Hort Innovation

Final Report

IPM program for the macadamia industry – University of the Sunshine Coast

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Delivery partner:

University of the Sunshine Coast

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Summary

This project is part of a larger IPM program (MC16003-8) and investigated behavioural and electrophysiological responses of insects to odours identified in conjunction with NSW DPI. Macadamia have a wide variety of insect pests, some of the most significant are the macadamia seed weevil (*Kuschelorhynchus macadamiae*), the fruitspotting bug (*Amblypelta nitida*) and *Leptocoris rufomarginata*.

Initial studies focused on *K. macadamiae* and response to odours from macadamia. Unsurprisingly, both large and small nuts attracted the weevils in olfactometer studies, although none of the more volatile compounds from the headspace and solvent extractions showed behavioural responses. On the other hand, key fatty acids (e.g. myristic acid, palmitoleic acid) are attractive to the weevils. These are the fatty acids that are higher in immature nuts. As nuts mature, their fatty acid profile changes, and become dominated by other acids (such as oleic acid). Weevils avoid the odour of this acid. Pure palmitoleic acid is expensive, and so an alternative source of this acid was found in commercially available sea buckthorn oil. A series of field trials were run, at the CTH, Alstonville. These trials tested macadamia oil spiked with a variety of fatty acids and at various times of year. No weevils were trapped in any of these trials. A recent APVMA permit allowing the use of indoxacarb (PER86827) to control the pest have lessened the urgency to control this pest.

Fruitspotting bug (*A. nitida*) is a major pest of macadamia. The banana spotting bug (*A. lutescens lutescens*) has commercial lure for its control based on an aggregation pheromone, and so *A. nitida* may also use such a strategy. The pheromone in *A. lutescens lutescens* is produced by the male and has been shown to attract both male and female bugs. When kept in culture with males, female fruitspotting bugs avoid the odour of groups of males, but not if females are kept in same-sex enclosures for several days - suggesting a male-produced odour that allows females to make decisions about behavior. Females are not attracted to the odours of a single male bug, suggesting that multiple males are needed before the putative pheromone is produced, perhaps in a mating lek. Aerations of were made of unmated *A. nitida* bugs individually and in groups. The only odours detected were those that have been reported previously, and do not show any behavioural or electrophysiological responses in previous trials associated with this project.

No pheromones or attractant odours are known for Leptocoris bug but other genera in the family produce odours containing monoterpenes; aerations above groups of Leptocoris bugs showed monoterpenes. Y-tube olfactometer assays determined that the bugs either avoided or ignored most of the compounds when tested individually, but when blended in the same relative proportions as found in the headspace over live bugs, they were significantly attracted to the blend. Electrophysiological studies with antennae showed clear detection of several of the major components of the blend. Field tests were conducted to test this blend in the field, but no bugs were trapped.

Keywords

Macadamia; Integrated Pest Management; Macadamia seed weevil; *Kuschelorhynchus macadamiae*; Fruitspotting bug; *Amblypelta nitida*; Leptocoris bug; *Leptocoris rufomarginata*

Introduction

The Australian macadamia industry annually brings more than $400 million economic value to local communities. There are a number of pests impacting on the productivity of the macadamia industry in Australia. The pests include flower and foliage pests (such as macadamia lace bug (*Ulonemia* spp.), felted coccid *(Eriococcus ironsidei*), macadamia flower caterpillar (*Cryptoblabes hemigypsa*) and mites and thrips species), kernel and post-harvest pests (such as fruit-spotting bugs (*Amblypelta* spp.) and macadamia seed weevil (*Kuschelorhynchus macadamiae*), banana fruit caterpillar (*Tiracola plagiata*) or kernel grub (*Assaria seminivale*)) and pests attacking the branches and trunk (such as bark beetles (*Cryphalus subcompactus*) or *Xyleborus* sp.) (Bright, 2016). Pest management strategies in the past have been developed for single pest species. These strategies, particularly for fruit-spotting bugs (*Amblypelta* spp.) covered a number of approaches, including monitoring tools, chemical and biological control, cultural control and a pilot study of an area wide management approach (Huwer et al, 2016b). However, no truly integrated strategy has been developed to date that has taken more than 1 or 2 of the key-pests into account. One of the key pests of macadamia, and the initial focus of this project was the macadamia seed weevil (*K. macadamiae*) (Bright, 2016; Fay et al, 1998). As the project developed, other key pests of macadamia also became a focus of the project.

Insects use infochemical cues (e.g. pheromones, kairomones) to identify and locate mates and host plants, and these can be used to manipulate their behaviour (Wyatt, 2013). Manipulating these cues can be used to attract and kill, to disrupt mating behaviour, or to attract natural enemies. Infochemicals as monitoring tools can delimit pest distributions, facilitate early pest detection, and improve knowledge of pest phenology and dispersal. Monitoring strategies using these techniques will also help further reduce the chemical load in the environment (Brockerhoff et al, 2010). This research investigated behavioural and electrophysiological responses of the insect pests of macadamia to odours from host plants and volatiles produced by conspecifics. The potential for using these compounds as monitoring or control tools was also be investigated.

This project is part of a larger IPM program with different components. The larger IPM program brings together a team of highly experienced researchers, specifically in pest management in macadamias and in IPM extension and adoption.

Methodology

This research project studied behavioural and electrophysiological responses of three species of macadamia pest insects, the macadamia seed weevil (*Kuschelorynchus macadamiae*), the fruit-spotting bug (*Amblypelta nitida*) and Leptocoris bug (*Leptocoris rufomarginata*) to a variety of plant and insect odours. Laboratory experiments and field trials for each species are described separately (See Appendix 1).

Key components of the methodology of this study were:

1. Identification of the key components of pheromone/kairomone chemicals which have potential as lures for use in a monitoring program
2. Testing of insect behaviours in response to volatiles, including:
    a. response to volatiles using electro-antennograms (EAG) to determine which chemical compounds the insect can detect.
    b. examining the responses to fractions of a compound mixture separated using gas chromatography (GC-EAD)
    c. attraction to identified compounds by (GC-EAD) using Y-tube and four-arm olfactometer studies
3. Testing of trap designs suitable for use with macadamia seed weevil
4. Field testing of pheromone/kairomone lure/trap combination on research station trials at CTH. Compare with the emergence times from overwintering larvae collected in unsprayed areas as a predictor of activity and treatment timing.

Electroantennography or EAG is a technique used to measure the average output of the antenna to the brain for a given odour. The antenna is removed from the insect and attached to two electrodes that allow the voltage between them to be measured while an odour is puffed on the antenna. A deflection in the voltage indicates that the odour is detected by receptor neurons in the antenna. The technique is also applied in screening of insect pheromones by examining the responses to fractions of a compound mixture separated using gas chromatography (GC-EAD).

An electrophysiological response to an odour, as detected by EAD, provides no information as to whether or not the compound is attractive, repellent or otherwise, merely that there are receptor cells on the insect's antenna that detect the chemical. Following on from EAD studies it is vital to undertake further laboratory studies before a field trial. Compounds identified by EAD as giving a response were tested in Y-tube and four-arm olfactometer studies. The appropriate bioassay for each species depends upon the behaviour of that particular species.

Primarily, these two techniques differ in the number of choices tested simultaneously. Charcoal filtered air is delivered into the olfactometer, coming simultaneously from all arms of the olfactometer. Response of the insects to different odours is tested by applying test compounds or blends to absorbent paper in one arm of the olfactometer, with the control arm having blank solvent. Insects were released one at a time at the non-test arm of the olfactometer and their behaviour recorded for 10 minutes using a video camera. For each individual the time spent in each arm zone was recorded.

Field trials were conducted to validate attractiveness of compounds identified in laboratory studies, and assess trap design, trapping density and placement of traps. The trials were conducted in macadamia orchards in the Northern Rivers region of NSW and coordinated with NSW DPI. Unfortunately, none of the trapping programs conducted were successful in trapping the test insects, and future studies are needed to refine lure development and odour release if the compounds identified in the field are to become part of a successful IPM program for macadamia pest insects.

Outputs

Hayes, RA, 2018, "Odour attractants for *Sigastus* weevil", oral presentation at Australian Macadamia Society Consultants meeting, The Oaks, Caloundra, June 2018

Hayes RA, 2019, Brisbane, Macadamia IPDM Program Reference Group meeting, Fortitude Valley, February 2019

Hayes RA, 2019, IPDM Program for the Macadamia Industry Researchers meeting, Ecosciences Precinct, Dutton Park, October 2019

Hayes RA, 2020. IPDM Program for the Macadamia Industry Researchers meeting, virtual meeting, September 2020

Outcomes

No outcomes to report

Monitoring and evaluation

The Monitoring and Evaluation (M&E) plan for this research was developed as part of the overall M&E plan for the Macadamia IPDM program. As such, the relevant section of that plan is shown below:

Program Details	Performance Measures	Evaluation Methods
Monitoring and Attractants • Development and testing of lures to aggregate pests and optimised timing of pesticide application • Development of MSW lure	• Number, type and efficacy of lures developed – including MSW lure	• Program Milestone reports with details of activities undertaken and issues

The Key Evaluation Questions for this research were the development and testing of lures, including the development of a lure for *K. macadamiae*. The performance measure is the number, type and efficacy of these lures.

Electrophysiological, behavioural and trapping studies have been conducted on three pest insect species of concern in macadamia orchards. Target compounds have been identified and tested in field situations for two of these (*K. macadamiae* and *L. rufomarginata*). While no new compounds were identified for *A. nitida*, clear behavioural evidence supporting a male-produced odour was demonstrated. Unfortunately, no lures tested in field trials for any of the species of interest were successful in trapping insects.

As noted in the midterm review of this project (Cunningham, 2019) "The development of effective odour lures to attract insect pests is no easy task. It is not at all unusual for the R&D process to takes 5-10 years from initial studies to something that works in the field, depending on the complexity of the lure and chemical structure of the components … the expectation of having conducted behavioural, electrophysiological (electroantennogram, EAG) research, volatile analysis, and field trials, to explore the feasibility of such a lure, identify tentative attractants, and show some attraction in the lab and (hopefully) field … is in line with what is feasible. Delivering a fully functional field effective lure for MSW is not, for good reason." In line with this, the data presented in this Final Report on behavioural, electrophysiological and trapping studies for the three target species demonstrate a satisfactory result of the present study.

Recommendations

Kuschelorhynchus macadamiae

- Macadamia seed weevil continues to be a pest of macadamia orchards, despite the permitted use of indoxacarb as a control strategy. This is especially for the farms that desire to maintain an organic certification. Laboratory studies demonstrated attraction to several fatty acids characteristic of immature nuts. Future studies on this species should focus on trap design and deployment as the traps trialed in the current study failed to trap any insects.

Amblypelta nitida

- Although no pheromone produced by either male or female fruitspotting bugs has been identified to date (in this or previous studies), the behavioural data provide strong support for a male-produced odour to attract females. Further work on identification of this odour may well yield a pheromone trapping system comparable to that for the congener *A. lutescens lutescens*. As an extremely significant pest insect of macadamia and a wide variety of other crops, such work remains valuable.

Leptocoris rufomarginata

- The lack of success of the trapping trial for Leptocoris bug is not a true indication of the lure's potential effectiveness, as the trial was limited to one site late in the activity season. This was due to the laboratory testing concluding late in the timeframe of the project, with only a very limited window available for a field test. More extensive testing should occur at multiple locations, and earlier in the season.
- Single compound tests of the terpene blend showed no attractiveness for *L. rufomarginata*, whereas the blend of all nine components was attractive. Electrophysiological tests demonstrated responses to four of these compounds (3-carene, α-terpinene, camphene and γ-terpinene). Strategic testing of a blend of these four components may show equal attractiveness to the full blend of nine and is worth testing in both laboratory and field – a simpler blend would be cheaper and easier to manipulate than the full blend.

Refereed scientific publications

No scientific publications to report

References

Aldrich JR, Blum MS, Lloyd HA, Evans PH and Burkhard DR. 1979. Novel exocrine secretions from two species of scentless plant bugs (Hemiptera: Rhopalidae). *Entomol. Exp. Appl*. 26:323-331.

Aldrich JR, Carroll SP, Lusby WR, Thompson MJ, Kochansky JP and Waters RM. 1990a. Sapindaceae, cyanolipids, and bugs. *J. Chem. Ecol*. 16:199-210.

Aldrich JR, Carroll SP, Oliver JE, Lusby WR, Rudmann AA and Waters RM. 1990b. Exocrine secretions of scentless plant bugs: *Jadera, Boisea, Niesthrea* species (Hemiptera: Heteroptera: Rhopalidae). *Biochem. Syst. Ecol*. 18:369-376.

Baker JT, Blake JD, MacLeod JK, Ironside DA and Johnson IC. 1972. The volatile constituents of the scent gland reservoir of the fruit-spotting bug, *Amblypelta nitida*. *Aust. J. Chem.* 25: 393-400.

Bright J. 2019. *Macadamia plant protection guide 2019-20. NSW DPI Management Guide.* NSW Department of Primary Industries. Orange, NSW. pp 87.

Brockerhoff EG, Liebold AM, Richardson B and Suckling DM. 2010. Eradication of invasive forest insects: concepts, methods, costs and benefits. *NZ J. For. Sci*. 40 (suppl): S117-S135.

Cunningham, JP. 2019. *MC18005: Independent midterm review of the Macadamia Integrated Pest Management Program*. Agriculture Victoria Research. pp 19.

El-Sayed AM. 2021. The Pherobase: Database of Pheromones and Semiochemicals. http://www.pherobase.com. Accessed 9 February 2021.

Fatima T, Snyder CL, Schroeder WR, Cram D, Datla R, Wishart D, Weselake RJ and Krishna P. 2012. Fatty acid composition of developing Sea Buckthorn (*Hippophae rhamnoides* L.) berry and the transcriptome of the mature seed. *PLoS ONE* 7(4): e34099. doi:10.1371/journal.pone.0034099

Fay HAC, De Faveri SG, Storey RL and Watson J. 1998. *Sigastus* weevil - an emerging pest of macadamias in north Queensland. In: *Proceedings of the Sixth Workshop for Tropical Agricultural Entomologists*, May 1998, Darwin, Australia

Jennings D and Oberprieler R. 2018. A review of the Tribe Cryptoplini (Coleoptera: Curculioninae), with *revision* of the genus *Menechirus* Hartmann, 1901 and description of a new genus associated with *Macadamia*. Diversity. 10(3): 71. doi:10.3390/d10030071

Gallagher E, O'Hare P, Stephenson R, Waite G and Vock N. 2003. Macadamia problem solver and bug identifier. The State of Queensland Department of Primary Industries. Brisbane. pp 215.

Huwer R, Maddox C, Bright J, Hickey M, Newton I and Alt S. 2016a. *Fruitspotting bugs 2016. NSW DPI Management Guide.* NSW Department of Primary Industries. Orange, NSW. pp 34.

Huwer R, Maddox CDA, Newton I, Hayes RA, Chambers D, Fay H, Khrimian A, Wright C, Hickey M, Bright J, Robertson D, Purdue I, Liang W, Morris S, Wilcox B, Govender A and Llewellyn R. 2016b. A multi target approach to fruitspotting bug (MT10049). Hort Innovation, Sydney, NSW. pp 247.

Olsson SB and Hansson WS. 2013 Electroantennography and single sensillum recording in insect antennae. In: Touhara K (ed.) *Pheromone Signaling: Methods and Protocols*. Methods in Molecular Biology, 1068. pp. 157 – 177.

Schwarz J, Gries R, Hillier K, Vickers N and Gries G. 2009. Phenology of semiochemical-mediated host foraging by the western boxelder bug, *Boisea rubrolineata*, an aposematic seed predator. *J. Chem. Ecol.* 35:58-70.

Shi, R. 2017. Potential ways to increase the value of the macadamia industry. Presentation to the 2017 International Macadamia Research Symposium. 12-15 September 2017. Hawaii, USA.

VSN International. 2018. Genstat for Windows 19th Edition. VSN International, Hemel Hempstead, UK.

Wyatt TD. 2013. *Pheromones and Animal Behavior: Chemical Signals and Signatures*. Cambridge University Press. Cambridge, UK.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

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Appendix 1 Detailed methods and results

Macadamia Seed Weevil (*Kuschelorhynchus macadamiae)*

Originally described as *Sigastus* sp., but now described as *Kuschelorhynchus macadamiae* (Jennings & Oberprieler, 2018) the macadamia seed weevil is considered one of the most important pests of macadamia in the southern part of the production area (Bright, 2019). Studies on a kairomone lure based on the volatile chemistry of immature macadamia nuts were the initial focus of the work on the weevil. Shi (2017) reported on changes in relative levels of fatty acids in macadamia nuts as they mature. Immature nuts, which are favoured for oviposition by *K. macadamiae* have higher levels of myristic acid, palmitoleic acid and linoleic acid. As the nut ages, the relative concentration of oleic acid increases compared to the other fatty acids.

Behavioural responses to macadamia odours: A four-arm olfactometer was used to examine the response of unmated male and female weevils to key fatty acid odours. Humidified air flowed through each arm of the olfactometer at a flow rate of ≈ 250 mL/min. One arm of the olfactometer contained the test odour, myristic acid (2 µL, Sigma) or oleic acid (2 µL, Sigma), and the other three arms were control (blank). Weevils were observed for five minutes after first movement, and the mean time spent in the arm with odours was compared to the mean time spent in the blank arms. Weevils spent significantly more time in the olfactometer arm containing myristic acid and palmitoleic acid, which are characteristic of immature nuts, than the blank (Mann-Whitney: U = 8.0, P = 0.038; and U = 2.0, P = 0.002 respectively) (n = 7 for each) (Figure 1a, b). In contrast, weevils significantly avoided the arm containing oleic acid odours compared to the control (blank) arm (Mann-Whitney: U = 8.0, P = 0.003) (n = 9) (Figure 1c). Pure palmitoleic acid is expensive, and so for use in a trap, it is necessary to find an alternate source of this compound. Oil from the fruit of the sea buckthorn (*Hippophae rhamnoides*) is a known source of this acid (Fatima et al, 2012), and so the response to this oil (2 µL, Kavalia Power) was also examined. Although there was not significant attraction to the oil (Mann-Whitney: U = 6.0, P = 0.222) (n=6), there was a trend suggesting this (Figure 1d). The commercial oil is a mixture of berry and seed oil, and concentrations of these acids vary between the different parts of the fruit, which may have impacted on the result.

The response of weevils to frass produced by adult feeding on macadamia nuts was also assessed (Figure 2), following anecdotal reports suggesting weevils were attracted to the frass of other weevils (Maddox, pers. comm.). As previously, weevils (n = 9) were observed in the olfactometer with one arm containing a frass sample (≈ 10 g), and the other three blank. The mean percentage of time spent in the arm with frass odours (32.1 ± 9.8 %) did not differ significantly from time spent in the blank arms. (22.6 ± 3.3 %) (Mann Whitney: U = 26.0, P = 0.556), This was the same for each sex independently. There was no evidence that weevils preferred the odours of frass to the control arms of the olfactometer.

The response of weevils to extracts produced by pressing the kernel of small and large macadamia nuts (variety A4) was also examined in the olfactometer. When tested alone, the small kernel extract was not significantly attractive to the weevil (Mann Whitney: U = 7.0, P = 0.886) (Figure 3a). In a three-choice assay, comparing small and large kernel extracts to blank, again there was no significant attraction to the nut extracts (Kruskall-Wallis: H = 3.52, P = 0.172) (Figure 3b). This lack of attraction may be due to interference between the two nut sizes, however, because when the time spent in the two nut quadrants were combined, the attraction of weevils to nuts over all was significant (Mann Whitney: U = 4.0, P = 0.026) (Figure 3c).

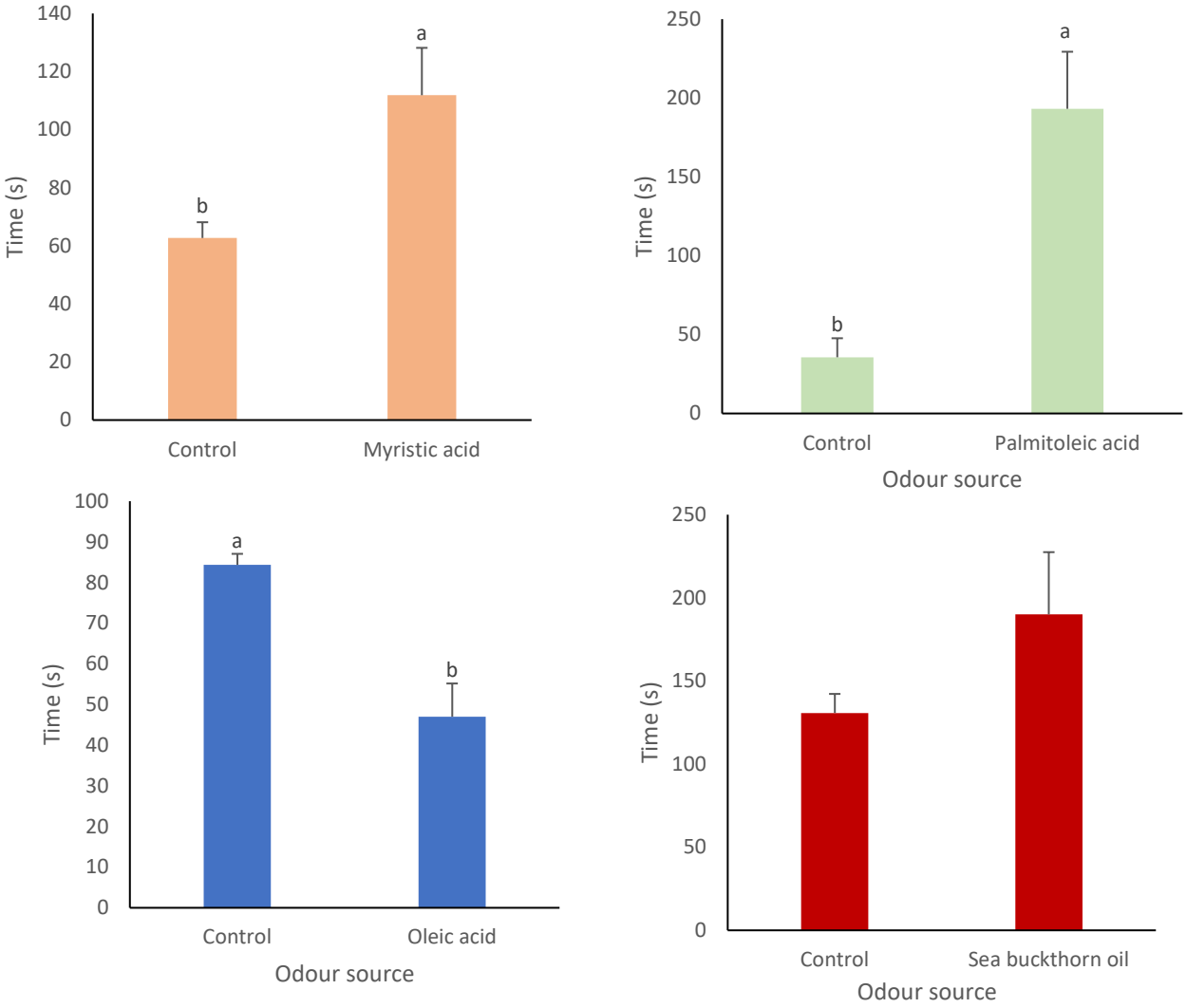


Figure 1: Time (in seconds) spent in the arm containing fatty acid samples compared to a blank arm (Mean ± SEM). Different letters above columns indicate significant differences

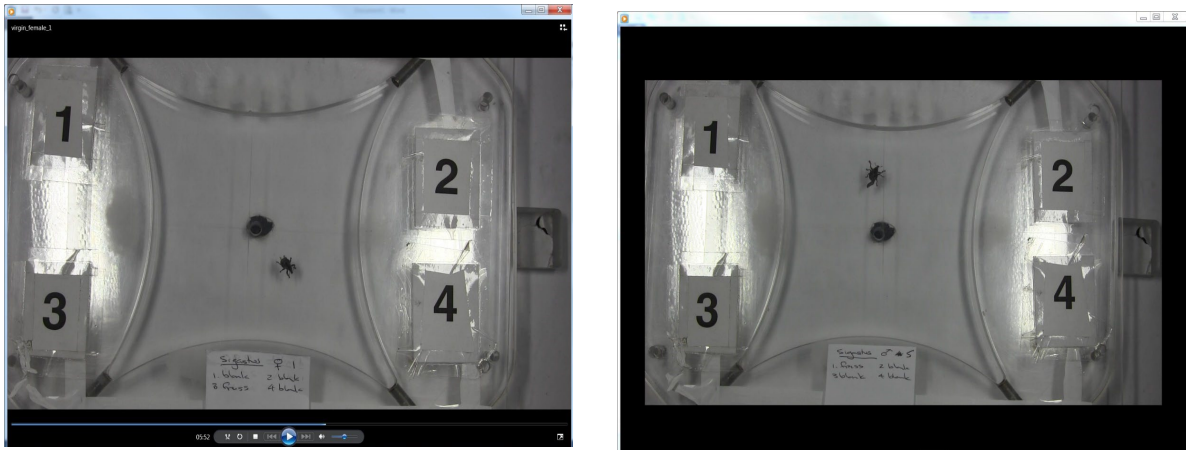


Figure 2: Behavioural analysis of attraction of *K. macadamiae* weevil to odours in an olfactometer

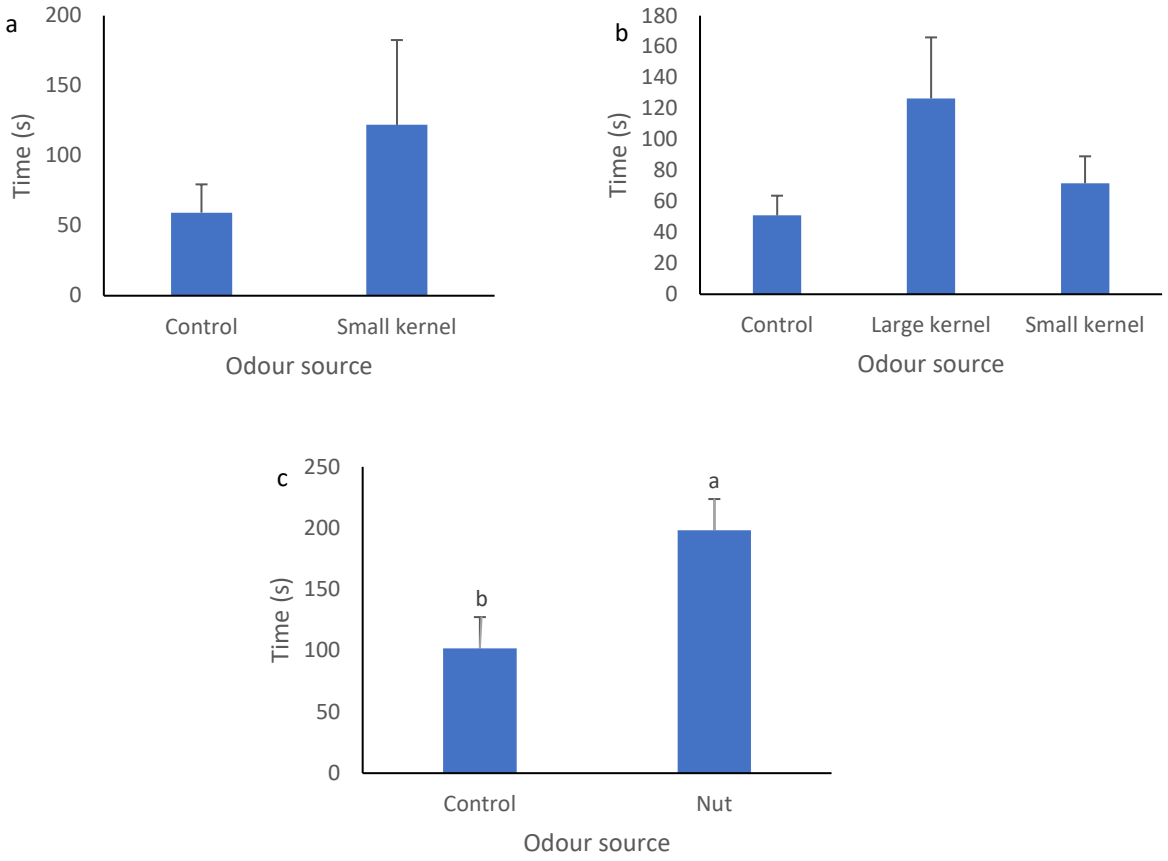


Figure 3: Time (in seconds) spent in the arm containing a) small kernel extract, b) small versus large kernel extract or c) total time in nut quadrants compared to a blank arm (mean + SEM). Different letters above columns indicate significant differences

Chemistry of nut odours: The limited success of weevil response to nut odours prompted an examination of the chemistry of these odours. The pressed oil and flesh of small and large kernels and shells of macadamia nuts (variety A4) were examined, both by headspace examination and solvent extraction. The headspace of each replicate was sampled at 250 mL/min for 18 h. Laboratory air was drawn through a charcoal trap over the sample and then through a thermal desorption tube preloaded with Tenax TA (35/60 mesh) (Markes International Ltd.). For solvent extraction, samples were extracted with hexane (Sigma) for 30 min at a rate of 0.5 g/mL.

Headspace samples were thermally desorbed from the tubes using a TD-100 thermal desorption unit (Markes International Ltd.), both they and the liquid samples (1 µL) were introduced into a gas chromatograph (GC) (Agilent 6890 Series) coupled to a mass spectrometer (MS) (Agilent 5975) and fitted with a silica capillary column (Agilent, model HP5-MS, 30 m × 250 mm internal diameter × 0.25 µm film thickness). Data were acquired under the following GC conditions: carrier gas He at 51 cm/s, split ratio 13:1, transfer-line temperature 280 °C, initial temperature 40 °C, initial time 2 min; rate 10 °C/min, final temperature 260 °C, final time 6 min. The mass spectrometer was held at 230 °C in the ion source with a scan rate of 3.89 scans/s. Tentative identities were assigned to peaks with respect to the National Institute of Standards and Technology (NIST) mass spectral library. Mass spectra of peaks from different samples with the same retention time were compared to ensure that the compounds were indeed the same. Compounds identified in the headspace samples included the green leaf volatiles (C6 aldehydes, alcohols and esters), monoterpenes (including α-pinene, α-phellandrene and α-terpinene) and 1,8-cineole. There was no evidence of the fatty acids in these samples (Figure 4). The hexane extract samples, on the other hand, were dominated by the saturated and monounsaturated C16 and C18 fatty acids and a phytosterol (β-sitosterol) (Figure 5).

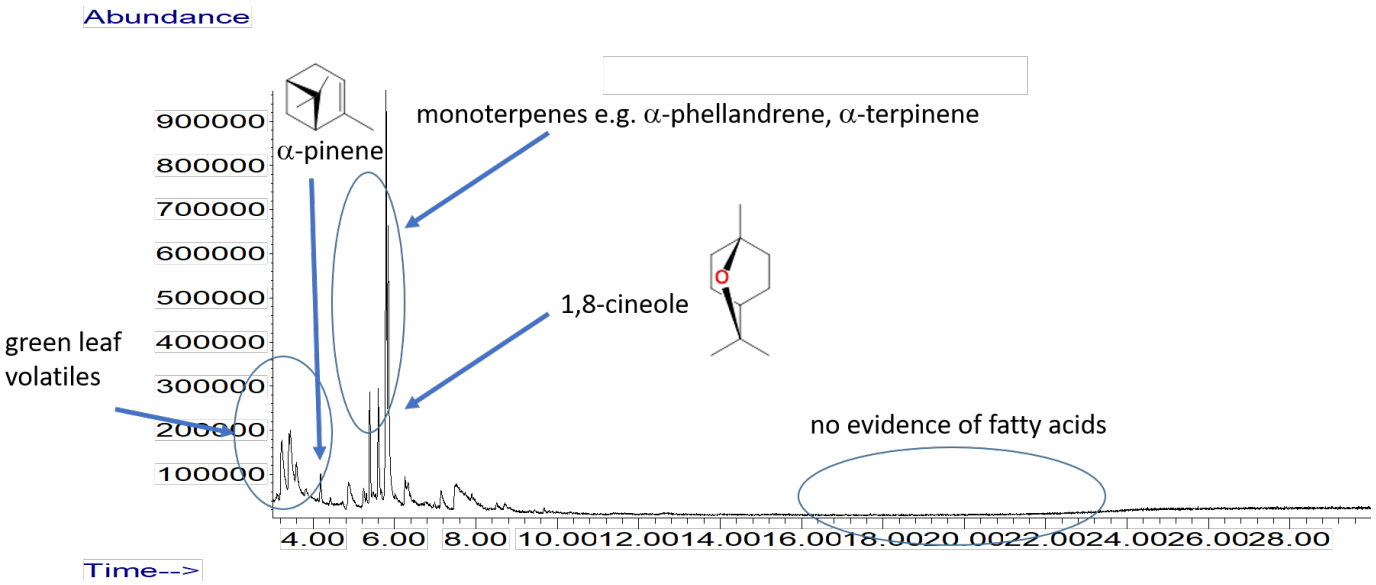


Figure 4: Total ion chromatogram of the headspace of small macadamia kernel (variety A4), showing compounds identified, and no evidence of fatty acids.

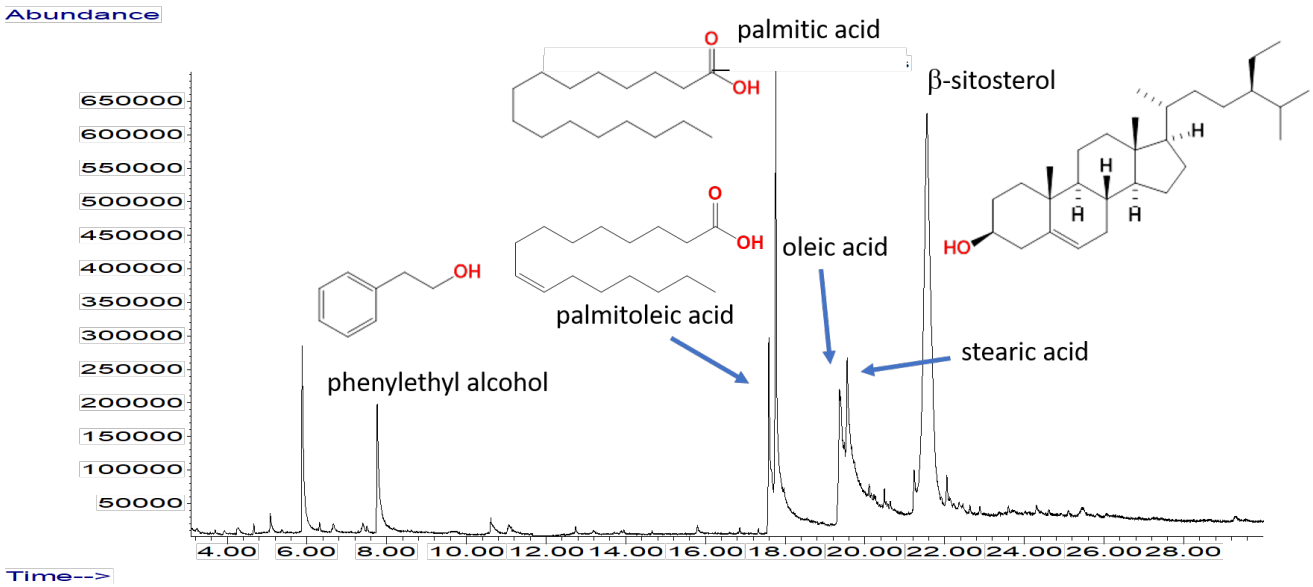


Figure 5: Total ion chromatogram of the hexane extract of small macadamia kernel (variety A4), showing compounds identified.

Field trials: A preliminary field trial was conducted at the research macadamia orchard at the NSW Centre for Tropical Horticulture (CTH), Alstonville (28.847° S, 153.456° E). Initial field trials were used to assess the response of weevils in the nut production season. Traps consisted of a roll of corrugated cardboard attached to a macadamia tree, that had been dosed with an odour treatment (Figure 6). Traps were deployed, one trap per tree, for a period of ten days on two occasions, 11 – 23 Oct and 23 Oct – 2 Nov 2018. Treatments were prepared in commercial macadamia oil (Proteco Gold) or commercial sea buckthorn oil (Kavalia Power) and consisted of:

- control (blank)
- macadamia oil (500 µL)
- macadamia oil + oleic acid (200 µL/mL) (500 µL)
- macadamia oil + myristic acid (200 µg/mL) (500 µL)
- sea buckthorn oil (500 µL)

No weevils were trapped during either of the trial periods. As stated above, the goal of the system is to trap over-wintering adult weevils, before populations increase as new nuts form. This initial field trial was used to demonstrate that the lures are not suitable for use during the period when there are plenty of competing odours present in the field.

a b c

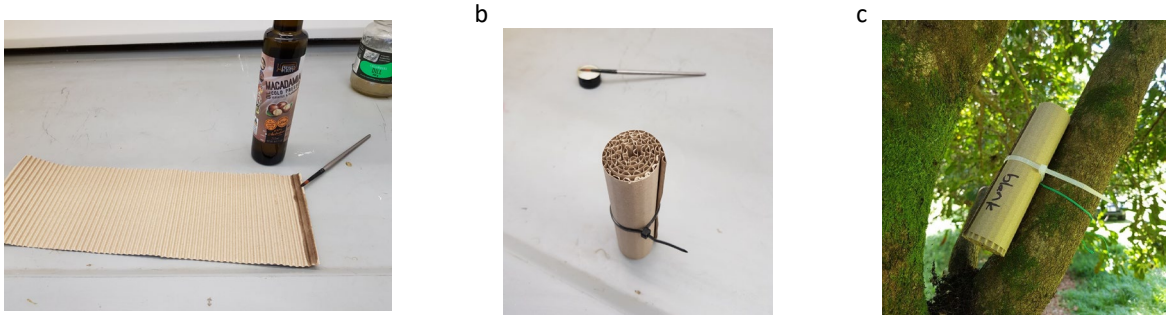


Figure 6: Traps used to assess field attractiveness of potential kairomones for *K. macadamiae*. a) application of test odour to corrugated cardboard, b) assembled trap, c) trap deployed in a macadamia tree.

A third field trial was conducted between 25 March – 29 April 2019, when no small nuts were present to compete with trap odours. As before, traps consisted of a roll of corrugated cardboard attached to a macadamia tree, that had been dosed with an odour treatment. Traps were deployed, one trap per tree, on the north-east face of the tree, attached to a branch in the outer canopy, close to flowers in bloom. Treatments were:

- control (blank)
- macadamia oil (500 µL)
- macadamia oil + oleic acid (200 µL/mL) (500 µL)
- sea buckthorn oil (500 µL)

A fourth field trial was conducted at Rijks macadamia farm, Rous Road, Alstonville (28.866°S, 153.396° E). The grower here reported to NSW DPI of aggregations of macadamia seed weevils, especially on trees with “out of season” flowering and some early nut set. Weevils were observed to be feeding during the day, but “hiding” at night in curled leaves. The same traps as deployed in the three previous trials were used, with the cardboard rolled as a cylinder, as well as fluted cardboard wrapped directly around the branch as an alternate harbourage. The field trial was conducted from 15 May to 18 June 2019. Treatments were:

- control (blank) cylinder
- control (blank) around branch
- sea buckthorn oil (500 µL) cylinder
- sea buckthorn oil (500 µL) around branch

No macadamia seed weevil was found in any of the traps deployed in either of trials three or four. There is no evidence at all for attractiveness of fatty acid and oil odours to *K. macadamiae* under field conditions.

Electrophysiology: Electrophysiological responses of *K. macadamiae* were tested for:

- sea buckthorn oil
- oleic acid
- palmitoleic acid
- myristic acid

To date results have been inconclusive with no indication of olfactory detection of any of the odours by the weevils (Figure 7).

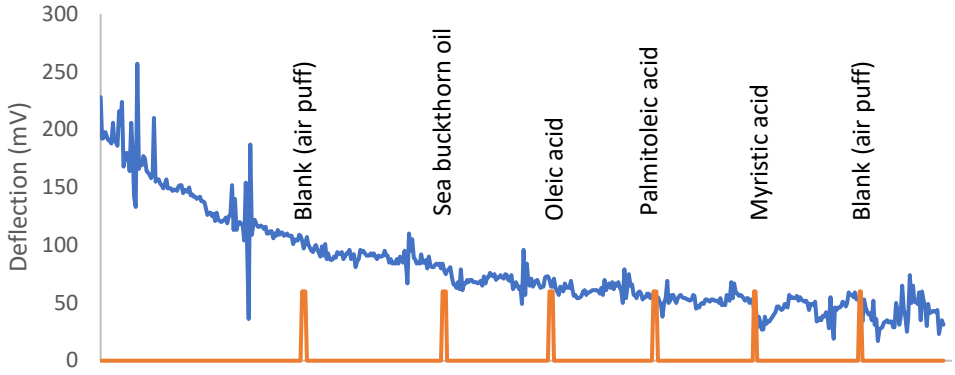


Figure 7: Representative electrophysiological response (blue) of the antenna from a *Kuschelorhynchus macadamiae* antenna to fatty acid and oil samples. Orange peaks indicate when odour was administered.

With the identification of indoxacarb (PER86827) as a key measure for control of macadamia seed weevil when applied to match-head sized nutlets, there has been an elimination of egg-laying by adult females, and a concomitant reduction in losses caused by this pest (Bright, 2019). At the Macadamia IPDM program review workshop in October 2019 it was decided that this shift in concern for this pest meant that MC16007 should broaden the focus of the project and focus on other insect pests of macadamia.

Fruitspotting bug (*Amblypelta nitida*)

Fruitspotting bug is a pest of a wide range of horticultural crops, and a major pest of the macadamia industry (Bright, 2019; Gallagher et al, 2003; Huwer et al, 2016a). The congeneric banana spotting bug (*Amblypelta lutescens lutescens*) has had a commercial lure developed by Queensland Department of Agriculture and Fisheries for its control based on an aggregation pheromone, and so it seems likely that *A. nitida* may also use such a strategy. The pheromone in *A. lutescens lutescens* is produced by the male and has been shown to attract both male and female bugs. The investigation for such a pheromone in *A. nitida* is thus a worthy approach. Previous work by members of the program team (Huwer et al, 2016b) initially thought that they had identified a target pheromone for *A. nitida*, but field trials gave very inconclusive results. Further basic research into the behaviour of the bug is thus warranted.

A four-arm olfactometer was used to determine preference or avoidance of odours. No visual or tactile cues were available to the test subject, so any preference was based entirely on olfactory cues. Responses of female *A. nitida* were tested in the four-arm olfactometer (Figure 8) to odours produced by green beans (*Phaseolus vulgaris*) (their laboratory food source) and by a group of four male *A. nitida* feeding on green beans. Individual female bugs were placed in the olfactometer for ten minutes, and the amount of time spent in each sector was ascertained and assumed to relate to their preference for the odours in that sector.

Statistical analyses were performed using Genstat version 19.1.0.21390 (VSN International, 2018). Differences between treatments was analysed by a one-way ANOVA, and Fisher's Protected LSD, or a Student's t-test, when there were only two choices.

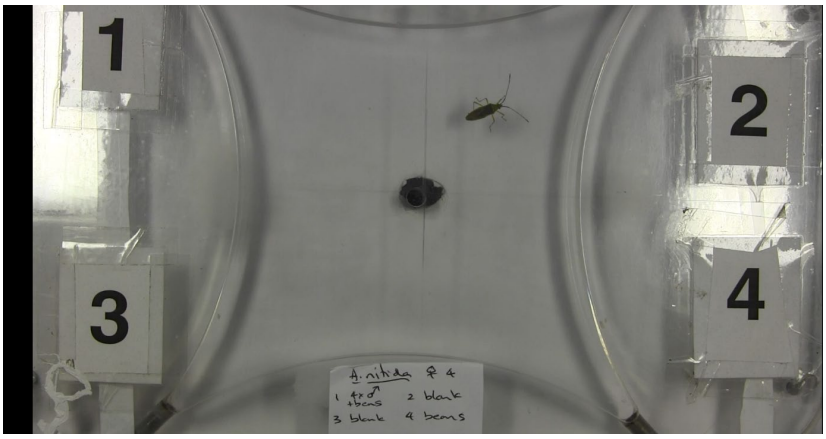


Figure 8: A female *Amblypelta nitida* in the olfactometer arena. The amount of time spent in each sector of the arena, of a ten-minute assay was measured to determine any preference

Female *A. nitida* that had been maintained in cages with male bugs and food showed a significant avoidance of treatment sectors (ANOVA: $F_{2,8} = 152.96$, $P < 0.001$), and spent significantly less time in the sector with males than either of the other choices, spending on average twice as much time in the sector with the food than that with the males. Overall, however, they spent an average of 446.7 ± 3.2 s (74.4 % of total time) in the control sectors (Figure 9a).

When females were tested that had been housed separately from males for a period of at least 48 hours the response was different. When the treatments were considered separately, the avoidance of the treatment sectors disappeared, there was now no significant difference between control or either of the treatments (ANOVA: $F_{2,26} = 0.13$, $P = 0.883$) (Figure 9b).

If, however, the time spent in the two treatment sectors was added together, so a two-choice test between treatment and control, then female *A. nitida* were found to spend significantly more time in the combined treatments than the control (blank) (Student's t-test: $t = -2.41$, $P = 0.036$) (Figure 9c)

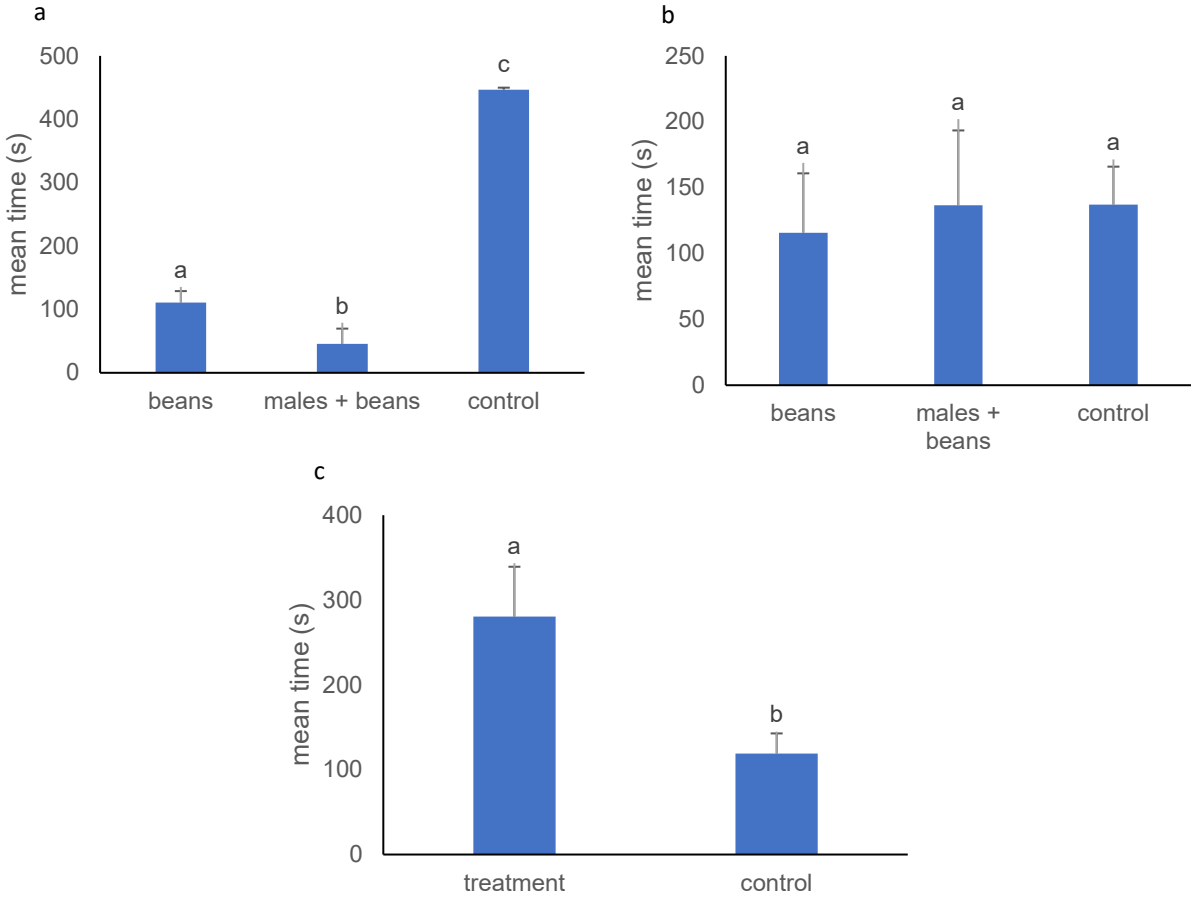


Figure 9: Mean time (in seconds) spent in the arms of the four-arm olfactometer a) female *A. nitida* maintained with males; b) female *A. nitida* denied access to males for at least 48 hours before testing; c) female *A. nitida* denied access to males for at least 48 hours before testing, combined treatment sectors compared to control sectors. Different letters above columns indicate significant differences

The results from the olfactometer bioassays were somewhat surprising, with *A. nitida* females that had been previously maintained with males appearing to actively avoid males, but also avoiding the food source (without males). In many insects, females avoid males once they have mated. The *A. nitida* females that had been previously separated from males did not appear to show any attraction or repulsion to any single treatment, showing that they are no longer repelled by males, but nor were they attracted to them. They were only separated from males for 48 hours before the test, and they may show more attraction to males if they are separated from males for longer, or by using virgin females. These results warrant further investigation.

Anecdotally (Khrimian, pers comm) male-produced aggregation pheromones in some species of Hemiptera (e.g. Brown Marmorated Stink Bug *Halyomorpha halys*) can only be isolated using single male bugs i.e. they won't produce a pheromone if they are in groups, as the competing males may avoid producing a pheromone in a group. Responses of female *A. nitida* were thus tested in the four-arm olfactometer to odours from a single male *A. nitida* feeding on a green bean. Individual female bugs were again observed for ten minutes in the olfactometer.

Female *A. nitida* that had been separated from males continued to show avoidance of males that was seen previously when tested against a single male (ANOVA: $F_{2,8} = 13.58$, $P = 0.006$) (Figure 10). This is in direct contrast to the previous results. Contrary to our expectations it would appear likely that a single male produces an insufficient amount of any putative pheromone to attract females, and if a pheromone does exist it is only produced by males when in a group with other males. These results are perplexing and warrant further investigation.

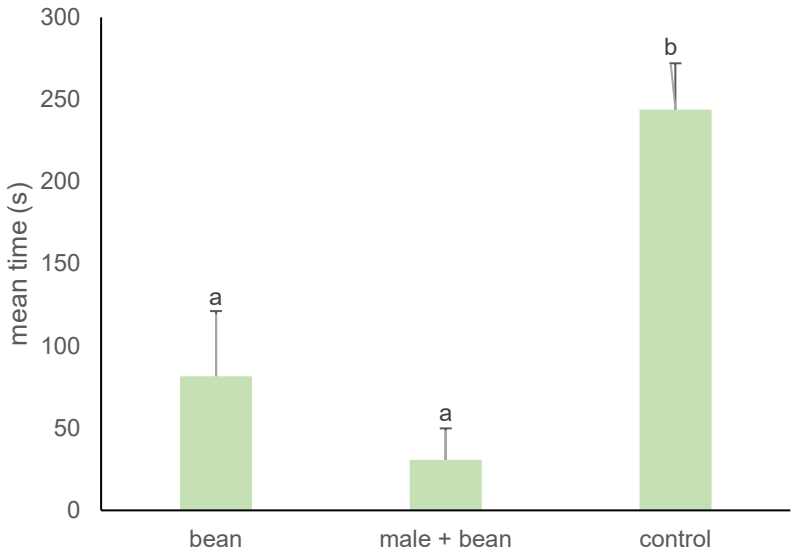


Figure 10: Mean time (in seconds) (± SEM) that *A. nitida* females spent in the arms of the four-arm olfactometer. Different letters above columns indicate significant differences.

With lack of evidence of male-produced pheromones, the research instead decided to look at whether or not there was any evidence of odours produced by either sex of the bug, especially if they had never had the chance to mate. Groups of four unmated males or females as well as replicate individual male and female bugs were placed in a flask with a food source (a bean). The headspace of each replicate was sampled at 250 mL/min for 18 h. Laboratory air was drawn through a charcoal trap over the sample and then through a thermal desorption tube preloaded with Tenax TA (35/60 mesh) (Markes International Ltd.).

Headspace samples were thermally desorbed from the tubes using a TD-100 thermal desorption unit (Markes International Ltd.) and were introduced into a gas chromatograph (GC) (Agilent 6890 Series) coupled to a mass spectrometer (MS) (Agilent 5975) and fitted with a silica capillary column (Agilent, model HP5-MS, 30 m × 250 mm internal diameter × 0.25 µm film thickness). Data were acquired under the following GC conditions: carrier gas He at 51 cm/s, split ratio 13:1, transfer-line temperature 280 °C, initial temperature 40 °C, initial time 2 min; rate 10 °C/min, final temperature 260 °C, final time 6 min. The mass spectrometer was held at 230 °C in the ion source with a scan rate of 3.89 scans/s. Tentative identities were assigned to peaks with respect to the National Institute of Standards and Technology (NIST) mass spectral library. Mass spectra of peaks from different samples with the same retention time were compared to ensure that the compounds were indeed the same (Figure 11).

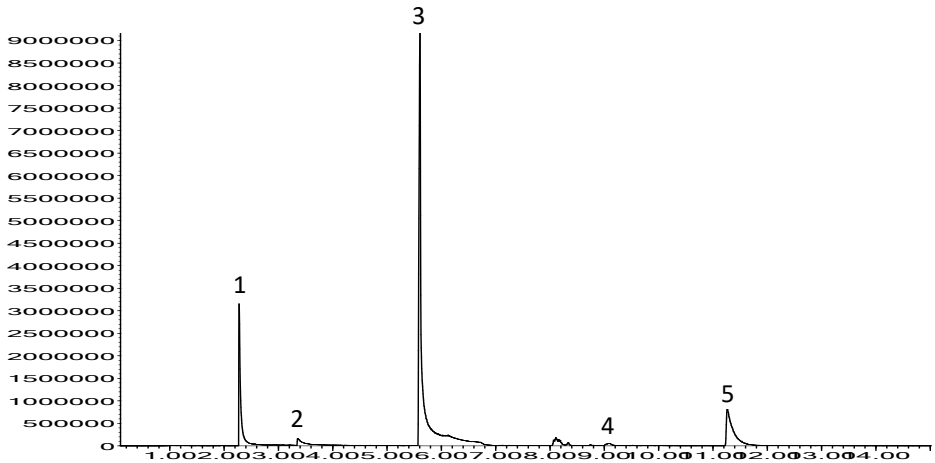


Figure 11: Total ion chromatogram of the headspace of an unmated female *A. nitida*. Compounds detected are: 1 - hexanal, 2 - hexanol, 3 – hexyl acetate, 4 – hexyl butanoate, 5 – hexyl hexanoate

Compounds identified in the headspace samples have all been previously identified from the scent gland of this species (Baker et al, 1972), with no behavioural activity demonstrated to them previously (Huwer et al, 2016b).

Leptocoris bug (*Leptocoris rufomarginata*)

Another insect that is described as an emerging pest in macadamia is *Leptocoris* bug (Bright, 2019). There are two species of these bugs in Australia – *Leptocoris rufomarginata* and *L. tagalica* both of which have been found in macadamia (Gallagher et al, 2003). *Leptocoris* (Figure 12a) is a genus of true bugs (order Hemiptera, suborder Heteroptera, family Rhopalidae, subfamily Serinethinae) with native hosts such as foambark (*Jagera pseudorhus)* and the exotic golden rain tree (*Koelreuteria elegans*). *Leptocoris* will occur as a large aggregation of bugs on macadamia when the alternate hosts have no crop (Bright, 2019). No pheromones or attractant odours are known for *Leptocoris* but other members of the family including *Boisea rubrolineata*, *B. trivittata*, *Jadera antica*, *J. haematoloma*, *J. hinnulea*, *J. obscura*, *J. sanguinolenta* and *Niesthrea louisianica* have been studied (Aldrich et al, 1979, 1990a, 1990b, Schwarz et al, 2009). Despite the common name for the family of scentless plant bugs, all the studied species produce odours consisting of monoterpenes (such as 3-carene, β-pinene, myrcene and limonene) and several also produce six and eight carbon aldehydes (El-Sayed, 2019).

a b

Figure 12: a) *Leptocoris rufomarginata* bug resting on a macadamia nut (Photo: Dalton Baker); b) aeration setup for investigation of volatile compounds in the headspace of *Leptocoris* bugs.

The headspace above ten mixed-sex groups of four individual bugs (with a small twig of macadamia as a food source) was sampled to see if they were producing detectable quantities of volatile compounds (Figure 12b). Bugs were placed in a flask and laboratory air was drawn through a charcoal trap over the sample and then through a thermal desorption tube preloaded with Tenax TA (35/60 mesh) (Markes International Ltd.). Headspace of each replicate was sampled at 250 mL/min for 18 hours. Analysis of the headspace by GC-MS was as described above.

Four of the *Leptocoris* aerations showed clear evidence of volatile compounds. Unfortunately, at this stage there is no clear method for determining sex of adult bugs. It may be that in the groups with no volatiles detected, the group may consist of all females, the sex unlikely to produce an odour; or they may be in such a physiological state as to not producing an aggregation pheromone.

Fourteen compounds were detected in the headspace of the four groups of *Leptocoris* bugs (Table 1, Figure 13). All of the compounds detected were monoterpenes, a compound class that has been shown previously to form part of the odour produced by Rhopalid bugs (Aldrich et al, 1979, 1990a, 1990b, Schwarz et al, 2009). Ten of these compounds were detected in at least 75 % of aerations examined, and these compounds are among the most common in reported studies (e.g. β-pinene, myrcene and limonene).

Nine of the compounds detected were tested individually in a two-choice manner in a Y-tube olfactometer (Figure 14) for response of bugs to the odour. Each compound (2 µL, 100 mg/mL in ethanol) was tested in a ten-minute bioassay, with test compound against an ethanol blank to see if they had a preference. Air was pulled through the olfactometer at a flowrate of ≈ 1L/min. Each assay was videoed, and time spent in each arm of the olfactometer, as well as time in the "pre-choice" arm was scored. While bugs are in this arm of the olfactometer, they are exposed to both treatment and control odours, as the air from each arm is mixed here.

Table 1: Compounds detected by GC-MS in the headspace above a group of *Leptocoris rufomarginata* bugs. Tentative identities were assigned by library search and match with Kovats Retention index. Compounds are listed by retention time

Compound #	Retention time (min)	Tentative identity	% occurrence
1	3.731	3-carene	50
2	3.988	monoterpene A	75
3	4.115	α-thujene	50
4	4.185	α-pinene	100
5	4.415	camphene	100
6	4.815	monoterpene B	50
7	4.896	β-pinene	100
8	5.240	myrcene	75
9	5.399	α-phellandrene	75
10	5.602	α-terpinene	100
11	5.800	limonene	100
12	6.198	β-phellandrene	50
13	6.322	γ-terpinene	100
14	6.808	isoterpinolene	100

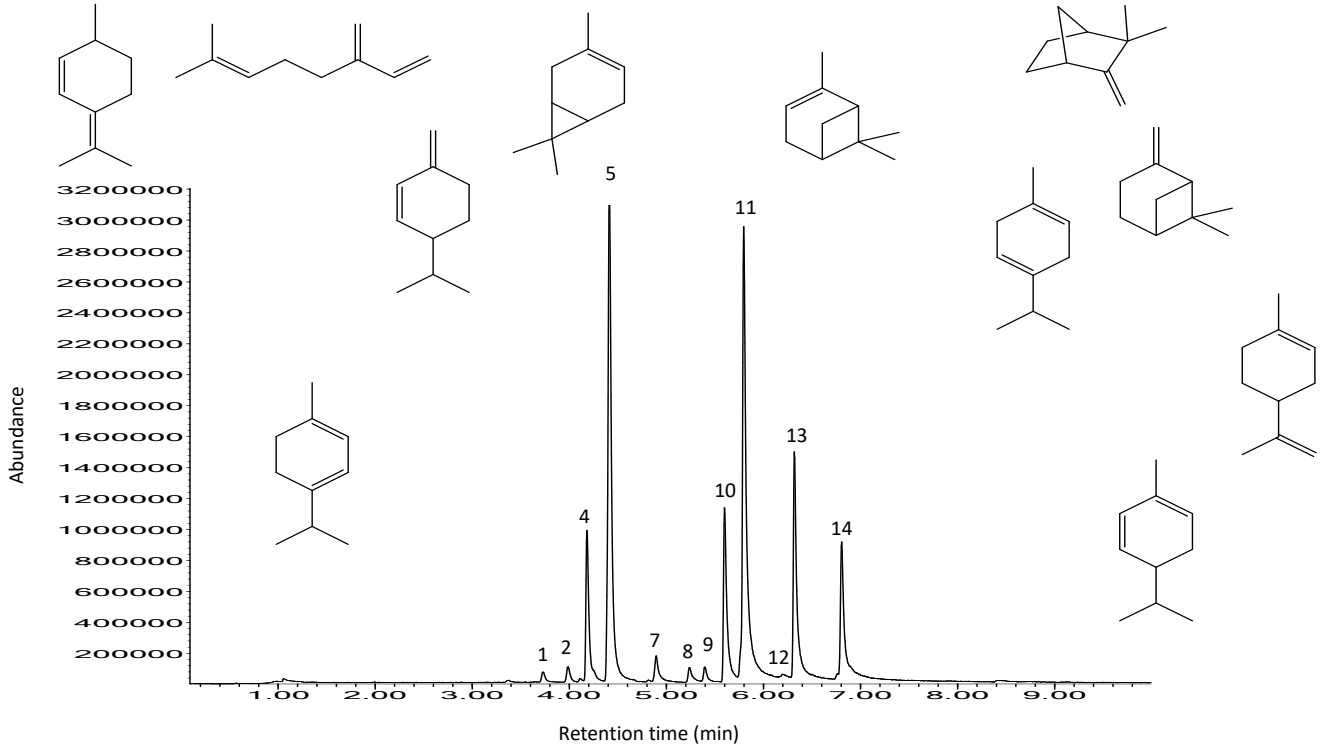


Figure 13: Total ion chromatogram of the headspace above a group of *Leptocoris rufomarginata* bugs. Numbers above a peak refer to the tentative identity of compounds as listed in Table 1. Compounds detected are:

1	3-carene	9	phellandrene
2	monoterpene A	10	α-terpinene
4	α-pinene	11	limonene
5	camphene	12	β-phellandrene
7	β-pinene	13	γ-terpinene
8	myrcene	14	isoterpinolene

A blend of the nine compounds was prepared in ethanol (100 mg/mL total), with each component at the relative concentration detected in the headspace of the aerations, and this blend was tested in the olfactometer (Table 2). For each assay the time to first choice for any arm was also determined (Table 3).

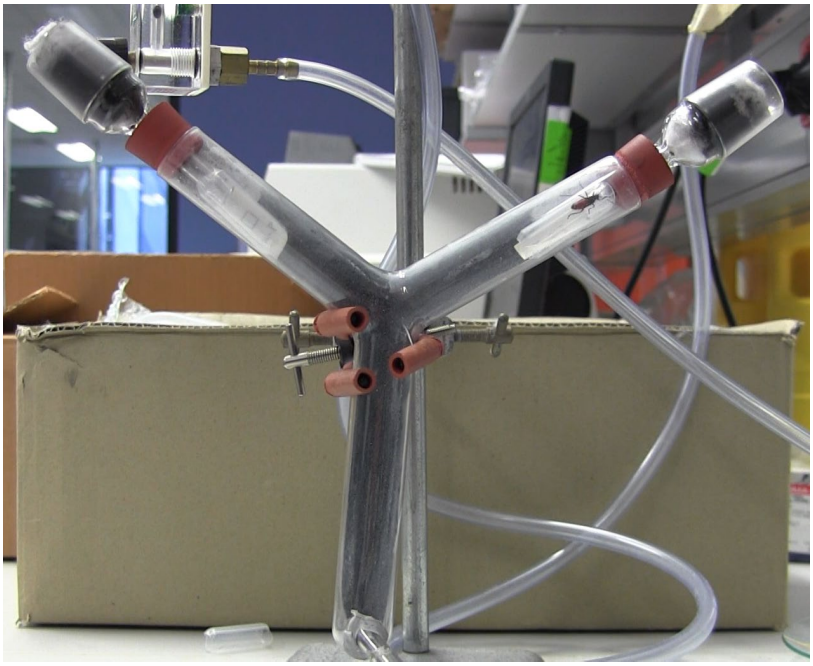


Figure 14: Y-tube olfactometer showing *Leptocoris* bug having chosen one arm in a two-choice test.

Table 2: Relative concentration of the nine components of the terpene blend, with a final concentration of 100 mg/mL in ethanol.

Compound name	Relative concentration (%)
3-carene	0.19
α-pinene	4.70
α-phellandrene	1.02
α-terpinene	14.73
β-pinene	4.22
camphene	17.11
γ-terpinene	21.56
limonene	35.43
myrcene	1.03

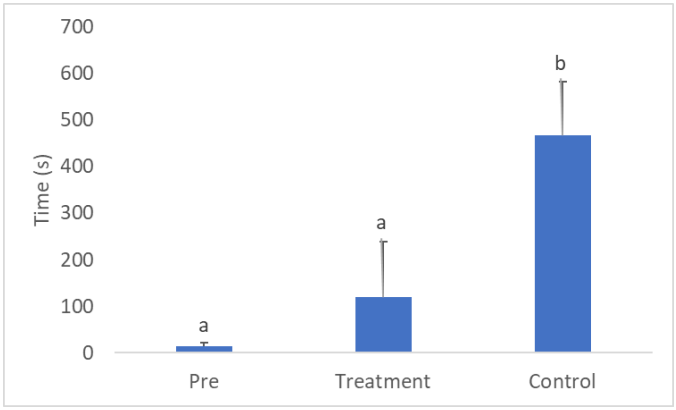
There was no significant difference in the mean time it took for bugs to make their first choice for treatment or control, regardless of compound or blend (ANOVA: $F_{9,39} = 0.67$, $P = 0.729$) (Table 3). The majority of the bugs (86.1 %) made a choice for either treatment or control within one minute of the trial commencing.

In the assays with single compounds against ethanol, there was frequently no significant difference between treatments (Figure 15, Table 4). There was a high level of variance in the responses of the bugs, which makes it hard to demonstrate a clear choice. There was only one compound, 3-carene, that was significantly avoided, in this assay the bugs showed a significant preference for the control over the treatment arm. In the case of one other compound (α-pinene), there is a trend demonstrating possible avoidance, and for α-terpinene there is a trend for choosing the treatment over the control. For most compounds tested there is no suggestion at all of preference for either arm of the olfactometer. The case is quite different in respect to the terpene blend, which has the nine components in the same relative proportions as was detected in the headspace of live bugs. Here there is a clear,

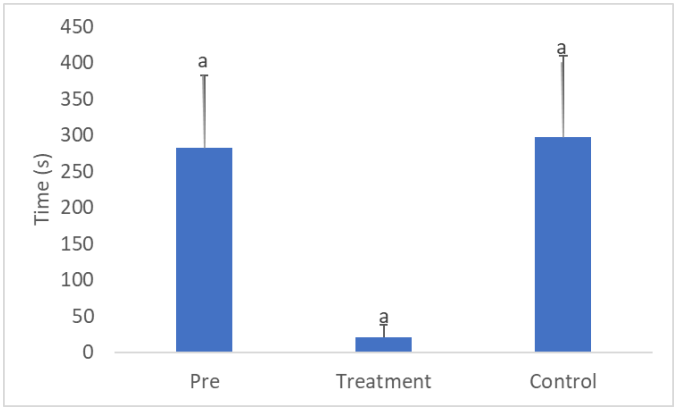
significant attraction of the bugs to the treatment arm of the olfactometer, and in fact no bug ever entered the control arm in any trial with this blend. This is not surprising, it is quite common for synergies between different components of pheromone blends, meaning that there is no behavioural response unless several components (or even all) of the natural blend are present in the correct relative amount.

Table 3: Mean (± SEM) time of *Leptocoris* bugs to make a first choice for either treatment or control arm of a Y-tube olfactometer.

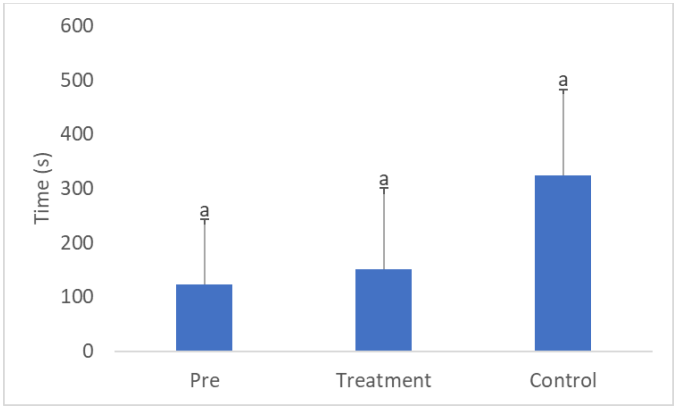
Compound name	Mean (± SEM) time to make first choice (s)
3-carene	14.4 ± 6.8
α-pinene	9.25 ± 2.0
α-phellandrene	5.25 ± 1.3
α-terpinene	55.0 ± 22.3
β-pinene	91.0 ± 80.4
camphene	47.7 ± 15.0
γ-terpinene	45.0 ± 32.1
limonene	89.5 ± 80.5
myrcene	27.6 ± 8.1
terpene blend	22.0 ± 1.7



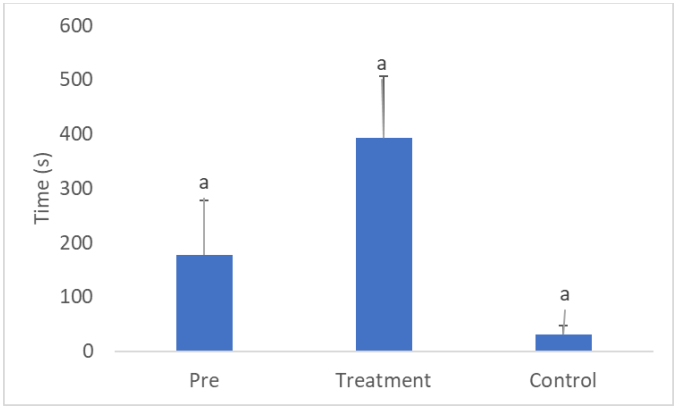
3-carene



α-pinene



α-phellandrene



α-terpinene

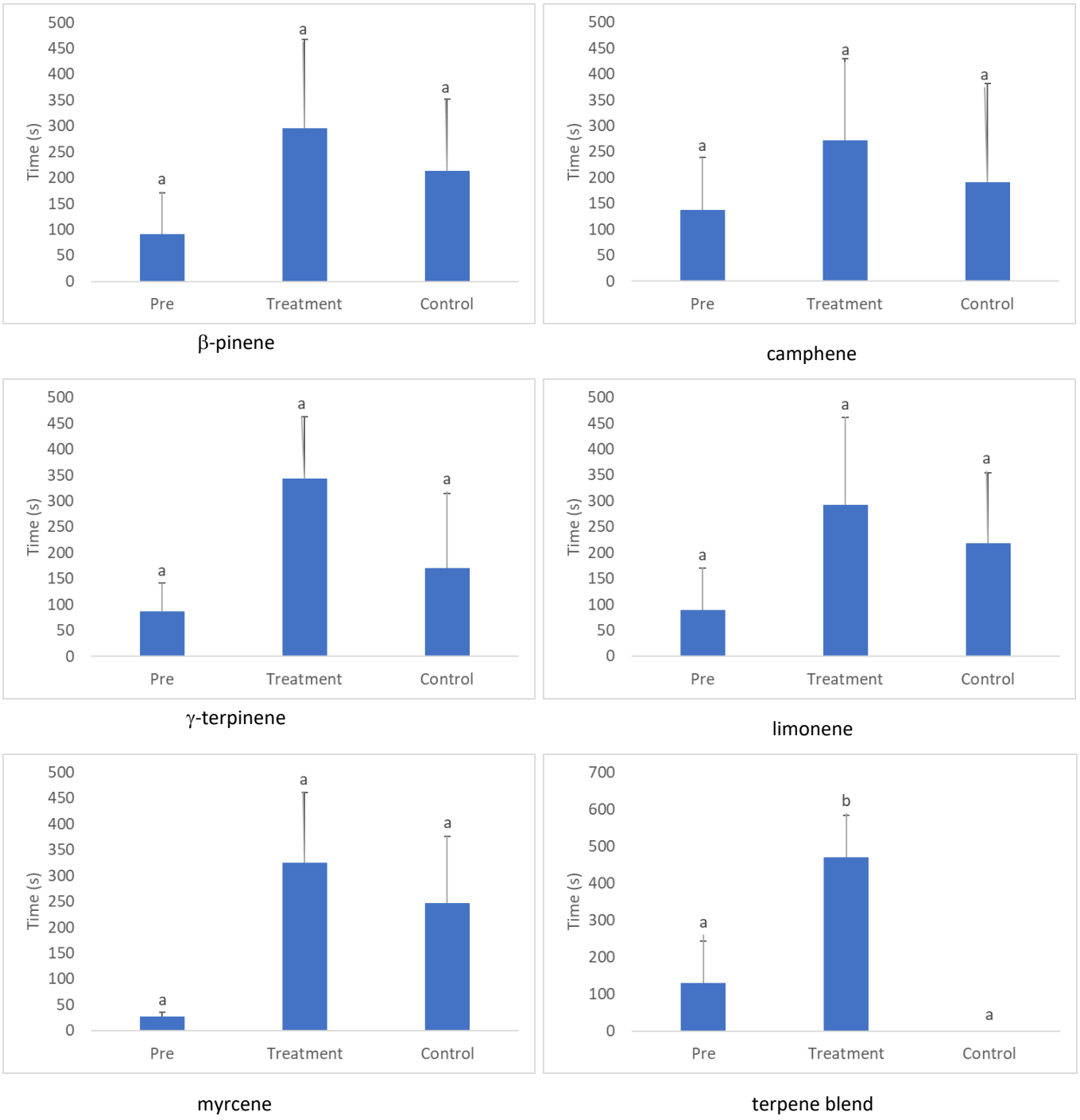


Figure 15: Response of *Leptocoris* bugs in a Y-tube olfactometer, showing time spent in each arm. Each compound is shown on a separate graph. Columns sharing a letter are not significantly different.

Electrophysiology is an invaluable technique to quickly and quantitatively assess the response of the olfactory system to odour stimuli. Electroantennography is performed by measuring the change in electrical potential along the excised antenna as the antenna is exposed to potential olfactory stimuli. A response caused by depolarization of the olfactory sensory neurons gives a characteristic shape (Olsson and Hansson, 2013).

Antennae of *L. rufomarginata* were excised under phosphate buffered saline (pH 7.4, Sigma) and mounted in electroconductive gel (Medtel) across two electrodes connected to an EAG Combiprobe manipulator (Syntech) in a constant stream of humidified air (flow rate = 19.9 mL/s) at ambient temperature (~22 °C). Air and solvent blanks were puffed (14.8 mL of air over 0.5 s) over the antenna (Stimulus controller CS-55, Syntech) which was held

directly in the stream of air. Chemical standards (100 mg/mL in ethanol) and solvent blanks (~50 µL) were introduced on 5 mm filter paper disc in a glass Pasteur pipette. The signal from the antenna was amplified and filtered (IDAC-2 Signal Acquisition Controller, Syntech), and data collected and analysed using software developed for electroantennography (GcEad V4.4, Syntech). DC voltage displacements were recorded for the different test stimuli and compared with a blank air response. Air was used as the standard stimulus tested at the start and end of the brace of stimuli to check for signal deterioration.

Table 4: ANOVA analysis for difference between treatments in two-choice olfactometer

Compound name	ANOVA analysis
3-carene	$F_{2,14} = 6.02$, P = 0.015
α-pinene	$F_{2,14} = 2.54$, P = 0.134
α-phellandrene	$F_{2,11} = 0.58$, P = 0.58
α-terpinene	$F_{2,8} = 4.24$, P = 0.071
β-pinene	$F_{2,11} = 0.58$, P = 0.579
camphene	$F_{2,8} = 0.19$, P = 0.83
γ-terpinene	$F_{2,11} = 1.35$, P = 0.307
limonene	$F_{2,11} = 0.59$, P = 0.575
myrcene	$F_{2,14} = 2.04$, P = 0.173
terpene blend	$F_{2,11} = 6.38$, P = 0.015

Positive electrical responses were observed for 3-carene, α-terpinene, camphene and γ-terpinene (Figure 16). The size of the electrical response was relatively small, possibly due to the age of the bugs being tested. Future studies need to concentrate of using younger animals, to maximise the electrophysiological responses.

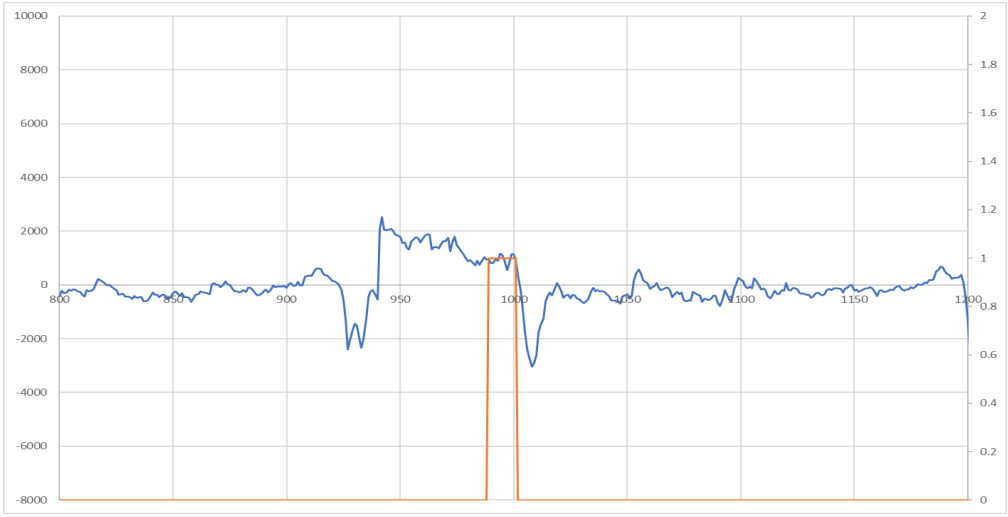


Figure 16: Electroantennographic response (blue trace) to a puff of γ-terpinene (orange trace). Depolarisation of the nerve fibre in the antenna demonstrates that the compound is detected by the insect.

Using the terpene blend as tested in the laboratory a field trapping study was conducted to evaluate the efficacy of this mixture. The nine-component blend had the same relative ratios of the compounds as measured in the headspace above the bugs (Table 2). The blend or water (as a control) (1 mL) were placed in small Ziploc bags (75 × 50 mm) and traps hung in trees close to a golden rain tree known to contain bugs at Laidley (-27.6 °S, 152.4 °E). Despite the traps being in a location where bugs were present, no bugs were trapped in this trial.