Behavioral Responses of Plum Curculio (Coleoptera: Curculionidae) to Different Enantiomer Concentrations and Blends of the Synthetic Aggregation Pheromone Grandisoic Acid

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ABSTRACT The plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), is an important pest of fruit in North America. Males produce an aggregation pheromone (grandisoic acid) that attracts both sexes of the northern univoltine and the southern multivoltine strains. Grandisoic acid ((1*R*,2*S*)-1-methyl-2-(1-methylethenyl)-cyclobutaneacetic acid) is a chiral molecule containing one chiral center. A synthetic racemic mixture will contain two optical isomers that are mirror images of each other with equal amounts of (+)- and (−)-enantiomeric isomers. Male plum curculio only produce the (+) enantiomer. Some enantiomers can have antagonistic effects on the attraction of weevils to pheromones. An understanding of the effect of both enantiomers on the behaviour of plum curculio is needed to develop more efficient trap baits. Behavioural bioassays were conducted in a dual-choice still-air vertical olfactometer using a quantity of 1.5 ml of both (+) and (−) synthetic enantiomers and the racemic mixture of grandisoic acid with live female responders to determine which concentration and enantiomeric purity is the most attractive and if there is an antagonistic effect of the unnatural (−) enantiomer. Results indicated that plum curculio were attracted to low concentrations of the (+) enantiomer at 72% enantiomeric excess, but that strains were attracted to different concentrations of the (+) enantiomer (2×10^{-7} mg/ml for univoltine, 2×10^{-9} mg/ml for multivoltine).

RÉSUMÉ La charançon de la prune, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), est un ravageur important des fruits de l'Amérique du Nord. Les mâles produisent une phéromone agrégative – l'acide grandisoïque – qui attire les males et femelles de la souche univoltine du nord et de la souche multivoltine du sud. L'acide grandisoïque ((1*R*,2*S*)-1-methyl-2-(1-methylethenyl)-cyclobutane-acetic acid) est une molécule chirale contenant une centre chiral, qui résulte dans la possibilité de deux enantiomères. La version synthétique de cette phéromone contient au départ des quantités égales de l'énantiomère (+) et (−), cependant la phéromone produite naturellement par les mâles contient seulement l'enantiomère (+). Certains énantiomères peuvent empêcher l'attraction aux phéromones chez les autres charançons. Pour cette raison une bonne compréhension du comportement des charançons est nécessaire afin de développer des appâts plus efficaces. Des bio-essais ont été effectués dans un olfactomètre vertical à deux choix, sans courant d'air, avec une quantité de 1.5 ml de chaque énantiomère et du mélange racémique d'acide grandisoïque, avec des femelles vivantes comme répondeurs afin de déterminer la concentration et niveau de pureté le plus attirant pour les femelles, et si l'énantiomère (−) a un effet antagoniste. L'énantiomère (+) de l'acide grandisoïque avec un excès énantiomérique de 72% a été le plus attirant pour les femelles matures vierges. Les deux souches ont été attirées par de faibles concentrations de cet énantiomère, mais à des concentrations différentes (2×10^{-7} mg/ml pour la souche univoltine, 2×10^{-9} mg/ml pour la souche multivoltine).

KEY WORDS aggregation pheromone, attractant, repellent, odour, olfactometer

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One of the most important native pests of fruits in eastern North America is the plum curculio, *Conotrachelus nenuphar* (Herbst), 1797 (Coleoptera: Curculionidae). Damage at harvest from this pest can reach up to 90% of pome and stone fruit (Vincent and Roy 1992). There are two reproductively incompatible strains of this insect (Padula and Smith 1971; Zhang and Pfeiffer 2008, 2010), a northern univoltine strain with an obligatory diapause, and a multivoltine southern strain with a facultative diapause (Racette et al. 1992).

Plum curculio, like most insects (Bernays and Chapman 1994), uses olfactory cues to locate conspecifics and fruit for mating, feeding, and oviposition (Butkewich et al. 1987, Butkewich and Prokopy 1993, Leskey et al. 2005). Virgin males produce grandisoic acid (1R,2S)-1-methyl-2-(1-methylethenyl)-cyclobutaneacetic acid (Eller and Bartelt 1996), which is presumed to be the major component of its aggregation pheromone. This component has been shown to be attractive to both strains and both sexes (Eller and Bartelt 1996). Furthermore, plum curculio have also been shown to respond to Grandlure, the aggregation pheromone of the boll weevil, *Anthonomus grandis* Boheman, 1843 (Tumlinson et al. 1969), in previous tests (Hock et al. 2014). Grandlure contains grandisol, and Curculionidae species that respond to Grandlure also usually produce multiple component pheromones (Hardee et al. 1974, Eller et al. 1994, Hedin et al. 1997, Cross et al. 2006, Armstrong 2010, Szendrei et al. 2011). For example, the boll weevil (Tumlinson et al. 1969), the strawberry blossom weevil, *Anthonomus rubi* Herbst, 1795 (Innocenzi et al. 2001), and the pepper weevil, *Anthonomus eugenii* Cano, 1894 (Eller et al. 1994, Addesso and McAuslane 2009), all produce multiple component pheromones. The various components of these aggregation pheromones have also been shown to be attractive at different concentrations or ratios for the boll weevil (Tumlinson et al. 1969), the pecan weevil, *Curculio caryae* Horn, 1873 (Hedin et al. 1997), and the strawberry blossom weevil (Innocenzi et al. 2001). It is thus possible that plum curculio also produces a multiple component aggregation pheromone with as-yet unidentified secondary compounds, e.g., compounds in addition to the primary component of grandisoic acid, and that attraction to these compounds depends on their concentration (Leskey et al. 2009a,b). The attractant isolated from virgin male plum curculio by Eller and Bartelt (1996) was the optically active, naturally occurring (+) enantiomer of grandisoic acid [(+)GA]. However, it should be noted that the synthetic grandisoic acid in the form of a racemic mixture [containing equal amounts of both the (+) and (−) enantiomer] was also found to attract plum curculio in some field studies (Eller and Bartelt 1996, Piñero et al. 2001, Piñero and Prokopy 2003, Leskey et al. 2005). The attractiveness of the individual (+) and (−) enantiomers, however, has to date not been evaluated in the field or in laboratory bioassays, with most studies using a synthetic racemic version (Eller and Bartelt 1996; Piñero et al. 2001, 2011; Piñero and Prokopy 2003; Leskey et al. 2005). In some laboratory studies, the racemic version has been found to be unattractive as well

(Akotsen-Mensah 2010). Different enantiomers have been shown to elicit different responses in other beetle species (Tumlinson et al. 1977, Dickens and Mori 1989) and even small amounts of an unnatural enantiomer can be antagonistic in some species (Lindgren et al. 1994). Enantiomeric excess (ee) is a measure of enantiomeric purity, which is known to affect the response of weevils like the boll weevil (Dickens and Mori 1989). Electroantennogram (EAG) studies performed on adult plum curculio using formulations with decreasing amounts of the negative enantiomer, (−)GA, and increasing amounts of (+)GA have also indicated an increase in the magnitude of response with increasing percent of (+)GA (Leskey et. al. 2009b).

Despite the quantified attraction provided by grandisoic acid dispensers in the field, trap captures throughout the season using these baits remain unreliable, especially when used as a sole component in baits, or after petal fall because of competition from attractive odours in the orchards (Prokopy et al. 2002, Leskey and Wright 2004). A reliable and efficient method for attracting, aggregating, and retaining plum curculio invading apple orchards has been difficult to provide (Prokopy et al. 2000, Leskey et al. 2008), but progress has been made by using the trap tree method of Piñero et al. (2011). However, captures can still be increased and damage to neighbouring trees reduced if a highly attractive odour could be identified for use as a trap bait. This would also decrease the number of trees and fruit sacrificed in the trap tree method, as well as reduce the amount of chemical insecticides applied.

In light of the influence that pheromone enantiomers, concentrations, and purities have in insect behavior (Tumlinson et al. 1977, Dickens and Mori 1989, Lindgren et al. 1994, Hedin et al. 1997, Innocenzi et al. 2001, Leskey et. al. 2009b), this study attempts to determine which synthetic grandisoic acid mixture is most attractive to adult plum curculio and at what concentration, and to investigate possible antogonistic effects of the negative enantiomer. Laboratory bioassays were conducted in a large upright dual-choice still-air olfactometer using both univoltine and multivoltine strains. The synthetic pheromone volatiles tested included a racemic mixture, (±)GA, of grandisoic acid (Eller and Bartelt 1996) and different purities of its two synthetic enantiomers, (+)GA and (−)GA. Different quantities and enantiomeric purities of grandisoic acid were tested to determine 1) which enantiomer or mixture is the most attractive and the concentration (mg/ml) at which the enantiomer or mixture is most attractive; 2) if the enantiomeric purity (percent ee of (+) vs (–) enantiomer) has an effect on plum curculio response, i.e., if there is an antagonistic effect of the (–) enantiomer; and 3) if the response between the two strains is similar.

Materials and Methods

Plum Curculio. Female plum curculios from both strains were used as per Hock et al. (2013, 2014). For univoltine plum curculio, larvae were obtained from

infested apples (*Malus* sp.) collected in late June to early July 2009 from unsprayed orchards. They were kept outdoors in the ground in emergence cages. Emerging adults were collected daily and separated by sex (Thompson 1932), then transferred to overwintering cages (Le Blanc 1992), and placed under natural conditions (e.g., outdoors) throughout the winter. Overwintered adults were removed from cages the following spring, placed in 2-liter plastic containers (152 mm in height, Ø = 125 mm at the base, 152 mm at the top). Containers were put in environmentally controlled chambers (model Sanyo LR-350H, with three active neon lights [32 W] = 400 lumens) at 25 ± 2°C, 70% relative humidity (RH), and a photoperiod of 16:8 (L:D) h to mimic optimal summer conditions (Amis and Snow 1985). They were fed immature apples, *Malus pumila* Borkhausen, 1803 'McIntosh' and given water (Hock et al. 2013, 2014). A laboratory population of plum curculio using some of the collected wild individuals from 2009 was also established as per Hock et al. (2013, 2014) based on the procedure of Hoffmann et al. (2007). Multivoltine plum curculio were obtained from a laboratory-reared population at the Appalachian Fruit Research Station (Kearneysville, WV) established in 2001 and augmented annually with wild individuals (Hock et al. 2013, 2014). Multivoltine plum curculio were held under similar conditions as the univoltine plum curculio (25 ± 2°C, 70% RH, and a photoperiod of 16:8 [L:D] h) and kept in environmental control chambers, as described in Hock et al. (2013, 2014). All females used were sexually mature and virgin. Experiments were conducted during a simulated scotophase, as plum curculio is known to be more active at night (Smith and Flessel 1968; Racette et al. 1990, 1991; Chouinard et al. 1993).

Olfactometer. The olfactometer used is as per Hock et al. (2013, 2014), which consisted of a large (Ø = 105 mm inner, 50 mm in height) round Pyrex glass container (Corning Inc., Corning, NY) with three apical openings (Ø = 24 mm inner, 60 mm in height). The central opening served as a point of introduction for insects into the arena, with a stopper to prevent the plum curculio from leaving after introduction. The two lateral openings were used as connectors to the jars containing the odour sources and were covered with standard nylon mosquito screening to prevent plum curculio from escaping and reaching the odour sources (Hock et al. 2013, 2014). A 500-ml standard glass Mason jar (Bernardin Ltd.., Richmond Hill, ON, Canada) was placed upside-down on each of the lateral openings on either side of the top of the olfactometer (Hock et al. 2013, 2014), one side (odour zone) containing the test odour source (i.e., synthetic pheromone) and the other containing the control (air) (Tafoya et al. 2003, Tinzaara et al. 2007, Addesso and McAuslane 2009). Each jar was secured to the olfactometer using ParafilmM (Sigma-Aldrich Canada Ltd. Oakville ON, Canada).

Experimental Conditions. Tests were conducted as described by Hock et al. (2013, 2014) during scotophase in an observation room held at 25 ± 2°C and 70% RH (Smith and Flessel 1968, Racette et al. 1991, Chouinard et al. 1993). A red filter (LEE Filter, red primary no. 106, Son-Art Production, Saint-Hyacinthe, QC, Canada) was used to cover a neon light (40 W) that served as the sole source of light during experiments, as plum curculio activity and behaviour are not perturbed by red light (Prokopy et al. 1995). A still-air setup was used, as is customary for plum curculio in behavioural and olfactometer experiments (Prokopy et al. 1995; Leskey et al. 1996, 2001; Leskey and Prokopy 2000, 2001; Akotsen-Mensah 2010) because of the fact that plum curculio are easily disturbed by air (Leskey and Prokopy 2001, Akotsen-Mensah 2010). 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; other positions were considered as no choice having been made and were disregarded in analysis (Tinzaara et al. 2007, Altuzar et al. 2007, Akotsen-Mensah 2010, Hock et al. 2013, 2014).

Odour zones were randomized after each replicate to limit any bias associated with the environment of the olfactometer. The entire olfactometer was dismantled prior to each new experiment, washed with Sparkleen soap (Fisherbrand, Pittsburgh, PA), rinsed with acetone and hexane (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) to remove any lingering odours prior to the next experiment, and then air-dried (Tinzaara et al. 2007, Amborgi and Zarbin 2008, Addesso and McAuslane 2009, Akotsen-Mensah 2010, Hock et al. 2013).

Source Material. All synthetic pheromones were kept in a freezer at − 21°C until use. Synthetic solutions were made by dissolving the granular pheromone in heptane (Sigma-Aldrich Inc., St. Louis, MO). All test solutions used filled 1.5-ml microcentrifuge tubes (Sigma-Aldrich Inc., 3050 Spruce Street, St. Louis, MO) and had a cotton wick protruding to allow dispersion of the odour. The microtube was then placed inside a 500-ml Mason jar, which was then attached to the olfactometer. Synthetic pheromone volatiles used in this study included (+)GA and (−)GA, as well as (±)GA.

The (±)GA and its enantiomers were obtained from the University of Quebec at Chicoutimi, QC, Canada. Different purities of enantiomers were obtained from (±)GA as per Hock et al. (2014), according to the methods described in Procedure for Oxidation of Racemic Grandisol.

Procedure for Oxidation of Racemic Grandisol. Grandisoic acid was prepared by sequential oxidation of grandisol, the active ingredient of commercial Grandlure (Bedoukian Research, Danbury, CT). N-methylmorpholine (NMO, 3,400 mg, 29.0 mmol) was added to a dried solution of 2,240 mg of grandisol (14. 5 mmol) in methylene chloride (CH_2Cl_2, 25 ml). The mixture was chilled with ice water, after which the catalytic amount of tetrapropylammonium perruthenate (TPAP, 255 mg, 0.726 mmol) was added to a dried solution of grandisol CH_2Cl_2. The mixture was stirred for 60 min at room temperature or until the reaction was complete, as shown by using thin-layer

chromatography. The solution was evaporated under reduced pressure to give a black oily residue (Hock et al. 2014).

The second oxidation step was conducted by dissolving crude grandisal in a mixture of t-butanol (30 ml), water (5 ml), and 2-methyl-2-butene (31 ml). The mixture was chilled to 0°C. An aqueous solution (20 ml) containing sodium chlorite (2,360 g, 26.1 mmol) and monosodium phosphate (3,800 mg, 27.6 mmol) was added drop-wise over a period of 10 min. The mixture was vigorously agitated for a period of 30 min. Work-up was performed by evaporating the solvent and adding 50 ml of $NaOH_2N$. The mixture was washed with CH_2Cl_2 (4×50 ml). The aqueous phase was acidified with aqueous HCl 10% to pH 4. The product was extracted with CH_2Cl_2 (5×50 ml) and dried with $MgSO_4$ (Hock et al. 2014).

Enantiomeric Purification. Enantiomeric purification was performed by co-crystallization of grandisoic acid. One gram of grandisoic acid was dissolved in hot ethanol-water mixture (50:50) and 965 mg of quinine was added. The solution was allowed to chill in a cold room ($-18°C$) for 24 h. The crystals obtained were filtered and re-crystallized three other times in a hot ethanol–water mixture (50:50). The mother liquors were combined each time. After four re-crystallization steps, the solid was dissolved in CH_2Cl_2 and (−)GA was extracted with $NaHCO_3$. The aqueous layer was washed with CH_2Cl_2 and neutralized with aqueous HCl 10%, which precipitates grandisoic acid. The latter was extracted with CH_2Cl_2, dried with $MgSO_4$, and evaporated under reduced pressure. The mixture was acidified with aqueous HCl 10% and grandisoic acid was extracted with CH_2Cl_2. The organic phase was washed with aqueous HCl 10%, dried with $MgSO4$, and evaporated under reduced pressure (Hock et al. 2014).

The optical rotation of the pure enantiomer is 47.9°, and the optical rotation obtained herein was +34° and +24.7° for the (+)-enantiomer, which results in an ee of 72 and 51.6% (+)GA, respectively. The optical rotation was −32° for the (−)-enantiomer, resulting in an ee of 66.8% (−)GA. The optical rotation of a racemic mixture is 0° with a resulting ee of 0% (+)GA (50:50 ratio of both enantiomers).

Pheromone Mixtures and Concentrations. A range of concentrations was chosen to determine the optimal concentration to which the plum curculio would give the greatest response. Concentrations were chosen based on preliminary tests done in our laboratory. While an attempt was made to ensure similar concentrations were tested between strains, the limited amount of grandisoic acid enantiomers available at the same purities occasionally resulted in unequal numbers of concentrations being tested. For univoltine females, vials of 1.5 ml with concentrations of 2×10^{-3}, 2×10^{-6}, 2×10^{-7}, 2×10^{-8}, 2×10^{-9}, and 2×10^{-10} mg/ml of (+)GA (72% ee) in a heptane solvent were used as test solutions. This amounts to doses of 3×10^{-3}, 3×10^{-6}, 3×10^{-7}, 3×10^{-8}, 3×10^{-9}, 3×10^{-10} mg, respectively. For multivoltine females concentrations, concentrations of 2×10^{-6}, 2×10^{-7},

2×10^{-8}, 2×10^{-9}, and 2×10^{-10} mg/ml of (+)GA (72%) were used (corresponding to doses of 3×10^{-6}, 3×10^{-7}, 3×10^{-8}, 3×10^{-9}, 3×10^{-10} mg, respectively). Based on the results of this experiment, tests using the most attractive concentration found for both strains were also conducted using a (+)GA solution with a lower enantiomeric purity of 51.6% (+)GA to see if the purity of solution [% ee (+)GA] would affect plum curculio response.

Regarding (−)GA at 66.8% ee, vials of 1.5 ml at concentrations of 20, 2, 0.2, and 2×10^{-7} mg/ml were tested for univoltine plum curculio. For multivoltine females, only the 2×10^{-7} mg/ml concentration was tested [because of low (–)GA amounts available]. The (±)GA mixture was tested at concentrations of 2×10^{-7} and 4×10^{-7} mg/ml for univoltine plum curculio (doses of 3×10^{-7} and 6×10^{-7} mg, respectively), and 2×10^{-9} and 4×10^{-9} mg/ml multivoltine (doses of 3×10^{-9} and 6×10^{-9} mg, respectively). These concentrations were chosen based on the results of the experiments using (+)GA, as we wished to determine if the same concentrations or higher concentrations found attractive to plum curculio using (+)GA would also be attractive using (±)GA.

The average number of replicates was 17 (range 9–41); however, occasionally replicate number was higher. Also, because there were occasional deaths of plum curculio during rearing within the selected age groups, and because not all plum curculio responded during trials, there were times when replicate number was lower (Hock et al. 2014).

Pheromone Enantiomeric Purity. Depending on how well the enantiomers of grandisoic acid can be separated, each solution has a different ratio of enantiomeric purity of these enantiomers. Purification of (±)GA into (+)GA usually leads to small quantities (g) of the purified enantiomer, and always with at least a small percent of (−)GA present as well (e.g., a mixture of 72% ee (+)GA will also contain 14% (−)GA). To see if plum curculio responded differently to different purities of (+)GA, the following purities were tested for both strains: 66.8% ee (–)GA, 0% (+)GA (*i.e.*, (±)GA), 51.6% ee (+)GA, and 72% ee (+)GA. Each enantiomeric purity was tested at the most attractive concentration found for each strain: the 2×10^{-7} mg/ml for univoltine females, 2×10^{-9} mg/ml for multivoltine females.

Statistical Analysis. SPSS statistical software (SPSS Inc., 2006, Chicago, IL) was used to analyze all data as per Hock et al. (2013, 2014). Comparisons between test and control odour choices were analyzed using a chi-square test ($P = 0.05$), as were the responses between experiments; a Yates continuity correction was applied when necessary (Siegel and Castellan 1988). Comparisons between two experiments were done using a Mann–Whitney U Test ($P = 0.05$). Comparisons between more than two trials or experiments were done using a Kruskal–Wallis Test ($P = 0.05$), if a significant difference was found a Mann–Whitney U Test with a Bonferronni correction (Shaffer 1995) was conducted to determine which groups were significantly different from each other ($P = 0.05$).
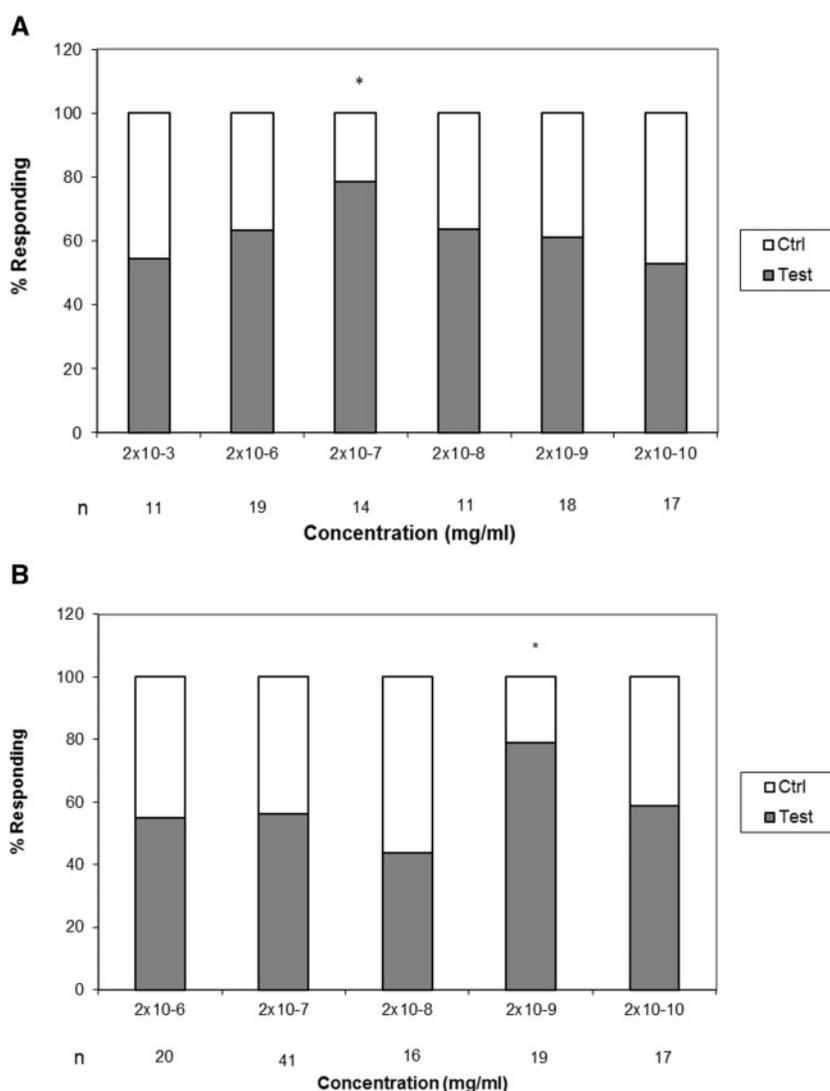
Fig. 1. Percent of female plum curculio responding to 1.5 ml of different concentrations of the positive enantiomer of grandisoic acid [(+)GA] at the same percent of enantiomeric excess (72% ee) versus the control (Ctrl), in a vertical still-air dual choice olfactometer. An "*" denotes a significant difference between test and control (Ctrl) at $P = 0.05$, according to the chi-square test. Replicates for each concentration are indicated below each graph (n). (A) Univoltine females. (B) Multivoltine females.

Results

Attraction of different grandisoic acid mixtures and concentrations. *Female Response to 72% ee (+)GA.* For the univoltine strain, there was a significant difference only for the concentration of 2×10^{-7} mg/ml ($\chi^2 = 4.571$; df $= 1$; $P = 0.033$), with more females responding to this concentration than the control. None of the other concentrations were found to be significantly attractive when compared with the control (2×10^{-3}: $\chi^2 = 0.091$, df $= 1$, $P = 0.763$; 2×10^{-6}: $\chi^2 = 1.600$, df $= 1$, $P = 0.206$; 2×10^{-8}: $\chi^2 = 0.818$, df $= 1$, $P = 0.366$, 2×10^{-9}: $\chi^2 = 0.889$, df $= 1$, $P = 0.346$; 2×10^{-10}: $\chi^2 = 0.059$, df $= 1$, $P = 0.808$; Fig. 1A).

For the multivoltine strain, there was only a difference in response for the 2×10^{-9} mg/ml concentration versus control ($\chi^2 = 6.638$; df $= 1$; $P = 0.012$), with this concentration proving more attractive than the control (Fig. 1B). There was no significant difference for any of the other concentrations between test and control odours (2×10^{-6}: $\chi^2 = 0.200$, df $= 1$, $P = 0.655$; 2×10^{-7}: $\chi^2 = 0.610$, df $= 1$, $P = 0.435$, 2×10^{-8}: $\chi^2 = 0.250$, df $= 1$, $P = 0.617$; 2×10^{-10}: $\chi^2 = 0.529$, df $= 4$, $P = 0.467$; Fig. 1B).

Female Response to 51.6% ee (+)GA. Because 2×10^{-7} mg/ml (+)GA was found to be the most attractive concentration for univoltine plum curculio in the previous experiments, and 2×10^{-9} mg/ml (+)GA

Table 1. Female response to different enantiomeric purities and concentrations of the (+)enantiomer ((+)GA), the (−)enantiomer ((−)GA), and the racemic mixture ((±)GA) of grandisoic acid

Strain	Mixture	Concentration (mg/ml)	% Purity (ee)	χ^2	P	df
Univoltine	(+)GA	2×10^{-7}	51.6	1.000	0.317	1
	(−)GA	20	66.8	2.000	0.157	1
	(−)GA	2	66.8	0.053	0.819	1
	(−)GA	0.2	66.8	5.400	0.020	1
	(−)GA	2×10^{-7}	66.8	4.000	0.045	1
	(±)GA	2×10^{-7}	0	0.333	0.564	1
	(±)GA	4×10^{-7}	0	0.4000	0.527	1
Multivoltine	(+)GA	2×10^{-9}	51.6	2.882	0.090	1
	(−)GA	2×10^{-9}	66.8	0.059	0.808	1
	(±)GA	2×10^{-9}	0	2.882	0.090	1
	(±)GA	4×10^{-9}	0	0.000	1.000	1

Chi-squared (χ^2), significance (P), and degrees of freedom (df) are given for a level of significance of $P = 0.05$.

was the most attractive concentration for multivoltine plum curculio, these test concentrations were therefore used to test the response of plum curculio to (+)GA at a lower enantiomeric purity of (+)GA. For univoltine females, there was no significant difference between response to 2×10^{-7} mg/ml (+)GA at 51.6% ee and the control, and the same held true for the multivoltine strain, with no difference in response between 2×10^{-9} mg/ml at 51.6% ee versus the control (Table 1).

Female Response to 66.8% ee (−)GA. For univoltine females, there was no significant difference in response to the 20 or the 2 mg/ml concentrations of (−)GA at 66.8% ee versus the control. However, there was a significant antagonism to the lower concentrations of 0.2 and 2×10^{-7} mg/ml, with more females choosing the control in both cases. For multivoltine females there was no significant difference in response between 2×10^{-9} mg/ml (−)GA at 66. 8% ee and the control (Table 1).

Female Response to (±)GA. Based on the results of the experiments regarding the most attractive concentrations of (+)GA at the ee tested, concentrations of 2×10^{-7}, 4×10^{-7} mg/ml univoltine), and 2×10^{-9}, 4×10^{-9} mg/ml (multivoltine) of (±)GA were tested. We wished to determine if the same or higher concentrations found attractive to plum curculio using (+)GA would also be attractive using (±)GA. There was no significant difference in response between any of the (±)GA concentrations tested versus the control for univoltine females. The results were the same regarding multivoltine females for both concentrations of (±)GA, with no significant difference between test and the control (Table 1).

Comparison of Female Response to Different Enantiomeric Purities of (+)GA. Female responses to (+)GA, (−)GA, and (±)GA of different ee from the above experiments at the most attractive concentration for each strain were compared to determine the effect of enantiomeric purity on response. For univoltine females, concentration of 2×10^{-7} mg/ml was used, while for multivoltine females, 2×10^{-9} mg/ml was the concentration used. Responses to the following test

odours were compared: 72% ee (+)GA, 51.6% ee (+)GA, (±)GA (0% (+)GA), and 66.8% ee (−)GA. For univoltine females, there was a steady decrease in response from the purest solution of (+)GA (72% ee) to the solution with the least amount of (+)GA, e.g., 66.8% ee (−)GA, with a significant difference in response between purities (K = 10.416; df = 3; $P = 0.015$). The 72% ee (+)GA significantly differed from the 66.8% ee of (−)GA (Z = 20.500; $P = 0.002$), but not to the intermediate purities of (+)GA (51.6% ee (+)GA: Z = 94.000, $P = 0.346$; (±)GA (0% ee): Z = 53.000, $P = 0.059$; Fig. 2A). The situation regarding multivoltine females was similar, with more females responding to the highest enantiomeric purity (72% ee) at a concentration of 2×10^{-9} mg/ml (+)GA than any other purity. There was a significant difference when comparing purities (K = 11.831, df = 3, $P = 0.008$), with the 72% ee differing from all others (51.6% ee (+)GA: Z = 81.500, $P = 0.003$; (±)GA: Z = 81.500, $P = 0.003$; 66. 8% (−)GA: Z = 110.000, $P = 0.050$; Fig. 2B).

Discussion

Among all concentrations tested for (+)GA at the 51.6 and 72% ee, only the 2×10^{-7} mg/ml concentration at the 72% ee elicited a significant attractive response from univoltine females, and only the 2×10^{-9} mg/ml concentration at the 72% ee elicited a significant attraction for multivoltine females. This suggests that plum curculio response to (+)GA is dependent on the concentration and enantiomeric purity of this synthetic enantiomer. The results also indicate that the two strains seem to respond to the same (i.e., a high) enantiomeric purity, but to different concentrations of (+)GA. Differences in pheromone response of different weevil strains have previously been reported for plum curculio (Hock et al. 2014) and for other insects (Fletcher et al. 1968, Messina and Dickinson 1993). Responding to different concentrations of pheromone components between strains may serve as an adaptive advantage in areas where plum curculio strains overlap, as univoltine and multivoltine plum curculio cannot produce viable offspring when intermating (Padula and Smith 1971, Zhang et al. 2008, 2010). Concentration-dependent responses from plum curculio have been reported for synthetic lures (Prokopy et al. 2004, Hock et al. 2014), as well as for conspecifics (Hock et al. 2014) where the attraction of females to two males was significantly greater than to one or five males for both plum curculio strains. This situation is similar to the concentration-dependent responses that are seen in other beetles, such as the cigarette beetle, *Lasiderma serricorne* (F., 1792) (Coleoptera: Anobiidae) (Coffelt and Burkolder 1972), the boll weevil, *A. grandis* (Hardee et al. 1974), and the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) Olivier, 1790 (Hallett et al. 1999).

Enantiomeric purity can be of importance if a synthetic form of grandisoic acid is to be used as an attractant for plum curculio since neither (±)GA, which contains both (+)GA and (−)GA in equal amounts, nor (−)GA or the (+)GA with the lower enantiomeric purity (55.6% ee) were found to be significantly attractive to
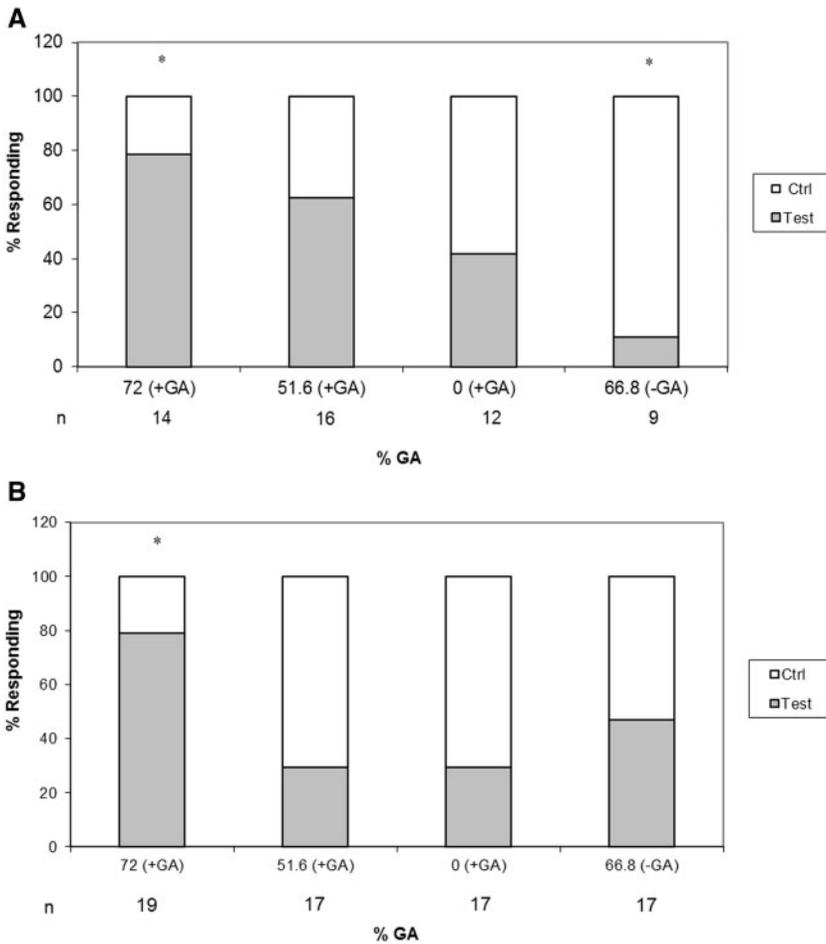
Fig. 2. Percent of female plum curculio responding to different percentages of enantiomeric excess of grandisoic acid enantiomers (%GA) versus the control (Ctrl) in a vertical still-air dual choice olfactometer. Enantiomeric purities indicated correspond to the positive ($(+)$GA) and negative ($(-)$GA) enantiomers. An * denotes a significant difference between test and control (Ctrl) at $P = 0.05$ according to the chi-square test. Replicates for each concentration are indicated below each graph (n). (A) Univoltine female response tested using 1.5 ml at a concentration of 2×10^{-7} mg/ml of grandisoic acid mixtures. (B) Multivoltine female response tested using 1.5 ml at a concentration of 2×10^{-9} mg/ml of grandisoic acid mixtures.

plum curculio in our olfactometric studies at the concentrations tested. Comparing the response of females of both strains to different purities of $(+)$GA revealed a significant attractive response only for the 72% ee $(+)$GA, and all other purities eliciting no attraction at the concentration tested. Enantiomeric purity has been shown to affect the response of other weevils, such as the boll weevil (Dickens and Mori 1989) and the strawberry blossom weevil (Cross et al. 2006). For example, the $(-)$- and $(\pm)$-grandisol were both found to elicit much weaker EAG responses than $(+)$-grandisol in the boll weevil, with $(-)$-grandisol having a tendency to inhibit responses to $(+)$-grandisol (Dickens and Mori 1989). Males of the strawberry blossom weevil produce only the $(+)$ or R-enantiomer of lavandulol, but the S-enantiomer has been found not to be significantly repulsive, and the racemic mixture was found to be equally attractive (Cross et al. 2006). Test concentrations of 1.0 and 10 mg/ml of $(+)$GA and of Grandlures,

the synthetic form of boll weevil pheromone (also containing grandisol), have both been found to elicit significant responses from plum curculio in EAG studies (Hock et al. 2014). However, EAG studies of adult plum curculio using formulations with increasing amounts of $(+)$GA [and conversely decreasing amounts of $(-)$GA] have also shown an increase in the level of response (Leskey et. al. 2009b). As only the $(+)$ enantiomer is emitted naturally by male plum curculio, the general unattractiveness of the synthetic $(-)$ enantiomer and the lack of attraction to synthetic $(\pm)$GA in olfactometric experiments at the concentrations tested may be explained by this; i.e., $(+)$GA is the natural enantiomer and plum curculio will respond primarily to $(+)$GA.

As mentioned earlier, the response to grandisoic acid is also highly dependent on concentration (Hock et al. 2014), similar to what is seen in other beetles (Zhuge et al. 2010, Lee et al. 2011). This could also explain the

difficulty in observing consistent long-term attraction in the field when solely grandisoic acid dispensers (using a standard formulation of (±)GA) are used without any host plant volatiles to augment plum curculio response (Piñero et al. 2001, Piñero and Prokopy 2003, Prokopy et al. 2003, Leskey and Wright 2004, Leskey et al. 2005). At a given concentration, the amount of (+)GA present in a racemic mixture may not be enough to attract plum curculio. In addition, increasing the amount of grandisoic acid in lures (racemic mixture) does not necessarily increase attraction, as noted in field studies by Prokopy et al. (2004) and Leskey and Zhang (2007). Previous studies have shown that in the laboratory, females are attracted to low concentrations (0.003 mg per rubber septum) of (±)GA (Leskey and Prokopy 2001), while high concentrations of 3.00 mg (per rubber septum) was less attractive to females. Field studies using twice the amount of grandisoic acid (2 mg/day) present in lures also resulted in no increase in attraction (Prokopy et al. 2004). This could be because of the fact that increasing the overall concentration of the lure results in a respective increase in concentration of both (+)GA and (−)GA present in the mixture. Our results regarding (−)GA indicate that even small concentrations (−)GA at high purities (66.8% ee) may have an a antagonistic or repellent effect on both multivoltine and univoltine females at least under laboratory conditions, especially since a greater number of univoltine females chose the control over 0.2 mg/ml and 2×10^{-7} mg/ml (−)GA (Fig. 2A). Akotsen-Mensah (2010) also found no attraction of males or females to grandisoic acid lures (25 mg lures with a release rate of 0.04 mg/h) either alone or in conjunction with other lures (e.g., benzaldehyde, plum essence) in his behavioural tests. It may be that at low concentrations of (±)GA, the amount of (+)GA present in the mixture may not be enough to significantly attract plum curculio, while at very high concentrations the amount of (+)GA present may be offset by the antagonistic effects of (−)GA. Inhibition of response of beetles by small amounts of the unnatural isomer is also seen in field bioassays of the Japanese beetle *Popillia japonica* Newman, 1841 (Tumlinson et al. 1977). Thus plum curculio are similar to other beetles in that concentration of pheromone is a key factor in behavioural responses.

It should be noted however, that the response to synthetic pheromones is less clear than what is seen for naturally produced male-odours. Akotsen-Mensah (2010) noted that plum curculio did not respond to grandisoic acid pheromone lures in olfactometer trials, while two mature live males were found to be attractive to female plum curculio (Hock et al. 2013, 2014). This could result from the fact that only a single pheromone compound was tested by Akotsen-Mensah (2010), e.g., synthetic (±)GA, and plum curculio may produce secondary pheromone compounds (i.e., compounds in addition to the primary compound of grandisoic acid) similar to the boll weevil (Tumlinson et al. 1969), the pepper weevil (Eller et al. 1994), and strawberry blossom weevil (Innocenzi et al. 2001). Therefore the attraction of females to a single pheromone compound

would differ from that of the complete pheromone blend that is produced naturally by males.

Currently, the use of (±)GA in combination with benzaldehyde is sufficient to attract plum curculio activity in the field at the beginning of the season (Piñero and Prokopy 2003; Leskey et al. 2005, 2008; Piñero et al. 2011). However more research is needed to establish an active space for a given pheromonal bait that will limit plum curculio activity to traps or trap-trees, and diminish the damage to neighbouring 'non-baited' trap trees. A more effective bait could also decrease the number of trees sacrificed in the trap-tree method by increasing attraction of plum curculio. Nonetheless the response to synthetic grandisoic acid components in the field may differ from results in a laboratory setting, as was found for (±)GA durint laboratory studies done by both Akotsen-Mensah (2010) and herein. Therefore in the future, tests using a high enantiomeric purity of (+)GA should be performed in the field. In addition, tests with high enantiomeric purities of the other enantiomer, (−)GA, should also be attempted to determine if this enantiomer has a repellent effect in the field, as well as its possible role as a repellent in a push–pull strategy. As (±)GA is attractive to plum curculio in apple orchards, especially in combination with host plant volatiles (Piñero and Prokopy 2003, Piñero et al. 2011), combinations of the natural enantiomer (+)GA at the most attractive concentration (2×10^{-7} mg/ml for univoltine plum curculio, 2×10^{-9} mg/ml for multivoltine plum curculio) along with attractive host plant volatiles (*i.e.*, plum essence, Akotsen-Mensah 2010) may increase trap captures. Continued research in the use of grandisoic acid and optimization of the plum curculio pheromone is necessary to develop more efficient trap baits for monitoring or control (e.g., attracticides) and to improve the efficiency of captures to attract, aggregate, and retain plum curculio throughout the season. Because of the difficulty in collecting pheromone samples, full analysis and quantification of the pheromone produced by plum curculio remains to be completed, inhibiting rapid progress in finding more attractive pheromone combinations.

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