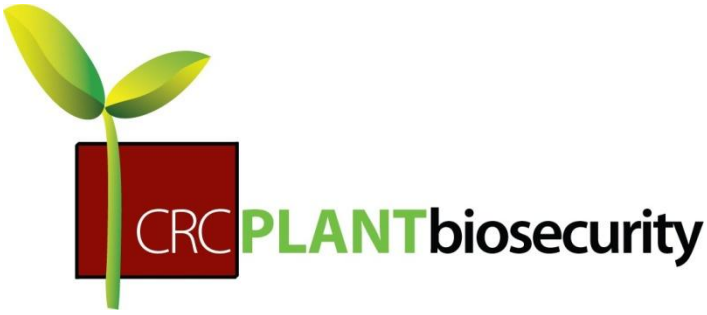

Cooperative Research Centre
for National Plant Biosecurity

Final Report

CRC20081

Biosensor based detection of grain pests

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1. Executive Summary

The aim of this project was to develop a technology based on insect-derived olfactory receptors that is applicable to use as biological detectors of insects in stored grains. These detectors will be suitable for use as sensors in a highly sensitive biosensor device. Such devices will be the equivalent of a detector dog that can perform real time detection and identification of a wide range of volatile chemicals but in a cheap, robust and constantly available machine form and with added knowledge on the identity of the chemicals detected. We envision that such biosensors would be utilised by end-users in an integrated approach to management of storage pests, in order to develop treatment thresholds that maintain market access but minimise development of phosphine resistance.

In the course of this project we have successfully identified and produced good candidates for the front end detectors of a biosensor device capable of detecting the stored grain pest *Tribolium castaneum* (Red flour beetle).

2. Aims and objectives

The aim of the *Biosensor-based Detection of Grain Pests* project was to identify biological detectors and biological signals associated with *Tribolium* beetles, which could subsequently be utilised to develop a senstive pest biosensor for use in a grain storage context. This would provide a range of economic/trade and environmental benefits to Australian bulk grain companies as current detection methods are unreliable leading to overuse (and loss of efficacy) of phosphine to remove storage pests for export.

This project aimed to determine the feasibility of developing a biosensor that can detect insects in grain with high sensitivity and speed. The primary planned outcome of the project was to demonstrate detection of an insect derived odour by a *Tribolium* chemoreceptor expressed in insect cells. In addition the project was to demonstrate proof-of-concept and establish the technical protocols for adapting this approach for other biosecurity applications. Milestones required to attain these goals included the discovery of specific volatile-receptor combinations relevant to *Tribolium*, and subsequent receptor production and assay development using cell lines expressing these receptors.

3. Key findings

Target volatiles

This project relies on isolation of a receptor that insect pests utilise to detect volatiles used for communication between themselves. We have used available knowledge from scientific literature and also designed, built and completed

olfactometer assays to identify the volatiles that should be used as the target for receptors.

Behavioural studies using a Y-tube olfactometer

Initial experiments were designed to test the behaviour of *Tribolium castaneum* in the presence of various volatiles such as those from infested grains. Using a Y-tube olfactometer designed for this project (Figure 1), we confirmed that both adults and larvae respond to the odour of infested flour more than clean flour. While both life stages prefer flour infested with their own species or *Ryhzopertha dominica* (Lesser grain borer). Interestingly there was a differential response to flour infested with *Sitophilus granarius* (Granary weevil) (Figure 2) with only larvae choosing *Sitophilus*-infested grain.

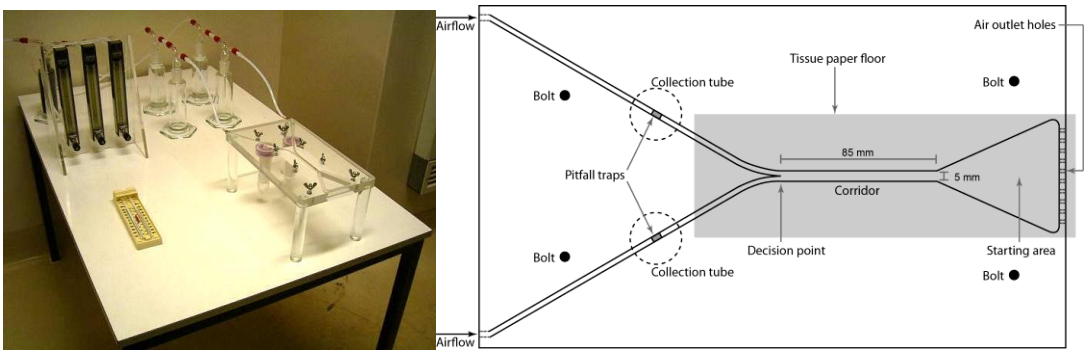

Figure 1. Y-tube olfactometer set-up with controlled airflow and sample bottles (left) and the plan for the test arena (right).

These behavioural results indicate that *T. castaneum* has olfactory receptors suitable for detecting clean and infested food sources. Rather than work with the complex odour of an infestation, our work focused on finding individual receptor:odour pairs that could be used to detect infestations.

In order to identify the most appropriate compounds to use as target volatiles we tested the responses of adults and larvae to available chemicals that are known to be produced by *T. castaneum* or *R. dominica* as pheromones. To date we have tested methyl-1,4-benzoquinone (MBQ), a volatile important for aggregation and stress/defence response; 1-pentadecene (C15:1), oil that coats the beetles; 4,8-dimethyldecanal (DMD), aggregation pheromone of *T. castaneum*; and Dominicalure 1 and 2, commercial aggregation pheromones of *R. dominica*.

Results from these assays have shown adults and larvae consistently respond behaviourally to DMD, but not to the other chemicals.

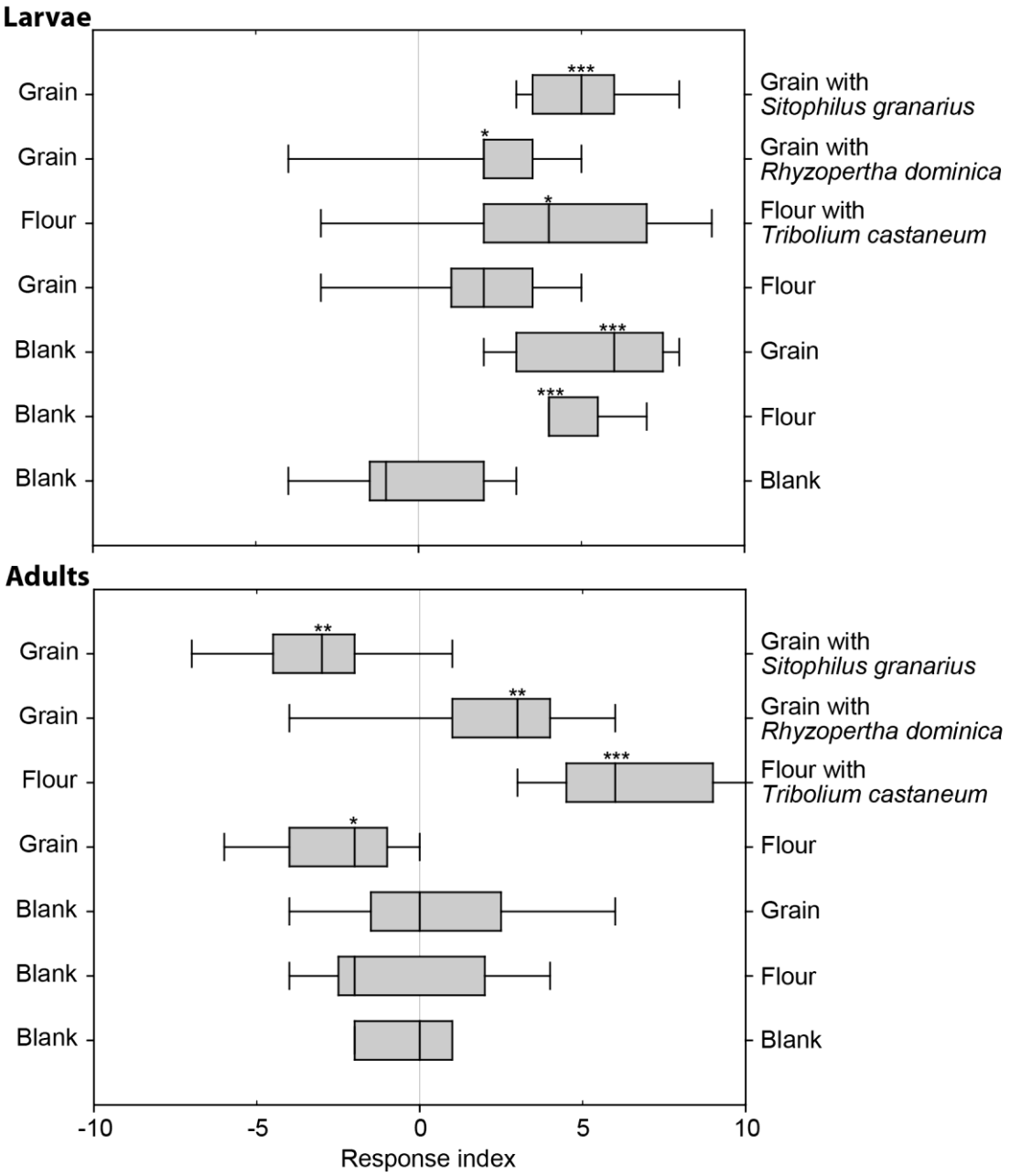

Figure 2. Box and whisker plot of the responses of adult and larval *T. castaneum* in two-choice olfactometer studies. Response index: -10 = all individuals repelled, 0 = no apparent attraction or repulsion, and 10 = all individuals are attracted. The two compounds involved in each two-choice test are shown to the left and right of each plot (blank indicates empty sample bottle). Results are based on nine independent groups of ten individuals with significant differences indicated: * $P < 0.05$,** $P < 0.01$, and *** $P < 0.001$ (Mann-Whitney U-test).

Behaviour towards pure DMD

If one particular life stage, or sex, was capable of sensing DMD than others, it is likely to be an ideal source for a highly sensitive DMD receptor. In using our behavioural experiments we have for the first time compared the sensitivity for DMD among male beetles, female beetles, and late-stage larvae. Females are

attracted to about 100-fold less DMD than larvae, or 200-fold less than males (Figure 3). Males may be less sensitive because, unlike females, they produce DMD. Therefore, it is likely that a female-specific receptor (or a receptor expressed more highly in females) could be the best target for high DMD sensitivity.

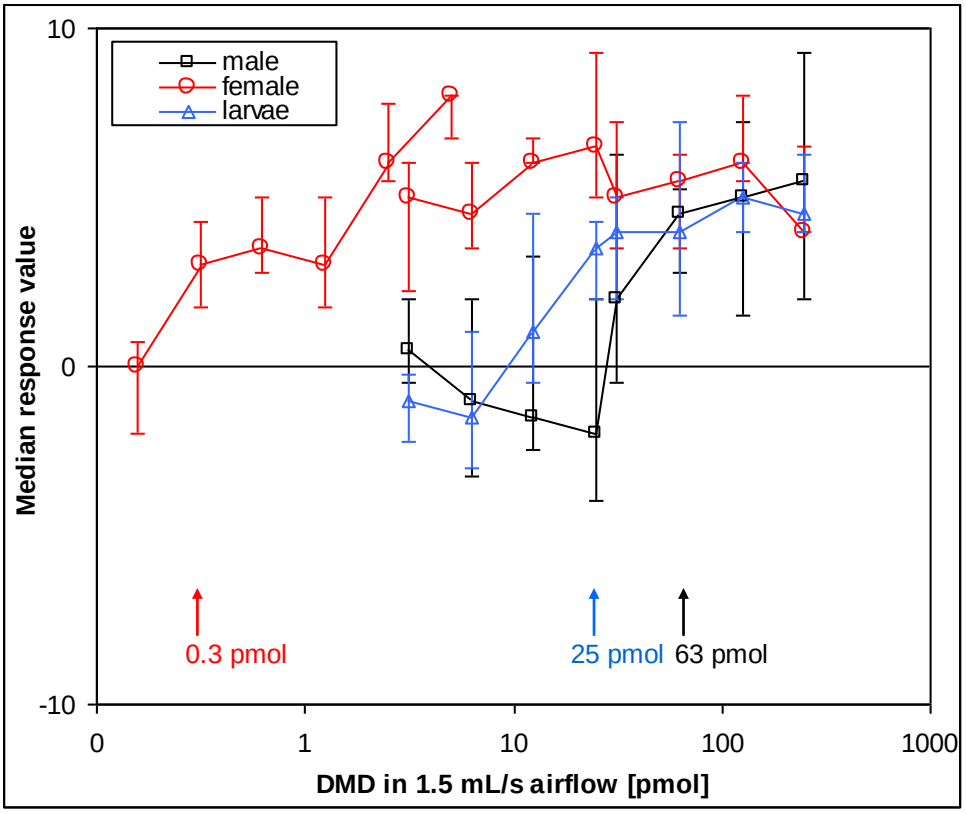

Figure 3. Median response of unmated *T. castaneum* to synthetic DMD in Y-tube olfactometer: females (red circles), males (black squares), or larvae (blue triangles). Response calculated as the number of beetles that crawl towards the DMD minus the number that crawl towards the blank sample (in groups of ten beetles). Error bars indicate the inter-quartile range from 8 replicates. The lowest significantly attractive concentration is indicated for each group.

Electrophysiological recordings from *T. castaneum* antennae

In addition to behavioural studies we have also adapted a method to allow electrophysiological recordings (electroantennograms or EAGs) from adult and larval antennae of *Tribolium* in response to these odours. EAGs have previously been reported for a closely related species, *T. confusum* (Verheggen et al. 2007), and more recently for *T. castaneum* (Deul et al. 2011). However, neither of the methods described in these papers worked with our laboratory equipment and beetle strain. Instead, we used modified methods for the adults and developed a new method for larvae. Our EAG experiments confirm that *T. castaneum* detects all tested chemicals with their antennae (Figure 4) despite not seeing consistent behavioural responses with these chemicals, with the exception of DMD (see

above). For all four volatiles we see electrophysiological responses in both adults and larvae suggesting *T. castaneum* can detect all of these volatiles with its olfactory system and hence would contain receptors responding to these odours.

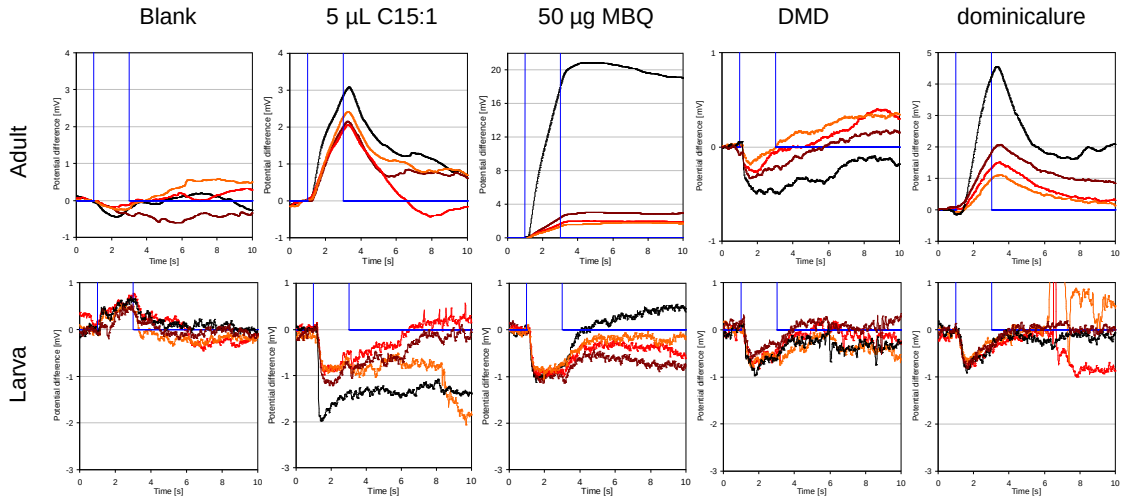

Figure 4. An array of EAG results with *T. castaneum* adults (upper row) or larvae (lower row). EAG recordings were made with exposure to: clean air (blank), 1-pentadecene (C15:1), MBQ, DMD, or Dominicalure. The antennae were exposed to the sample odour for 2 s, 1 s after recording began (indicated by the blue line). Four sequential recordings were made for each test: with the black, brown, red and orange trace relating to the first, second, third and fourth recordings, respectively. The horizontal axes indicate the experiment time (0-10 s) and the vertical axes show the antennal potential difference (mV).

Finding Candidate Odorant Receptors

The *T. castaneum* genome encodes 259 intact odorant receptor proteins (Engsontia et al. 2008). As it is not possible to screen each of these genes for responses to our target volatiles, we initially chose a subset that have previously been shown to be expressed in both adults and larval heads (as both life stages responded to our target volatiles in EAG experiments). This list expanded as we tested receptors with closest sequence similarity to those that showed differences in initial experiments. Previous published studies have only used crude RT-PCR experiments to show the expression of these receptors, based on the presence or absence of a visible amplicon using gel electrophoresis. We applied quantitative RT-PCR (qRT-PCR) techniques, which required substantial work up for appropriate total RNA extractions, cDNA amplification and reference gene selection and optimisation. Using this more sensitive technique, we were able to measure transcripts for receptors that could previously not be detected in the beetle.

Quantitative RT-PCR (qRT-PCR) for putative pheromone receptors of *T. castaneum*

Using sequence information provided by the *Beetlebase* website, primers were designed against 83 odorant receptors of *T. castaneum*. Primers were designed over intron/exon boundaries where possible, and produced an amplified product

between 100-400bp in length. TcRPS6, was used as a reference gene for qRT-PCR experiments which were carried out on a Rotorgene 6000 Series machine with results analysed using the Rotorgene software (Corbett Life Sciences). Receptors involved in pheromone reception often show sex-specific expression bias, so we have compared the expression levels of male and female beetles for 83 receptors. We found three receptors (TcOr7, TcOr3, TcOr84) with relatively higher expression in male beetles, and one expressed more highly in females, TcOr24 (Figure 5); these receptors are key candidates as pheromone receptors of *T. castaneum*.

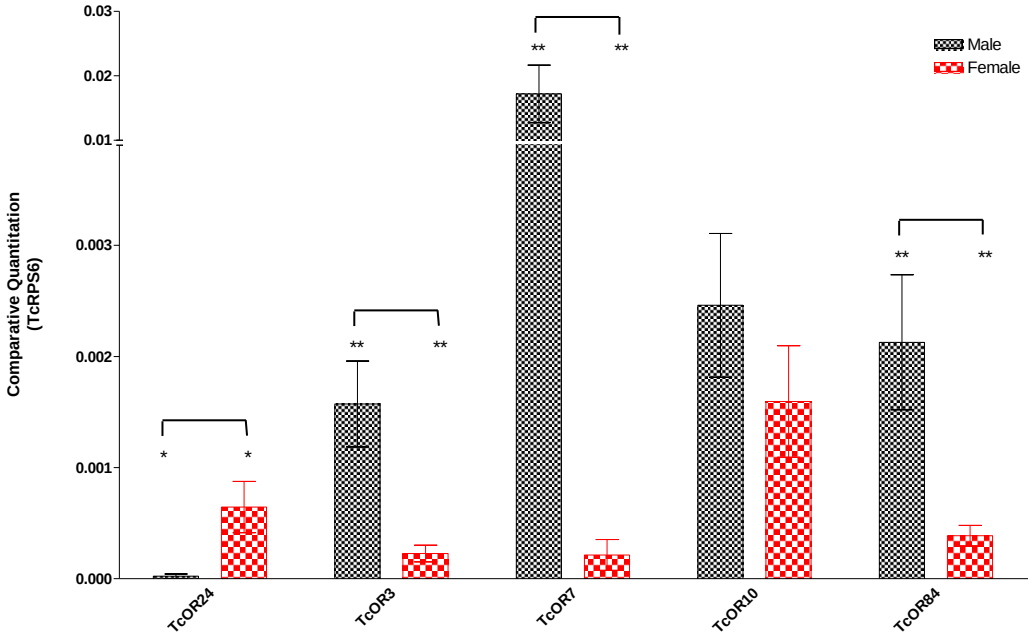

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Figure 5. Comparative quantitation of TcOR24, TcRO3, TcOR7, TcOR10 and TcOR84 gene transcript levels between whole male and female beetles. Total RNA was extracted from beetles and 1ug used in oligo dT primed reverse transcription reactions, and transcript levels investigated using qRT-PCR. Results shown are Mean ± SEM. Experiments were conducted in technical duplicates and results display biological replicates ($n \geq 3$). ** unpaired t-test, $P<0.01$; *unpaired t-test, $P<0.05$

Our experiments with EAG had shown that responses to DMD, but not MBQ, were eliminated if the antennal terminal club was removed. We therefore examined the transcription of these sex-biased ORs in the terminal club compared to the rest of the antenna. OR7 was predominantly transcribed in the terminal club (Figure 6), providing further evidence that OR7 is a candidate DMD receptor.

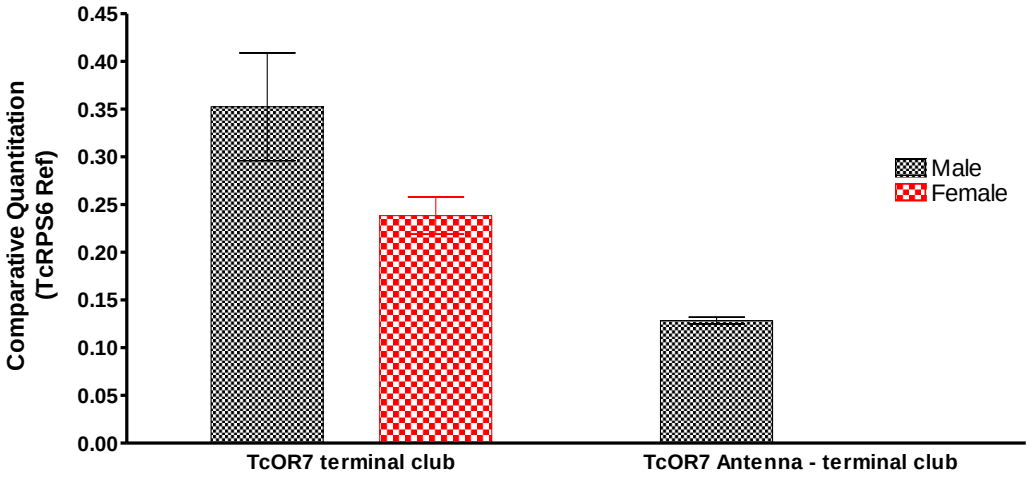

Figure 6. Comparative quantitation of TcOR7 gene transcript levels between the terminal club of the male and female beetles, compared to the rest of the antenna. Quantitative RT-PCR experiments were conducted in technical duplicates. Mean ± SEM (n =2)

During the course of this project we have been privileged to unpublished information from Professor Cobb at the University of Manchester, regarding putative DMD receptors. His group recently found that OR7 and OR10 are expressed in only one sex of the *T. castaneum* adult beetles (using non-quantitative methods). Our results (using qRT-PCR) showed OR7, was expressed more highly in males, but OR10 appeared to be expressed similarly in each sex (Figure 5). Furthermore, the Cobb group attempted unsuccessfully to publish experimental results that implicate OR7 or OR10 in DMD detection: they utilised *T. castaneum* RNA interference (RNAi) in combination with behavioural studies, and expression in *Xenopus* oocytes with patch-clamp analysis.

Characterising candidate ORs - Calcium-imaging assays

The *in vitro* calcium-imaging assay (Kiely et al, 2007) observes individual insect cells as they respond to chemical odours in solution. The cells are transfected to express the OR of interest and when activated, calcium influx into cells occurs, and this is monitored by a Ca^{2+}-dependent fluorescent dye. CSIRO have tested four genes using this system for responses to DMD (aggregation pheromone of *T. castaneum*). The candidate ORs were transiently expressed in *Sf*9 cells before the cells were tested in calcium imaging assays. The challenge in this approach is making enough observations of an infrequent event: a cell that is expressing the OR and responds to DMD. To date, it has not been possible to observe a consistent significant response to DMD through transient expression of a target OR.

To improve the quality of the experimental output, the number of cells expressing the OR needs to be increased. For this purpose, we developed *Sf*9 monoclonal stable cell lines for OR7 or OR10. We have been able to maintain and store these cell lines and have confirmed that the target OR genes are transcribed. However, it has not been possible to observe a consistent significant response to DMD. To

date, the only significant effect from expressing target ORs has been with OR7 stable Sf9 cell lines with transient expression of *T. castaneum* Orco added. These cells allowed successful detection of influx of Ca-ions, but did not respond to DMD. It is possible that these lepidopteran cells are not suitable for our coleopteran ORs.

We examined an alternative approach for improving the proportion of cells expressing a target OR: the baculovirus expression system (Invitrogen). This method may be more effective as at the optimal (early) post-infection time-point, the infection efficiency will be much higher while maintaining cell viability. Results with TcOR10 with a GFP reporter have shown successful high expression of the receptor with minimal cell death 32 hours post infection (Figure 7). To date, it has not been possible to detect a significant response to DMD from cells infected with baculovirus for OR7 or OR10.

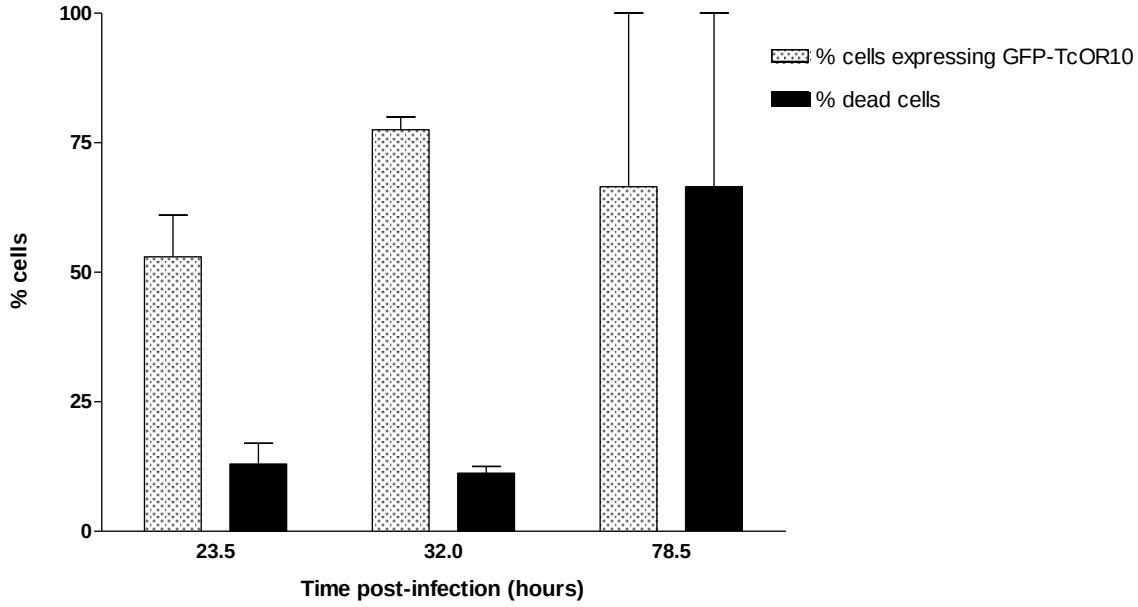

Figure 7. Baculovirus expression time course: 1:100 - budded viruses (His6GFP_TcOR10): 30 million cells in 20 ml. Cells were counted on a haemocytometer.

RNA interference assays

RNAi has been identified as an ideal method for testing whether or not an OR gene is involved in the detection of certain odours. The method has been used by Matthew Cobb's group at the University of Manchester (Engsontia et al. 2008) to demonstrate TcOrco is essential for a behavioural response to DMD. TcOrco is a conserved insect co-receptor that is required to heterodimerise with all other OR proteins to form functional olfactory receptor complexes.

We have replicated this work by preparing double-stranded RNA (dsRNA) for the TcOrco gene or GFP2 (control), injecting pupae and allowing them to mature. The resulting adults were tested in the Y-tube olfactometer to examine if knock down of TcOrco was achieved and changed behavioural responses to DMD. DMD was used as the test odour, since it is the only pure chemical available that can elicit

close to 100% attraction. The results with males showed that TcOrco RNAi consistently prevented attraction to DMD compared to the negative control.

To test whether the RNAi protocol could reduce the level of receptor transcripts, qRT-PCR was used (Figure 8). Total RNA was extracted from approximately one week old male adults (n=3) and qRT-PCR was used to monitor levels of TcOR7 in beetles injected with either dsGFP (as a negative control) or dsTcOR7. Additionally, to test the specificity of the RNAi with respect to othr ORs, expression levels of a different OR were also examined in these beetles. An approximate 40% decrease in the transcript levels of TcOR7 due only to dsTcOR7 was seen in this case, and no decrease in transcription of TcOR54 was detected. We were unable to test the behaviour of these beetles due to time constraints but this could be completed in a follow-up project to replicate the results of our collaborators.

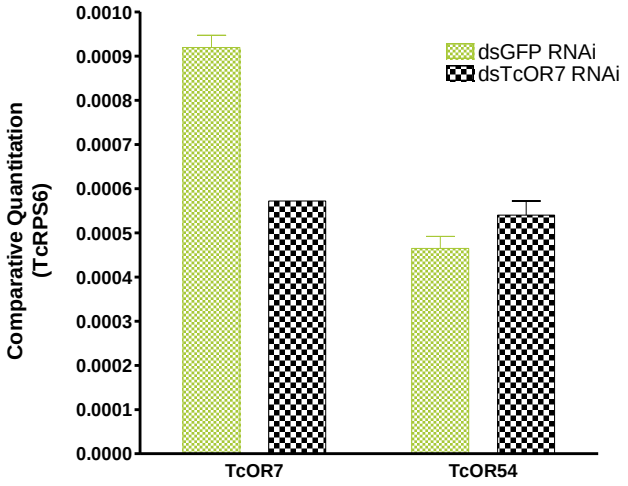

Figure 8. Comparative quantitation of two OR genes, TcOR7 and TcOR54, in male beetles either injected with dsGFP RNA (green bars) or dsTcRO7 RNA (black bars). A decrease in transcript level of TcOR7 was shown only in the dsTcOR7-injected beetles, whereas no change was seen in other OR transcripts, such as TcOR54 shown here. Technical duplicates are shown. Mean ± SEM (n=2).

Investigating *T. castaneum* odorant binding proteins (OBPs)

In addition to targeting the use of membrane-bound receptors as recognition elements for a sensing device, we have acknowledged the potential of associated proteins involved in the olfaction pathway of insects, including the odorant binding proteins (OBPs). In general, OBPs are believed to have a lower specificity and sensitivity (compared to the olfactory receptors) for their volatile ligands, but have some potential advantages in their stability and solubility in a biosensor

platform. In addition, they are known to increase the solubility of the volatile ligands, making the volatile ligands available to their relevant olfactory receptors. Primers were designed for qRT-PCR for six OBPs with assays carried out as above. Some OBPs did not contain introns and therefore optimal primers could not be obtained. Results for OBP10 and OBPC16 (both containing introns) showed similar quantities in both males and females (Figure 9). Results with OBPC3 indicated that not only was there a large amount of this OBP in the beetles, there was also a bias towards transcription in the males over the females. This result indicates this OBPC3 may play a role in pheromone binding. DMD is predominantly released by male beetles and this OBPC3 could play a role in solubilising the volatile compound prior to its release. Therefore, OBPC3 may also have potential as a molecular detectors of DMD.

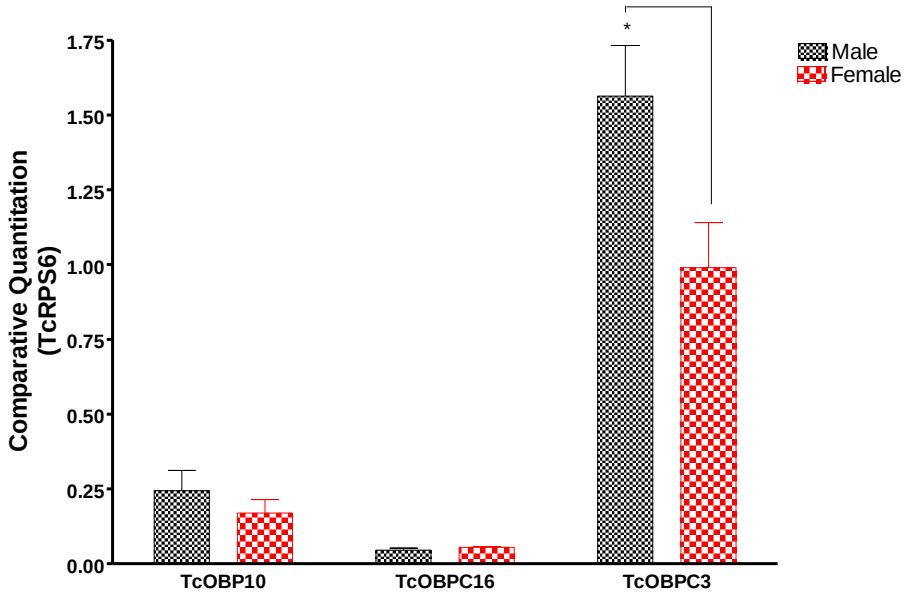

Figure 9. Comparative quantitation of OBP10, C16 and C3 gene transcript levels between whole male and female beetles. Experiments were conducted in technical duplicates.

We have expressed four OBPs, (C17, C16 ,10 and C3) in *E. coli,* and have evidence that OBPC3 might be involved in pheromone binding due to the sex-biased transcription seen using qPCR (Figure 9). We are currently investigating the ability of these OBPs to bind DMD and retain this pheromone in aqueous solution. DMD is hydrophobic and highly volatile: an aqueous solution of DMD in a vial will have a quantity of DMD present in the air above the solution (headspace). The presence of OBPs in the solution, if they are able to bind DMD, will reduce the amount of DMD that becomes volatile and that is able to be detected in the headspace. Further experiments can be designed to determine the specificity and affinity of these OBPs for DMD. This preliminary result with headspace analysis (Figure 10) corroborates the qRT-PCR results and suggests that OBPC3 (and perhaps the other tested OBPs) is a good candidate for DMD

binding. Ultimately, this sort of protein may be useful for developing a detector, for transferring DMD from an air sample to a solution for analysis, or both.

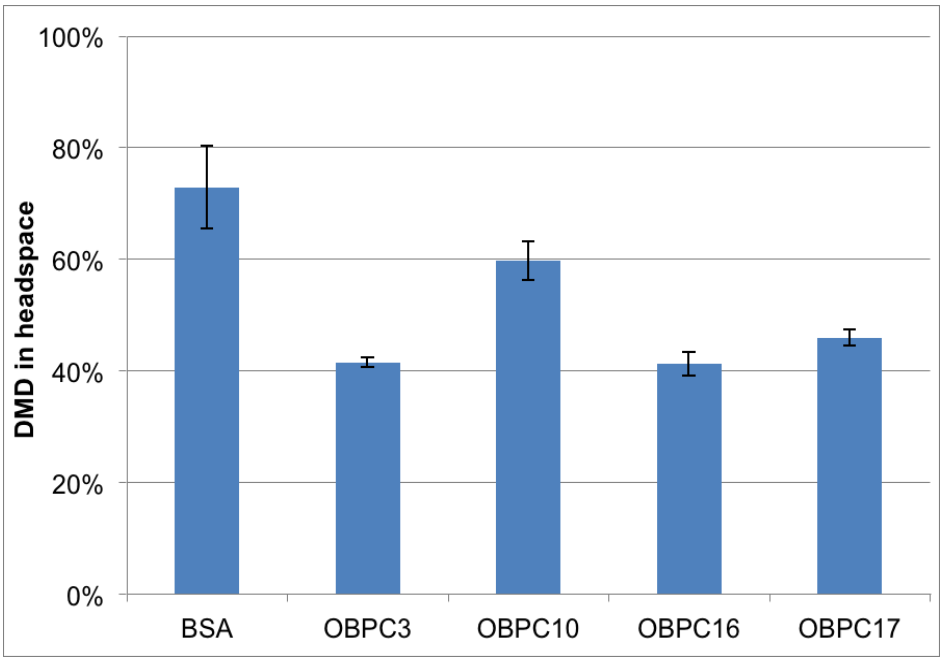

Figure 10. Volatility of 0.1 µM DMD in phosphate buffered saline with OBP protein added to 1 µM. The amount of DMD adsorbed to solid-phase microextraction fibre (PDMS) in the headspace is presented as a proportion of the amount when no protein is present. The mean of two replicates is presented with error bars indicating the individual observations.

Summary

- Adult and larvae *T. castaneum* respond to infested flour more than clean flour.
- Both adults and larva respond behaviourally to DMD with female adults displaying the highest sensitivity to the compound.
- All reported volatiles associated with *T. castaneum* did not show behavioural responses, but nevertheless, produced a physiological response detected by EAG indicating that the beetles have receptors for all these volatiles.
- Male beetles express at least three receptors at higher levels than females and females express at least one receptor more highly than males. These are candidate pheromone receptors.
- As yet we are unable to show a calcium ion response by activating any of these candidate pheromone recepotors expressed in Sf9 cells using either stably transfected or baculovirus infected cells.

- The OBPs we expressed and purified are capable of solubilising (which indicates direct binding to) DMD, and therefore also make good candidates for use in a biosensor platform.

- DMD has been identified as a major component of volatiles produced by grain infested with *T. castaneum* (Seitz & Sauer 1996) and we have shown beetles have a strong behavioural response to this volatile. Therefore, this potential receptor or combination of receptors/ligand pair (perhaps in conjunction with OBPs) would be the primary candidates to focus on for a detection device.

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4. Implications for stakeholders

- The implication of finding putative pheromone receptors of the stored grain pest, *T. castaneum*, means we have candidates for the biorecognition component of a biosensor device.

- The development of a grain pest biosensor would allow greater control and efficiency for grain pest management applications. This would facilitate the maintenance of access to export markets and minimise the level of phosphine-resistance in stored grain pests, which is a key threat to market access.

- Capabilities developed through the course of this project can potentially be applied to other stored grain pests, and indeed, to other applications requiring monitoring of volatiles.

5. Recommendations

This project has identified four volatiles that are associated with the presence of beetles that would be appropriate targets for detection of insects in stored grains. Candidate olfactory receptors that could later be developed into sensors for a biosensor device have been thoroughly examined in the beetle. Through collaborations we have identified the ligand/receptor pair DMD/TcOr7, however we have been unable to produce cell lines in which receptor-related Ca-influx could be detected. Therefore an alternative method to *in-vitro* calcium imaging in *Sf9* may be required for further OR characterisation.

We recommend two parallel efforts in the next phase of this research. The first effort draws on the results of the current research and would concentrate on developing a transduction platform that would transduce a signal from an insect receptor to an electronic device. This project would see us investigate methods of converting the odour-induced change in insect receptors and, for example, could be based on different reporting approaches such as detecting current produced by the ion channel feature of insect ORs or optical/mass changes at a surface due to DMD:OR interactions. Insect receptors would be beneficial in a biosensor as they are likely to be highly specific and highly sensitive to insect derived odours, for this, and many other applications. In addition, this avenue would investigate the binding capacity of the odorant binding proteins as additional/alternative recognition components in the proposed biosensor device.

The second effort would draw on the Cybernose® capability currently available at CSIRO. CSIRO are developing a prototype biosensor for the defence industry and have now built a large library of nematode receptors that can be screened for responses to target infestation odours, such as those identified in this current project. CSIRO has also developed enabling IP in nematode receptor transduction and a detection platform that is the core technology for developing a prototype biosensor specifically for the grains industry.

6. Abbreviations/glossary

ABBREVIATION	FULL TITLE
CRCNPB	Cooperative Research Centre for National Plant Biosecurity
cDNA	Complementary DNA
DMD	4,8 – Dimethyl Decanal
DNA	Deoxyribosenucleic acid
EAG	Electroantennography
GFP	Green Flourescent protein
OBP	Odorant binding protein
OR	Odorant receptor

Orco	Odorant co-receptor
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcription

7. Plain English website summary

CRC project no:	CRC20081
Project title:	Biosensor based detection of grain pests
Project leader:	Alisha Anderson
Project team:	Bradley Stevenson, Kelly Hill and Richard Glatz
Research outcomes:	The experimentally derived information regarding the proteins involved in the perception of the *T. castaneum* produced volatile DMD, will aid in the development of a biosensor platform for the detection of this stored grain insect. The key information uncovered during the course of this project included: • Adult and larvae *T. castaneum* respond to infested flour more than clean flour. • Both adults and larva respond behaviourally to DMD with female adults displaying the highest sensitivity to the compound. • The antennae of *T. castaneum* responded to four beetle odours, including the aggregation pheromones for *Rhyzopertha dominica* and itself, but only its own pheromone caused a significant behavioural response. • Male beetles express at least three receptors at higher levels than females, and females express at least one receptor more highly than males. These are candidate pheromone receptors. • Insect *Sf9* cells are not suitable for the expression and characterisation of *T. castaneum* olfactory receptors using calcium influx assays. • The OBPs we expressed and purified are capable of solubilising (which indicates direct binding to) DMD, and therefore also make good candidates for use in a biosensor platform.

Research implications:	• The implication of finding putative pheromone receptors of the stored grain pest, *T. castaneum*, means we have candidates for the biorecognition component of a biosensor device. • The development of a grain pest biosensor would allow greater control and efficiency for grain pest management applications. This would facilitate the maintenance of access to export markets and minimise the level of phosphine-resistance in stored grain pests, which is a key threat to market access. • Capabilities developed through the course of this project can potentially be applied to other stored grain pests, and indeed, to other applications requiring monitoring of volatiles.
Research publications:	Richard Glatz, Kelly Bailey-Hill. (2011) Mimicking nature's noses: from receptor deorphaning to olfactory biosensing. *Progress in Neurobiology*. 93(2): 270-96. Kelly Bailey. (2011) Making Sense of it all: A review of olfactory biosensing. In *Nanotechnology in Australia: Showcase of Early Career Research*. Pan Stanford Publishing Pty Ltd. Editors - Deborah Kane, Adam Micolich, and James Rabeau. Chapter 12, 375- 408. Kelly Hill, Bradley Stevenson, Alisha Anderson, Sylwek Chyb and Richard Glatz (2011). Sex-biased transcription of olfactory receptors of the beetle *Tribolium castaneum.* Annual Scientific Meeting of the Australasian Association for Chemosensory Science. Matakana, New Zealand. Oral presentation. Bradley Stevenson, Kelly Hill, Cécile Faucher, Lijun Cai, Alisha Anderson, Richard V. Glatz, and Sylwek Chyb (2011). Sniffing out grain infestations with the Red Flour Beetle. Sixth International Symposium on Molecular Insect Science, Amsterdam. Poster presentation Kelly Bailey-Hill, Bradley Stevenson, Alisha Anderson, Sylwek Chyb and Richard Glatz (2011). Investigating the receptors involved in chemical communication for the pest beetle *Tribolium castaneum. CRCNPB Science Exchange*, Barossa Valley, South Australia. Poster presentation. Winner of Board of Directors Poster Prize. Bradley Stevenson, Kelly Bailey-Hill, Alisha Anderson, Sylwek Chyb and Richard Glatz (2011). Sniffing out grain infestations with the red flour beetle. *CRCNPB Science Exchange*, Barossa Valley, South Australia. Oral presentation. Kelly Bailey, Bradley Stevenson, Richard Glatz and Sylwek Chyb (2010). A beetle biosensor. *Proceedings of the*

	Biosensor world congress. Glasgow, Scotland. Poster presentation. Sylwek Chyb, Kelly Bailey and Richard Glatz. (2009) Developing new tools to detect the troublesome Tribolium. *CRCNPB Science Exchange*. Sunshine Coast, Queensland. Sex-specific quantitation of odourant receptors of *Tribolium castaneum* - Kelly Hill, Bradley Stevenson, Alisha Anderson, Sylwek Chyb and Richard Glatz. *In preparation* Behaviour of adult or larvae *Tribolium castaneum* to odours of infested wheat. Bradley Stevenson, Lijun Cai, Cécile Faucher, Alisha Anderson, Sylwek Chyb. *In preparation.*
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