dSO Avoidance Assay

We used the protocol of Fernandez et al. (2014) where, under uniform light conditions, responder flies were placed in a binary-choice T-maze with a choice of entering a vial with ambient air (air vial) or one containing dSO. After 1 min, we counted the number of flies in each vial and had a minimum of 9 replicates for each different treatment in each experiment. Emitters were agitated by vortexing 15 s on, 5 s off, repeated 3 times (for a total of 1 min), and removed, leaving only residues produced or emitted by the stressed flies. When testing the response to the odours from non-stressed individuals, the emitters were transferred in and out of vials using a phototaxic response, instead of mechanical manipulation, leaving only residues produced by the non-stressed flies.

Performance Index (PI) The performance index (PI) is the relative response of responder flies to a vial that previously contained stressed or non-stressed flies and a control air vial, providing a measure of avoidance to dSO (Brenman-Suttner et al. 2018; Dahanukar and Ray 2011; Fernandez et al. 2014; Fernandez et al. 2017; Krause Pham and Ray 2015; Suh et al. 2004, 2007; Turner and Ray 2009). The PI is calculated by subtracting the number of responder flies in the experimental vial from the number in the air vial, divided by the total number of flies used in the assay, and multiplied by 100. In our experiments, a positive PI value indicates avoidance of the experimental vial, whereas a negative value indicates attraction. Thus, complete avoidance of the experiment vial would give a PI of 100, while a PI of 0 represents would result from an equal (50/50) distribution between the air and experimental vials and indicate no preference.

Parameters Evaluated

Age, Sex, and Mating Status We examined the effect of emitter age by testing the response of 20 3–4-day-old mated flies (15 male and 15 female) to dSO produced by 70 mixed-sex,

mated individuals that were either 2–5 days, 1, 2, 3, 4, 5, 6 or 7 weeks old.

We determined the effect of sex of mated emitter on dSO production by testing the PI of either 20 mated male or female responders to dSO from 70 male, 70 female, or 35 of each sex responders.

Brenman-Suttner et al. (2018) reported that the response of 7-day-old flies was stronger than older individuals (up to 30 days old), but as they did not examine younger flies we compared the response of 3–4- and 7–10-day-old mated individuals to dSO from 3–7-day-old emitters.

We also assessed the effect of mating status and sex of responders by measuring the PI of 15 or 30 flies to the dSO from 20 or 70 mixed-sex emitters.

Diel Periodicity To test whether the time of day affects the emission of, and response to, dSO we used adults reared under a 12:12 LD cycle in different incubators, the first with lights on at 08:00 and the other at 04:00. This allowed us to run assays simultaneously, with both 15 male and female responders being tested to the dSO from 20 mixed-sex emitters at ZT4-ZT8 or ZT8-ZT12 in their respective LD cycles.

dSO Emission and Persistence Following Stress We measured the emission of dSO at different times following stress, where 20 flies were either left undisturbed for 1 min (no stress), or vortexed with 3 bouts of mechanical agitation (as described in the section dSO Avoidance Assay), and then allowed to rest undisturbed for 10 s, 1 min, 1 or 2 h and then transferred using phototaxis into the experimental vial. They were removed after 1 min, again using phototaxis, and the vial assayed against an air control. We determined the persistence of the dSO by leaving the vials in which 20 mixed-sex flies had been removed after 3 bouts of mechanical agitation open to allow for its dissipation. Those vials were assayed against an air control, using 15 responders.

Emitter Density and Type of Stress Fernandez et al. (2014) showed that as few as 10 flies can be used as a source of dSO, but it is unknown if lower densities produce enough to elicit a response. Therefore, we examined the behaviour of 15 responders when tested with volatile cues produced by 2, 5, or 10 mixed-sex flies, or individual males and females.

To determine if the duration of stress affected the PI, we tested the response to dSO produced by 20 flies that had been exposed to 1, 2, or 3 bouts of mechanical agitation, or transferred from one vial to another by banging, compared with an air control.

We also examined the effect of different stresses by determining the PI of 15 mixed-sex responders to a vial that had contained 20 flies that were either (i) unstressed (ii) had been mechanically agitated as described above or (iii) held together without food or water for 12 h when compared with an air control.

dSO Profiles

We collected the Volatile Organic Compounds (VOCs) from unstressed and stressed adults of *D. melanogaster, D. simulans, and D. suzukii*. For each species, we transferred 100 mixed-sex 3–7-day-old flies into a 20 mL glass container and allowed them to equilibrate with the surroundings for 2 h. A sec glass vial with a silicone septum was then screwed to the lid of the first file to create a closed system. We used a technique similar to that reported by Farine et al. (2012) to collect pheromone volatiles and cuticular hydrocarbons from Drosophila, but with a 65 µm PDMS/DVB fused silica SPME fiber (Supelco Analytical, Bellefonte, PA) (Chen et al. 2017). This capillary column is not suitable for CO_2 retention, but as CO_2 has previously been determined to be an avoidance compound for *D. melanogaster*, it still allowed us to identify some of the other emitted compounds as dSO candidates.

After 30 min flies were vortexed (for a min), the 65 µm PDMS/DVB fused silica SPME fiber was immediately inserted into the septum and left for 15 min then analyzed by GC–MS. The same procedure was used to collect VOCs from non-agitated flies. There were 3 biological replicates for *D. melanogaster, D. simulans,* and two for *D. suzukii,* with three experimental replicates in each biological one.

Identification of the VOCs was performed using a 5975C inert XL EI/CI MSD with a triple-axis detector coupled to a 7890A GC system (Agilent Technologies). Compounds were desorbed at 260 °C by pulsed-splitless injection using a 0.75 mm i.d. liner (Supelco, Bellefonte, PA) onto a DB-5MSDG column (30 m × 0.25 mm, 0.25 µm). Helium was used as the carrier at a flow rate of 1 mL/min with a column head pressure of 12.445 psi (1 psi = 6.895 kPa). The oven gradient started at 35 °C for 1 min and then increasing at 10 °C/min to 260 °C where it was held for 2 min. Full scan spectra were acquired between 30 and 350 amu (1 amu = 1 g/mol) at a rate of 4.51 scans/s. Spectral features were deconvoluted and identified using Agilent MassHunter Unknowns Analysis (version B.08.00) with the Mass Spectral Search Program from the National Institute of Standards and Technology (NIST) (version 2.0 g, December 4, 2012). For each species, compounds were retained if they were present in at least 2 of the 3 experimental replicates for each biological replicate. The peak areas of retained compounds were $\log_{10}$ transformed to normalize peak areas between experiments and across species. CO_2, which has already implicated as a dSO component, was not suitably retained by this GC–MS method.

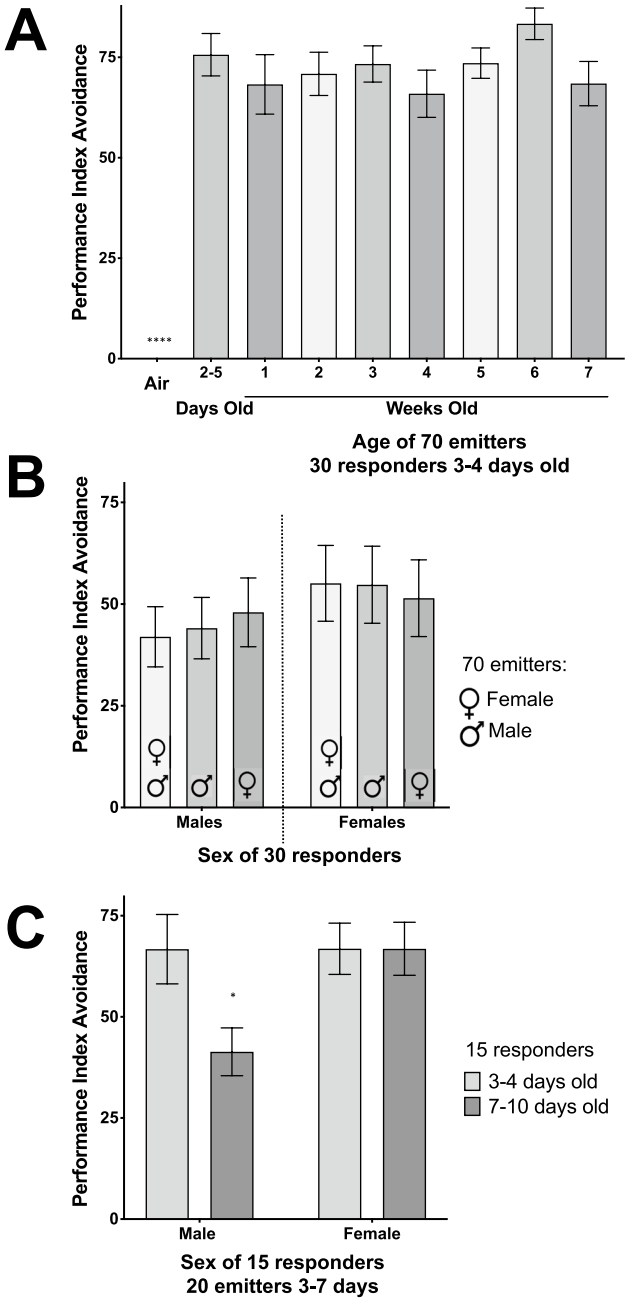

Fig. 1 The effect of age (A) and sex (B) of emitters and of responders (C) on the response of *Drosophila melanogaster* adults to dSO. (**A**) Increased age of emitters: The age of emitters had no effect on the response by the 3–4-day-old responders (70 emitters, 30 responders: One-way ANOVA $F_{8,81}=42.64$, $P<0.0001$). Emitters of all ages led to a significantly higher avoidance than the air vial (Tukey's post hoc test: $P<0.0001$ with all ages different from air, but not from each other; n = 20, 8 and 9, respectively for air, 2–5-day-old and 1–7-week-old). (**B**) Sex of responders and emitters: There was no difference in the avoidance of a dSO vial when testing 70 emitters that were males and females individually or mixed sex (Two-way ANOVA: $F_{2,43}=0.01077$, $P=0.9893$, n = 7–9). No sex effect of the 30 responders was observed (Two-way ANOVA: $F_{1,43}=1.626$, $P=0.2091$, n = 7–9). (**C**) Age of responders: Older males had a significantly lower response than younger males (20 emitters 3–7-day-old, 15 responders 3–4- and 7–10-day-old, Two-way ANOVA: $F_{1,32}=3.362$, $P=0.076$ for effect of age; Sidak's *post-hoc* test comparing age $P=0.0422$, only between old and young males. For all graphs: Bars represent the mean ± s.e.m.; * $P<0.05$, **** $P<0.0001$, n = 9 replicates for all treatments

Statistical Analysis

We confirmed that the data were normally distributed (Anderson–Darling, D'Agnotino-Pearson tests), and had equal standard deviations (Bartlett and Brown-Forsythe tests) prior to applying parametric tests and used the arbitrary alpha level of 0.05 for all statistical tests (Wasserstein and Lazar 2016). One-way and Two-way ANOVAs were used, followed by Tukey's or Sidak's *post-hoc* tests (as appropriate) to correct for multiple comparisons in GraphPad Prism (version 7.0a for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com). We used Welch's t-tests to confirm that the response to air was not significantly different from 0: as this was always the case, the values were not included.

Results

Emitters: Age and Sex

Neither age or sex of emitters affected the response of 30 mixed-sex flies to 70 2–5-day-old, or 7-week-old emitters (Fig. 1A; $F_{8,81}=42.64$, $P<0.0001$; with a post-hoc showing the only difference was with the air vial) or to 70 mixed- or single-sex emitters (Fig. 1B; $F_{2,43}=0.01077$, $P=0.9893$). As expected from the results of previous experiments, the PI of responders was not affected by their sex (Fig. 1B; $F_{1,43}=1.626$, $P=0.2091$).

Responders: Age, Sex, and Mating Status

Overall, the response of 3–4- and 7–10-day-old adults to dSO from 3–7-day-old emitters was not significantly affected by age ($F_{1,32}=3.362$), sex ($F_{1,32}=3.41$) or age by sex interaction ($F_{1,32}=3.357$; Fig. 1C) with $P>0.07$ in all cases. However, as a visual inspection of the data suggested an effect of age in males only, and the 0.07 P value was close to the arbitrary alpha cut-off of 0.05, we performed a Sidak's *post-hoc* analysis that revealed that older males had a significantly lower response than younger ones (Fig. 1C; $P=0.0422$).

Neither the sex ($F_{3,38}=28.01$, $P<0.0001$, with post-test showing the only difference was with air vial; Fig. 2A; and $F_{1,32}=0.9248$, $P=0.3434$; Fig. 2B) or mating status ($F_{1,32}=4.098$, $P=0.0514$; Fig. 2B) affected the PI of 30 responders to dSO produced by 70 emitters. We repeated the experiments measuring the response of 15 responders to 20 emitter flies (10 of each sex), as similar densities had been used in other studies (Brenman-Suttner et al. 2018). In this case, both sex ($F_{1,29}=7.191$, $P=0.0120$) and mating status ($F_{1,29}=8.088$, $P=0.0081$) significantly affected the PI of responders, with females showing a greater

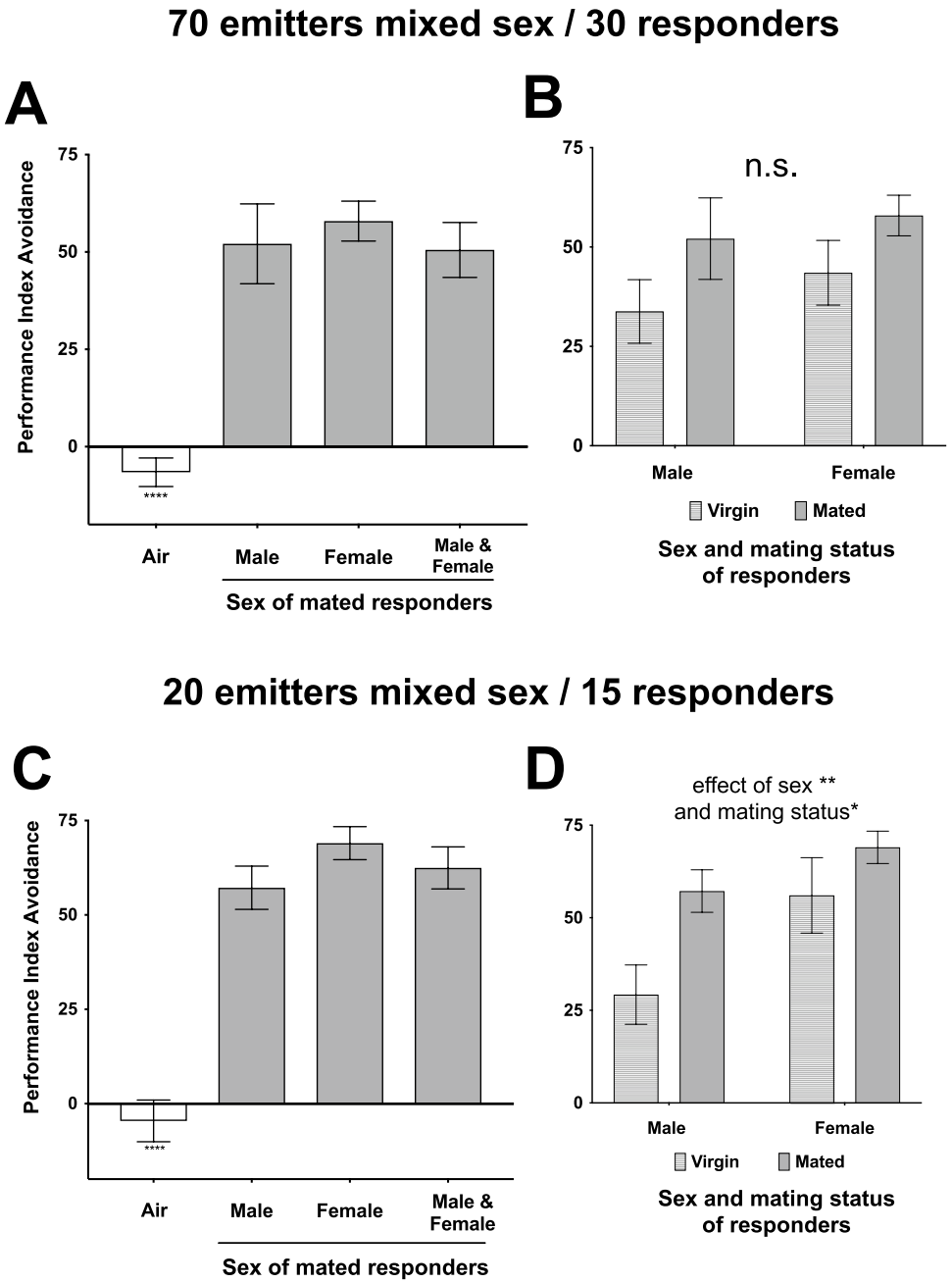

Fig. 2 The effect of sex (**A** and **C**) and mating status (**A** and **D**) on the response of *Drosophila melanogaster* adults to dSO at two different densities of emitters and responders. (**A** and **C**) Effect of sex in mated flies. Regardless of their sex, the PIs of 30 **(A)** or 15 (**C**) responders significantly differed to the vials containing dSO emitted by 70 (**A**) or 20 (**C**) emitters, compared to the vial containing ambient air (One-Way ANOVA in (**A**): $F_{3,38}=28.01$, $P<0.0001$, and in (**B**): $F_{3,32}=40.89$, $P<0.0001$, with Tukey's *post-hoc* test: $P<0.0001$, indicated by ****, in both **A** and **C**). The avoidance of the air vial itself is not statistically different from 0 (no preference displayed, one sample t-test: (**A**: $t_{14}=1.791$, $P=0.09$, **C**: $t_8=0.8271$, $P=0.4321$). However, there is a strong avoidance of dSO, with no statistical difference, when mated responder males and females were tested independently or when the sexes are mixed when tested (**A**: One-way ANOVA: $F_{3,38}=28.01$, $P<0.0001$, n = 15 for air, 9 for all other treatments; **C**: One-way ANOVA: $F_{3,32}=40.89$, $P<0.0001$, n = 9). Despite a visible trend with 70 emitters (**B**), there is no statistically significant effect of the mating status (Two-way ANOVA: $F_{1,32}=4.098$, $P=0.0514$), or sex (Two-way ANOVA: $F_{1,32}=0.9248$, $P=0.3434$), on the avoidance of dSO by the 30 responders. (**D**) With 20 emitters, the mating status of the 15 responder flies did affect their dSO avoidance in a statistically significant manner. Both male and female virgin responders displayed a lower PI index than their mated counterparts (Two-way ANOVA: $F_{1,29}=8.088$, $P=0.0081$). Sex of the responder flies also affected their dSO avoidance. The females displayed a higher avoidance of dSO than males in both the virgin and mated treatments (Two-way ANOVA: $F_{1,29}=7.191$, $P=0.0120$). For all graphs: Bars represent the mean ± s.e.m.; ns = not significant; $*P<0.05$, $**P<0.01$, $***P<0.001$. (**A**) and (**B**) are from the same experimental data set, (**C**) and (**D**) are from the same experimental data set: different comparisons were performed among groups

response than males and mated individuals of both sexes being more responsive than virgins (Fig. 2D).

Based on the results above and those of Fernandez et al. (2014), in the following experiments, we tested two combinations: 30 responders and 70 emitters (slightly stronger avoidance compared to 20 emitters, although the difference was not statistically significant; Fernandez et al. 2014), or 20 emitters and 15 responders (more sensitivity as shown in Fig. 2).

Time of Day

The time of day (Fig. 3A) did not affect either the emission of, or response to, dSO (Fig. 3B; $F_{3,64}=0.3224$, $P<0.8091$), although in all cases females had significantly higher responses than males (Fig. 3B; $F_{1,64}=8.084$, $P<0.006$).

dSO is Volatile and Emitted for a Limited Time

The level of avoidance behaviour observed by both sexes was significantly affected by the time elapsed between generating the dSO source and testing (Fig. 4A; $F_{2,60}=41.43$,

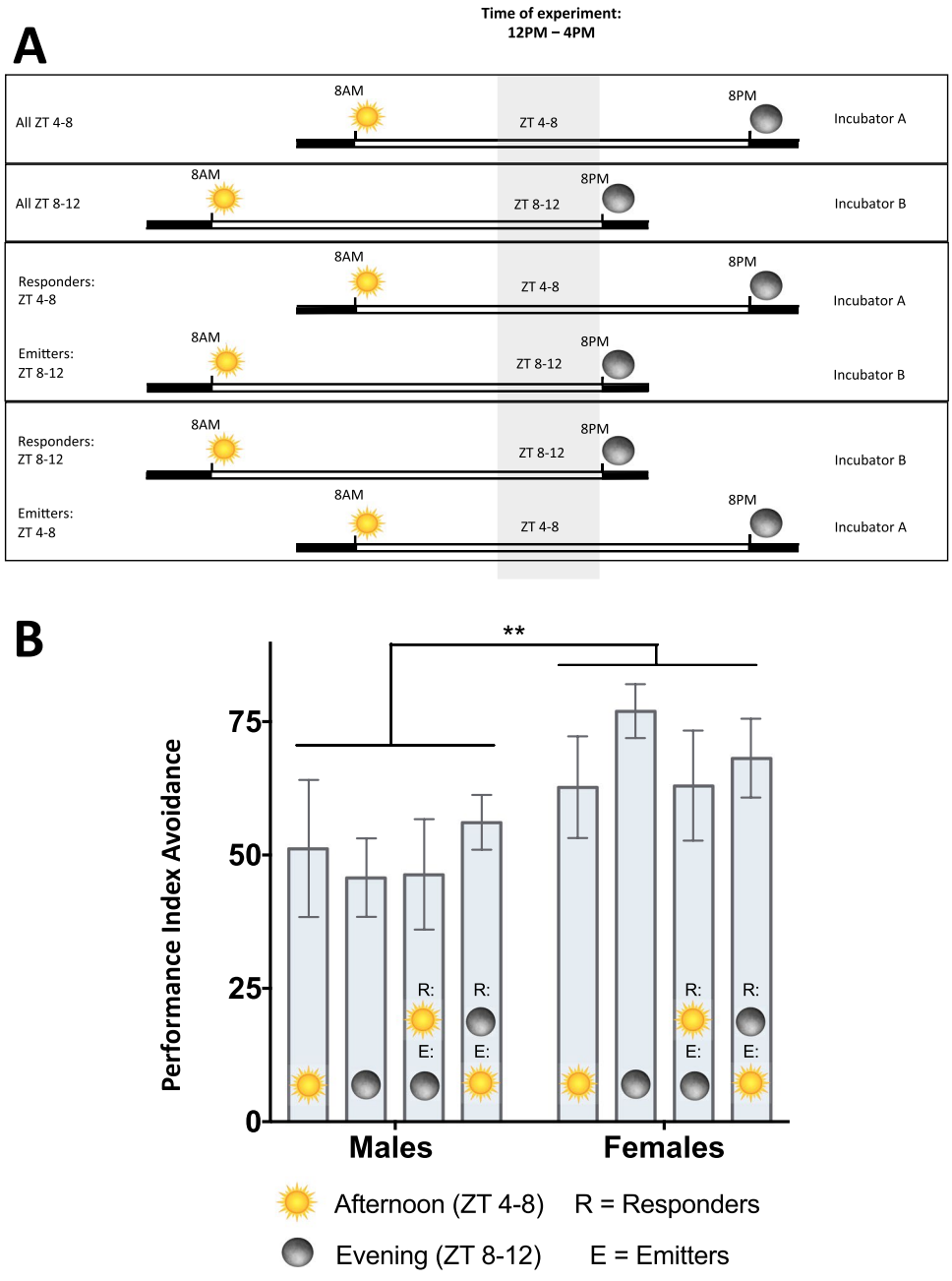

Fig. 3 The effect of the time during the photophase that emitters and responders are tested on the response of *Drosophila melanogaster* adults to dSO. (**A**): Experimental design used to determine the potential effect of time of day (ZT4-8 and ZT8-12) on both the emission and reception of dSO by *D. melanogaster*. The experimenter performed all of the experiments at the same time, indicated here as Eastern Standard Time, in shaded grey. The time of the photophase when the flies were tested is indicated as ZT (Zeitgeber): time after which the lights were on. To allow testing different parts of the photophase (light–dark cycle) of the flies simultaneously, two incubators were used (A and B), and their photophase was offset by 4 h, as indicated in the diagram. (**B**): Time of day has no influence on the emission of or response to dSO (Two-way ANOVA: $F_{3,64}=0.3224$, $P<0.8091$) in 3–7-day-old flies. The females displayed a higher avoidance of dSO than the males indicating a sex effect (Two-way ANOVA: $F_{1,64}=8.084$, $P<0.006$). Bars represent the mean ± s.e.m.; ** $P<0.01$, and n = 9 replicates of 20 emitters and 15 responders for all treatments

P < *0.0001*). Both males and females showed a significant level of avoidance when exposed to a dSO source 1 min after it was generated but not after 2 min, indicating the olfactory cue is very volatile. However, both sexes emit dSO for at least 1 h following agitation (Fig. 4B; $F_{4,80}=36.14$, $P<0.0001$), with females having significantly higher PIs than males ($F_{1,80}=6.446$, $P<0.0001$).

Emitter Density

There was no difference in the responses to either sex ($t_{16}=0.3575$, $P=0.3627$) so the data were pooled. The PI of responders was not significantly affected whether 1 or 10 emitters were used to generate the odour cue (Fig. 5A; $F_{4,92}=4.2$, $P=0.0038$).

dSO is Emitted Under other Stressful Conditions, Including Simple Transfer and Occupancy of an Empty Dry Vial for 12 h

Just the mechanical transfer of flies from one vial to another results in the same level of avoidance by responders as vortexing emitters one to three times in a min, all of which were significantly higher than the air vial (Fig. 5B; $F_{4,45}=29.45$, $P<0.0001$).

A significant difference was also observed in the response to unstressed flies (transferred using their response to light) compared to those stressed mechanically for one min or held without food/water for 12 h (Fig. 5C; $F_{3,118}=101.8$, $P<0.0001$). Again, female responders showed a significantly higher avoidance than males (Fig. 5C; $F_{1,118}=7.257$, $P=0.008$).

The Avoidance of dSO is not Species-Specific

Drosophila melanogaster, D. simulans, and *D. suzukii,* adults all exhibited some level of response to odours from both conspecific and heterospecific sources, but there were significant interspecific differences (Fig. 6; $F_{8,71}=10.5$, $P<0.0001$). For example, the level of response observed for either *D. melanogaster* and *D. suzukii* did not differ between the three sources, but the PI for *D. melanogaster* was always significantly higher than *D. suzukii* (main effect of responders – $F_{2,71}=33.08$, $P<0.0001$, Tukey's *post-hoc* test comparing differences in performances for responders regardless of emitters $P \leq 0.0013$). *Drosophila simulans* was the only species that showed the highest response to the conspecifics odour source, although the response to heterospecific cues was only significant for those from *D. suzukii* (Tukey's *post-hoc* test, $P<0.05$).

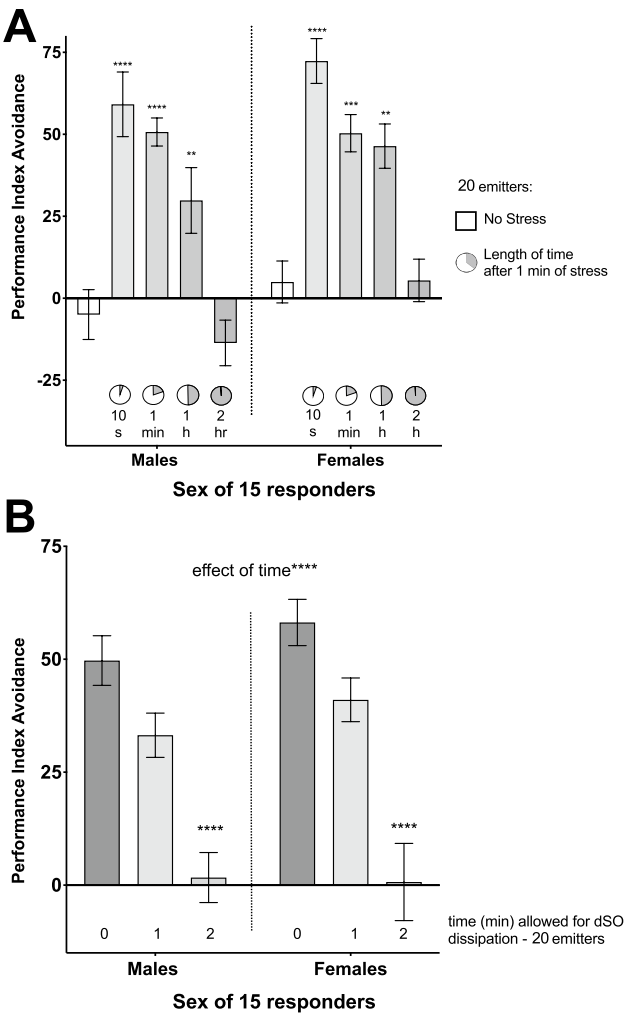

Fig. 4 dSO dissipates quickly, but its emission lasts for up to 1 h. (**A**) Avoidance of vials that had contained 20 emitter flies that had been stressed 10 s, 1 min, 1 or 2 h prior to occupying the stress vial. The 15 responder flies strongly avoid vials that had been occupied by flies that were stressed by mechanical agitation 10 s, 1 min, or 1 h prior to occupying the stress vial for 1 min. However, the responder flies do not display any preference when presented with the choice between an empty vial or a vial that has been occupied by flies that have not been stressed, or that were mechanically agitated 2 h prior to occupying the vial (Two-way ANOVA, effect of time duration after stress: $F_{4,80}=36.14$, $P<0.0001$, Tukey's *post-hoc* comparison to unstressed indicated on graph). Females had a higher avoidance than males in all treatments (Two-way ANOVA, effect of sex: $F_{4,80}=6.446$, $P<0.0001$). (**B**) Avoidance of vials that had contained 20 emitter flies mechanically agitated for 1 min, but were left in the open air for increasing time. The 15 responders did not avoid the stress vial when it was left open for 2 min to the point where it was not significantly different from 0 (Two-way ANOVA – main effect of length dissipation time: $F_{2,60}=41.43$, $P<0.0001$, One sample t-test – difference from 0: $t_8=0.08186$, $P=0.9368$) Tukey's *post-hoc* test comparison to 0 min indicated on graph. For all graphs: n = 15 flies 3–7-day-old for 0 min of dissipation time, n = 9 for both 1 and 2 min of dissipation time, and bars represent the mean ± s.e.m.; **** $P<0.0001$

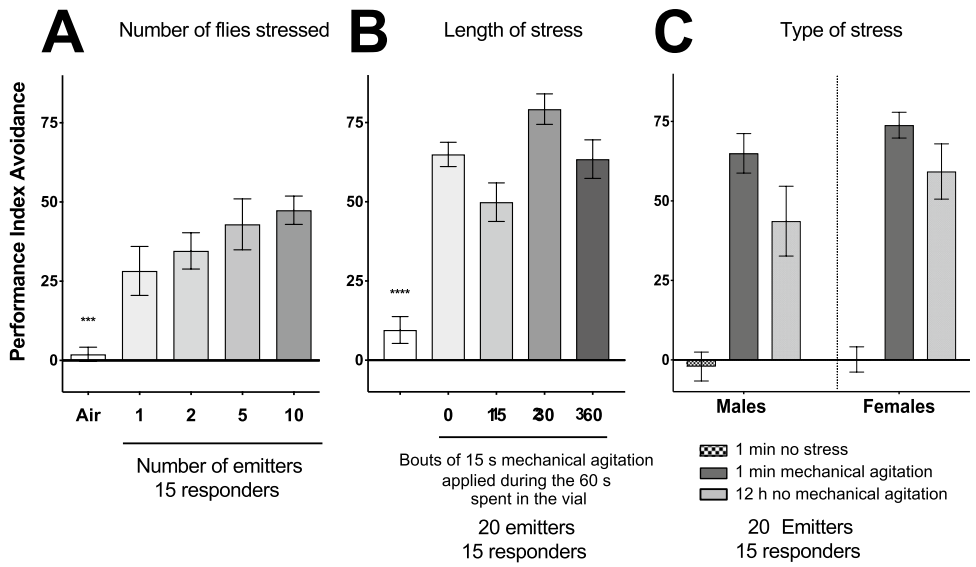

Fig. 5 The effect of density and the form of stress emitters were subject to on the response of *Drosophila melanogaster* adults to dSO. (**A**) Number of emitters: All treatments had a significantly higher PI than the air control, and are not different from each other (One-way ANOVA and Tukey's *post-hoc* test: $F_{4,92}=4.2$, $P=0.0038$, n = 13, 16, 13, 11 and 15, respectively for air, 1, 2, 5 and 10 emitters). Number of emitters is indicated, and there were 15 responders. (**B**) Avoidance of vials occupied 1 min by emitters mechanically agitated for different length of time: All times of mechanical agitation of the 20 emitters lead to a significantly higher avoidance by the 15 responders than that of the air vial (One-way ANOVA and Tukey's *post-hoc* test: $F_{4,45}=29.45$, $P<0.0001$, n = 12, 10, 9, 8 and 11, respectively for air, 0, 1, 2 and 3 bouts of agitation, with 5 s rest in between), with no statistical difference between no agitation – just transfer (=0 bouts of agitation) – or 3 bouts of 15 s of mechanical agitation (Tukey's *post-hoc:* $P=0.9995$). (**C**) Avoidance of vial occupied for either 1 min by emitters stressed by mechanical agitation, or 1 min by non-mechanically agitated emitters, or for 12 h by non- mechanically agitated emitters. Responders had no preference to a vial that has been previously occupied by flies that were non-mechanically agitated and had spent 1 min in the vial (see Results section for details on "non-mechanically agitated"). However, if those non-agitated flies spent 12 h in the vial, responders avoided that vial, although not as strongly as their avoidance of vials in which flies had been agitated (Two-way ANOVA: main effect of stress $F_{2,84}=84$, $P<0.0001$, comparison of two types of stress—Tukey's *post-hoc* test*:* $P=0.0183$). 15 responder and 20 emitter flies, n = 9 repeats of biological replicates. For all graphs: 3–7-day-old flies were used in each trial. Bars represent the mean ± s.e.m.; *** $P<0.001$, **** $P<0.0001$

dSO Profile is Species Specific

A total of 54 compounds were putatively detected across all species and treatments and major differences in the volatile profiles of stressed and unstressed flies were observed, but there was only minimal overlap in the compounds detected from the different species (Fig. 7). Branched and aliphatic alkanes, particularly those containing less than 20 carbons ($C_n<20$) were found in the volatiles of stressed flies, regardless of species (Fig. 7).

Dicussion

Our results support the previous finding that whenever they are subjected to different types of stress, *D. melanogaster* adults emit olfactory cues that affect the behaviour of conspecifics (reviewed in Dahanukar and Ray 2011). In part, this is probably the result of general physiological changes, as bees that had been vortexed also increase the production of CO_2 (Bateson et al. 2011), a major component of dSO (Suh et al. 2004). Our findings also suggest that the techniques frequently used to transfer flies in experiments examining many different aspects of *Drosophila* biology result in the release of dSO. Thus, while the olfactory cue dissipates quickly (Fig. 5B), stressed flies may continue to emit for at least an hr (Fig. 5A). As this could be an important confounding factor we would suggest that anyone researching *Drosophila* behaviour use a non-mechanical approach when transferring flies, such as the light response we used here, or negative geotaxis, as proposed by Trannoy et al. (2015).

The response to alarm signals is contextual, probably associated with trade-offs between the associated costs and benefits related to future reproduction, as clearly shown in the responses of different stages of the green peach aphid to (*E*)-β-farnesene (Montgomery and Nault 1978). This is also the case for *D. melanogaster* responding to dSO. For example, while both virgin and mated individuals would benefit by leaving and thus avoid potential danger, virgins leaving a site with conspecifics could decrease their chances of acquiring a mate, which is important given the mean longevity

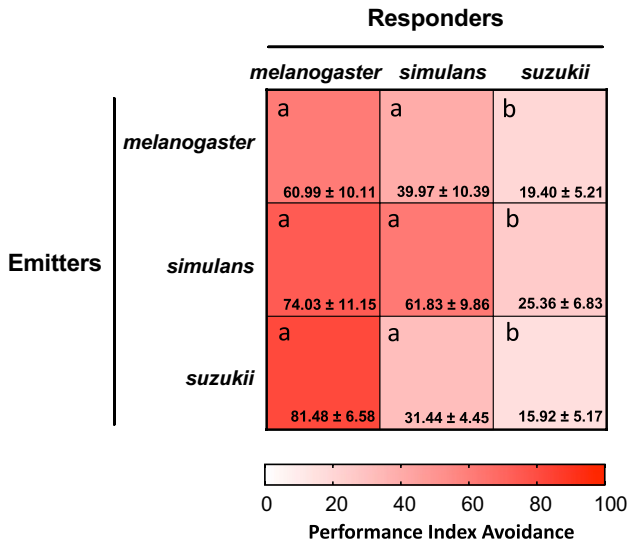

Fig. 6 A heat map comparing the response of *Drosophila melanogaster, D. simulans* and *D. suzukii* adults to the dSO emitted by conspecific and heterospecific adults. *Drosophila melanogaster* avoided dSO emitted by all species, including itself. *D. simulans* and *D. suzukii* also avoided dSO emitted by its own and the other two species (Two-way ANOVA: main effect of responders $F2_{,\ 71} = 33.08$, $P < 0.0001$). Numbers in the lower right corner of each treatment are mean ± s.e.m. Treatments with the same letter in the upper left corner are not statistically different from each other in Tukey's *post-hoc* tests; n = 12 replicates of 15 responder and 20 emitter flies for *D. melanogaster* emitters with *D. suzukii* responders and *D. suzukii* emitters with *D. melanogaster* responders, n = 8 replicates of 15 responder and 20 emitter flies for all other treatments

of *Drosophila* adults under field conditions is 3–7 days (Rosewell and Shorrocks 1987).

Furthermore, the results from several of our experiments show that mated males exhibit lower response levels than mated females of the same age (Figs. 2B, 3B, 4), which again could relate to aspects of reproductive success. The more mates a male acquires, the higher his potential lifetime reproductive output (e.g. Royer and McNeil 1993), albeit up to a point (Douglas et al. 2020), thus a lower response to dSO might increase his chances of encountering another receptive female. On the other hand, by leaving a dangerous site mated females may reduce short-term oviposition opportunities but would increase future oviposition opportunities. We cannot eliminate the possibility that mated females are also responding to increased levels of CO_2, a component of dSO, as increased infection rates, decreased survival, and/or abnormal development of progeny may result from high concentrations of CO_2 (also known as hypercapnia – Azzam et al. 2010; Nicolas and Sillans 1989; Sharabi et al. 2009). Finally, the absence of an age-related response to dSO by females (Fig. 1C) is not surprising, for although the highest daily egg output occurs during the first week (Tatar et al. 1996), moving away from potential danger or high concentrations of CO_2 could extend future opportunities to oviposit. In contrast, mating opportunities for males decline with age (see Ruhmann et al. 2018), so a lower response by older males (Fig. 1C) would be expected given they have less to lose with respect to future reproduction opportunities than younger individuals.

Physiological and behavioural changes relating to age (see reviews by Brenman-Suttner et al. 2019 and Kumar Chaudhary and Rizvi 2019) and mating (Miller et al. 2014; Ruhmann et al. 2018) have been well documented in *D. melanogaster.* Thus, age-related changes observed in the responses to dSO (our results Fig. 1C, Brenman-Suttner et al. 2018) or between virgin and mated flies (Fig. 2B, D) could also involve changes in the sensitivity of the olfactory system reported with mating (Ziegler et al. 2013), and age (Cook-Wiens and Grotewiel 2002). However, the lack of effect of age on the emission of dSO suggests that, as other behaviours underlying escape of danger (Simon et al. 2006), dSO avoidance might be more robust to the effect of aging.

Our results clearly show that all three species produce alarm cues (Fig. 6) and that each one responds to both conspecific and heterospecific volatiles. However, there are marked differences as *D. melanogaster* and *D. simulans* showed high responses to both conspecific and heterospecific cues, whereas *D. suzukii* exhibited low responses regardless of the source. This pattern is similar to the interspecific responses to CO_2, for while *D. melanogaster* and *D. simulans* avoid high levels of CO_2, *D. suzukii* does not (Krause Pham and Ray 2015). This may be related to differences in foraging behaviour, as *D. suzukii* prefers younger fruits that emit higher levels of CO_2 (Krause Pham and Ray 2015). Therefore, the high avoidance observed for *D. melanogaster* and *D. simulans* probably involved a response to both CO_2 and other components of the dSO, whereas *D. suzukii* only responds to the other components. Furthermore, the benefit gained by responding to a rapidly dissipating conspecific alarm cue would be of limited value to *D. suzukii*. Females attack fresh fruit so the abundance and distribution of available feeding/oviposition sites under natural ecological conditions would result in low spatial densities of conspecific adults. Similarly, even if present in the same habitat with *D. melanogaster* and *D. simulans*, due to their feeding and oviposition behaviours they would not be in close proximity, and thus one would not expect a high response of *D. suzukii* to heterospecific danger cues. In contrast, *D. melanogaster* and *D. simulans* adults feed on fermenting fruits, resulting in higher densities and a greater probability of detecting dSO, so responding to intraspecific cues indicating a nearby source of danger would be advantageous. In addition, when *D. melanogaster* and *D. simulans* co-exist on the same food source they form species-specific clusters but are often in close proximity (centimeters apart) (Soto-Yeber et al. 2018). This suggests neither species avoid unstressed

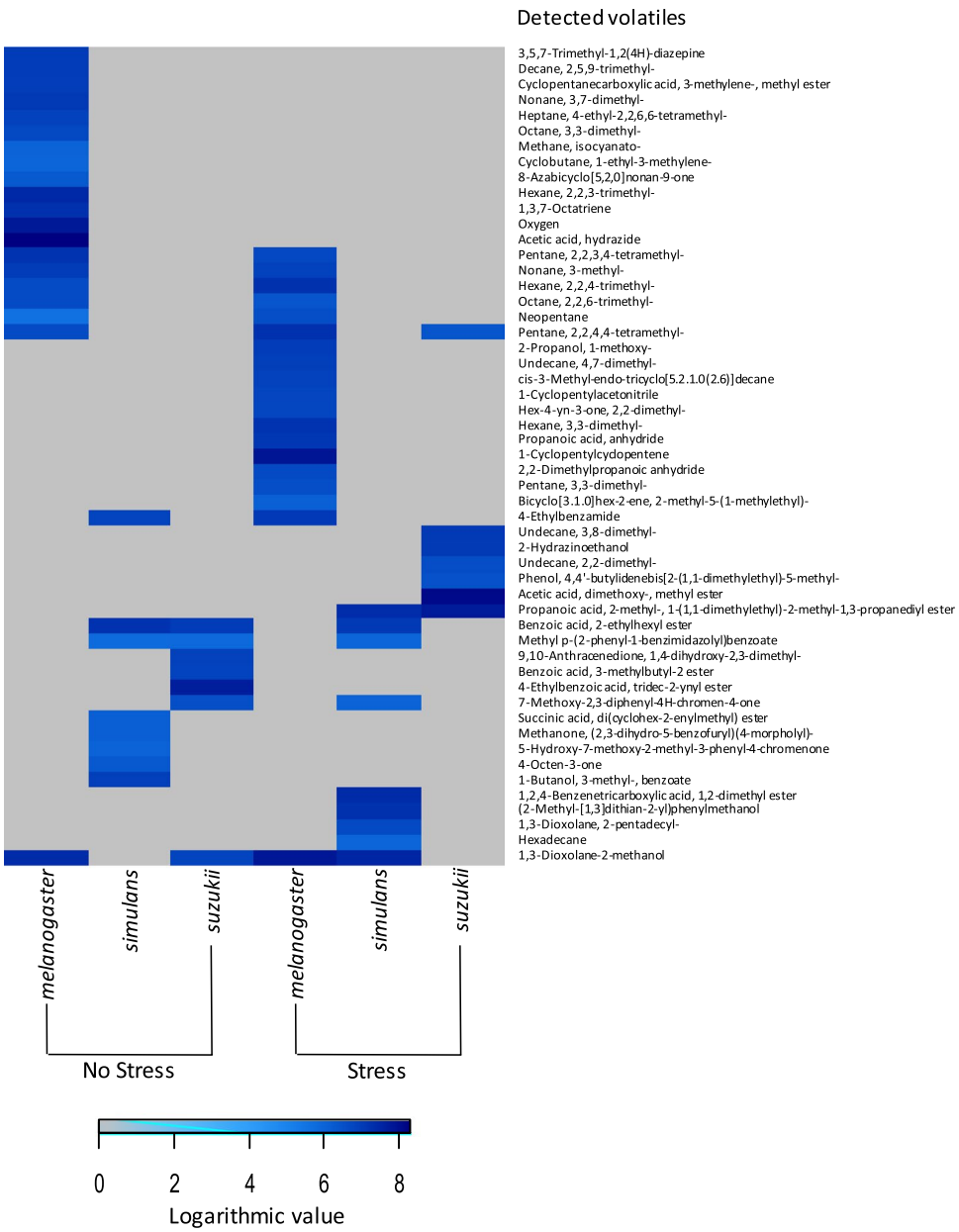

Fig. 7 A heat map comparing the normalized dSO volatiles emitted by *Drosophila melanogaster*, *D. simulans* and *D. suzukii* under stressed and non-stressed conditions. The volatiles emitted from *D. melanogaster*, *D. simulans* and *D. suzukii* were collected by SPME and analyzed by GCMS (NIST mass spectral database, version 2.0) in both unstressed and mechanically stressed conditions. A clear distinction is observed between stressed and unstressed in all three species. However, only a limited overlap across species was observed. Detected volatiles included smaller and branched alkanes ($C_n < 20$). In total there were 32, 15, and 14 compounds detected in *D. melanogaster*, *D. simulans* and *D. suzukii,* respectively. Color intensity corresponds to the $\log_{10}$ of the detected component peak areas in the GCMS chromatogram

heterospecifics, but given the proximity, they would benefit from responding to interspecific danger signals.

One would expect a higher response to conspecific rather than heterospecific cues if dSO contained other compounds in addition to CO_2 as proposed by Suh et al. (2004) and Enjin and Suh (2013). However, this was only observed in *D. simulans* (Fig. 6), even though there are marked intra- and interspecific differences between unstressed and stressed *D. melanogaster* and *D. simulans* (Fig. 7). Further research is required to determine if the high responses to heterospecific dSO are just modulated by CO_2 or if there are other common components.

Flies can release cuticular hydrocarbons into the air (Farine et al. 2012) and are detected through either olfaction or taste (Ferveur 2005) and the volatiles collected from each species following stress contained alkanes ranging from the C5 – C16 (Fig. 7). There were few shared compounds at the interspecific level, but similar sized classes of alkanes were found. However, as cuticular hydrocarbons have an important role in mating, including avoidance of heterospecifics (Billeter et al. 2009), the compounds released are sufficient to result in the observed heterospecific responses.

Additional research is required to identify which compounds, produced by each species, affect avoidance behaviours. This study provides information that would be useful when establishing protocols for further analyses, including using different collection methods. The list of compounds we identified is not exhaustive, and we cannot exclude the

fact that other important dSO volatiles might not have been retained. We used a type of capillary column that does not efficiently separate permanent gases and more polar compounds. For example, the ion of CO_2 (identified as a dSO component – Suh et al. 2004) was detected in the "void volume", however, there was no peak resolution.

We now know that dSO emission is not affected by age, sex, or time of the day, but that it is produced in response to simple mechanical agitation (Fig. 5B), similar to the release of alarm cues by agitated zebrafish (Mathuru et al. 2012) and does not require vortexing or electric shocks (Suh et al. 2004). Responder flies also avoided vials where 20 unagitated flies had been held without food or water for 12 h (Fig. 5C), which could have resulted in the release of dSO, although there may be other metabolism by-products resulting from starvation. Under such conditions, conspecifics would avoid sites unsuitable for feeding or oviposition, similar to the release of volatile cues by starved mosquito larvae that renders the site unattractive to females as an oviposition site (Zahiri et al. 1997).

While dSO is a short-lived volatile cue (Fig. 4B), flies emit dSO for up to 1 h following stress (Fig. 4A), similar to the release of (*E*)-β-farnesene by stressed aphids (Vandermoten et al. 2012). Although the amount of dSO emitted increases with fly density (Fernandez et al. 2014), the response to one stressed fly elicits the same avoidance response as 10 individuals, so one could potentially examine individual variability in emission as a function of age, mating status, or the level/type of stress experienced.

Once the exact composition of the dSOs produced by different species has been determined, it would be possible to evaluate their potential as a component of pest management programmes. While it could have potential against species, at least for *D. melanogaster* as our results suggest such an approach would not be effective against *D. suzukii.* Clearly, different aspects of the habitat would need to be considered as canopy cover affects factors such as light intensity, temperature, and humidity (Soto-Yeber et al. 2018) which, in turn, would affect the distribution of adults.

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Author Contributions Data acquisition and analysis were carried out by RTY, EL, ISM, SC, ISM, TM, AFG and MS; Protocols elaborated by TM, AFS and JNM; the first draft was written by RTY, EL and AFS and the final draft edited by RTY, TM, AFS and JNM.

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Data Availability Upon request

Declarations

Ethical Note No approval is required from the Western's Animal Care Committee or the Provincial and Federal regulatory bodies to study invertebrates. However, we provided appropriate rearing conditions and anesthetized flies using CO_2 or cold anaesthesia when manipulating flies for colony maintenance. Stress treatments to produce dSO resulted in no mortality.

Preprint Finally, the behavioural portion of this manuscript can be found on bioRxiv—a preprint server for biology, operated by Cold Spring Harbor Laboratory: Yost et al. (2021) Is *Drosophila melanogaster* Stress Odorant (dSO) really an alarm pheromone? bioRxiv:534719 doi:10.1101/534719.

Conflicts of Interest None

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