Dose reduction and alternatives to the phenol pheromone in monitoring and management of the grass grub *Costelytra zealandica*

Running title: Alternative lures for grass grub

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Abstract:

BACKGROUND: Endemic New Zealand grass grub *Costelytra zealandica* is a pest of introduced pasture that uses phenol as a sex pheromone. The pheromone could be used to monitor and manage grass grub populations, but the irritating properties and toxicity of phenol for human handlers, as well as the possible ecotoxicological effects, pose obstacles to the deployment of the pheromone. This study aimed to limit the use of phenol by dose-response studies and investigation into alternative attractants and synergists to phenol.

RESULTS: No difference in trap catch was seen across the range of 1-100 mg of phenol, while rates below this (0.001-0.1 mg) caused a large drop in catches. Our results indicated that 1 mg

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loading in lures was enough to indicate beetle presence over one week. 4-Hydroxybenzaldehyde and *p*-cresol proved unattractive in this study, both as single attractants and as synergists with phenol. Phenyl acetate, phenyl benzoate and diphenyl carbonate all formed phenol under hydrolytic conditions to act as successful propheromones, while phenyl acetate was found to be as attractive as phenol on its own.

CONCLUSION: This study described several ways to reduce or avoid the use of phenol in the field while maintaining lure effectiveness.

1 INTRODUCTION

The endemic grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae) is a significant pest of introduced pasture systems in New Zealand. An infestation of grass grub can reduce pasture yield by 6% for every 100 larvae/m^2, as a result of larval feeding.[1] Adult grass grubs can also cause considerable damage to horticulture through defoliation, for example of kiwifruit, blueberry and grapevine crops.[2]

The sex pheromone of *C. zealandica* was the first of the Scarabaeidae family to be discovered, when grass grubs were initially found to be attracted to the phenolic resin Durez 12687.[3] Identified as phenol,[4] the unusual structure of the grass grub pheromone is due to its bacterial origin. It is produced by *Morganaella* sp. in the colleterial glands of the female grass grub, by catabolism of the amino acid tyrosine.[5] Since then, many more scarab pheromones have been discovered, varying widely in structure and in method of action.[6] However, many of those in melolonthine scarabs are hypothesized to be derived from amino acids.[7]

Success so far in using phenol as a control tactic has been limited. While there has been some success in using it to predict the beginning of the flight time to apply pesticides effectively,[8] it has proved not to be effective in causing mating disruption[9] or in predicting population numbers.[10]

Also, despite its former use as a bactericidal ingredient in carbolic soap over several decades, phenol is more toxic and a has a more negative public reputation relative to the majority of pheromones used to suppress pest populations.[11, 12] As semiochemical control methods are intended to be safer, more publically-acceptable methods of pest control than pesticide usage, we thought it useful to investigate potential ways to minimize the exposure to phenol.

The present study investigated ways to minimize or avoid the use of phenol, due to concerns over the negative perception. Dose-response in field catches was investigated, demonstrating the minimum quantity of phenol that could be used without reducing catch, which would allow handlers to limit their exposure.

Alternative compounds were also tested for their attractiveness to *C. zealandica*. Attractive compounds might be used either as synergists to improve the efficacy of traps (reducing phenol use), or as substitutes to eliminate the use of phenol completely. Prior investigations have shown that grass grubs are attracted to 4-hydroxybenzaldehyde (Fig. 1),[13] so these experiments were repeated to see if this chemical could viably be used as an alternative to phenol. Phenyl acetate and *p*-cresol (Fig. 1) were also investigated, both as attractants and as synergists to phenol.

Propheromones are substances which can be chemically converted to a pheromone, in situations where the pheromone is unstable or, as in this case, unsafe to handle. The term propheromone was coined by Pickett et al in 1993.[15] Highly reactive pheromones, such as aldehydes[14] and conjugated dienes[15] can be difficult to utilize in the field. However, using a propheromone allows the pheromone to be released slowly as the reaction occurs to produce it *in situ*, such as by cleavage in sunlight. This allows the use of pheromones which would not otherwise be feasible. Phenyl acetate, phenyl benzoate and diphenyl carbonate (Fig. 1), which can all be chemically converted into phenol under certain conditions, were investigated as potential propheromones. These compounds were tested for their release rate from the

dispenser and for their phenol-producing reaction rate. Propheromone release rates should be slower than that of the active substance to prevent ineffective loss, and the conversion rates within the lures should be fast enough to generate a minimum threshold of pheromone. If an effective propheromone could be found, this would theoretically be equivalent to using phenol without the need to handle it.

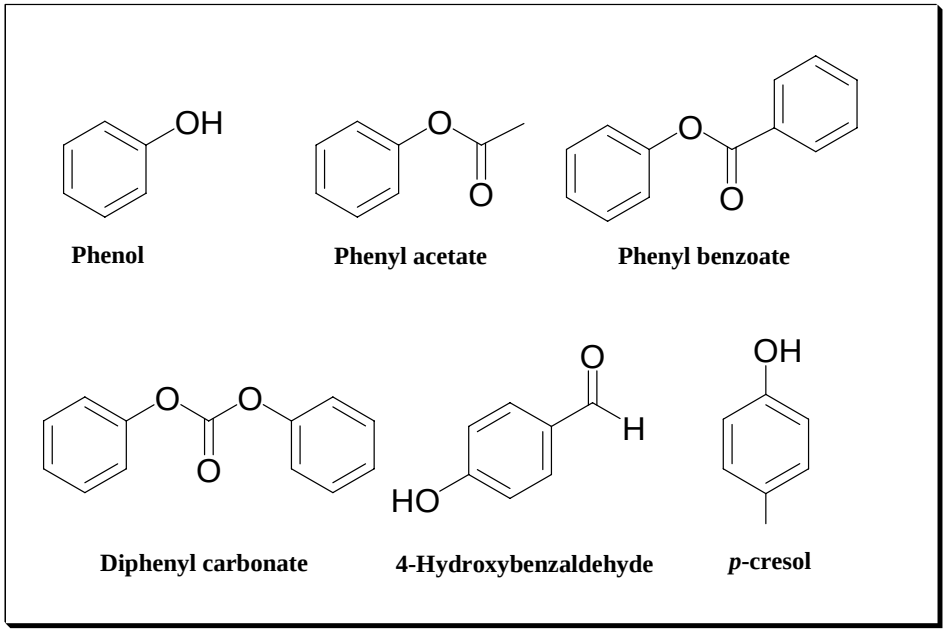


Figure 1: Chemical structures of the pheromone of New Zealand grass grub *Costelytra zealandica* and alternative attractants tested.

2 METHODS AND MATERIALS

2.1 Chemicals

All chemicals used in this research were >98% chemical purity, from Sigma-Aldrich. The acetone used as a solvent in the propheromone trials was purchased from Mallinckrodt and was 99.5% chemical purity.

2.2 Field trapping

All experiments were conducted in Canterbury, New Zealand. Lures were placed in water traps (2 L plastic containers 17 × 17 × 9 cm filled with c. 0.8 L of fresh water), which were covered with 2.5 cm square wire mesh to prevent bird predation, and draining holes to keep water level steady (Fig. 2).[16] Beetles would fly into the trap and be unable to exit the water. Treatments were in place at least 30 min before the expected start of the flight period, which begins 20 min after dusk, to enable the male beetles to orientate toward an attractant source. The lures were made up in heat sealed clear polyethylene bags (5 cm x 6 cm; 100 μm thick), containing a white piece of polyester felt (4 cm x 2.5 cm x 2 mm) unless otherwise stated, which was impregnated with the active ingredient. All trapping trials used three replicates unless otherwise stated, with traps laid out 7 m apart, with 25 m between each replicate in a randomized complete block design.

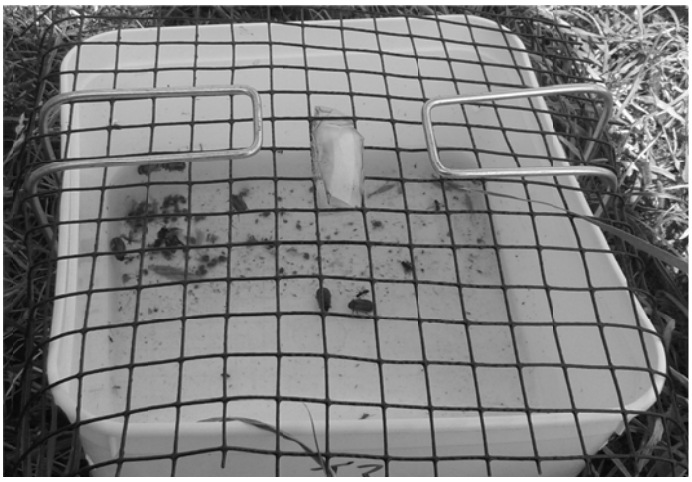


Figure 2: Trap used for field trapping experiments of New Zealand grass grub *Costelytra zealandica* in Canterbury, New Zealand.

2.3 Dose-response experiments

To determine the effect of dose on catch, and the minimum dose needed, traps were set out containing phenol dissolved in 100 µL acetone at increasing doses (0, 0.001, 0.01, 0.1, 1, 10, and 100 mg). Trials were carried out over two consecutive nights in November 2006.

To determine the effectiveness of the doses over time (i.e. longevity), the experiment was repeated with the polyethylene bag lures and run over two weeks in November 2015, with traps being checked every other day, but lures were not replaced.

2.4 Evaluation of other attractants/synergists

To evaluate the reported ability of 4-hydroxybenzaldehyde to act as an attractant and as a synergist to phenol[13], treatments were made up with: phenol (0.5 mmol) in water (800 µL); 4-hydroxybenzaldehyde (0.5 mmol) in water (800 µL); and phenol (0.25 mmol) in water (400 µL) and 4-hydroxybenzaldehyde (0.25 mmol) in water (400 µL) in individual dispensers. Five replicates of each were placed in polyethylene bags as described above and another five replicates of each were placed on dental rolls (1 cm diam. x 3.5 cm) to check for any effect of the dispenser. Five replicates of a negative control contained both a polyethylene bag and a dental roll. The traps were monitored over five consecutive nights in November-December 2011.

Phenyl acetate and *p*-cresol were tested as individual attractants by placing out traps containing the compound (1.06 mmol), or as synergists by placing out traps containing both the compound (0.53 mmol) and phenol (0.53 mmol), in separate bags. The positive control was phenol (1.06

mmol), and a blank bag served as a negative control. The traps were monitored for two weeks in November 2015.

2.5 Evaluation of propheromones

Three phenyl esters were selected as potential propheromones. Phenyl acetate, phenyl benzoate and diphenyl carbonate (Fig. 1) were all measured for release rate and hydrolysis rate. Release rate was measured by preparing a lure sachet containing only the putative propheromone (100 mg) (i.e., not under hydrolysis conditions), and weighing the sachet each day to gravimetrically measure the release rate.

The fastest hydrolysis conditions were determined by preparing a vial of each propheromone under different potential hydrolysis conditions, i.e. basic, acidic or neutral. The rate was analyzed every other day by taking the solution (1 μL) and diluting it 1000-fold in acetone, and then analyzing on a gas chromatograph-mass spectrometer (GC-MS). A Varian 3800 GC coupled to a Saturn 2200 MS (Varian Walnut Creek, CA, USA) was used. Injections were splitless for 0.6 min, and helium was used as the carrier gas with a constant flow of 1 mL/min. The GC oven was equipped with a DB-5ms column, with dimensions of 30 m x 0.25 mm id x 0.25 μm film thickness. The GC oven temperature program was 40°C (held for 2 min) to 280°C at 10°C/min for the DB-5ms column (held for 15 min). The injector temperature was 250°C, while the transfer line was maintained at 280°C. All MS analysis was carried out in electron impact mode using an ionization voltage of 70 eV, and a mass range of 29 m/z to 399 m/z. The reaction was measured quantitatively by integrating the peak area of both the starting material and phenol as the reaction product, and calculating the proportion of phenol present relative to the starting material. As this was done simply to see which putative propheromones hydrolyzed sufficiently (>1% hydrolysis) under the given conditions, this method was considered sufficient.

2.6 Trapping trials with putative propheromones

Molecules that formed phenol under hydrolysis conditions were selected as propheromones to test for attraction. Propheromone lures were made up in solution of 800 µL acetone + 200 µL water. Phenyl acetate (1mmol) was tested under acidic conditions (10 µL H_2SO_4), while phenyl benzoate (1 mmol) and diphenyl carbonate (0.5 mmol) were tested under basic conditions (0.8 eq and 1.6 eq NaOH respectively). Half the amount of diphenyl carbonate was used, as hydrolysis of 1 mol diphenyl carbonate yields 2 mol phenol. No felt was used in propheromone lures to allow the reaction to occur in solution. The traps were checked every other day for two weeks in November 2015, and the randomized order of the treatments was changed every seven days.

2.7 Cleavage of phenyl acetate by sunlight

Three replicates of phenyl acetate (100 mg) were placed in PE bags and left in the sun for 4 days. The PE bags were extracted with acetone (3 mL) and the extract analyzed by GC-MS using the same program as above. The amount of phenol produced as a percentage of the amount of phenyl acetate remaining was calculated by their relative peak areas.

2.8 Statistical Analysis

Catches were log-transformed to stabilize the variance and subjected to ANOVA for lure treatment effects using Minitab v. 16 (www.minitab.com). Tukey's Least Significant Difference tests were used *post hoc* to separate treatments with a family-wise error rate of 5%.

3 RESULTS

3.1 Dose-response of phenol in field tests

A logarithmic series of phenol doses between 0.001 mg and 100 mg was tested for a dose response (Fig. 3). Doses above 1 mg did not increase trap catch, but below the threshold of 1 mg loading the catch was greatly reduced, and not significantly different from the zero dose.

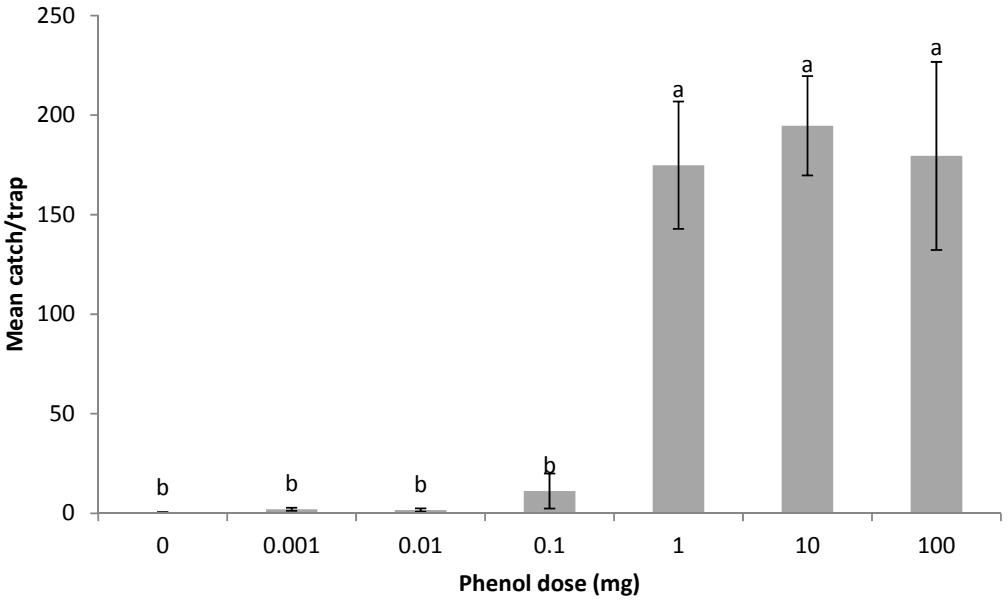


Figure 3. Dose-response catch per trap (n=3) of the New Zealand grass grub, *Costelytra zealandica* (± SE), with 0.001 – 100 mg phenol, dissolved in 100 µL acetone and released from polyethylene bags in the field 20-21 November 2006. The same lower-case letter indicates no significant difference (α= 0.05), according to the Tukey's test.

3.2 Longevity

In testing the longevity of the doses, the 1, 10 and 100 mg lures were all equivalently effective for the first 9 days, after which the 1 and 10 mg had reduced catch, while the 100 mg lure was still catching at the end of the 15 days over which the experiment was run (Fig. 4). The mean release rate of phenol from the 100 mg lure was gravimetrically determined as 2.74 mg/d (±

SEM, 0.192), which is higher than the the reported 229 ng produced by the female beetle in a day.[5]

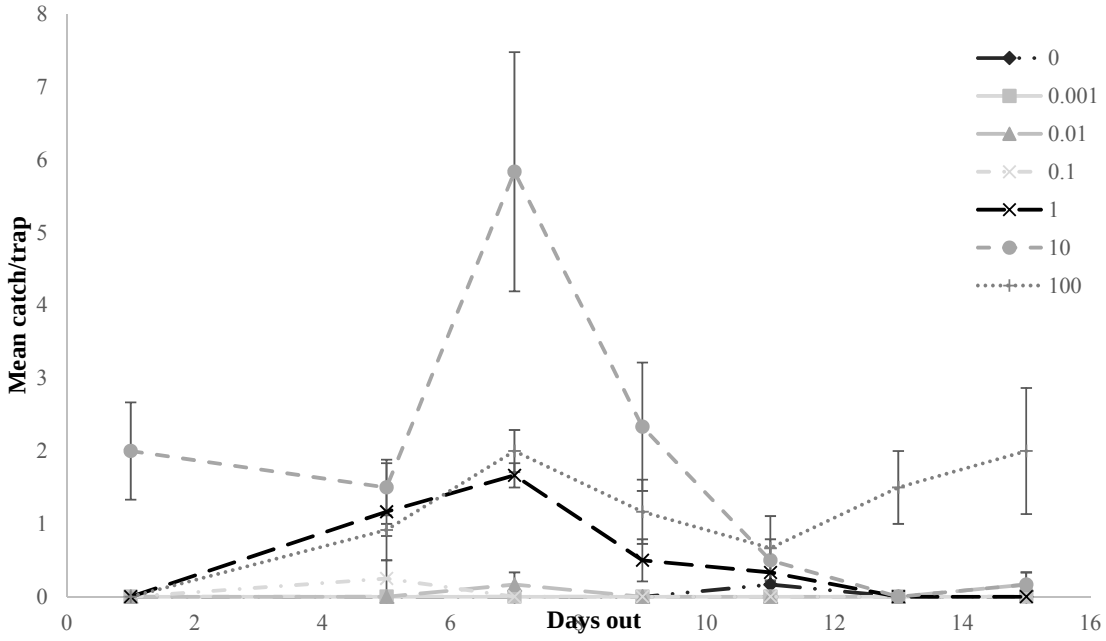


Figure 4. Mean catch per trap of New Zealand grass grub *Costelytra zealandica* over time to phenol at loadings from 0.001 to 100 mg in polyethylene bags (n = 3). Traps were monitored from 12-26 November 2015.

3.3 Alternative attractants and synergists

Chapman (2009) reported catches of grass grubs to acetone or ethanol solutions of 4-hydroxybenzaldehyde.[13] Our experiments did not confirm the finding that 4-hydroxybenzaldehyde was attractive to grass grubs on its own, and we did not find it to be synergistic to phenol (Fig. 5). Catches from 4-hydroxybenzaldehyde alone were not significantly different from those in the control, and catches from the combination of phenol and 4-hydroxybenzaldehyde were not significantly different from catches with phenol alone.

The 4-hydroxybenzaldehyde did not appear to be either attractive or acting synergistically (F=44.2; df=3, 31; P<0.001). To test whether the PE bag dispenser biased the results, dental rolls were used as an alternative dispenser in parallel. Lure type did not have a significant effect on catch (F=1.7529, p=0.18731), so the results from the two dispenser types were combined.

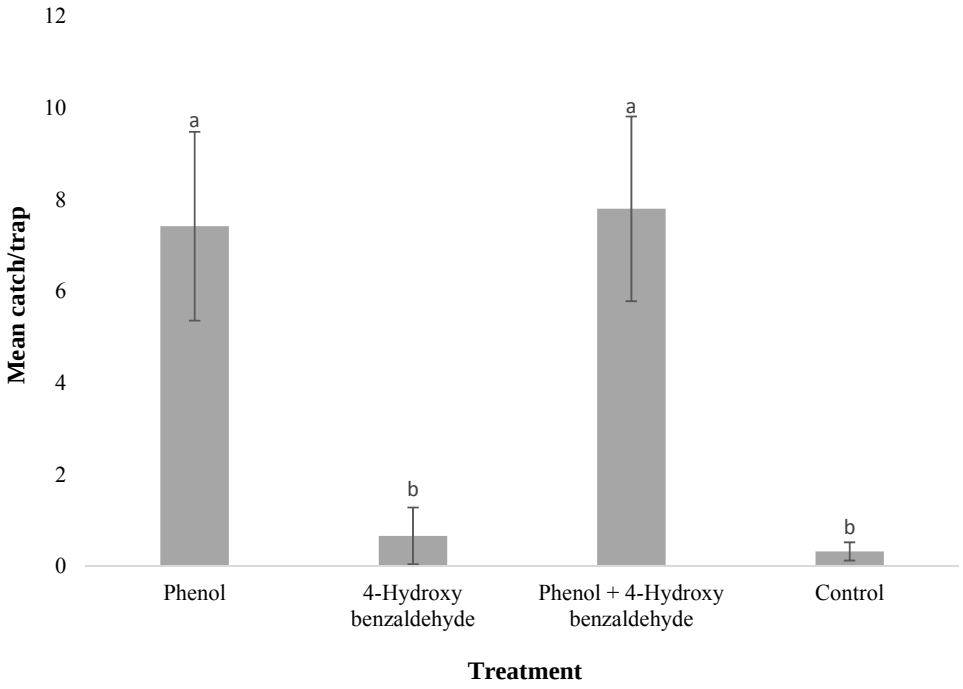


Figure 5. Mean catch of the New Zealand grass grub *Costelytra zealandica* (± SE) to lures with 4-hydroxybenzaldehyde as an individual attractant and in combination with phenol (n = 10), from 29 November – 4 December 2011. The same lower-case letter indicates no significant difference (α= 0.05), according to Tukey's test.

Two phenol derivatives, phenyl acetate and *p*-cresol were also tested as grass grub attractants and as synergists to phenol. Phenyl acetate was as effective as phenol, and the combination of the two resulted in trap catches that were not significantly higher (Fig. 6). *p*-Cresol did not

catch significantly more than the negative control, and combination of *p*-cresol with phenol decreased the attractiveness of phenol (F=18.08; df =6, 120; P<0.001).

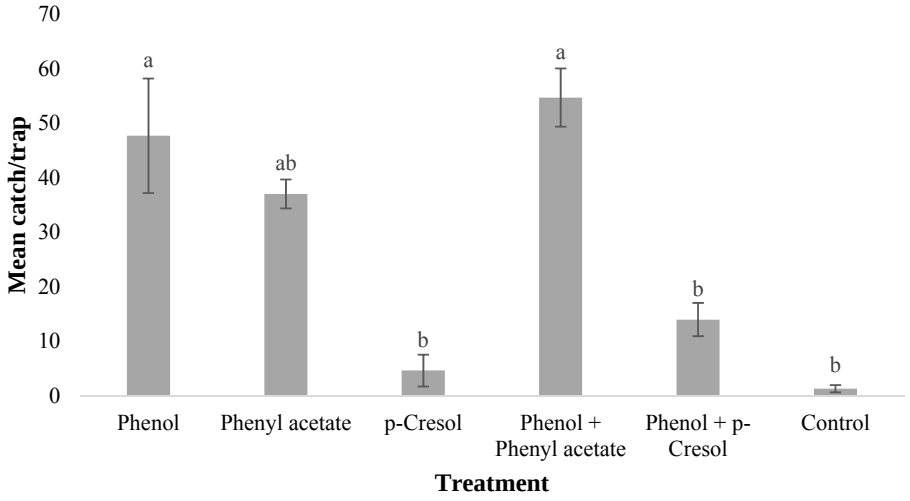


Figure 6. Mean catch per trap of the New Zealand grass grub *Costelytra zealandica* (± SE) with phenyl acetate and *p*-cresol as individual attractants, and in combination with phenol (n = 3). Traps were monitored from 12-26 November 2015. The same lower-case letter indicates no significant difference (α= 0.05), according to Tukey's test.

3.4 Hydrolysis and release rates of propheromones

Phenyl acetate, phenyl benzoate and diphenyl carbonate all had a fast enough hydrolysis rate for >1 mg of phenol to be produced from 100 mg of propheromone per day (Fig. 7, Table 1), meaning that the minimum amount of phenol required for maximum catch (1 mg) according to the dose response experiment (Fig. 3) would be reached. Phenyl acetate had a fast enough release rate (25 mg/day) that there was concern that this might affect its longevity in the field, while the release rate of phenyl benzoate and diphenyl carbonate (0.38 mg/day and 0.33 mg/day respectively) (Table 1) was believed to be slow enough that they should last the length of the flight period

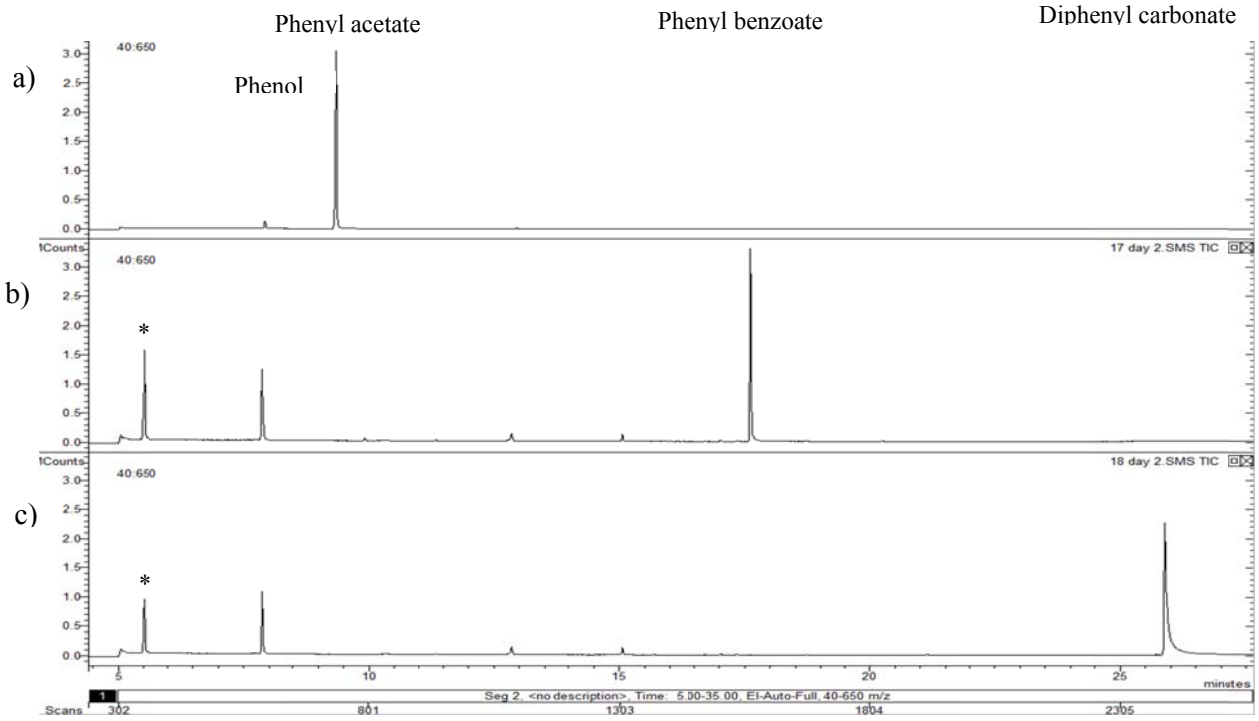


Figure 7: Comparison of representative chromatographs showing the hydrolysis of a) phenyl acetate under acidic conditions, and b) phenyl benzoate and c) diphenyl carbonate under basic conditions, after two days, in acetone. * = byproduct formed by dimerization of acetone under alkaline conditions.

Compound	Release rate (mg/day (± SE))	Catalyst	Hydrolysis rate (%/day)*
Phenol	2.74 (± 0.19)	-	-
Phenyl acetate	25.05 (± 1.19)	10 µL H_2SO_4	1.7
Phenyl benzoate	0.38 (± 0.06)	0.8 eq NaOH	8.5
Diphenyl carbonate	0.33 (± 0.033)	1.6 eq NaOH	18.8

Table 1. Hydrolysis and release rates of phenol and putative propheromones. The release rate is measured gravimetrically under neat conditions. The hydrolysis rate is measured as the percentage of starting material converting to phenol per day under the catalytic conditions used in the field.

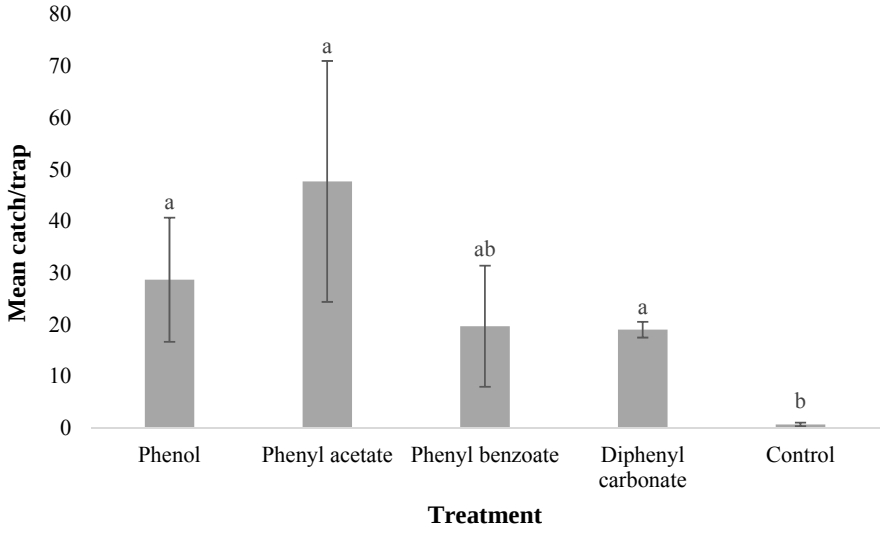
3.5 Cleavage of phenyl acetate by sunlight

Cleavage of the acetate bond in sunlight to form phenol from phenyl acetate occurred at a maximum rate of 0.1% after four days across three replicates. This rate is far too low to suggest that the attractiveness of phenyl acetate could be due to its reaction to form phenol *in situ* – for this to be the case, the 150 mg of phenyl acetate used in the trapping trial would have to react at a rate of >1% to form enough phenol to give the results seen in figure 6.

3.6 Trapping trials with propheromones

When the propheromones were tested in the field, phenyl acetate caught more than the positive control, but not significantly so. Phenyl benzoate and diphenyl carbonate also had catch rates statistically equivalent to phenol (Fig. 8a) (F=5.40; df =4, 85; P<0.001). The longevity of the propheromones was measured across two weeks (Fig 8b), and all 3 propheromones continued to have catch equivalent to phenol for the duration of the trial, despite concerns that phenyl acetate would release too quickly and therefore not last the two weeks.

a)



b)

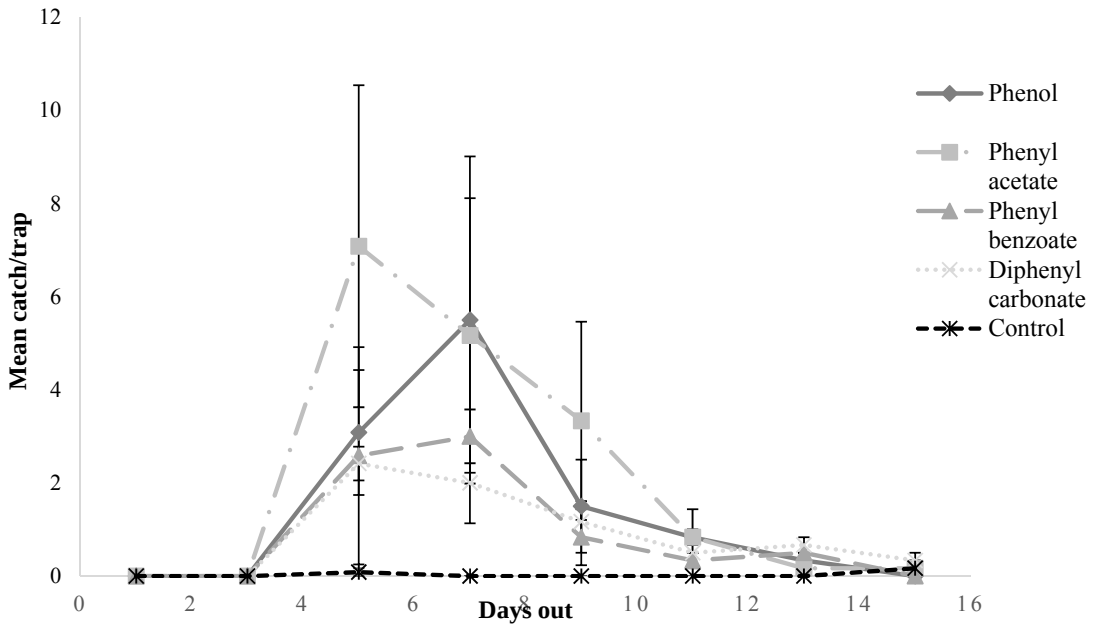


Figure 8: Catch of New Zealand grass grub *Costelytra zealandica* with propheromone treatments tested in polyethylene bags; a) the mean catch over the entire flight (± SE) and b) the variation in catch over time (± SE). Hydrolysis conditions were created with acid for phenyl acetate and with base for phenyl benzoate and diphenyl carbonate (n = 3). Traps were monitored from 12-26 November 2015. There is no significant difference between bars with the same letter (α= 0.05), according to Tukey's test.

4 DISCUSSION

The dose-response trapping experiment showed that traps loaded with 1 mg were as effective as those loaded with higher doses, and that at this loading the lure remained effective for well over a week, while at 100 mg loading the lure remained effective for the 2-week duration of the trial. As previous trials have used up to 1 g phenol as an attractant,[16] this finding means that if this technique is commercialized, the minimum amount of phenol can be used, limiting potential handler exposure.

4-Hydroxybenzaldehyde proved unsuccessful as an attractant on its own or as a synergist to phenol in our hands, even across different dispenser types. Chapman[13] showed in 2009 that 4-hydroxybenzaldehyde was attractive to *C. zealandica* both on its own and with an additive effect to phenol when placed in separate dispensers. We were unable to replicate these results, however water was used as a solvent in our experiments whereas Chapman used acetone and later ethanol as solvents. This suggests that, contrary to the assertion in that manuscript that 4-hydroxybenzaldehyde is attractive regardless of solvent used, perhaps there is some effect in play with regard to the solvent.

p-Cresol was also inactive as an attractant, and actually decreased catch when tested in combination with phenol. This decrease in trap catch is due to the presence of *p*-cresol, rather than the lower quantity of phenol, as the quantity of phenol used was still higher than the minimum required as shown by our dose-response study. *p*-Cresol also could not have affected the release rate of phenol, because the two were placed in separate dispensers. This therefore suggests that *p*-cresol somehow lowers the attractiveness of phenol, possibly by acting as a repellant or by masking the signal of phenol in some way. Phenyl acetate was attractive on its own and in combination with phenol. A suggested explanation for the attractiveness of phenyl acetate was that it is easily cleaved to form phenol. It was thought that this may be able to be catalyzed by the UV in sunlight, so the possibility was investigated. The rate of reaction under sunlight was shown to be only 0.1% after four days, meaning 1500 mg of phenyl acetate would be needed to give the 1 mg phenol, the minimum dose necessary as per our dose response trial. This is much higher than the quantities used in this study, so catalysis via sunlight is unlikely to be the cause of the attractiveness of phenyl acetate. Another possible explanation of the action of phenyl acetate could be that it, after impinging on the antennae, is hydrolyzed by an esterase on the way to the dendrite, forming phenol at the antennae. A similar mechanism occurs in the cabbage looper moth *Trichoplusia ni,* whose pheromone, *Z*-7-dodecenyl acetate, is hydrolyzed

upon reaching the dendrite.[17] If *C. zealandica* also has hydrolyzing esterases in the sensillum lymph, it could explain the attractiveness of phenyl acetate.

As mentioned previously, a good propheromone should diffuse slowly or not at all from the dispenser, and cleave quickly under the reaction conditions to give the pheromone. For this reason the potential candidates were tested for suitability as propheromones and screened for release rate and hydrolysis rate under different conditions. As seen in the dose-response experiment, only 1 mg of phenol is needed to obtain good trap catches. So if 100 mg of starting material is used, it needs only to hydrolyze fast enough for 1 mg of phenol to be produced at the onset of trapping. Phenyl acetate, phenyl benzoate and diphenyl carbonate were all able to meet these criteria. When tested as propheromones all were effective (Fig. 8a). The fast diffusion of phenyl acetate from the PE bags was anticipated to give it too short a longevity to be useful in the field. However, this expectation was not reflected in the results, which showed that phenyl acetate, when used as a propheromone, continued catching for the entire two weeks of the trial. This may mean that the hydrolysis rate of phenyl acetate is fast enough that enough phenol has formed to continue to be effective by the time the phenyl acetate has all dissipated. There was no real difference between treatments with time.

This research has outlined multiple avenues through which the use of phenol in insect lures could be reduced or eliminated. It is quite feasible that the attractant and propheromones described here will find use in improved monitoring or mass trapping applications of *C. zealandica*, although more research is required to achieve this.

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