

Olfactometer Responses of Plum *Curculio conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae) to Host Plant Volatiles, Synthetic Grandisoic Acid, and Live Conspecifics

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Abstract The plum curculio *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae) is a major pest of pome and stone fruit, but will also attack other fruits. Males produce the aggregation pheromone grandisoic acid; emitting only the (+)-enantiomer which is attractive to both sexes of the univoltine and multivoltine strains, while the synthetic racemic mixture contains optical isomers with equal amounts of (+)- and (–)-enantiomers. Synergy between odours can increase trap captures and improve monitoring techniques, therefore tests were performed in a dual-choice olfactometer with odours attractive to plum curculios according to literature to determine 1) under what physiological conditions (mating status, age, starvation period) these odours are attractive or repulsive, 2) if the (+)-enantiomer or the odour of live males synergizes with host plant volatiles, and 3) if there is a difference in response between plum curculio strains. Females were exposed to: benzaldehyde; trans-2-hexenal; apples; extracts of: plums, apples, blueberries; grandisoic acid; and live males. Plum essence

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was found to be the most attractive host-plant odour for both immature and mature virgin females, and immature whole apples were attractive to starved females, while trans-2-hexenal, McIntosh apple essence, benzaldehyde along with the combination of benzaldehyde and plume essence was found to be repulsive. Starvation, age, and mated status all influence response to odours. No synergistic or additive effects were observed between any of the odour combinations tested, including the combination of both the natural and synthetic pheromone and plum essence or apples.

Keywords Hostplants · aggregation pheromone · semiochemicals · attractants · repellents · synergy

Introduction

The plum curculio *Conotrachelus nenuphar* Herbst (Coleoptera: Curculionidae) is an important native North American pest of pome and stone fruits such as apple, plum, and peaches, but it can also be a pest of other fruit such as blueberries. This insect is capable of damaging 90% of fruit at harvest (Vincent and Roy 1992) and has two strains which cannot produce viable offspring together (Padula and Smith 1971; Zhang and Pfeiffer 2008; Zhang et al. 2010). The northern univoltine strain (found in Canada and the northern part of the US) has a mandatory reproductive diapause, while the southern multivoltine strain (found in the mid and southern parts of the US) has a facultative diapause (Racette et al. 1992).

Like many insects (Bernays and Chapman 1994), plum curculio use olfactory cues to locate fruit for feeding and oviposition purposes (Butkewich and Prokopy 1993; Butkewich et al. 1987). An aggregation pheromone [(+)-(1*R*,2*S*)-1-methyl-2-(1-methylethenyl)cyclobutaneacetic acid], called grandisoic acid is produced by males of the species and is attractive to both sexes and strains (Eller and Bartelt 1996). The pheromone produced by virgin male plum curculio is the (+)-enantiomer of grandisoic acid, (+)GA (Eller and Bartelt 1996). However the synthetically produced racemic mixture of grandisoic acid containing equal amounts of both the (+)- and (–)-enantiomers, was also found to be attractive in some studies (Piñero et al. 2011), weakly attractive in other studies (Pinero and Prokopy 2003; Leskey et al. 2005; Leskey et al. 2008), and unattractive in others (Leskey and Prokopy 2000; Akotsen-Mensah 2010). Male-produced aggregation pheromones are produced by weevils such as the boll weevil *Anthonomus grandis* Boheman (Tumlinson et al. 1969), the pepper weevil *Anthonomus eugenii* Cano (Eller et al. 1994), and the strawberry blossom weevil *Anthonomus rubi* Herbst (Innocenzi et al. 2001). These weevils produce multi-component aggregation pheromones, indicating that this may be the case for plum curculio as well (Leskey et al. 2009a, b; Hock et al. 2014, 2015).

Pheromones have been shown to synergize with host plant volatiles resulting in increased attraction for many weevil species (Landolt and Phillips 1997), such as the boll weevil (Dickens 1989), the red palm weevil *Rhynchophorus ferrugineus* Olivier (Poorjavad et al. 2009), the palm weevil *Rhynchophorus palmarum* L. (Rochat et al. 1991), and the banana weevil *Cosmopolites sordidus* Germar (Tinzaara et al. 2007). Synergistic effects between certain host-plant volatiles and pheromones have also previously been demonstrated for plum curculio (Leskey et al. 2005; Akotsen-

Mensah et al. 2010). For example, benzaldehyde and plum essence have been shown to attract plum curculio both in the laboratory and in the field (Butkewich and Prokopy 1997; Prokopy et al. 1995; Leskey et al. 2001), and have been used as trap baits in attempts to more accurately monitor this pest (Prokopy and Leskey 1997; Pinero and Prokopy 2003; Leskey et al. 2005). However, competition from natural odour sources present within the orchard causes drops in trap captures (Leskey and Wright 2004b), specifically after petal fall (Prokopy et al. 2002; Leskey and Wright 2004a). Until recently, there was no reliable and efficient method for monitoring large numbers of plum curculio invading apple orchards (Prokopy et al. 2000; Leskey et al. 2008; 2009). However, Piñero et al. (2011) have developed a trap-tree approach that can attract plum curculio to specific trees baited with pheromones, thereby limiting the need to apply insecticide in the rest of the orchard. The trap-tree approach comes with some drawbacks however, as it requires the sacrifice of several trees along the perimeter rows of orchards, and also incurs damage to non-baited neighbour trees which are near the baited trees (Leskey et al. 2008; Piñero et al. 2011). Improvements in the trap-tree approach can still be made by finding a more attractive odour source which would limit plum curculio damage to neighbouring trees, as well as more efficiently maintain attraction, aggregation, and retention of adult plum curculio within specific baited trap-tree canopies in apple orchards (Leskey et al. 2008; Piñero et al. 2011). Alternatively, any volatiles severely repellent to plum curculio can be used as part of a push-pull strategy: certain trees are baited with attractive volatiles while neighbouring trees are treated with the repellent volatiles (Cook et al. 2007, Leskey et al. 2014). This results in plum curculio being ‘pushed’ away from the neighbouring trees, and ‘pulled’ towards the baited trees, thereby limiting the amount of damage in unbaited-trees near the baited trees in the ‘trap-tree zone’. This method could also be a useful component of attract and kill strategies, whereby the baited tree is further treated with pesticides (Leskey et al. 2008).

Much is still not understood regarding plum curculio attraction to odours, conspecifics, and synthetic pheromones. An understanding of the various factors contributing to the attraction of plum curculio to different odours can be used to further improve monitoring techniques, as was done for similar weevil species (Klassen et al. 1982; Jutsum and Gordon 1989; Ridway and Inscoe 1990; Smart et al. 1994). Various physiological factors have been shown to be important in the response of other weevils. For example, younger boll weevils produce more pheromone than older weevils (Spurgeon 2003), and mated males are less attractive than virgin males in the rice weevil *Sitophilus oryzae* Linnaeus (Phillips and Burkholder 1981). An understanding of similar conditions in plum curculio can result in advancements of management techniques, as was achieved with the boll weevil (Tumlinson et al. 1969; Dickerson et al. 1987).

In the current study, tests were done in order to determine the behavioural responses of plum curculio to various synthetic and natural host-plant odours under different physiological conditions (*i.e.* different mating status, ages, and starvation periods). The synthetic host plant volatiles tested in experiments are naturally present in the principal host plants of plum curculios (Alm and Hall 1986; Polavarapu et al. 2004; Lafleur et al. 2007). Other non-host plant volatiles known to elicit responses from plum curculio were also tested, such as (+)GA and live males (Eller and Bartelt 1996; Hock et al. 2014, 2015). Attractive natural or synthetic host-plant volatiles were also examined for

synergy with attractive natural or synthetic pheromone odours. Synthetic odours of plums and benzaldehyde are known to be attractive to plum curculio in the field (Akotsen-Mensah 2010; Piñero et al. 2011), and are likely candidates for synergy with such pheromone components as those emitted by live males (Hock et al. 2014) as well as synthetic (+)GA (Hock et al. 2015).

Experiments were designed to determine 1) which of the plant volatiles tested are the most attractive or repulsive to curculios and under which physiological conditions (mating status, age, starvation period), 2) if host plant volatiles increase attraction or synergize with the synthetic grandisoic acid or with live males and under what conditions, and 3) if there is a difference in the responses between univoltine and multivoltine female curculios. We predicted that starved females would respond more strongly than fed females to both host plant odours and combinations of host plant odours and synthetic pheromones or conspecifics since these volatiles are supposed to signal the presence of a suitable food source (Landolt and Phillips 1997). We also hypothesized that females would respond more strongly to natural odours of volatiles and conspecifics, since they may contain more complete chemical formulas than what is produced synthetically (Leskey et al. 2009b). Some small differences in responses between mated and virgin females as well as between strains was also expected based on previous observations (Hock et al. 2014, 2015).

Materials and Methods

Plum Curculio Both strains of plum curculio were used and treated as in Hock et al. (2013, 2014, 2015). Univoltine plum curculio were obtained from infested apples (*Malus* sp.) collected in late June early July 2009 from unsprayed orchards in Saint-Bruno-de-Montarville, Québec, Canada (45.55028°N, 73.31917°W). Insects were kept in emergence cages (Hock et al. 2013) and emerging adults were collected daily and immediately separated by sex (Thompson 1932) then transferred in overwintering cages (Le Blanc 1992) and placed outside throughout winter to simulate a natural diapause period. Overwintered adults were removed from the cages the following spring and placed in 2 L plastic containers. Plum curculio were given water and small thinning (*i.e.* immature) apples that had been collected during the summer from Saint-Bruno-de-Montarville, Québec, Canada, and stored until use for feeding. Adults were placed in environmental control chambers at 25 ± 2 °C, 70% R.H., with 16 L:8D to mimic optimal summer conditions (Amis and Snow 1985) and some adults were also used to establish a laboratory colony that does not need to undergo winter diapause (Hock et al. 2013). Multivoltine curculios were taken from a laboratory population established at the Appalachian Fruit Research Station (Kearneysville WV) in 2001 and augmented annually with wild individuals (Leskey et al. 2010; Hock et al. 2014). Adults were reared in the laboratory at 25 ± 2 °C, and a 14L:10D photoperiod on a diet of green thinning apples and water as per the methods of Amis and Snow (1985). Newly emerged adults were held in mixed-sex groups of 100 individuals, and were allowed to mate and lay eggs in thinning apples, and subsequent emerging larvae were placed in 500 ml jars containing soil to undergo pupation. Jars were shipped to the Institut de recherche et de développement en agroenvironnement (IRDA; St-Hyacinthe, Quebec, Canada) and then placed in environmental control chambers (25 ± 2 °C, 70%

R.H., 16 L:8D) (Hock et al. 2014). Emerging adults were collected daily, separated by sex and held under the same conditions as the univoltine strain.

Physiological Status of Plum Curculios Tested Fed female curculios of both strains were used as responders unless otherwise noted, and were always sexually mature except for one experiment, where different age groups of immature (two to eight days old), mature (17–22 days), and old (30–36 days) females of the univoltine strain were tested in order to determine the effect of age on response to the most attractive host plant volatile (*e.g.* plum essence). In addition, females were usually fed except in experiments where we wished to test if nutritional status had an effect on response. In such experiments females were starved (*e.g.* for either 24, 48, or 72 h) prior to experimentation. All females were also virgin except for one experiment where mated univoltine female response to plum essence was tested. In this case females from the ‘mated’ modality were held with males for two weeks prior to experiments to allow for mating. All females from ‘mated vs. virgin’ experiments were dissected at the end of the experiment to assess their physiological status (mature/immature and mated/virgin). After the olfactometric tests, females were placed in 70% ethanol and held at 4°C (Hoffmann et al. 2004; Hock et al. 2014). Their status was assessed using the criteria from Hoffmann et al. (2004), this involved examining oocytes and spermatheca. Results revealed that all dissected females were mature having developed oocytes, and females in the ‘mated’ modality had copulated possessing sperm in the spermatheca as evidenced by its opaque and cloudy appearance (Hoffmann et al. 2004). Regarding volatiles tested, although an attempt was made to test the same odours on strains, occasionally limited numbers of plum curculio were available resulting in some experiments being conducted only on a single strain. This was due to sporadic outbreaks of fungi in rearing colonies and the difficulty in transporting plum curculio during the winter (*i.e.* shipping multivoltine plum curculio from WV long distances to Qc.) (Hock et al. 2014, 2015).

Olfactometer A vertically orientated still-air olfactometer identical to Hock et al. (2013, 2014, 2015) was used. This olfactometer takes advantage of the insect’s natural tendency to crawl and climb up trees (Racette et al. 1991) and the fact that plum curculio are most active in the absence of wind (Chouinard et al. 1992; Prokopy et al. 1995), especially under humid and warm conditions during the evening (Lafleur and Hill 1987; Chouinard et al. 1993). The olfactometer consisted of a large (inner $\varnothing = 105$ mm, 50 mm in height) round, dome-shaped Pyrex® glass container (Corning Inc., Corning, New York, USA) (Hock et al. 2013). Two lateral openings on the very top of the dome (inner $\varnothing = 24$ mm, 60 mm height) were used as connectors to the odour-containing jars. The central opening at the apex (inner $\varnothing = 24$ mm, 60 mm height) was the point of introduction for plum curculio into the arena. Odour jars consisted of 500 ml standard glass Mason™ jars (Bernardin LTD., Richmond Hill, On., Canada); one jar contained the ‘test’ odour source and the other the control (air). The jars were placed upside-down, one on each lateral opening and Parafilm®M (Sigma-Aldrich Canada Ltd.) was used to secure the jars to the olfactometer. The lateral openings as well as the mouth of the Mason jars were covered with standard nylon mosquito screening to prevent insects from joining odour sources or escaping. The olfactometer was placed in an observation room held at ca. 25 ± 2 °C and 70% R.H.

(Smith and Flessel 1968). Experiments were conducted during a simulated scotophase (night), by gradually dimming the lights so that experiments could be conducted during the day, leaving the photoperiod to occur at night (*i.e.* an altered light cycle). This was done, starting from the time curculio's were immature, because plum curculio are known to be more active during the night (Chouinard et al. 1993) and have been shown to produce pheromone during scotophase (Hock et al. 2014). A red filter (LEE Filter, red primary no. 106®, Son-Art Production, Saint-Hyacinthe, Qc, Canada) covered a neon light (40 W) that served as the sole source of light during experiments since plum curculio activity and behaviour are not disturbed by red light (Prokopy et al. 1995). Odour zones were randomized after each replicate to limit any bias associated with the environment of the olfactometer. The entire olfactometer was dismantled daily, prior to each new experiment, washed with Sparkleen™ soap (Fisherbrand, Pittsburgh, Penn., USA), rinsed with acetone and hexane (Sigma-Aldrich Canada Ltd.) to remove any possible lingering odours or contamination, and air-dried.

Experimental Conditions Each female served as a replicate and the time of each replicate was always recorded. At the beginning of each trial one female was introduced into the olfactometer and left for 30 min, after which its position was noted and the female removed. Only insects found within a radius of 10 mm or within the tubes leading to the odour jars (e.g. test or control odour zones) were used for statistical analysis. All other positions were considered as no choice having been made and were disregarded in analysis (average of approximately 35%) (Tinzaara et al. 2007; Altuzar et al. 2007; Akotsen-Mensah 2010). The average number of replicates was 20, however occasionally replicate number was higher or lower due to occasional deaths of plum curculio during rearing, and because not all plum curculio responded during trials (Hock et al. 2014, 2015).

Volatiles Tested Both synthetic and natural host plant odours were selected based on volatiles present in various plum curculio host-plants (*i.e.* plums, apples, and blueberries) that are known to elicit behavioural responses (Leskey et al. 2001, 2005; Akotsen-Mensah 2010). Odours tested were synthetic plum essence which was the most attractive synthetic host plant volatile in pre-tests – plum being the preferred host plant for plum curculio (Leskey and Wright 2007), trans-2-hexenal which is a green leaf volatile (saturated/monosaturated six-carbon alcohols/aldehydes) present in many plants (Metcalf and Metcalf 1992) and a natural aroma characteristic of apples (Corbo et al. 2000) that curculios can detect (Hock et al. 2014) and respond to (Prokopy et al. 2003), blueberry essence because blueberries can be host plants for the plum curculio (Mampe and Neunzig 1967; Alm and Hall 1986; Polavarapu et al. 2004; Lafleur et al. 2007) especially in North Carolina, New Jersey and Delaware where plum curculio can be a major pest (Jenkins et al. 2006), an apple ester and McIntosh apple Essence both of which are based on one of the most attractive hosts for curculios (Quaintance and Jenne 1912; Leskey and Wright 2007). Fruit essences, like plum essence (Akotsen-Mensah 2010) were essentially commercial blends of concentrated fruit juices. Benzaldehyde was also tested since it is a natural component of unripe apples, apple blossoms, and plums and has been shown to be attractive to plum curculio in field studies both alone and in various odour combinations (Leskey et al. 2001; Leskey et al. 2008; Akotsen-Mensah et al. 2010). Thinning apples are a favourite food source for plum curculio and

are commonly used to rear laboratory populations (Quaintance and Jenne 1912; Leskey and Wright 2007). Previous studies (Leskey and Prokopy 2001) found punctured fruit to be the most attractive to plum curculio compared to un-punctured fruit, we therefore decided to test the response of fed females and females starved for 24 h to five un-punctured and five punctured thinning apples as well.

Tests for Synergy We also tested the following for synergy: benzaldehyde + plum essence, two males + plum essence, two males + five un-punctured apples, (+)GA + plum essence. Field tests have shown that the combination of plum essence and benzaldehyde is also attractive to multivoltine females (Akotsen-Mensah et al. 2010); which is why we chose to test this odour combination on multivoltine females. Male plum curculio used in experiments were always mature and virgin. Since two mature virgin males was previously shown to be attractive to virgin female plum curculio of both strains (Hock et al. 2013, 2014), this quantity of males was used to see if the combination of males and host plant volatiles would give a greater response than either odour alone. Males were placed in a 500 ml odour jars with either apples or microcentrifuge tubes containing the synthetic host plant volatiles. We also tested (+)GA in conjunction with plum essence on univoltine females in order to ascertain if there would be increased attraction or synergy between these two compounds, since other compounds (*i.e.* benzaldehyde) have been shown to synergize with the racemic mixture of grandisoic acid (Piñero et al. 2001; Akotsen-Mensah et al. 2010). We chose to test only (+)GA (and not the (–)-enantiomer) since it is the synthetic version that most closely corresponds to the pheromone produced naturally by males in the wild (Eller and Bartelt 1996), but also based on the fact that electroantennogram (EAG) experiments showed that plum curculio are able to detect this molecule (Hock et al. 2014). Furthermore, the (–)-enantiomer was found not to be attractive to plum curculios in previous bioassays (Hock et al. 2015). Solutions of synthetic (+)GA were made by diluting the granular form in heptane (Sigma-Aldrich Ltd.). A concentration of 2×10^{-7} mg/ml of (+)GA with an optical purity (*i.e.* enantiomeric excess) of 72% was used for univoltine females, since this was the concentration and purity of (+)GA found to attract the most females in previous laboratory studies (Hock et al. 2015).

Test Odour Source Material All synthetic odours were kept in a freezer at -21 °C until use. For synthetic odours 1.5 ml microcentrifuge tubes (Sigma-Aldrich Canada, Ltd.) were filled and a cotton wick was inserted through a hole at the top and left protruding in order to allow dispersion of the odour. Tests using more than one synthetic odour source were done by placing a micro tube of each odour in the same olfactometer test jar. Details of synthetic host plant volatiles are as follows: plum essence (Milne Fruit Products Inc. 804 Bennett Avenue, Prosser, WA, USA), trans-2-hexenal (Sigma-Aldrich Canada Ltd.), blueberry essence (Kerry Inc. Ingredients and Flavours, 555 Rutherford, Granby, Qc., Canada), McIntosh apple essence (Kerry Inc. Ingredients and Flavours, 555 Rutherford, Grandby, Qc., Canada), apple ester (Metarom Neotech Inc., 5000 Armand Frappier, St-Hubert, Qc., Canada), and benzaldehyde (Sigma-Aldrich Ltd.). These volatiles were obtained as liquids and tested as neat solutions, since previous laboratory and field studies have shown that similar concentrations elicited a response from plum curculio, and are attractive or repulsive depending on the odour being tested (Leskey et al. 2001, 2008; Akotsen-Mensah 2010,

Leskey et al. 2014). Natural fruit odours tested consisted of thinning McIntosh apples which were collected from apple orchards in Saint-Bruno-de-Montarville, Québec, Canada during the summer of 2010 and kept in cold storage (5 °C) until use in experiments. Prior to the beginning of each experiment apples were washed to remove any pesticides present. ‘Punctured’ apples consisted of apples with two holes (one old made 24 h prior, and one fresh made immediately before the start of the experiment) made using a sterile pin in order to simulate plum curculio feeding injuries (old and fresh, respectively) (Leskey and Prokopy 2001). The synthetic pheromone (+)GA was obtained from the University of Quebec at Chicoutimi (Qc, Canada) as per the methods described in Hock et al. (2014). Briefly, racemic grandisol was obtained from commercial grandlure (Bedoukian Research, USA) and directly converted to racemic grandisoic acid using pyridinium dichromate in dimethylformamide. Resolution of grandisol was obtained in gas chromatography using β -cyclodextrin stationary phase to show that it was racemic. Using simple gradient condition at pH 4.5 in high performance liquid chromatography with a β -CD column, selectivity was observed for grandisoic acid. Direct separation of grandisoic acid was optimized to selectivity $\alpha = 1.1$ and APCI-MS analysis was performed to confirm the identities of grandisoic acid enantiomers (Hock et al. 2014, 2015).

Statistical Analysis SPSS statistical software (SPSS Inc. 2006, Chicago, Illinois) was used to analyze all data. Comparisons between choices (test vs. control) were analyzed using a Binomial test ($P < 0.05$); with each success (x) having a 50% chance of succeeding ($p = 0.5$). Comparisons between two experiments or treatments were done using a χ^2 contingency Table ($P = 0.05$) (Sokal and Rohlf 1995).

Results

Fed, Mature, Virgin female Response to Host Plant Odours Significantly more univoltine females responded to plum essence compared to the control ($n = 26, x = 19, P < 0.0001$). Trans-2-hexenal was found to be significantly repulsive ($n = 14, x = 1, P < .001$) as was McIntosh apple Essence ($n = 15, x = 4, P = 0.042$), while neither blueberry essence ($n = 18, x = 6, P = 0.071$), Apple ester ($n = 25, x = 10, P = 0.097$), or benzaldehyde ($n = 12, x = 5, P = 0.193$) elicited a significant response (Fig. 1). There was also no significant difference in the response of fed univoltine females to either unpunctured ($n = 12, x = 3, P = 0.054$) or punctured ($n = 9, x = 2, P = 0.070$) apples vs. the control (Fig. 1). For multivoltine females, only plum essence and benzaldehyde were tested, with plum essence being significantly more attractive than the control ($n = 29, x = 20, P = 0.019$), and benzaldehyde was significantly repulsive compared to the control ($n = 16, x = 1, P < 0.001$) (Fig. 1). The odour combination of plum essence and benzaldehyde elicited a significant repulsive response from multivoltine females ($n = 12, x = 1, P = 0.003$). Multivoltine females did not show any significant difference in response to the apple modalities vs. the control (*i.e.* unpunctured apples: $n = 11, x = 7, P = 0.161$; punctured apples: $n = 11, x = 7, P = 0.161$) (Table 1). There was a significant difference between host plant odours for both univoltine ($\chi^2 = 22.1, df = 7, P = 0.002$) and multivoltine ($\chi^2 = 25.3, df = 4, P < 0.001$) females.

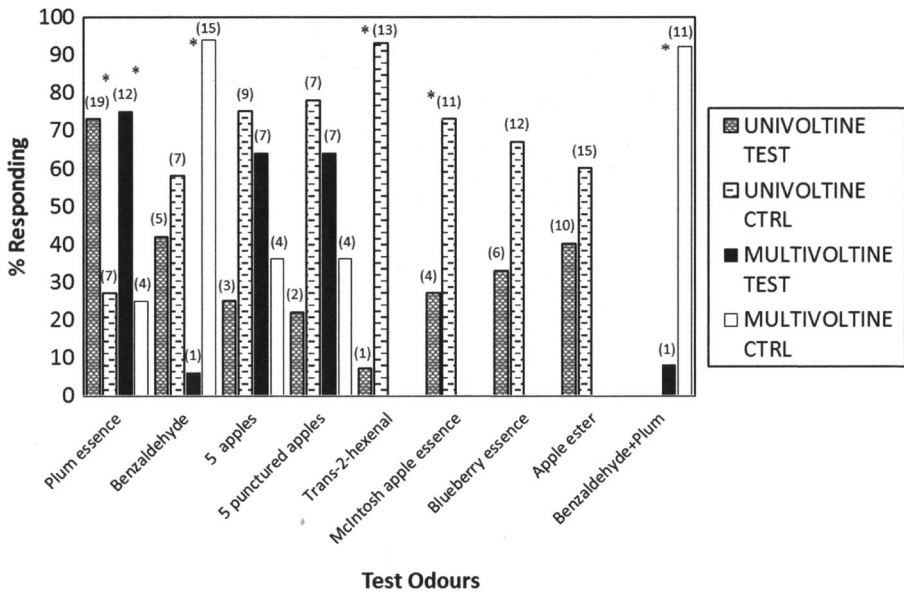


Fig. 1 Percent of mature, fed, virgin univoltine female responding to different host plant volatiles in a dual-choice olfactometer. Synthetic host-plant volatiles (1.5 ml): plum essence, trans-2-hexenal, McIntosh apple essence, blueberry essence, apple essence, benzaldehyde, benzaldehyde + Plum = benzaldehyde and plum essence. Non-synthetic host plant volatiles 5 apples (thinning), 5 punctured apples (thinning), ctrl = control. * denotes significant differences between test and control odours (Binomial test, $P < 0.05$); numbers in parentheses denote number of curculios responding

Influence of Physiological factors on Female Responses to Host Plant Odours.

Age For univoltine females the response of immature females to plum essence was statistically significant ($n = 66, x = 40, P = 0.022$) (Fig. 2). Significantly more mature females were also found in the test jar with the odour of plum essence than in the control jar ($n = 33, x = 23, P = 0.011$). There was no significant difference in the response of old females to plum essence vs. control ($n = 45, x = 22, P = 0.117$) (Table 1). There was no significant difference between ages for univoltine females ($\chi^2 = 3.53, df = 2, P = 0.171$), but given that mature females responded to plum essence in a significant manner and in greater proportion than other ages did, mature females were used in subsequent tests.

Mated Status Significantly more fed univoltine virgin females were attracted to plum essence than the control ($n = 20, x = 15, P = 0.015$) (Table 1). There was no significant difference in the response to plum essence vs. control from mated females ($n = 16, x = 11, P = 0.07$) (Table 1). There was no significant difference between mated status for univoltine females ($\chi^2 = 0.173, df = 1, P = 0.677$) therefore virgin females were used in all following tests.

Starvation Significantly more fed univoltine females were found in the test jar containing plum essence than in the control jar ($n = 11, x = 9, P = 0.027$). There was no difference in response for plum essence vs. control for females starved for 24 ($n = 15, x = 7, P = 0.196$), 48 ($n = 16, x = 7, P = 0.174$), or 72 h ($n = 8, x = 6,$

Table 1 Response of plum curculio to natural and synthetic (1.5 ml) host plant volatiles and pheromones. (+)GA = (+) enantiomer of grandisoic acid, ee = enantiomeric excess, NA = not applicable. * denotes significant differences between test and control odours (Binomial test, $P < 0.05$), where x = number of successes (*i.e.* curculios responding to test odour), n = total number of plum curculio responding to odours (*i.e.* number of replicates / trials) and p = probability of successes (*i.e.* probability of choosing the test odour) which was 0.5

Olfactometric Tests		Binomial Statistic Results ($P < 0.05$, $p = 0.5$)					
		Univoltine Plum Curculio			Multivoltine Plum Curculio		
Physiological State of Females	Odour	x	P	n	x	P	n
		<i>Test Responses</i>			<i>Test Responses</i>		
Virgin, fed, immature / old	plum essence	40	0.022*	66	NA	NA	NA
		22	0.117	45	NA	NA	NA
Virgin, fed, mature	plum essence	19	0.001*	26	12	0.028*	16
Fed, mature, mated	plum essence	11	0.070	16	NA	NA	NA
Virgin, mature, and starved: (0 h),	plum essence	(9)	(0.027)*	(11)	(20)	(0.019)*	(29)
24 h,		7	0.196	15	8	0.167	18
48 h,		7	0.174	16	4	0.121	12
72 h		6	0.109	8	9	0.027	11
Virgin, fed, mature	McIntosh apple essence,	4	0.042*	15	NA	NA	NA
	blueberry essence,	6	0.071	18	NA	NA	NA
	apple ester	10	0.097	25	NA	NA	NA
Virgin, fed, mature	trans-2-hexenal	1	<0.001*	14	NA	NA	NA
Virgin, fed, mature	benzaldehyde	5	0.193	12	1	<0.001*	16
Virgin, fed, mature	benzaldehyde and plum essence	NA	NA	NA	1	0.003*	12
Virgin, fed, mature	5 thinning apples,	3	0.054	12	7	0.161	11
	5 thinning punctured apples	2	0.070	9	7	0.161	11
Virgin, mature, and starved 24 h	5 thinning apples,	12	0.002*	13	9	0.153	15
	5 thinning punctured apples	10	0.016*	12	5	0.246	9
Virgin, fed, mature	2×10^{-7} mg/ml 72% ee (+)GA and plum essence	7	0.193	12	NA	NA	NA
Virgin, mature, and starved 72 h	2×10^{-7} mg/ml 72% ee (+)GA and plum essence	4	0.038*	16	NA	NA	NA
Virgin, fed, mature	2 males and plum essence	10	0.092	15	12	0.120	20
Virgin, fed, mature	2 males and 5 thinning apples	5	0.092	15	7	0.174	16
		8	0.121	12	5	0.193	12

Table 1 (continued)

Olfactometric Tests		Binomial Statistic Results ($P < 0.05, p = 0.5$)	
		Univoltine Plum Curculio	Multivoltine Plum Curculio
Virgin, mature, and starved 24 h	2 males and 5 thinning apples		

$P = 0.109$). For multivoltine females the situation was similar, with fed females giving a significant response to plum essence ($n = 16, x = 12, P = 0.028$) while those starved for 24 ($n = 18, x = 8, P = 0.167$), and 48 h ($n = 12, x = 4, P = 0.121$) gave no significant response, but those starved for 72 h also giving a significant response ($n = 11, x = 9, P = 0.027$) (Table 1). There was no significant difference between starvation periods for univoltine females ($\chi^2 = 4.44, df = 3, P = 0.218$) but there was a significant difference for multivoltine females ($\chi^2 = 8.81, df = 4, P = 0.032$) females.

Significantly more univoltine females starved for 24 h responded to un-punctured ($n = 13, x = 12, P = 0.002$) and punctured apples ($n = 12, x = 10, P = 0.016$) than to the control (Fig. 3), though there was no significant difference in response of starved univoltine females between punctured and un-punctured apples ($\chi^2 = 0.476, df = 1, P = 0.490$). Starved multivoltine females did not exhibit a significant difference in response to the un-punctured ($n = 15, x = 9, P = 0.153$) or punctured apples ($n = 9, x = 5, P = 0.246$) (Table 1); and there was also no significant difference in response of starved multivoltine females between punctured and un-punctured apples ($\chi^2 = 0.0457, df = 1, P = 0.831$).

Odour Combinations Two Males + Apples Since no difference in the response to punctured vs. un-punctured apples was observed, un-punctured apples were used in conjunction with two mature virgin males to test the response of females to this odour combination. For univoltine females, neither those fed ($n = 15, x = 5, P = 0.092$) (Fig. 4) or starved for 24 h ($n = 12, x = 8, P = 0.121$) gave a significant response compared to

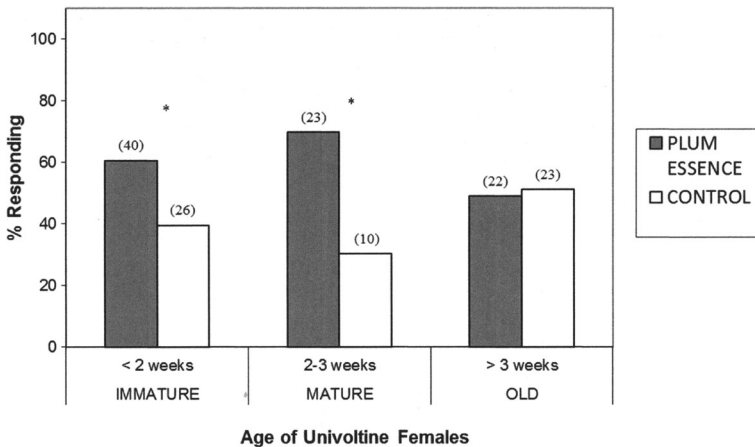


Fig. 2 Percent of fed univoltine females of different age groups (immature, mature, old) responding to plum essence (1.5 ml) in a dual-choice olfactometer. * denotes significant differences between test and control odours (Binomial test, $P < 0.05$); numbers in parentheses denote number of curculios responding

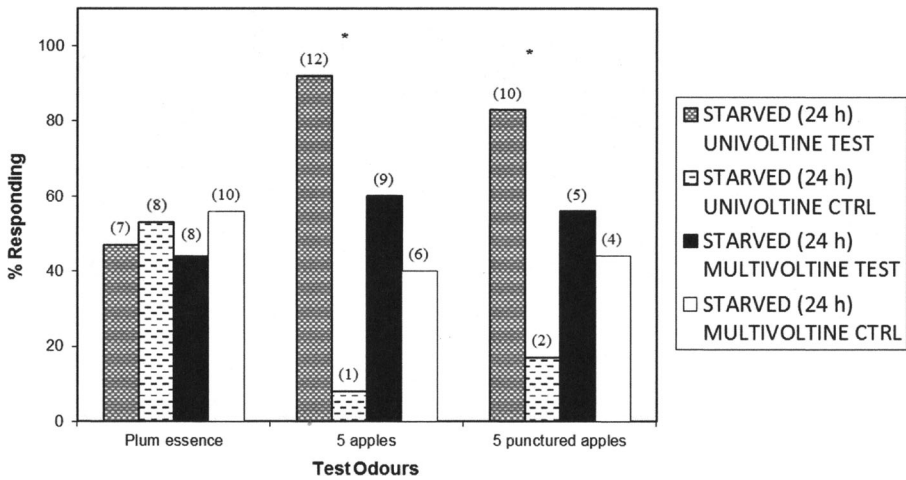


Fig. 3 Percent of univoltine and multivoltine females starved for 24 h responding to host plant volatiles in a dual-choice olfactometer. 5 apples (thinning), 5 punctured apples (thinning), ctrl = control. * denotes significant differences between test and control odours (Binomial test, $P < 0.05$); numbers in parentheses denote number of curculios responding

the control. The situation was similar for multivoltine plum curculio with neither of these odour combinations eliciting a significant response from fed ($n = 16, x = 7, P = 0.174$) or starved ($n = 12, x = 5, P = 0.193$) females vs. the control (Table 1).

Two Males + Plum Essence The addition of two males to plum essence did not enhance the response of univoltine females ($n = 15, x = 10, P = 0.092$) compared to the control (Fig. 4). For virgin multivoltine females, the response to the odour combination

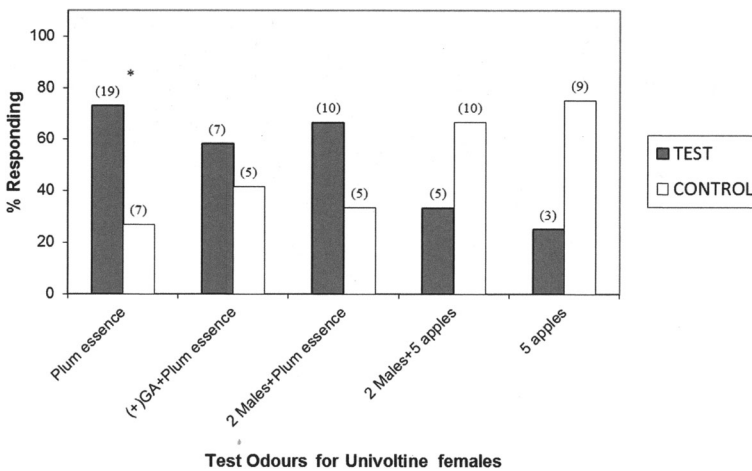


Fig. 4 Percent of fed univoltine females responding to natural and synthetic pheromones and host plant volatiles, alone and in combinations, in a dual-choice olfactometer. 5 apples (thinning), 2 males = two mature virgin univoltine males, (+)GA = 2×10^{-7} mg/ml (+) enantiomer of grandisoic acid at 72% optical purity. * denotes significant differences between test and control odours (Binomial test, $P < 0.05$); numbers in parentheses denote number of curculios responding

of plum essence with two males was similarly non-significant ($n = 20$, $x = 12$, $P = 0.120$) (Table 1).

(+)GA with Plum Essence There was no significant difference between fed female response to control vs. (+)GA in combination with plum essence ($n = 12$, $x = 7$, $P = 0.193$) (Fig. 4). Although there was no significant difference in response to plum essence for any of the starvation periods mentioned previously for univoltine females (*Influence of Physiological factors on Female Responses to Host Plant Odours. Starvation*), there was a significant difference for multivoltine females. Furthermore, numerically more univoltine and significantly more multivoltine females chose plum essence when starved for 72 h than any of the other starvation periods. This was therefore the starvation period chosen to gauge the influence of starvation on univoltine plum curculio response to the concentration of 2×10^{-7} mg/ml of (+)GA in the presence of plum essence. There was a significant repulsion to the combination of (+)GA and plum essence from females starved for 72 h ($n = 16$, $x = 7$, $P = 0.038$) (Table 1).

Discussion

Of the synthetic volatiles tested, only plum essence elicited the strongest attraction from mature, fed, virgin females of both strains, which supports the bioassay results of Akotsen-Mensah (2010). Leskey and Prokopy (2001) found that bioassay responses of female plum curculio to the odour of fresh wild plums (*Prunus americana* Marshall) were significantly stronger compared to the control. Furthermore, volatiles from ‘Stanley’ plum also elicit strong electroantennogram responses (EAG) from plum curculio (Leskey et al. 2009a; Hock et al. 2014), with some plum cultivars (European *P. domestica* L.: ‘Stanley’, ‘Japanese’ *P. salicina* Lindl.: ‘Formosa’, and ‘Santa Rosa’) being preferred over American cultivars (*P. americana*) (Leskey et al. 2001, 2005; Leskey and Wright 2007). Plum essence was also found to be highly attractive to plum curculio in field trials (Coombs 2001; Whalon et al. 2006; Akotsen-Mensah et al. 2010), indicating its possible use as a component in baited traps. Starvation (24, 48) did not increase attraction to plum essence in the current study, supporting the results of Akotsen-Mensah et al. (2010), indicating that fed plum curculio will still respond strongly to attractive synthetic host plant volatiles in the field. However multivoltine curculios starved for 72 h did respond strongly to plum essence, indicating that plum essence may be used throughout the season as a trap bait and is not limited to being used either solely on starved individuals that exit winter diapause at the beginning of the season or solely on fed individuals later on in the season.

Mated univoltine females did not significantly respond to plum essence. Observations have been made on plum curculio (Leskey et al. 2001; Akotsen-Mensah 2010) and on other beetles (Ocellachain and Pyan 1977; Phillips and Burkholder 1981; Rochat et al. 1991) where mating status has not been shown to affect response. Conversely, there are cases with other insects, as well as plum curculios, where differences in attraction after mating have been observed (Shu et al. 1998; Qi and Burkholder 1982; Walgenbach et al. 1983; Hock et al. 2014). Plum essence could be signifying severely damaged fruit which might mean that the fruit is unsuitable for

oviposition, *i.e.* that other females have already deposited their eggs in that particular fruit, since an increase in the amount of larvae within a single fruit can decrease larval survival (Butkewich et al. 1987). Studies have shown that female plum curculio can discriminate against heavily damaged fruit for oviposition purposes (Butkewich et al. 1987) perhaps due to the type or quantity of volatiles released from the damaged tissue, which may signify an acceptable food source for virgin females who are looking for males and feeding sites, but an unusable site for mated females looking to oviposit. Using plum essence to attract virgin females, who have just exited winter diapause, towards attract-and-kill traps would reduce the amount of females that could mate and therefore also reduce damage due to oviposition as well as the subsequent ‘summer’ generation of plum curculios.

The phenology of host plants is synchronized with the physiology of plum curculio (Leskey et al. 1996, 2001, 2010) and the attraction of curculios to odours is greatest when volatiles are taken during petal fall (Quaintance and Jenne 1912; Whitcomb 1933; Steiner and Worthley 1941), the phenological stage when plum curculio are found in greatest numbers on host plants and are ready to lay eggs (*i.e.* sexually mature) (Hoffmann et al. 2004; Leskey et al. 2010). Thus the changes in plum curculio physiology around this time (from immature to mature states) may increase their preference for host plant odours such as those represented by plum essence, while conversely later on in the season older plum curculios would have access to older and perhaps less suitable fruit (regarding oviposition for example) which could be a reason for the decline in attraction to plum essence for aging univoltine curculios. Effect of age on response to host plant volatiles has been found in the pepper weevil (Addesso and McAuslane 2009) and the maize and granary weevil, *Sitophilus zeamais* Motschulsky and *Sitophilus granarius* L. respectively (Wakefield 1998). It is possible that the other synthetic host plant volatiles tested (benzaldehyde, trans-2-hexenal, McIntosh apple essence, apple ester, blueberry essence) did not “represent” the correct phenological stage that is the most attractive to mature plum curculio, even though similarly formulated synthetic odors (*e.g.* plum essence, benzaldehyde) have been found to elicit responses at similar concentrations in previous studies (Leskey and Prokopy 2001; Leskey et al. 2008; Akotsen-Mensah 2010). However, the concentrations used did elicit a significant repulsion for some synthetic odours (McIntosh apple essence, benzaldehyde, trans-2-hexenal, benzaldehyde + plum essence), indicating that these volatiles may be used in a push-pull strategy as repellents instead of attractants (Leskey et al. 2014).

For example, benzaldehyde is attractive to plum curculio in apple orchards alone and in various odour combinations (Pinero and Prokopy 2003, Leskey et al. 2008), but in our laboratory experiments benzaldehyde elicited either no response, or it caused repulsion, depending on the strain of plum curculio. Our results are in accordance with those of Akotsen-Mensah (2010) who found that pure benzaldehyde as well as benzaldehyde lures were inhibitory to multivoltine plum curculio in bioassays, both alone and in combination with racemic grandisoic acid or plum essence. Furthermore, the combination of benzaldehyde and racemic grandisoic acid was also unattractive to multivoltine curculios in peach orchards (Leskey 2006; Akotsen-Mensah 2010). The authors cited differences in environmental conditions (*i.e.* release rate of volatiles) and physiological conditions of plum curculio (lab-reared *vs.* wild insects, strain used) as possible causes for the observed differences between the results observed in laboratory

and other field trials. It is likely that these are also the reasons for the differences seen between field studies and our results; since the concentration tested in our olfactometric experiments is similar to that of other laboratory and field experiments (Leskey et al. 2008; Akotsen-Mensah 2010). Given that benzaldehyde is currently used as an attractant in the field at a release rate of 228 mg/day/tree in a trap tree strategy to control plum curculio (Leskey et al. 2014), using it as repellent as well may not be the best strategy, at least not in apple orchards. Using a completely different odour (*i.e.* trans-2-hexenal) as a repellent might be more effective.

In our studies, trans-2-hexenal was repulsive to univoltine females. In other insects trans-2-hexenal has been found to be attractive (Landolt and Phillips 1997), and such green leaf volatiles can be used for host-plant recognition (Metcalf and Metcalf 1992; Bernays and Chapman 1994) as well as to enhance the effects of pheromones among certain insects (Dickens et al. 1990). EAG studies indicate that plum curculio are capable of detecting and responding to 0.05 mL of evaporated trans-2-hexenal (Hock et al. 2014). In the field, a release rate of 40 mg/day/tree (Prokopy et al. 2003) of trans-2-hexenal was found unattractive, and trans-2-hexenal can be repulsive at high concentrations in laboratory bioassays (Leskey et al. 2001). Thus trans-2-hexenal may have a possible use as a repellent for plum curculio, protecting trees as the ‘push’ component in a push-pull approach (Leskey et al. 2014).

Punctured apples were supposed to simulate feeding scars left by plum curculio (Leskey and Prokopy 2001) and were as attractive to starved (24 h) univoltine females as un-punctured apples, indicating the attraction of starved plum curculio to natural whole-fruit odours, supporting the results of Leskey and Prokopy (2001). Using the complete volatile combinations present in whole fruit (Leskey et al. 2009a, 2009b, Leskey et al. 2010) may improve baits *vs.* a single plant volatile, as is currently used in apple orchards (Piñero et al. 2011). For example, multiple volatile combinations of host plant odours have been found more attractive than host plant and pheromone combinations in peach orchards (Akotsen-Mensah 2010).

In some field trials, plum curculio pheromones synergize with host-plant volatiles such as benzaldehyde (Piñero et al. 2001; Pinerio and Prokopy 2003; Leskey et al. 2005), and many weevil pheromones have been observed to synergize with host plant volatiles (Landolt and Phillips 1997). Males advertise their presence to females by indicating an available food source nearby for feeding and oviposition, increasing their chances of attracting and mating with females (Landolt and Phillips 1997). In our study, the combination of five apples and two males did not enhance the attraction of plum curculio, whether fed or starved, neither did the combination of two males and plum essence or two males and (+)GA. Although it is possible that males may have fed on apples during the testing of the five apples + two males odour combination, Leskey and Prokopy (2001) found that females did not respond differently to the odour combinations of plums + males *vs.* mechanically injured plums, though females preferred both of these odors over the control. This indicates that plum curculio may not readily differentiate between mechanical injuries to fruit and injuries caused by other plum curculio. Though pheromone emissions were not collected from the males during these tests, two males (in the presence of apples) were shown to produce grandisoic acid during scotophase in pre-tests done in our lab, and eight males have been shown to produce more pheromone during scotophase when in the presence of fruit (Hock et al. 2014). Two virgin mature males also elicited the greatest attraction from female plum

curculio of both strains compared to other numbers and ages of males (Hock et al. 2014). Males can produce pheromone from eight to 36 days of age, with an average peak between 12 and 20 days (Hock et al. 2014). This indicates that the males used in our studies were likely producing pheromone, and the lack of attraction of the fruit + male combinations supports previous laboratory findings; for example, although attractive separately, males or synthetic racemic grandisoic acid combined with whole fruit odours (*i.e.* plums) or plum essence gave a neutral effect in bioassays (Leskey and Prokopy 2001; Akotsen-Mensah 2010).

In our study, starved (72 h) univoltine female plum curculio were repulsed by the combination of (+)GA and plum essence indicating they have no interest in the odour of males and may avoid them. Starvation has also been shown to decrease responsiveness to pheromones in the southern green stink bug, *Nezara viridula* L. (Brennan et al. 1977). Female curculios require food to oviposit (McGiffen and Meyer 1986; Butkewich and Prokopy 1993), but it is unclear if they feed before mating. If females must feed prior to mating, this may prompt them to seek food before seeking males. Numerous males in the immediate vicinity may hamper feeding efforts by females, since males readily and repeatedly mate within short periods of time (Johnson and Hays 1969), especially in the presence of fruit odours (Leskey and Prokopy 2001). Conversely, it is possible that when combined, the mix of volatiles used in our study may simply be in the wrong combination or ratio for plum curculio. For example, field trials have shown that the three component combination of benzaldehyde, plum essence, and racemic grandisoic acid was less attractive than the ‘host plant only’ combination of benzaldehyde and plum essence, which showed an additive effect (Akotsen-Mensah 2010). Concentration and ratio of individual odours placed in a combination has been shown to be a very important factor in plum curculio (Leskey et al. 2001; Leskey and Prokopy 2001) and weevil response (Coffelt and Burkolder 1972; Hardee et al. 1974; Hallett et al. 1999). Our results support those of Leskey and Prokopy (2001) and Akotsen-Mensah (2010), suggesting that plum curculio are primarily attracted to fruit volatiles and only secondarily to the odour of conspecifics, indicating the importance of fruit odours as key components in trap baits.

In general, our results indicate that both plum curculio strains responded similarly to most host plant odours tested, especially regarding attractive odours like plum essence. This means that plum essence may possibly be used in baits to monitor both univoltine and multivoltine plum curculio, which would be more cost effective in regions where the two strains overlap than employing two different trap baits based on strain. However, the use of these baits may still depend upon many factors present in a field setting that we were unable to test in our olfactometer, including the type of fruit orchard being protected, since the combined lure of racemic grandisoic acid and benzaldehyde was found to be effective in apple orchards but not in peach orchards (Leskey and Wright 2004a; Leskey 2006; Akotsen-Mensah 2010). Therefore, field trials are the next logical step in determining the effectiveness of these odours within an orchard.

Given the results, any future olfactometric studies should focus on using mature virgin females responders to plum essence or natural odours and extracts thereof, and tests using synthetic grandisoic acid or live males should include the use of fed responders. The results of our experiments demonstrate when plum curculio are most attracted to certain odours (*e.g.* mature, virgin), and therefore give an indication of which odours may work best as baits (*e.g.* plum essence) or antiaggregation odours

(e.g. *trans*-2-hexenal) throughout the season. This information can improve monitoring techniques by helping to develop better trap baits, or by the use of repellent volatiles for tree protection (Leskey et al. 2014; Gökçe et al. 2014).

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