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Oviposition by the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), on five citrus types in a laboratory

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Abstract

Frugivorous insects use visual, chemical and tactile cues to find a suitable host for oviposition. However, these cues can vary greatly among fruit cultivars and condition, changing their susceptibility to fruit fly oviposition. The aims of this study were to (1) determine the effects of ripeness stage and damage on oviposition propensity by sexually mature, mated female oriental fruit flies, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), on five citrus types (species and cultivars) under choice and no-choice conditions and (2) describe the oviposition behaviour of *B. dorsalis* on ripe fruit of the same five citrus types that were either damaged or undamaged under no-choice conditions. All tests were conducted in the laboratory. The citrus types tested were *Citrus sinensis* (L.) Osbeck cv Delta Valencia orange, *Citrus sinensis* (L.) Osbeck cv Glen Ora Late navel orange, *Citrus limon* (L) (Burm.f.) cv Eureka lemon, *Citrus paradisi* (Macfad.) cv Star Ruby grapefruit and *Citrus reticulata* (Blanco) cv Nadorcott mandarin. Peel physical properties and essential oil composition were determined for each citrus type and stage. Oviposition propensity of *B. dorsalis* was significantly greater on damaged citrus but was not correlated with fruit diameter, peel thickness, oil gland density or oil gland size. A total of 45 aromatic compounds were found to be significant between the five cultivars investigated, and 6 of 11 compounds were significantly associated with over-ripe fruits. *Bactrocera dorsalis* spent a significantly greater proportion of time ovipositing in damaged citrus and showed higher aggression when oviposition occurred in undamaged citrus. These results suggest that the removal of damaged and fallen fruit is important for controlling this pest in citrus orchards.

KEYWORDS
essential oil, ethogram, fruit damage, fruit phenology

INTRODUCTION

Many intrinsic and extrinsic factors influence frugivorous insect acceptance of fruit as a host before oviposition occurs. These include quality and availability of hosts, egg load of the female and presence of other larvae in the fruit (Aluja & Boller 1992; Diatta et al. 2013; Fitt 1984; Minkenberg et al. 1992; Prokopy et al. 1994). Insects use specific response-inducing visual and chemical stimuli from fruit to select a suitable fruit for oviposition (Levinson & Haisch 1984; López-Guillén et al. 2009; Piñero et al. 2006). In addition, an insect must overcome physical and chemical defences of the fruit in order for oviposition to occur (Balagawi et al. 2005; Díaz-Fleischer & Aluja 2003; Seo et al. 1982; Sturm et al. 2003; Van Mele et al. 2009). The physical and chemical cues of a fruit are not static in

time, with implications for oviposition site location and selection (Cugala et al. 2014; Rattanapun et al. 2009). Fruit ripening is associated with a change in colour, shape and size, as well as in volatiles emitted by the plant and fruit (Al-Maiman & Ahmad 2002; Brown & Miller 1999; Cugala et al. 2014; Jain et al. 2001; Medlicott & Thompson 1985). The chemical environment within the fruit further changes during ripening as metabolic pathways increase the amount of free sugars, alter the pH and change the properties of the outer layer or peel (Al-Maiman & Ahmad 2002; Camacho-Vázquez et al. 2019; Sturm et al. 2003).

Among the true fruit flies (Diptera: Tephritidae), various species use host plant volatiles to locate oviposition sites on fruit (e.g., Fein et al. 1982). Green leaf volatiles may be used to find a host in a mosaic of different plants and for long-distance host detection (Light et al. 1988). However, fruit volatiles need to be present in specific ratios to stimulate host acceptance (Jang et al. 1997). Physical barriers of the peel, toxic oils present in the peel and defences induced by damage or oviposition may deter females from laying eggs (Diatta et al. 2013; Papachristos et al. 2008). Mango (*Mangifera indica* L.), which is a preferred host for some fruit flies, still poses challenges for oviposition (Migani et al. 2014) as a result of its physical characteristics such as pericarp toughness and ripeness (Rattanapun et al. 2009). Resin present in the skin of unripe mangoes may also force deposited eggs out of the fruit (Rattanapun et al. 2009).

Citrus is an even less favourable host for tephritid larval survival or development (Greany et al. 1983; Lloyd et al. 2013). This is largely due to the oils in the flavedo region of the peel being toxic to larvae and the physical defences of the albedo region, which inhibit the growth of fruit fly immatures (Papachristos et al. 2008). Eggs deposited in the pulp of citrus are able to develop, although poorly in comparison with mango and guava (*Psidium guajava* L.) (Papachristos et al. 2008; Papachristos & Papadopoulos 2009; Rattanapun et al. 2009). However, damage to the peel of citrus can attract fruit flies and assist oviposition into the pulp region (Diatta et al. 2013; Theron et al. 2017). Many changes occur in citrus peel during fruit maturation. In brief, the peel grows from the equator of the fruit (Iglesias et al. 2007). As the fruit grows in size, oil glands migrate from the equator to either end of the fruit (Knight 2001). During this period, the cells produce oil, filling the oil glands with a unique volatile blend (Knight 2001; Shaw 1979). At the same time, the peel colour changes from green, to yellow, to orange (depending on type), in response to hormones, nutrient availability and environmental conditions (Huff 1983; Iglesias et al. 2007).

Bactrocera dorsalis (Hendel), an invasive fruit fly of Asian origin with a wide host range, has expanded its distribution across the world, more specifically in Africa and Indian Ocean region, occupying natural and cultivated habitats (De Meyer et al. 2010; Zeng et al. 2018).

Bactrocera dorsalis was declared established in the north and north-eastern areas in South Africa in 2013 (Manrakhan et al. 2015) and has since been listed as a pest of some citrus types in the country. To develop effective management strategies of this pest on citrus, an in-depth knowledge of its biology and interaction with the crop is required. For this reason, there is a need to determine the susceptibility of certain citrus types to attack from *B. dorsalis*. In comparison with other fruit fly species, there is little work published on the cues used for host searching and acceptance by *B. dorsalis*, despite many publications showing the pest status of *B. dorsalis* on over 40 economically important commercial fruit species (Drew & Hancock 1994; Iwaizumi 2004; Mwatawala et al. 2006; Rwomushana et al. 2008). Currently, it is known that *B. dorsalis* (Hendel) responds strongly to yellow and white spheres (Vargas et al. 1991). Also, it responds to plant volatiles from ripe guava (*Psidium guajava*), starfruit (*Averrhoa carambola* L.), strawberry guava (*Psidium cattleianum* L.), orange (*Citrus sinensis* L.), papaya (*Carica papaya* L.), mango (*Mangifera indica* L.) and tropical almond (*Terminalia catappa* L.) (Cornelius et al. 2000; Jang & Light 1991; Kamala Jayanthi et al. 2014; Siderhurst & Jang 2006). Additionally, observations of citrus infestation in the northern regions of South Africa suggested that fruit showing signs of damage were more likely to be infested (Theron et al. 2017).

The aim of this study was to determine the effect of citrus type, ripeness stage and fruit damage on the likelihood of *B. dorsalis* oviposition under choice and no-choice conditions in the laboratory. Physical and chemical characteristics of the peel of each type were quantified to provide potential reasons for differences in oviposition propensity. This was complemented by observing the behaviours of gravid female *B. dorsalis* associated with oviposition site selection in the five citrus types at picking ripeness, in damaged and undamaged fruits, under laboratory conditions. It was anticipated that the physical and chemical defences of citrus types would lead to them being less susceptible to oviposition by *B. dorsalis* (relative to a control) but that fruit damage would bypass these defences.

MATERIALS AND METHODS

Source and treatment of flies

Fruit flies were obtained from the wild by using a protein bait for females (three-component BioLure, Chempac (Pty) Ltd, Suider Paarl, South Africa) and methyl eugenol (Farma Tech International Corp, North Bend, WA, USA) for males. Baits were placed in insecticide-free Chempac bucket traps. Traps were placed in various fruiting trees (mango, Kei apple [*Dovyalis caffra*], orange and guava) in private residential and commercial properties within short driving proximity of Citrus Research International (CRI). An

insect aspirator was used to extract live flies from the traps into a small, transparent sample vial. Then, the sample vials were placed inside a larger ventilated, sleeved container and inverted to release the flies. Moist cotton wool was used to provide water to the wild-caught flies during transport back to the research facility. These were used to start a laboratory culture at CRI, Nelspruit, South Africa. The culture was refreshed with wild flies continuously up to the 10th generation to boost colony numbers and maintain genetic variance similar to the natural population.

Flies used in this study were taken from the culture after being maintained in the laboratory for 25 generations. Eggs were collected using a pierced apple (*Malus domestica* Borkh. cv Golden Delicious). Apples were pierced with an entomological pin making several lines of holes around the apple. Afterwards, each line of holes was removed as a segment from the apple and carefully opened, exposing the eggs, which were brushed and washed out with filtered water. This solution, collected in small containers, was left to stand for a few minutes. Viable eggs were reared on a carrot-based diet specifically formulated by CRI to rear larvae. Larvae emerged from the media and pupated in washed, sterilised river sand, whereafter they were collected twice a week to form age-matched cohorts. Each cohort comprised 20 mL of pupae. Cohorts were maintained in ventilated plastic containers with unlimited access to food (4:1 sugar and yeast hydrolysate enzymatic [Amberex 1003, Juneau, AK, USA]) and water. A pilot study conducted prior to these experiments showed that females were mated and most likely to oviposit on fruit from 21 days of age. All flies used in these experiments were maintained under natural lighting conditions at $22.38 \pm 0.02°C$, until they were used for experiments.

Fruit collection

Experiments were conducted on five citrus types (species and cultivars): *Citrus sinensis* (Osbeck) cv Delta Valencia, *Citrus sinensis* (Osbeck) cv Glen Ora Late navel, *Citrus limon* (L) (Burm.f.) cv Eureka, *Citrus paradisi* (Macfad.) cv Star Ruby and *Citrus reticulata* (Blanco) cv Nadorcott. All citrus fruit were harvested directly from orchards and did not go through any packhouse procedures. Fruit were harvested at four different colour stages, based on the Colour Prints for Blemish and Appearance Standards (CRI 1997), and endorsed by the Department of Agriculture Forestry and Fisheries, South Africa. The colour sets range from one to eight, with one being the colour at full ripeness and eight the colour of fully green fruit. Four ripeness stages were harvested: green (colour set 8–7), colour break (colour set 5–4), ripe (colour set 3) and senescence (colour set 1). Fruit were provided by various growers throughout Mpumalanga and Limpopo provinces, South Africa. Fruit were transported back to CRI

directly after harvest. A sample of 10 fruit from each citrus type for each ripeness stage was placed separately in the climate room to determine natural infestation. No pests emerged from these fruit. Fruit were dipped in a chlorine solution (100 ppm) for 3 min, replicating the standard drench solution in a pack line, to remove dust and prevent fungal growth. Fruit used for the study were stored at $9.50 \pm 0.01°C$ to prevent cold damage to lemons and the citrus at green and colour break. The citrus at green and colour break showed cold damage when stored at the recommended $4°C$. Fruit were placed in a climate room ($26.26 \pm 0.04°C$, $79.33 \pm 0.18\%$ relative humidity) in a ventilated container 24 h prior to experiments. Apples (*Malus domestica* cv Golden Delicious) were purchased from a supermarket and used in experiments as a positive control.

Citrus peel physicochemical properties

A reference sample of each fruit type and ripeness stage was used to determine the fruit diameter, flavedo and albedo thickness, and oil gland density and size. Fruit diameter was measured with a Vernier calliper at the equator. A thin slice of peel was cut with a surgical scalpel down the length of the fruit so that the equatorial region was situated in the centre of the slice. A 1-cm square was cut from the equatorial region of the peel slice. From the square, a 1-mm-thick slice was made through the flavedo layer exposing both oil glands near the surface and those slightly deeper in the flavedo. This thin 1-cm square of flavedo was used to count the number of oil glands under dissection microscope. This was replicated with three fruit of each citrus type at the four stages of ripeness to determine the oil gland density of the peel per square centimetres. Additional segments were cut at the equator region of the fruit, making thin transverse sections of the peel to determine the oil gland size. Oil gland size was measured using a Leica dissection microscope and LAS EZ software (Leica Microsystems, Ltd., Switzerland).

Fruit essential oils from citrus types and ripeness stages were obtained using Soxhlet extraction. A sample of five fruit for each citrus type and ripeness stage was used to obtain a single essential oil sample. Fruit were peeled using a store bought peeler, which permitted efficient separation of the flavedo from the albedo with minimal rupturing of oil glands. Fruit peels were placed in a metal sieve over 350 mL of water within a modified pressure cooker at the ARC-Institute for Tropical Plant Research, Nelspruit, South Africa. The water in the apparatus was heated to $100°C$ and the peels were steamed for 1 h. The steam, carrying essential oils, passed through a condensing tube. The collected condensation comprised water with essential oils floating on top. All essential oil was collected using a micropipette and placed into a 2-mL glass vial. Glass vials were sealed, labelled and stored in a cardboard box in a refrigerator ($4°C$) at CRI.

After each extraction, the equipment was washed thoroughly with hot soapy water and rinsed thoroughly with water. Three replicates were performed for each citrus type and ripeness stage. The aim of this process was to collect 1.5 mL of essential oil per replicate. However, green fruit, and in particular, those of Nadorcott, produced negligible amounts of oil, even when 7 and 10 fruit were used.

Chromatographic analysis

The essential oil samples were sent to the Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa, where they were dissolved in hexane. Sample separation was performed using a gas chromatograph (6890N, Agilent Technologies Network) coupled to an Agilent Technologies inert XL EI/CI Mass Selective Detector (MSD) (5975, Agilent Technologies Inc., Palo Alto, CA, USA). The GC–MS system was coupled to a CTC Analytics PAL autosampler. Aliquots (1 μL) of dissolved sample were analysed by GC–MS using the split (10:1) injection mode onto a Zebron 7HG-G027-11-GGA capillary column. The temperatures of the injector and the MS detector were 240°C and 150°C, respectively. The transfer line temperature was maintained at 250°C. Complete gas chromatography (GC) and mass spectroscopy (MS) parameters are given in Table 1. Compounds were identified using the NIST 95 and WILEY275 libraries, and reference to published records of compounds found in citrus peel essential oils. The concentrations of compounds were reported in terms of area percentage.

Oviposition propensity

Oviposition propensity by *B. dorsalis* was determined for each citrus type and ripeness stage (green, colour break, picking ripeness and senescence). Experiments were conducted in a no-choice and choice set-up in relation to the

TABLE 1 Analytical parameters for gas chromatography–mass spectroscopy (GC–MS) analysis.

Autosystem GC–MS	Parameters
GC 6890N	Carrier gas: Helium at 1 mL/min Column: ZB-Semi-volatile Zebron 7HG-G027-11-GGA 30 m × 0.25 mm × 0.25 μm Injector temperature: 250°C Split ratio: 1 μL at 10:1 Oven temperature programme: Initial temperature: 50°C for 2 min First ramp: 70°C at a rate of 2°C/min for 6 min Second ramp: 20°C/min for 3 min until 250°C
MS 5975	Electron ionisation (EI) mode: 70 eV Mass scan: m/z 40–650

Note: Numbers following GC and MS indicate model numbers of equipment used.

presence of fruit damage. Fruit damage was simulated by using an entomological pin to pierce a row of five holes, through to the pulp, in each quarter of the fruit peel. The pin was cleaned with ethanol (90% laboratory grade) between each fruit. In the no-choice set-up, *B. dorsalis* females were exposed to two fruit of the same cultivar at the same stage of ripeness, but with both fruit either intact or damaged. In the choice set-up, *B. dorsalis* females were exposed to one intact and one damaged fruit of the same cultivar at the same stage of ripeness. Choice experiments did not compare preference for citrus type or ripeness stage due to their seasonal availability. Fruit were suspended in the cages using rubber bands attached to metal hooks. Five females aged 21–25 days from a single cohort were placed in each container 30 min before fruit were introduced. Water was provided ad libitum during experiments. Fruit were exposed to females for 7 h between 7:30 and 16:00 on a test day. Each experiment was replicated five times with two different fly cohorts (10 replicates in total). Each fruit was dissected to record the number of stings per fruit, number of eggs per sting and the position of the eggs (flavedo, albedo or pulp).

Oviposition behaviour

Oviposition behaviour was recorded on the same five citrus types and Golden Delicious apples. Experiments were conducted in a no-choice set-up. A pilot study conducted for these experiments, using 1, 5, 10 and 20 females in a cage, showed that a single female in a cage with a fruit remained inactive for many days, whereas groups of 10 and 20 females spent a large proportion of the time fighting (results not shown). Based on these observations, five 21- to 25-day-old females were placed in a Perspex cage (50 × 50 × 50 cm) 20 min before a single ripe fruit was introduced. The fruit introduced to the cage was either damaged or undamaged, as described earlier. A mirror was placed behind the fruit to allow full view of the fruit without disturbing the cage. A video camera (Legria F450 HD, Canon, Netherlands) was placed on a tripod at an angle so that all surfaces of the fruit were visible. Ten replicates of each treatment were performed. No set of five females or fruit were used twice. Video files of behaviour were stored on an external 2-TB hard drive (Backup Plus, Seagate Technologies Inc., California, USA).

At a later date, female behaviour was scored from the video recordings. Videos were analysed using JWatcher (Version 1.0) (Blumstein et al. 2000), in which the proportion of time (total amount of time for a behaviour/total time on fruit) for each behaviour for each individual for each citrus type and treatment was quantified. Behavioural states that were observed were walking, tasting, probing, oviposition, aggression, grooming, feeding, stationary, ovipositor dragging, and wing beating or Shimmy (Table S1). These behavioural states are similar to those

described in a study of the Queensland fruit fly, *Bactrocera tryoni* (Nguyen et al. 2007). In most circumstances, only one of the five females made contact with the fruit, and the first female to arrive on the fruit represented the focal individual for the scoring of behaviour. Where more than one fly was on a fruit during an observation period, we scored interactions between the focal female and other flies (i.e., aggression; Table S1). Scoring of behaviour commenced as soon as a (focal) female made contact with the fruit. Scoring of the behaviour of the focal female on the fruit ran for 20 min, or until the female left the fruit after ovipositing. If no females were observed to interact with the fruit within 30 min of it being placed in the cage, observations were terminated. Personal observations showed that females not interacting with the fruit within 30 min were unlikely to initiate oviposition thereafter.

Data analyses

Physicochemical properties of fruit types were compared using linear models, with fruit type, ripeness and their interaction as effects. The 'car' library was used to run an analysis of variance ('Anova') with type III sums of squares to summarise the effects of each model. Post hoc Tukey's honestly significant difference tests (using the 'emmeans' procedure) were performed to identify homogeneous groups within each ripeness stage to draw out potential reasons for observed increases in oviposition into ripe fruit.

To evaluate the differences in citrus peel essential oil chemical profiles between fruit and degrees of ripeness, multivariate analyses were conducted in R (Version 3.5.2, 'Eggshell Igloo'; 2018) using packages vegan (Oksanen et al. 2019), MASS (Venables & Ripley 2002), pairwiseAdonis (Martinez Arbizu 2017) and indicspecies (De Cáceres & Legendre 2009). The Bray–Curtis distance with non-metric multidimensional scaling (NMDS) and 999 permutations was used to visualise differences in volatile profiles across treatments. NMDS ranks orders of data points to produce an ordination plot along two axes and does not require linearity among variables. Permutational multivariate analysis of variance (PERMANOVA) was implemented to determine the effects of fruit and ripeness on citrus peel essential oil chemical profile. Indicator species analyses were run using the Bray–Curtis distances to evaluate which citrus peel essential oil compounds were characteristic for each citrus type and ripeness stage.

To analyse oviposition propensity, data were converted to binary response variables (presence or absence of eggs) due to the high number of zeros. Only stings with eggs were used in the analyses as blind stings were difficult to detect so soon after oviposition. A generalised linear model with a binomial error distribution was used to analyse the effects of citrus type, stage of ripeness and fruit condition (damaged or undamaged) on the presence or absence of stings in fruit under choice and no-choice conditions. The minimal adequate model was determined by step-wise deletion of least significant terms. 'Anova' with type III sums of squares was run to summarise the effects of each minimal adequate model. Post hoc Tukey's tests were used to identify homogeneous groups. Pearson's correlation coefficient was used to determine the correlation between the number of eggs per fruit with fruit physicochemical properties (including limonene and linalool content, expressed as area percentage, and their ratio).

As noted earlier, fruit fly behaviours were scored using JWatcher (Version 1.0) and the same programme permitted first-order Markovian analyses to determine the probability of transition from one behaviour to the next. Four separate Markovian analyses were used to prepare kinetic diagrams for behaviours on damaged and undamaged citrus (all types pooled) and apples. Later, these kinetic diagrams were used as a basis to focus further analyses on the six most common behaviours: walking and tasting; probing; oviposition; aggression; grooming; and inactive. The time spent on each behaviour was analysed with a generalised linear model with quasibinomial error distribution, with citrus type and treatment (damaged or undamaged) as effects relative to the positive control (Golden Delicious). The time spent on each behaviour was analysed with reference to the total observation time using the 'cbind' function in R. Model summarisation was done using 'Anova' with type III sums of squares. Post hoc Tukey's tests were again used to identify homogeneous groups.

RESULTS

Physicochemical properties

There was a significant interaction between citrus type and growth stage on fruit size, peel thickness, gland density and gland size (Tables 2 and 3). Post hoc Tukey's tests indicated that within each ripeness stage, Eureka lemons were consistently among the smallest fruit (Table 3). Star Ruby grapefruit was the largest fruit when ripe and over ripe. Peel thickness tended to decline as fruit matured. Peel thickness of green and colour break Star Ruby grapefruit was greater than all other types, but this difference was lost by the ripe and over-ripe stages when peel thickness of the tested citrus types did not differ significantly. Gland density tended to decline as fruit matured, with the exception of Delta Valencia orange, where gland density was higher at colour break than at the green stage. Gland density of Delta Valencia orange, Eureka lemon and Star Ruby grapefruit was among the lowest across most growth stages. The highest gland density was always observed in Glen Ora Late navel oranges. Gland size peaked when citrus fruit reached the ripe stage. Gland size varied between citrus types when they were green, at colour break, or ripe, but when over ripe, there was no significant difference between them.

TABLE 2 Model summaries for linear models testing the effects of citrus type and stage of ripeness on measured physicochemical properties of fruit used to assess oviposition propensity by gravid female *Bactrocera dorsalis*.

Fruit property	SS	df	F	p	Fruit property	SS	df	F	p
Fruit size					**Peel thickness**				
Intercept	22 317.4	1	1601.045	<0.001	Intercept	22.8	1	13.555	<0.001
Type	289.5	4	5.193	**0.001**	Type	196.5	4	29.203	**<0.001**
Stage	272.5	3	6.515	**<0.001**	Stage	4.7	3	0.937	0.428
Type × Stage	4020.2	12	24.034	**<0.001**	Type × Stage	200.4	12	9.925	**<0.001**
Residuals	836.4	60			Residuals	100.9	60		
Gland size					**Gland density**				
Intercept	2.6	1	43.585	<0.001	Intercept	40 200.0	1	620.973	<0.001
Type	1.4	4	5.766	**<0.001**	Type	4147.0	4	16.015	**<0.001**
Stage	0.3	3	1.676	0.182	Stage	6140.0	3	31.616	**<0.001**
Type × Stage	5.8	12	8.233	**<0.001**	Type × Stage	5699.0	12	7.336	**<0.001**
Residuals	3.5	60			Residuals	3884.0	60		

Note: Fruit size and peel thickness were measured for each fruit exposed to flies, whereas a reference sample was taken for stage of ripeness, gland size and gland density. Significant effects are in bold type ($p < 0.05$).

A two-dimensional NMDS ordination analysis with the Bray–Curtis dissimilarity measure sufficiently described citrus peel essential oil compounds between fruit types and degrees of ripeness. There was a fair fit of the model to the data (stress = 0.2051) (Dugard et al. 2010), and the data showed significant differences among the chemical profiles (Figure 1). There was a significant effect of fruit type on the chemical profile ($r^2 = 0.6995$; $p < 0.001$). Eureka lemon was significantly different from Glen Ora navel orange and Star Ruby grapefruit in chemical profiles. There were no significant differences in chemical profiles for fruits of varying ripeness ($r^2 = 0.0558$; $p > 0.05$). There were significant essential oil compounds associated with the various fruit types ($p < 0.05$), including 16 associated with Eureka lemon, 7 associated with Nadorcott mandarin, 6 associated with Glen Ora navel orange, 2 associated with Delta Valencia orange and 14 associated with Star Ruby grapefruit (Table S2). Despite no overall difference in the chemical profile of fruit of varying ripeness, there were significant essential oil compounds associated with certain degrees of ripeness ($p < 0.05$), including three associated with green fruit, one associated with colour break fruit, one associated with ripe fruit and seven associated with over-ripe fruit (Table S3).

Oviposition propensity

Dissection of the fruit revealed that few had eggs in them. In the no-choice undamaged fruit, stings with eggs were found in Golden Delicious apples and ripe Eureka lemons (Table S4). In the no-choice damaged fruit, eggs were found in ripe Glen Ora Late navel, ripe Nadorcott mandarin and ripe and over-ripe Eureka lemon. In the choice experiments, eggs were found in damaged Golden Delicious apple, green damaged Nadorcott mandarin and ripe damaged Delta Valencia orange (Table S5). The number of eggs laid by *B. dorsalis* was not associated with peel thickness, gland size, limonene content or limonene:linalool ratio across the citrus types or ripeness stages (Table 3). However, the number of eggs was moderately correlated with increasing fruit size. Also, there were suggestive but weak negative correlations between the number of eggs with increasing oil gland density and linalool content. The main effect of stage of ripeness had a significant effect on the presence of stings ($\chi^2 = 25.07$, df = 3, $p < 0.001$), with stings being more likely in ripe fruit compared with other stages (Table 4). There was a significant interaction effect between citrus type and experiment (no-choice and choice) ($\chi^2 = 14.13$, df = 5, $p = 0.015$) on the presence of stings, with stings being significantly less likely in ripe, no-choice undamaged Eureka lemons compared with undamaged Golden Delicious apples. Females mostly laid eggs in damaged citrus under no-choice conditions; only two undamaged citrus fruit were found with stings. All oviposition sites in choice experiments were recorded from damaged fruit (Table 5). In fruit having stings and eggs, eggs were deposited in the albedo region of the fruit, except for one damaged green Nadorcott mandarin, where two eggs were recorded in the pulp region.

Oviposition behaviour

There were generally more events recorded for all behavioural states on damaged fruit compared with undamaged fruit. Overall, the probability of transition from probing to oviposition in damaged citrus (0.53) was higher than that of undamaged citrus (0.24) (Figure 2). Transition from feeding to probing was recorded in

TABLE 3 The mean (±1 SE) fruit peel properties of five citrus types at four stages of ripeness.

Ripeness	Citrus type	Fruit size (mm)	Peel thickness (mm)	Gland density (glands per cm²)	Gland size (mm)	Limonene (area %)	Linalool (area %)	Limonene:linalool ratio
Green	Delta	76.41 ± 1.66[b]	2.57 ± 0.24[a]	68.50 ± 4.36[a]	0.89 ± 0.14[b]	40.66	0.88 ± 0.04	48.40 ± 129.85
	Eureka	62.43 ± 0.56[a]	3.10 ± 0.48[a]	72.50 ± 4.38[a]	0.80 ± 0.07[ab]	20.78	0.16	
	Glen Ora	84.26 ± 1.42[c]	3.01 ± 0.12[a]	109.25 ± 4.65[b]	0.75 ± 0.09[ab]	92.10 ± 0.52	0.48 ± 0.26	132.77 ± 29.25
	Nadorcott	64.31 ± 2.39[a]	2.34 ± 0.29[a]	77.50 ± 6.26[a]	0.33 ± 0.04[a]	87.06 ± 1.28	1.93 ± 0.47	50.10 ± 10.38
	Star Ruby	76.39 ± 1.90[b]	12.52 ± 2.58[b]	88.00 ± 3.64[a]	0.52 ± 0.04[ab]	88.65	0.35	0.35
Colour break	Delta	74.70 ± 0.62[b]	2.39 ± 0.27[a]	100.25 ± 9.25[d]	0.80 ± 0.11[b]	45.87	0.68	67.45
	Eureka	67.28 ± 4.26[ab]	2.98 ± 0.44[a]	59.00 ± 3.16[a]	0.81 ± 0.08[b]	61.25 ± 3.92	0.00	
	Glen Ora	74.53 ± 1.88[b]	2.71 ± 0.31[a]	90.75 ± 3.65[cd]	0.11 ± 0.01[a]		19.34	1.00
	Nadorcott	65.51 ± 1.56[a]	1.76 ± 0.09[a]	75.75 ± 3.10[bc]	0.55 ± 0.05[ab]	15.28	0.00	0.00
	Star Ruby	72.51 ± 2.52[ab]	10.23 ± 1.61[b]	72.75 ± 4.41[ab]	0.71 ± 0.5[b]	60.60 ± 30.31	0.16	
Ripe	Delta	77.58 ± 1.62[bc]	2.58 ± 0.42[a]	50.5 ± 6.21[a]	1.14 ± 0.25[a]	61.45 ± 30.72	0.63 ± 0.33	101.32 ± 19.32
	Eureka	61.94 ± 0.60[a]	3.50 ± 0.21[a]	63.25 ± 4.43[a]	1.02 ± 0.31[a]	57.93 ± 7.79	0.00	
	Glen Ora	73.59 ± 2.23[c]	3.41 ± 0.35[a]	85.75 ± 3.21[b]	2.06 ± 0.25[b]	14.92	19.30	0.53
	Nadorcott	66.66 ± 1.07[b]	2.48 ± 0.27[a]	61.75 ± 3.41[a]	0.75 ± 0.07[a]	86.05 ± 0.95	1.96 ± 0.81	53.19 ± 22.47
	Star Ruby	107.65 ± 3.52[d]	4.16 ± 0.15[a]	49.25 ± 3.84[a]	0.79 ± 0.12[a]	91.97 ± 0.58	0.18 ± 0.09	347.17 ± 54.14
	Apple	62.81 ± 0.33						
Over ripe	Delta	84.14 ± 2.61[b]	3.67 ± 0.37[a]	54.25 ± 6.33[a]	0.82 ± 0.10[a]	92.00	1.09	1.09
	Eureka	62.00 ± 1.73[a]	2.05 ± 0.31[a]	53.50 ± 3.48[a]	0.75 ± 0.05[a]	42.39 ± 21.65	0.00	
	Glen Ora	78.64 ± 1.88[b]	2.18 ± 0.18[a]	85.75 ± 3.21[b]	2.06 ± 0.25[a]			
	Nadorcott	66.66 ± 1.07[a]	2.48 ± 0.27[a]	60.00 ± 2.54[ab]	0.75 ± 0.04[a]	92.10 ± 0.52		0.27
	Star Ruby	106.54 ± 2.56[c]	3.56 ± 0.65[a]	44.50 ± 2.77[a]	0.83 ± 0.05[a]	14.85	19.29	
r	Number of eggs	−0.37	−0.05	−0.21	0.02	0.10	−0.20	0.15

Note: Values for citrus types marked with the same superscript letter are not significantly different from each other within a ripeness stage (Tukey's honestly significant difference tests adjusted for comparing a family of estimates; no statistical comparisons were run on limonene or linalool content due to low and incomplete sample sizes). Values with no standard error presented are from a single observation. Pearson's correlation coefficient is shown for each fruit variable and the number of eggs per fruit (from the oviposition propensity experiment, calculated using the means for each). The size of ripe Golden Delicious apples is included for reference.
Abbreviations: Delta, Delta Valencia orange; Eureka, Eureka lemon; Glen Ora, Glen Ora Late navel orange; Nadorcott, Nadorcott mandarin; Star Ruby, Star Ruby grapefruit.
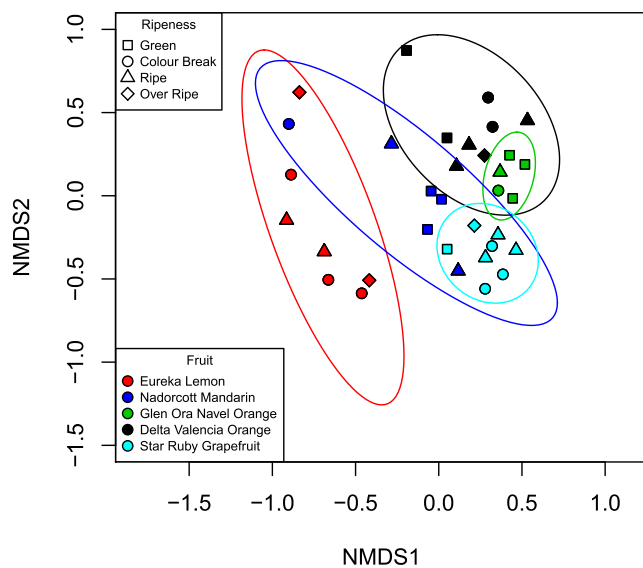
FIGURE 1 Non-metric multidimensional scaling (NMDS) ordination plot showing the relative similarity of essential oil composition of different citrus types and degrees of ripeness. Ellipses represent 95% confidence intervals. The treatments tested were degrees of ripeness denoted as Green, Colour Break, Ripe or Over Ripe and were tested on variants Eureka lemon, Nadorcott mandarin, Glen Ora Late navel orange, Delta Valencia orange and Star Ruby grapefruit.

damaged fruit but not in undamaged fruit. In damaged fruit, there was a transition from aggression to oviposition, while the reverse transition (oviposition to aggression) was recorded in undamaged fruit. In undamaged fruit, there were generally more transitions to aggression from other behavioural states compared with damaged fruit. Female *B. dorsalis* showed lower probability of transition from walking and tasting to probing in citrus (damaged: 0.27; undamaged: 0.40) than in apple (damaged: 0.59; undamaged: 0.75). The probability of transitions from probing to oviposition in apple was 0.48 and 0.13 in damaged and undamaged fruits, respectively (Figure S1).

Fruit type and damage treatment had varying effects on the behaviour of female *B. dorsalis* (Table 6). There was a significant interaction of fruit type and treatment on the proportion of time spent walking and tasting. Females spent a significantly larger proportion of time walking and tasting on damaged apple and Star Ruby relative to undamaged fruit of the same types (Figure 3). In contrast, females spent less time walking and tasting on damaged than undamaged Nadorcott. Fruit type influenced probing by females, with this behaviour significantly lower on Eureka lemons and Nadorcott mandarins than on the other fruit. There was a significant interaction of fruit type and treatment on the time spent ovipositing. Oviposition

TABLE 4 Mean (±1 SE) number of stings and eggs detected in citrus types at four stages of ripeness after exposure to five gravid female *Bactrocera dorsalis* under no-choice conditions (damaged or undamaged) in a laboratory.

Fruit type	Ripeness	Treatment	Number of stings	Number of eggs
Apple	Ripe	Damaged	0.40 ± 0.32	1.95 ± 1.49
		Undamaged	0.25 ± 0.13	4.10 ± 2.31
Eureka	Ripe	Damaged	1.75 ± 0.73	20.63 ± 8.68
		Undamaged	0.15 ± 0.11	2.05 ± 1.60
	Over ripe	Damaged	1.05 ± 0.49	9.70 ± 4.48
Glen Ora	Ripe	Damaged	1	9
Nadorcott	Green	Damaged	1	11
	Ripe	Damaged	0.20 ± 0.16	2.00 ± 1.63
Star Ruby	Green	Damaged	4	7.70 ± 2.60
	Ripe	Damaged	1	22

Note: Treatments with no stings or eggs are not presented. Values with no standard error presented are from a single observation. Ripe Golden Delicious apples were used as a control.
Abbreviations: Eureka, Eureka lemon; Glen Ora, Glen Ora Late navel orange; Nadorcott, Nadorcott mandarin; Star Ruby, Star Ruby grapefruit.

TABLE 5 Mean (±1 SE) number of stings and eggs detected in citrus types at four stages of ripeness after exposure to five gravid female *Bactrocera dorsalis* under choice conditions (damaged or undamaged) in a laboratory.

Fruit type	Ripeness	Treatment	Number of stings	Number of eggs
Apple	Ripe	Damaged	4	10
Delta	Ripe	Damaged	1	14
Nadorcott	Green	Damaged	0.15 ± 0.11	1.15 ± 1.08

Note: Treatments with no stings or eggs are not presented. Values with no standard error presented are from a single observation. Ripe Golden Delicious apples were used as a control.
Abbreviations: Delta, Delta Valencia orange; Nadorcott, Nadorcott mandarin.
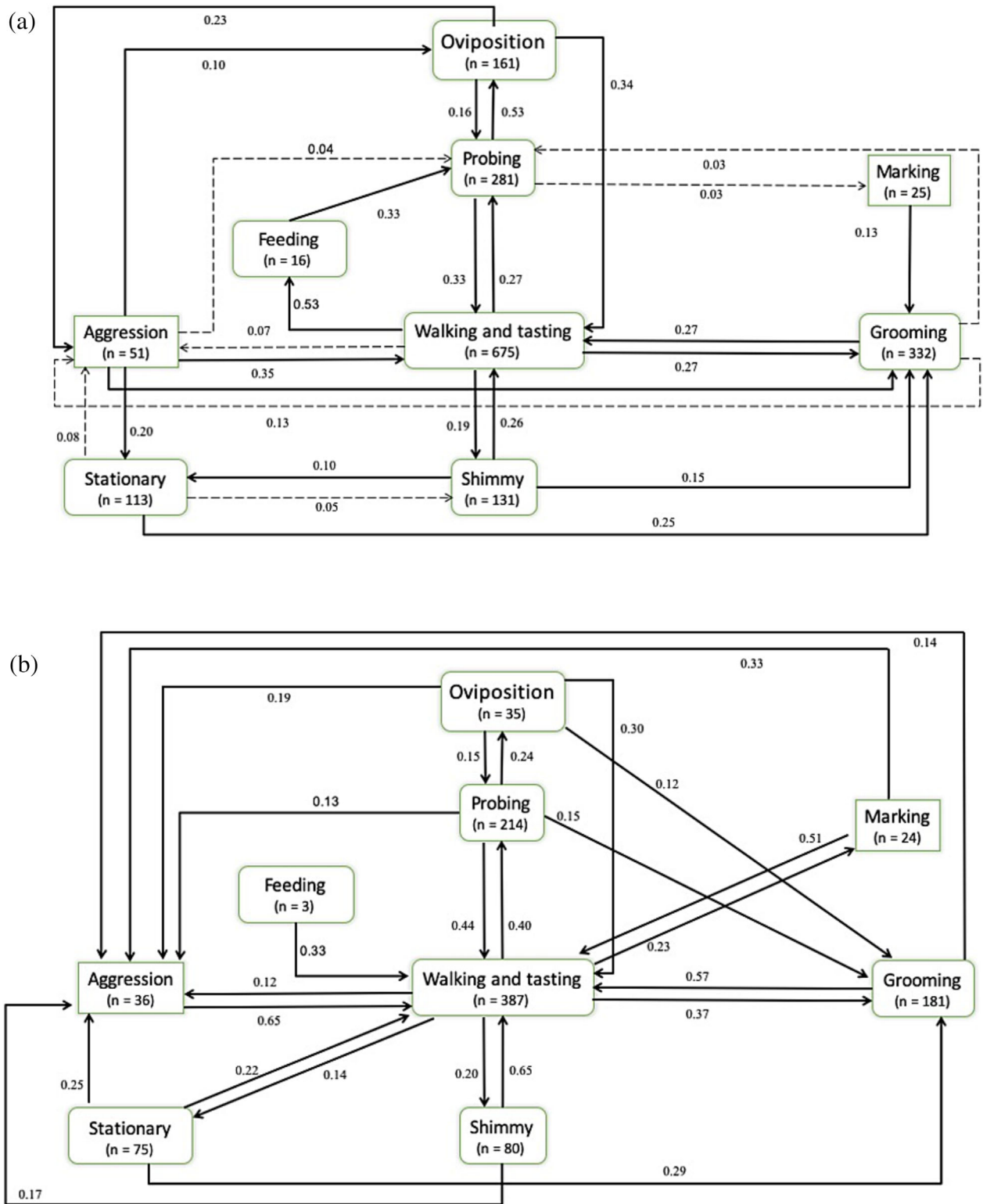
FIGURE 2 First-order Markovian analyses showing the probability of transition from one behaviour to another by gravid female *Bactrocera dorsalis* on (a) damaged and (b) undamaged citrus (all types pooled). Transitions with $p \geq 0.10$ are indicated by solid lines, and those with $0.09 \geq p \geq 0.03$ are indicated by dashed lines. Values in parentheses indicate frequencies of each behaviour observed.

behaviour on Eureka lemons, Nadorcott mandarins and Star Ruby grapefruits was absent on undamaged fruit, but females spent more than 30% of their time ovipositing into these fruit when they were damaged. Damage increased oviposition into apples, but to a lower extent. There was no significant effect of fruit type, treatment or

TABLE 6 Model effects for the six most frequent behaviours exhibited by female *Bactrocera dorsalis* on five citrus types and a positive control under no-choice damaged or undamaged conditions.

Behaviour	LR	df	p	Behaviour	LR	df	p
Walking and tasting				**Aggression**			
Type	5.606	5	0.346	Type	5.213	5	0.391
Treatment	0.653	1	0.419	Treatment	1.118	1	0.290
Type × Treatment	11.373	5	**0.044**	Type × Treatment	6.135	5	0.293
Probing				**Grooming**			
Type	12.722	5	**0.026**	Type	10.247	5	0.069
Treatment	2.123	1	0.145	Treatment	0.249	1	0.618
Type × Treatment	9.104	5	0.105	Type × Treatment	5.171	5	0.395
Oviposition				**Stationary**			
Type	5.606	5	0.346	Type	14.970	5	**0.010**
Treatment	0.653	1	0.419	Treatment	11.229	1	**<0.001**
Type × Treatment	11.373	5	**0.044**	Type × Treatment	28.448	5	**<0.001**

Note: Significant effects are in bold type ($p < 0.05$).
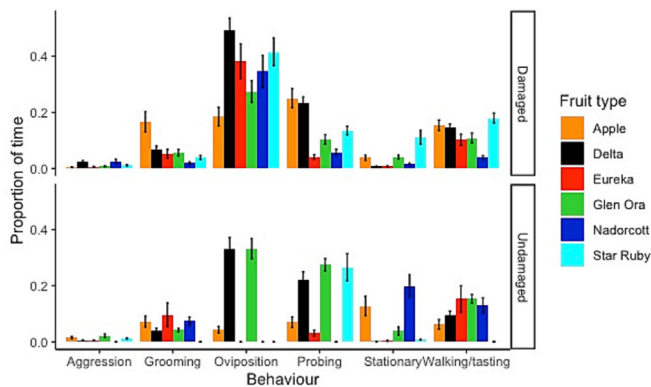
FIGURE 3 Mean (±1 SE) proportion of time female *Bactrocera dorsalis* spent on aggression, grooming, oviposition, probing, being inactive, and walking and tasting on (a) damaged and (b) undamaged citrus types and a positive control (Golden Delicious apple). Citrus types investigated were Golden Delicious apple, Delta Valencia orange, Eureka lemon, Glen Ora navel orange, Nadorcott mandarin and Star Ruby grapefruit. Each female was observed for 20 min.

the interaction thereof on the proportion of time spent exhibiting aggression or grooming. The interaction of fruit type and treatment had a significant effect on the proportion of time spent stationary. Females spent a higher proportion of time being inactive on damaged Star Ruby grapefruit compared with undamaged fruit of the same type, whereas they were stationary more often on undamaged than damaged apple and Nadorcott mandarin.

DISCUSSION

Bactrocera dorsalis females showed a preference for oviposition on damaged fruit across all types of citrus. The preference for damaged fruit was demonstrated under both no-choice and choice conditions. However, there were low levels of oviposition (stings and eggs) in intact (undamaged) ripe Eureka lemon and in Golden Delicious apple under no-choice conditions in the laboratory with both fruit types being evaluated as detached. Given that citrus fruit detached from a tree exhibit reduced defence due to changes in fruit quality and citrus peel (Diatta et al. 2013; Miller 1946), the low or lack of propensity of *B. dorsalis* to oviposit in intact detached citrus in our study shows the high resistance of citrus to this species. Oviposition by *B. dorsalis* was not quantified on attached citrus in this study and can be part of the host status determination process after the conduct of larval surveys to determine natural infestation of specific citrus type and condition. In surveys in commercial Eureka lemon orchards in South Africa, no eggs nor larvae of fruit flies (including *B. dorsalis*) were recorded on fruit that had just been harvested. This was despite the presence of fruit flies, including *B. dorsalis*, in the surveyed lemon orchards (Manrakhan et al. 2018). Theron et al. (2017) recorded no infestation of *B. dorsalis* on citrus fruit sampled on the ground (detached fruit) in South Africa despite the presence of the pest in the areas that they sampled. However, Theron et al. (2017) recorded infestation of *B. dorsalis* on a damaged, attached *Citrus sinensis* fruit. A fruit can be considered infested by a fruit fly if eggs or larvae are found inside the fruit. For a fruit to be classified as a host for a fruit fly, development to adulthood is required (Aluja & Mangan 2008). Here, we only investigated acceptance of a fruit for egg laying by *B. dorsalis* as a first step in understanding potential utilisation of South African citrus as a host by the species. Different citrus types were recorded as hosts of *B. dorsalis* in surveys carried out in areas where the species is present (Goergen et al. 2011; Moquet et al. 2021; Mwatawala et al. 2009; Rwomushana et al. 2008), but often in these host surveys, description of the conditions of the fruit samples from which *B. dorsalis* adults were reared was not clear. This creates uncertainty

into proper classification of the host status of different citrus types for *B. dorsalis*. In future surveys on hosts of this species, the conditions of the fruit sampled should be properly described as it is clear that for *B. dorsalis*, damage of a fruit type like citrus increases the chance of egg laying by this species, which can further influence utilisation of the resource.

Damage (either insect or mechanical) on citrus was previously shown to increase emissions of volatiles from the fruit (Kendra et al. 2011). An increase in volatile emission as a result of damage would most likely increase attraction of *B. dorsalis* to the oviposition site, eventually leading to the act of oviposition. In this study, a number of volatile organic compounds (VOCs) were identified from different citrus types, some of which (e.g., octane and β-pinene) were previously shown to attract *B. dorsalis* (Biasazin et al. 2014). Some citrus types such as lemon and grapefruit, when damaged, had more eggs and stings than others. Coincidentally, these citrus types had higher numbers of unique VOCs than other citrus types. Specific volatile compounds such as γ-octalactone emitted from mango (not found in this study) were found to elicit oviposition responses in *B. dorsalis* (Kamala Jayanthi et al. 2014). In citrus, limonene was identified as an oviposition stimulant for the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), while linalool was found to deter oviposition in this species (Ioannou et al. 2012; Papanastasiou et al. 2020). In addition, limonene was found to be emitted in higher amounts in citrus fruit that had mechanical or insect damage (Kendra et al. 2011). In our study, we found no association between limonene content of the essential oil and oviposition, but there was a suggestive trend for linalool content to reduce oviposition into citrus by *B. dorsalis*.

The stage of maturity was another condition that was found to affect oviposition propensity of *B. dorsalis* on citrus. Damaged fruit that were ripe had a higher number of stings and eggs of *B. dorsalis* than fruit at other maturity stages. Furthermore, *B. dorsalis* exhibited the same behaviour in other fruit types (Jang & Light 1991; Rattanapun et al. 2009; as *Bactrocera invadens* in Diatta et al. 2013). A higher oviposition propensity on ripe fruit was similarly exhibited by other fruit fly species, particularly those of a polyphagous nature like *B. dorsalis* (Cunningham et al. 2016; Greany et al. 1983; Joachim-Bravo et al. 2001). It has been suggested that polyphagous fruit flies like *B. dorsalis* use fruit ripening volatiles that are released in higher amounts in ripe fruit of many species as chemical cues for oviposition resources (Cunningham et al. 2016). In this study, we found no changes in chemical profiles of VOCs across maturity stages. However, it is possible that some volatiles were available in higher amounts at the riper stage and this was not quantified in the course of this study.

While olfactory cues play an important role in guiding a generalist tephritid to the location of a potential oviposition site, other cues emanating from the fruit that could, in nature, be either tactile (such as fruit skin characteristics) or physical (shape, colour and size) are additionally used by some species for final acceptance of that fruit as an oviposition site (Diaz-Fleischer et al. 2001). The toughness of the skin of mango, an important host for *B. dorsalis*, was found to influence egg laying in this species (Rattanapun et al. 2009). Here, though, we found no correlation between oviposition propensity of *B. dorsalis* and peel thickness or oil gland size of citrus. However, there was a weak but suggestive effect of increasing fruit size and oil gland density to suppress oviposition by *B. dorsalis*. It could be that in citrus, peel characteristics have a minor influence on egg laying by *B. dorsalis*. Alternatively, their effects might have been masked as a result of generally low egg laying across citrus types, or that physical peel characteristics of detached or damaged citrus fruit offer less resistance to *B. dorsalis* oviposition. In another species, *C. capitata*, thickness of the peel of citrus was not found to influence egg laying (Dias et al. 2018; Papachristos & Papadopoulos 2009), although there was a correlation between peel thickness and oviposition propensity in *Dacus persicus* (Leptoxyda) Hendel on milkweed (Sharma & Amritphale 2008). For *C. capitata*, oil gland density in the peel of citrus was negatively correlated with number of eggs laid (Papachristos & Papadopoulos 2009). In citrus, the characteristics of the peel might have a bigger influence on the survival of eggs and larvae of fruit flies than on egg laying. Hatchability of eggs of Caribbean fruit fly, *Anastrepha suspensa* (Loew), was found to be lower when eggs were deposited in the oil glands than in between oil glands (Greany et al. 1983). Oils contained in these oil glands were found to be highly toxic to fruit fly eggs and larvae (Greany et al. 1983; Papachristos & Papadopoulos 2009). It has also been suggested that properties such as pH and soluble solids found within the peel may also influence the oviposition propensity of fruit flies on certain fruits (Branco et al. 2000).

For the first time, we describe the oviposition behavioural sequences of *B. dorsalis* on damaged and undamaged citrus. We found transitions from probing (extension of the ovipositor and perpendicular positioning of it) to oviposition on a fruit, which increased when fruit was damaged. The probing to oviposition transition can be attributed to the presence of receptors in the ovipositor of females, which play an important role in final decision-making. Oviposition by *B. dorsalis* is a co-ordinated event with stimulants being perceived by both the antennae and ovipositor of the female (Vyas et al. 2022). The feeding to probing transition seen in damaged fruit, as well as increased walking and tasting activities, can possibly be attributed to further assessment of the fruit quality by the female using receptors on the mouthparts and other body parts. Female *B. dorsalis* can discriminate the presence of other fruit fly larvae in a fruit by avoidance to bore into the fruit,

possibly as a means of avoiding intraspecific competition (Prokopy et al. 1989). For *B. dorsalis*, the decision to oviposit is clearly taken on the fruit resource itself, and if the conditions are not appropriate, oviposition will not take place.

The findings of this study provide support for the importance of removing fallen or damaged fruit from orchards as they may provide a potential breeding site for *B. dorsalis*. Moreover, the results demonstrate that there is room for new management approaches for *B. dorsalis* such as manipulation practices to render the fruit even less susceptible to attack. The use of horticultural spray oils can be an example of such a manipulation practice. Petroleum-based horticultural spray oils coat the surface of the fruit, thus obstructing the release of VOCs and surface cues that are critical in host recognition by pest species (Kuhlmann & Jacques 2002; Lee & Kaiser 2022; Nguyen et al. 2007). Alternative manipulation options include the use of particle film sprays, such as kaolin, which alter the visual appearance and surface texture of the fruits (Saour & Makee 2004). Such sprays could be used to make fruit with superficial damage, such as wind damage, less desirable for oviposition. The benefits of using such methods to prevent invasion by pest species would need to be balanced against any potential effects on citrus yield, as kaolin sprays have been shown to decrease photosynthetic rate in Star Ruby grapefruit, though fruit development and quality were not affected (Jifon & Syvertsen 2003). The use of VOC cues using linalool derivatives may likewise prove effective in decreasing oviposition and fruit damage to citrus. Combinations of various tactile and chemical deterrents would need to be tested to prove efficacy in preventing oviposition on citrus by *B. dorsalis*.

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.