The attraction of the red flour beetle *Tribolium castaneum* to common environmental volatile compounds and its application to the management of stored product pests

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The red flour beetle *Tribolium castaneum* is an economically significant pest of stored products. New insights into the olfactory responses of this species could lead to the development of more effective lures to monitor this species and the development of new pest management strategies.

A large scale electroantennography (EAG) screen, testing the responses of *T. castaneum* antennae to 66 wheat germ oil and fungal associated volatile organic compounds (VOCs), revealed that *T. castaneum* respond to a wide range of compounds from these sources. When the individual VOCs that gave the strongest EAG responses (and blends of these compounds) were tested using behavioural bioassays, several of them were found to be significantly attractive to *T. castaneum*. This suggests that VOCs from these sources could be used by *T. castaneum* to locate stored products. These novel attractive VOCs could be used to improve the effectiveness of pest management lures for use against *T. castaneum* and other stored product insects.

The responses of *T. castaneum* to these VOCs were tested in two strains, an established laboratory strain and a recently caught wild population, and the responses of the two strains to the VOCs tested were found to be highly similar. This suggests that laboratory adaptation may not significantly affect the responses of *T. castaneum* to environmental VOCs.

In the course of conducting these experiments different bioassays for testing the attractiveness of odour sources to *T. castaneum* were tested and the Y-tube olfactometer bioassay was determined to be the most effective.

The bioassays used in this thesis also have potential as methods of deorphaning *T. castaneum* olfactory receptors when used alongside RNAi knockdowns of olfactory genes. This methodology was validated by knocking down the *T. castaneum* Orco orthologue gene (*Tc-or1*) and demonstrating that the resulting behavioural changes could be detected by the behavioural bioassays.

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Introduction

1.1 Introduction overview

Stored product insects are economically significant pests of a wide variety of food products, including globally important commodities such as cereal grains. Integrated pest management strategies are currently employed to control stored product insects, a key component of which is detection and monitoring of the insect pests. Traps and lures have been developed for this purpose, but there are several issues that can limit their effectiveness. This thesis aims to deepen our understanding of the olfactory mechanisms that mediate attraction to stored products in an important insect pest - the red flour beetle *Tribolium castaneum* - and to identify novel attractant compounds that could be incorporated into an improved lure for this species.

This introduction begins by explaining how stored product insects can lead to significant agricultural losses and describes some of the most important pest species. An overview of the current practices used in stored product insect pest management is then given, highlighting the importance of efficient insect monitoring in modern pest management strategies such as integrated pest management (IPM). This section also discusses the limitations of the lures currently used to monitor stored product insects and highlights the potential of foodstuff-associated volatile organic compounds (VOCs) as a source of novel attractant compounds that could improve the efficacy of these lures. The introduction explains why *T. castaneum*, as both one of the most important stored product insects and an extensively studied model laboratory system, is an ideal model system for undertaking this research. The introduction then summarises what is currently known about the attraction of *T. castaneum* to foodstuff-associated VOCs, the function of its olfactory system and the relevance of this in terms of developing improved lures to manage this pest species. The introduction also describes some of the techniques that have previously been used to study olfactory responses in *T. castaneum* and other insect species. It also explains how these techniques could be used to identify novel attractant compounds for use in lures and further develop our understanding of olfaction in this species. The introduction also briefly

discusses what is currently known about the mechanisms underpinning odour perception and olfactory-mediated behaviour in stored product insects and suggests how further research in this area could lead to more effective pest management. The possibility of laboratory adaptation and strain variation in the olfactory responses of *T. castaneum* and other stored product insects, and its relevance for pest management research are also discussed. Finally, the research aims outline the specific hypotheses addressed in this thesis, and the thesis overview summarises the contents of the remaining thesis chapters.

1.2 Stored product insects

Different animal species can feed on, and become pests of, crops and other human foodstuffs. Phytophagous insects in particular are considered to be the most significant biotic factor contributing to losses in grains (Kumar and Kalita, 2017) and are a significant contributor to global crop losses (Oerke, 2005). Indeed, it has been suggested that as much as one fifth of the world's total crop production is destroyed by herbivorous insects annually (Sallam, 1999). Certain important crops, such as wheat, can be especially susceptible to insect damage, with potential global losses for wheat estimated to be as high as 50% without physical, biological or chemical crop protection (Oerke, 2005). Damage to crops by insects and other pests can be separated into preharvest loss (damage that occurs while the crop is still in the field) and postharvest loss (damage that occurs in the rest of the supply chain, which includes farm storage, mills, food processors, distribution warehouses and retail stores) (Hagstrum and Flinn, 2014).

Insects that have adapted to infest stored products are referred to as stored product insects, and they are a significant contributor to postharvest losses, with damage by insects, mites and microorganisms accounting for an estimated global annual postharvest loss of 10-15% (Neethirajan et al., 2007). Losses due to just stored product insects can be as high as 20% in developing countries (Phillips and Throne, 2010), with the more severe rate of post-harvest loss attributed to factors such as the poor implementation of pest management technologies due to a lack of education (Kitinoja et al., 2011; Kumar and Kalita, 2017) and traditional storage structures made from local materials lacking adequate insect pest protection (Sallam, 1999; Kumar and

Kalita, 2017). Global losses due to insects are likely to worsen in the future due to climate change increasing insect metabolic growth rates (and therefore food consumption) (Dillon et al., 2010) and population growth rates (in temperate regions) (Deutsch et al., 2018). For rice, maize and wheat, losses due to insects are predicted to increase by 10%-25% per degree of global mean surface warming (Deutsch et al., 2018).

In addition to directly damaging food products through feeding, stored product insects also produce debris such as body fragments and faeces that can act as allergens to humans (Hubert et al., 2018) with exposure to infested grains being associated with a variety of different allergy symptoms including respiratory problems (Blainey et al., 1988). Many stored product insects also secrete chemicals into the food products they infest that can be carcinogenic or that impart an odour that makes the food unpalatable (Hubert et al., 2018). Of these toxic compounds, quinones are the most widely produced by insects (Weatherston, 1967) and are known to cause allergies and dermatitis in humans (Hubert et al., 2018). Quinone contaminated food sources have been demonstrated to be carcinogenic to animals in laboratory experiments (Lis et al., 2011). However, it is unclear if, even in heavily infested stored products, quinones can accumulate to such a degree to become a serious health hazard to humans (Hodges et al., 1996; Lis et al., 2011). Stored product insect infestations can also contribute to fungal infestations as the metabolic heat produced by feeding insects can raise the temperature of stored products, promoting fungal growth (Dunkel, 1988; Sallam, 1999). Stored product insects can also act as vectors of disease for stored grains by carrying pathogenic fungal spores and microbes on their bodies (Miller, 1995; Kumari et al., 2011; Yun et al., 2018). They can also carry mycotoxin producing fungi that can contaminate stored products and make them harmful to humans (Dunkel, 1988; Yun et al., 2018; Hubert et al., 2018).

Stored product insects can be categorised as primary (internal feeding) or secondary (external feeding) stored product insects (Charlton et al., 2009; Phillips and Throne, 2010). Primary stored product insects are capable of feeding on intact grains and their larvae develop inside grain kernels, whereas secondary stored product insects are incapable of feeding on undamaged kernels (primarily feeding instead on broken or rotten kernels, grain dust or

milled grain products) and their larvae develop outside of sound grain kernels (Phillips and Throne, 2010). Some of the most significant stored product insects, and whether they are primary or secondary pests, are listed in Table 1.1. Stored product insects consist primarily of species from the Coleoptera (beetles and weevils) and Lepidoptera (moths and butterflies) orders (Charlton et al., 2009). The order Coleoptera contains some of the most common and economically damaging stored product insects. Coleopteran pests can be particularly damaging owing to the wide variety of habitats that these species can infest, and because both larval and adult forms feed on stored products, unlike the Lepidoptera, where only larvae feed on stored products (Sallam, 1999).

Table 1.1 List of common stored product insects and whether they are classified as primary or secondary pests.

Table adapted from (Herrman, 1998).

Common name	**Scientific name**	**Order**	**Pest status**
Maize weevil	*Sitophilus zeamais*	Coleoptera	Primary
Rice weevil	*Sitophilus oryzae*	Coleoptera	Primary
Lesser grain borer	*Rhyzopertha dominica*	Coleoptera	Primary
Red flour beetle	*Tribolium castaneum*	Coleoptera	Secondary
Confused flour beetle	*Tribolium confusum*	Coleoptera	Secondary
Sawtoothed grain beetle	*Oryzaephilus surinamensis*	Coleoptera	Secondary
Khapra beetle	*Trogoderma granarium*	Coleoptera	Secondary
Rusty grain beetle	*Cryptolestes ferrugineus*	Coleoptera	Secondary
Angoumois grain moth	*Sitotroga cerealella*	Lepidoptera	Primary
Almond moth	*Cadra cautella*	Lepidoptera	Secondary
Indian-meal moth	*Plodia interpunctella*	Lepidoptera	Secondary
Mediterranean flour moth	*Ephestia kuehniella*	Lepidoptera	Secondary
Raisin moth	*Cadra figulilella*	Lepidoptera	Secondary

1.3. Current management strategies for stored product insects

As stored product insects can cause severe damage to stored commodities, efficient pest management practices are of great importance to the industry. Stored product insect management has a long history, with evidence of management strategies such as hermetic sealing of product jars and fumigation via burning sulphur being used in ancient Greece (Panagiotakopulu et al., 1995). Since then a variety of different management technologies have been developed with chemical control methods, including fumigation, becoming the main method of pest management in the 20th century (Hagstrum and Phillips, 2017). However, regulatory pressures, along with safety concerns and the increasing prevalence of pesticide resistance in stored product insects, has driven the use of alternative management strategies (Phillips and Throne, 2010; Hagstrum and Phillips, 2017). Modern stored product pest management strategies can involve the use of a variety of different chemical, physical and biological control measures to protect stored products against infestations and kill insects within stored product facilities alongside the use of traps and lures to monitor insect levels inside stored product facilities (Alder, 2010; Upadhyay and Ahmand, 2011; Hagstrum and Flinn, 2014). Integrated pest management (IPM) is currently promoted as a more economical method of pest control than the use of traditional chemical control techniques (Herrman, 1998). Although the concept of IPM has existed since the 1970s a variety of different definitions of this term can be found in the literature (Kogan, 1998). A consensus definition suggested by the same publication defines IPM as “a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment” (Kogan, 1998). A key aspect of this strategy is the use of decision thresholds to determine what action to take against a given insect species at different insect densities and detailed decision thresholds exist for the most common stored products pests (Hagstrum and Flinn, 2014). However, the effectiveness of this technique is reliant upon efficient

monitoring strategies and the ability to accurately estimate insect numbers within stored products.

One important method of protecting against stored product insect infestations is by ensuring food storage and processing facilities, and any equipment within, are thoroughly cleaned between shipments and that any residual food products are removed to reduce insect populations and remove residual food sources (Herrman, 1998; Phillips and Throne, 2010; Hagstrum and Flinn, 2014). Indeed, proper sanitation and the efficient removal of food debris has been described as the “primary preventative method(s) of stored product IPM” (Hagstrum and Phillips, 2017) and the “first line of defence” against insects in farms, grain elevators and warehouse facilities (Phillips and Throne, 2010). Poor sanitation in stored product facilities can also reduce the effectiveness of *T. castaneum* pest monitoring and control strategies. Food accumulation on surfaces has been shown to reduce the efficiency of insect monitoring traps baited with pheromone or kairomone lures (Campbell, 2013; Morrison et al., 2019). Residual food-sources can also act as an additional barrier preventing contact with chemical agents and provide a refugia from which insects can re-infest stored commodities (Morrison et al., 2019). The use of insect resistant packaging for stored products is another management strategy designed to prevent stored product insects from infesting stored products (Alder, 2010; Hagstrum and Flinn, 2014). However, there are numerous examples of insect pests being found inside packaged commodities (Athanassiou et al., 2011) and important stored product insects such as the lesser grain borer *Rhyzopertha dominica*, the rice weevil *Sitophilus oryzae*, the cigarette beetle *Lasioderma serricorne* (Riudavets et al., 2007) and *T. castaneum* (Hassan et al., 2016) can damage plastics commonly used for stored product packaging.

Once insects are detected in stored product facilities a variety of chemical, physical and biological pest control methods are available. Although there is pressure to reduce insecticide usage in the industry (Cox, 2004) contact pesticides and fumigants (gaseous pesticides) are still commonly used (Hagstrum and Phillips, 2017). Recent regulations restricting the use of many common fumigants, including the outright banning of the use of the ozone depleting methyl bromide, has promoted research into alternative physical, biological and chemical control methods (Bell, 2000; Fields and White, 2002).

Phosphine is currently the dominant fumigant worldwide (Hagstrum and Phillips, 2017). However, storage facilities need to be well sealed and heated to a sufficient temperature to allow the pesticide to vaporise for this type of fumigation to be effective (Upadhyay and Ahmand, 2011). There is also evidence of widespread resistance to phosphine and other pesticides in several important stored product insects in many different countries (Talukder, 2009). Contact pesticides, that are applied directly to surfaces and kill insects on contact are also used within stored product facilities (Upadhyay and Ahmand, 2011; Hagstrum and Phillips, 2017). However their usage is limited as they cannot be applied directly to processed foods or to surfaces that come into contact with them (Hagstrum and Flinn, 2014).

Physical control methods, which alter the environment to control stored product insects, are another common method of management. Temperature is a major method of physical control that can be used to manage stored product insects as it has been shown that lowering the temperature of an environment below 13°C or raising it above 35°C severely reduces the rate of development of most insects and leads to eventual death. Temperatures below -20°C and above 55°C can lead to death within minutes (Fields, 1992). Methods of cooling, such as cold air aeration, or freezing are not typically used for stored grains, owing to their high cost (Phillips and Throne, 2010). Heating to extreme temperatures has become more widely used in stored product facilities after the banning of the fumigant methyl bromide (Phillips and Throne, 2010; Hagstrum and Phillips, 2017), although there can be issues with ensuring that a uniform fatal temperature is reached across entire facilities (Phillips and Throne, 2010). The atmosphere within stored product facilities can also be modified by increasing carbon dioxide (CO_2) or reducing oxygen (O_2) concentrations to lethal levels. These methods can be very effective for treating stored product insects in cereal grains but rely on having airtight storage which would require the construction of facilities in many warehouses (Phillips and Throne, 2010). Entoleters, machines that use centrifugal forces to destroy insects, are also used in the milling industry, although they can cause damage to whole grain products (Phillips and Throne, 2010). Although physical control methods are less ecologically

harmful than chemical control methods they are often prohibitively expensive due to the energy cost (Alder, 2010).

There are also biological means for controlling stored product insects which involve the use of natural insect predators or parasitoids; or entomopathogenic fungi and nematodes (Herrman, 1998). There are examples of natural predators being used to successfully manage stored product insects, for example parasitic wasps have been used to manage stored product beetles within wheat storage bins (Flinn et al., 1996). However, this practice is not yet common in the industry, although there is active research into the effectiveness and economic efficiency of this management strategy (Schöller, 2010). Entomopathogenic fungi have been demonstrated to be capable of controlling a variety of stored product insect in laboratory experiments but there are issues with commercialisation of this technology (Rumbos and Athanassiou, 2017).

1.4. Stored product insect monitoring

Pest monitoring is another important component of modern stored product insect management, and a vital component of the decision thresholds used in integrated pest management (Wakefield, 2006; Neethirajan et al., 2007; Hagstrum and Flinn, 2014). Stored product insect monitoring has several purposes, including: detecting the presence of pest insects, estimating the pest densities at a particular site and determining the efficacy of different management strategies by comparing insect numbers before and after treatments (Trematerra et al., 2000; Heuskin et al., 2011). Effective monitoring can result in early pest detection, which reduces the damage that stored product insects can cause by allowing early intervention when infestations occur (Banga et al., 2018).

Stored product insect monitoring can include methods as simple as visually inspecting materials entering a facility or using traps and sampling probes to passively capture insects inside grains and other stored products. However these methods are time consuming, labour intensive and the results can be difficult to interpret as the capture rates of these traps and probes can be variable (Neethirajan et al., 2007; Banga et al., 2018). Typical traps used in

the stored product industry include pitfall traps, designed to capture walking insects inside cereal grain storage bins, and sticky traps, to capture flying insects in storage facilities (Phillips, 1997; Hagstrum and Flinn, 2014). Lures containing attractive semiochemicals are commonly used to improve the capture rates of traps for stored product insects, with synthetic versions of insect pheromones being the most common attractants used. Both sex pheromones (compounds typically produced by females to attract males for mating) and aggregation pheromones (pheromones produced by one sex to attract both males and females in order to exploit a specific resource such as food or an appropriate mating site), have been used as stored product insect lures (Heuskin et al., 2011). Lures containing pheromones in slow release formulations are available for around 30 of the most common stored product insects (Swords and Van Ryckeghem, 2010a; Swords and Van Ryckeghem, 2010b), and pheromone traps are commonly used as an “easy, efficient, and extremely sensitive way to detect insects in storage facilities” (Phillips, 1997). Pheromone traps can be effective at providing an early warning of pests as they can generally detect very low pest densities (Trematerra, 2012). However, as pheromones are, by their very nature, species specific, multiple different pheromones can only be combined in a multispecies lure if each component can be shown to not repel other insect species (Trematerra, 2012). For example, there is a commercial multi-species commercial lure containing the pheromones of the Indian meal moth *Plodia interpunctella*, the Mediterranean flour moth *Ephestia kuehniella*, the almond moth *E. cautella*, *Lasioderma serricorne*, the warehouse beetle *Trogoderma variabile*, the biscuit beetle *Trogoderma granarium*, *T. castaneum* and the confused flour beetle, *T. confusum* (Ven Ryckeghem et al., 1999). This is important as storage environments are often inhabited by different pest species and multi-species lures are likely to be more cost effective than multiple species specific ones (Trematerra, 2012). Additional semiochemicals, such as minor pheromone components or semiochemicals from other sources can also be added to pheromone lures to increase their attractiveness (Phillips, 1997). Food-based volatiles are sometimes used to enhance the attractiveness of pheromone lures (Cox, 2004), with grain derived oils often used in stored product insect traps as both a synergistic/additive attractant and as a trapping

medium for insects to fall into (Phillips and Throne, 2010). The addition of blends of food-based kairomones has also been demonstrated to increase the attractiveness of pheromone lures in important stored product insects such as *T. castaneum* and *S. oryzae* (Phillips et al., 1993; Phillips, 1997; Cox, 2004).

Contact pesticides can also be used in combination with attractive lures in an attract-and-kill or attracticide technique, where lures are used to attract pests towards an insecticide source (Savoldelli and Trematerra, 2011; Gregg et al., 2018). This attract-and-kill method has been tested in flour mills to help control a variety of different stored product insects (Savoldelli and Trematerra, 2011; Trematerra, 2012). Using insecticides and attractants together in this way allows for more targeted pesticide usage, reducing the amount of pesticide used overall, resulting in environmental and economic benefits (Cox, 2004; Trematerra, 2012).

Despite their widespread use in the industry, pheromone-based lures also have their limitations. The responses of stored product insects to pheromones or pheromone containing lures specifically have been shown to be influenced by biotic factors such as: insect age (Walgenbach and Burkholder, 1986; Obeng-Ofori and Coaker, 1990; Boughton and Fadamiro, 1996; Duehl et al., 2011), mating status (Walgenbach et al., 1983; Fedina and Lewis, 2007), strain (Rahalkar et al., 1985; Hawkin et al., 2011; Gerken et al., 2018), sex (Walgenbach and Burkholder, 1986; White, 1989), population density (Pierce et al., 1983; Walgenbach and Burkholder, 1986; Athanassiou et al., 2006), previous pheromone exposure (Obeng-Ofori and Coaker, 1990) and the presence of other species (Athanassiou et al., 2006). Environmental factors such as: the time of day (Obeng-Ofori and Coaker, 1990), landscape patterns (Campbell, 2013) airflow (Campbell, 2012), trap placement (Semeao et al., 2011), nutritional environment (Fedina and Lewis, 2007), food availability (Toews et al., 2005) and trap design (Phillips, 1997) can also affect the responses of stored product pest to pheromones. There are also reports from users in the food and pest management industry that the responses of important stored product insects, such as *T. castaneum*, to pheromone baited traps is limited (Campbell, 2012). Certain insect pheromones can also be difficult to synthesise on a cost-effective basis (Wakefield, 2006). The starting materials for synthesis reactions are often expensive, the reactions involved

are often complex, and intermediate reactions can produce unstable products, prohibiting the production of certain insect pheromones (Swords and Van Ryckeghem, 2010a).

Other methods of detecting stored product insects are also being developed including: E-nose odour sensors, X-ray imaging, thermal imaging and acoustic detection. However, these methods currently require the purchasing of expensive equipment that can require highly skilled workers or specially adapted facilities, limiting their usefulness (Banga et al., 2018).

As insect monitoring is such an essential component of modern integrated pest management practices any improvements in the effectiveness of detecting insects could significantly improve the ability to manage stored product insects. Improving attraction of stored product insects to traps will greatly increase the efficacy of monitoring by allowing insects to be detected at lower population densities, thus allowing pest control procedures to begin earlier, reducing the damage that insects can cause (Banga et al., 2018). It will also increase the effectiveness of stored product pest management practices generally, as more accurate monitoring will allow food producers to make more informed decisions about the effectiveness of the different strategies they employ. In particular, decision thresholds used to determine when to undertake specific management strategies can only be effective with accurate information about insect numbers in storage environments. If the traps used to monitor insects are not sufficiently attractive, pest insect populations could be underestimated leading to delays in starting appropriate treatments. As such, improving lure design will have a variety of pest management benefits.

1.5. Using semiochemicals to improve stored product insect lures

Due to the limitations associated with the use of pheromones discussed above, other semiochemicals have been proposed as alternative lures for stored product insects and other insects pests (Cox, 2004; Wakefield, 2006; Epsky et al., 2014; Nebapure and Chowdappa, 2015; Gregg et al., 2018). Semiochemicals produced by other organisms can provide insects with

important information that can be used to locate food sources or host-plants, assess food quality, and identify oviposition sites (Davis et al., 2013; Reinecke and Hilker, 2014). As a result many volatile organic compounds (VOCs) produced by plants (Bruce et al., 2005; Bruce and Pickett, 2011; Reinecke and Hilker, 2014; Carrasco et al., 2015) and microbes, such as bacteria, yeasts and fungi (Davis et al., 2013; Davis and Landolt, 2013), have been demonstrated to be attractive to a variety of different insect species.

For stored product pests the headspace volatiles of stored grains and grain-associated microorganism are a potential source of attractive semiochemicals. However, identifying which VOCs from these sources act as the attractive semiochemicals can be difficult as plant and fungal volatile emissions are complex mixtures, frequently comprising several hundred compounds (Bruce et al., 2005). It has been demonstrated in plants that only a few VOCs act as kairomones for insects, and that the attractive components may only be a minor component of the total volatile profile (Bruce and Pickett, 2011). Research into the responses of insects to plant derived VOCs has suggested that rather than responding to certain species specific compounds that a target plant produces, insects typically respond to the particular blend or ratio of ubiquitously produced volatiles that a specific plant produces (Bruce et al., 2005; Carrasco et al., 2015). However, there are examples of species with very specific plant hosts that respond strongly to taxonomically characteristic host compounds (Bruce et al., 2005). If the particular compounds from these sources that attract insects can be identified it may be possible to combine some of these volatiles into a novel blend that is more attractive to insects than naturally occurring plant blends, a process referred to as super-blending (Socorro et al., 2010; Gregg et al., 2018). Experiments have shown that many insects are more attracted to plant volatiles as blends than they are to individual volatiles (Visser, 1986; Bruce and Pickett, 2011), with the most attractive artificial blends of plant compounds typically containing four compounds, with additional compounds tending not to increase the attractiveness of blends further (Szendrei and Rodriguez-saona, 2010). The responses of insects to naturally occurring microbial blends are less well studied, but it is likely that they respond to these complex cues in a

similar way, with blend composition being more informative than individual volatiles (Davis et al., 2013).

Although food-associated volatiles have been shown to work synergistically with pheromones to increase their attractiveness to a variety of different insects (Gregg et al., 2018), including *T. castaneum* (Phillips et al., 1993), the use of food and plant derived semiochemicals is not currently a major component of stored product insect management. However, volatiles from foodstuffs (Cox, 2004), or plant and microbial volatiles, have been suggested as alternative lures for agricultural insect pests (Gregg et al., 2018). It has been suggested that VOCs from cereal grains and other foodstuffs could be used to improve the management of stored product insects specifically, either as an additional attractant for use with pheromone lures (Phillips, 1997; Cox and Collins, 2002; Cox, 2004; Trematerra, 2012) or on their own as an alternative to pheromones (Wakefield, 2006; Collins et al., 2007).

Plant VOCs have been used in attract-and-kill management strategies in a variety of different insect species (Gregg et al., 2018). Several food-based lures are already commercially available and have been demonstrated to be effective at managing fruit fly pests (Epsky et al., 2014; Biasazin et al., 2018; Lasa and Cruz, 2019) and lures using synthetic plant volatiles are being developed for managing pest noctuid moth species (Socorro et al., 2010). Specific compounds from cereals have been demonstrated to elicit significant electroantennography (EAG) or behavioural responses from a variety of stored product insects including: *T. castaneum* (Balakrishnan et al., 2017), *T. confusum* (Wenda-Piesik et al., 2017), the sawtoothed grain beetle *Oryzaephilus surinamensis* (Mikolajczak et al., 1984; Pierce et al., 1990; Collins et al., 2007), the merchant grain beetle *O. mercator* (Pierce et al., 1990), the rusty grain beetle *Cryptolestes ferrugineus* (Collins et al., 2007), the foreign grain beetle *Ahasverus advena* (Wakefield et al., 2005), the grain weevil *Sitophilus granarius* (Collins et al., 2007; Germinara et al., 2008; Piesik and Wenda-Piesik, 2015) and *S. oryzae* (Phillips et al., 1993).

Many insect species specifically use microbial volatiles to locate food sources such as decomposing plant material, fruits, nectar and animal tissues (Davis et al., 2013). In *D. melanogaster* (which uses overripe fermenting fruit for

feeding, mating and oviposition) yeast odours alone are enough to elicit attraction and yeast volatiles are more attractive than fruit volatiles on their own (Becher et al., 2012). Attraction to microbial VOCs can also be found in insects that do not feed on fungi or yeasts, but feed on sources that can be located through their close association with these organisms e.g. mosquitos responding to microflora associated with human skin (Takken and Knols, 1999; Carey et al., 2010). Indeed, insect attraction to fungal volatiles appears to be common to multiple insect taxa with field trials using volatiles from a ubiquitous fungal species attracting a diverse variety of insects (Davis and Landolt, 2013). In particular the common fungal volatile 1-octen-3-ol has been shown to be broadly attractive to hematophagous insects as a signal of fungal/mould infestations at potential oviposition sites (de Bruyne and Baker, 2008).

Despite fungi not being a primary food source for stored product insects, it is possible that volatiles produced by grain-associated fungi could be used by these insects to locate stored grains and other foodstuffs. Fungal odours may be especially important for secondary stored product pests (that are incapable of feeding on intact grains), as fungal volatiles could indicate that grains are in a condition that the insect can infest and utilise. It has even been theorised that certain stored product pests, such as *T. castaneum*, may have evolved primarily to respond to fungal volatiles rather than the volatiles of cereal grains (Ahmad, Daglish, et al., 2012). This can be explained by considering the evolutionary history of stored product insects. For example: beetles in the *Tenebrionidae* family (to which *T. castaneum* belongs) primarily feed on rotten tree bark and other decaying plant matter, and it is possible that this was the original food source of *T. castaneum* before it adapted to feeding on anthropogenic stored products (Dawson, 1977). It has been theorised that stored product insects such as *T. castaneum* may have first adapted to feed on rotten grains stored in the burrows of rodents, and other sources of rotten grains, before adapting to feed on mechanically processed grains stored by humans (Dawson, 1977). If this is the case then it is possible that the same olfactory receptors that were used to find fungi associated with rotting bark might have been adapted to finding fungi associated with rotting grains, and later to fungi found in the stored product warehouses. If fungal-associated

volatiles are used to identify grains in stored product warehouses it is possible that stored product insects may use a combination of fungal and wheat-produced volatiles to locate food sources and, as such, lures that use a combination of food and fungal volatiles may prove more effective.

The responses of stored product insects to fungal volatiles are less well studied than their responses to plant and foodstuff volatiles. Indeed, it has been suggested that interactions between microbial volatiles and insects is an understudied field across all insects (Beck and Vannette, 2017). However, certain stored product insects have also been found to be attracted to fungal produced odours or individual fungal compounds, including: *T. castaneum* (Ahmad, Daglish, et al., 2012), *O. surinamensis* (Pierce et al., 1991), *O. Mercator* (Pierce et al., 1991), *C. ferrugineus* (Pierce et al., 1991), the foreign grain beetle *Ahasverus advena* (Pierce et al., 1991), the squarenecked grain beetle *Cathartus quadricollis* (Pierce et al., 1991), the grain mite *Acarus siro* (Thomas and Dicke, 1972) and *Plodia interpunctella* (Būda et al., 2016). Yeast volatiles have also been shown to be attractive to the coffee bean weevil *Araecerus fasciculatus* (Yang et al., 2017).

Aside from the potential to develop more attractive lures, there are several other potential advantages to using lures based on environmental VOCs as opposed to pheromone-based lures. For example, there is the potential to attract species for which insect pheromones are not known or commercially available (Epsky et al., 2014). It has also been suggested that food-based lures can attract insects earlier than traps baited with pheromones (Epsky et al., 2014). This was demonstrated in the soft fruit pest medfly *Ceratitis capitate*, where traps with food-based lures captured flies in a mixed-fruit orchard earlier in the season compared to pheromone based lures (Papadopoulos et al., 2001). This effect is thought to be due to changes during the season in the response of the flies to the pheromone attractants reducing the attractiveness of pheromone containing lures (Papadopoulos et al., 2001). If the same effect is seen in stored product pests, then food-based lures may be more effective than pheromone attractants at certain times of the year. Unlike pheromones, which are usually species specific, volatiles from these sources are likely to be attractive to several different species (Collins et al.,

2007). There can also be 100-fold greater cost to use synthesised pheromones than cheaper kairomone chemicals (Metcalf, 1994).

1.6. *Tribolium castaneum* as a stored product pest

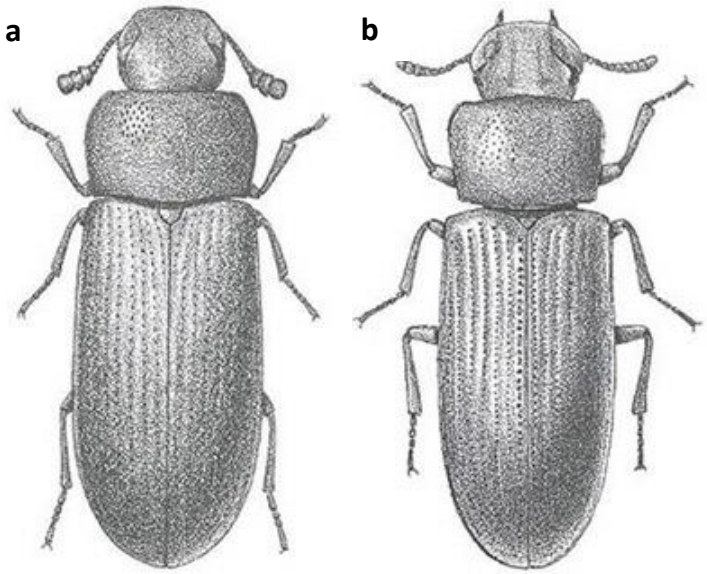

Figure 1.1 An illustration of the morphological differences between (a) *T. castaneum* and (b) *T. confusum* (Gorham, 1991).

Note the differences in the final antennal segments, and body shapes of the two species.

One stored product pest whose management could be improved with further research is *T. castaneum*, one of the most common and economically significant stored product insects (Herrman, 1998; Hagstrum and Flinn, 2014). It also has a sister species, *T. confusum*, that is also a stored product insect, and is virtually indistinguishable from *T. castaneum*, apart from some minor differences in its antennal and thoracic morphology (Fig. 1.1). *Tribolium castaneum* and *T. confusum* make particularly damaging pests owing to their broad cosmopolitan distribution and the wide variety of different foods they can infest. *Tribolium castaneum* and *T. confusum* are considered secondary-stored product pests noted for feeding on cereal flours, chopped nuts, seeds and a variety of dried food products (Bell, 2014). Infestations of *Tribolium spp.* can be particularly damaging as they lay their eggs on food sources, and all of the beetle's life stages can live and feed within the food. *Tribolium castaneum* and *T. confusum* also secrete toxic benzoquinones and other chemicals into the food they infest from stink glands on their prothorax and

posterior abdomen (Roth, 1941). Three key benzoquinone-related compounds have been identified from the stink gland secretions of *T. castaneum*: 2-methoxybenzoquinone, ethyl-1,4-benzoquinone (EBQ), and methyl-1,4-benzoquinone (MBQ). In contrast, *T. confusum* is only known to produce the latter two of these compounds (Li et al., 2013). Benzoquinones are among the most widely produced and effective offensive compounds produced by insects, owing to their toxicity and high volatility (Weatherston, 1967). Secreted benzoquinones have been shown to have broad antimicrobial properties, being effective at reducing the growth rate of a range of flour-associated bacteria (Yezerski et al., 2007). These benzoquinones have also been shown to be toxic to the beetles themselves (Yezerski et al., 2000), as well as other species (Lis et al., 2011), if there is not sufficient food media to absorb their secretions. These compounds can contribute to a conditioning effect, produced by severe *Tribolium spp.* infestations, that can render the food they infest unpalatable to humans. This conditioning is characterised by a depletion of the nutrient value of the medium, an accumulation of benzoquinones and other chemicals secreted by the beetles and the build-up of debris such as larval casts and dead individuals (Ghent, 1963). Insecticide resistance is also widespread in *T. castaneum* and *T. confusum* (Talukder, 2009) with *T. castaneum* in particular demonstrating resistance to all insecticides used for its control (Tribolium Genome Sequencing Consortium, 2008) driving the need to focus instead on alternative pest management strategies.

Both *T. castaneum* and *T. confusum* males produce an aggregation pheromone 4,8-dimethyldecanal (DMD) which is attractive to both males and females (Ryan and O'Ceallachain, 1976; Suzuki and Sugawara, 1979; Suzuki, 1981) and the use of lures containing this pheromone has become a key component of *Tribolium* spp. pest management. Synthetic DMD is available for use in monitoring (Swords and Van Ryckeghem, 2010a) and commercial lures and traps containing DMD are available from several different suppliers (Mullen, 1992; Hussain, 1994). These commercial lures typically contain DMD in combination with a food-based kairomone oil (Dissanayaka et al., 2018). However, there are reports from users in the stored product industry that the responses of *T. castaneum* to traps baited with commercial *Tribolium* lures

are limited (Campbell, 2012). This is supported by experimental evidence demonstrating that commercial lures only captured 2% of beetles released within 60 cm of the trap in a flour mill (Hawkin et al., 2011), while others have demonstrated that traps baited with commercial *Tribolium* lure are no more attractive to *T. castaneum* than empty traps under still air conditions (Campbell, 2012). It has even been demonstrated that fresh lures can be repulsive to *T. castaneum* (Hawkin et al., 2011).

Although the attraction of *T. castaneum* to DMD is well established (Suzuki, 1981) its attraction to food odours is less well known (Campbell, 2012). Considering that some commercial *T. castaneum* lures contain food-based attractants (Dissanayaka et al., 2018), there is great potential to increase the attractiveness of lures through a greater understanding of the responses of *T. castaneum* to specific food-related VOCs. One major barrier in improving the ability of these lures to attract *T. castaneum*, as well as other stored product insects, is a lack of knowledge about the specific odours that attract these insects to stored product environments (Campbell, 2012). As the common name of *T. castaneum,* the red flour beetle, implies, flour and other milled grains are a major food source for this species, however experimental data shows that the odours of flour are only marginally attractive to this species (Campbell, 2013). *T. castaneum* has also been shown to be more attracted to wheat kernels with other insects present, and to grains that were previously damaged by other pest species (Trematerra et al., 2000). When separate wheat kernel components were studied, internal germ volatiles were shown to be more attractive to *T. castaneum* than volatiles of the outer endosperm or bran portions (Seifelnasr et al., 1982). These preferences could be explained by the fact that *T. castaneum*, as a secondary stored product insect, is only capable of feeding on grains that are rotten, have been damaged by the infestation of other insects or have been mechanically processed by humans i.e. milled (Trematerra and Sciarretta, 2004). The presence of wheat germ volatiles could potentially be an indicator to the beetles that the grains are in a suitable condition to feed upon. *Tribolium castaneum* also exhibits a striking attraction to fungal odours, specifically the volatiles of fungi associated with cotton seed lint. Indeed, they were shown to be more attracted to these odours than to the odours of conventional food sources such as wheat (Ahmad,

Daglish, et al., 2012). Little is known about the specific blends of compounds from these sources that may be responsible for the attraction to cereal products. However, a recent study has used EAG to measure the depolarisation elicited by *T. castaneum* antenna to 94 volatile compounds, several of which are known to be produced by grain associated fungi or cereal grains and many of these compounds elicited strong responses (Balakrishnan et al., 2017). Although the attractiveness of compounds and the EAG response compounds elicit are not directly correlated, these data do suggest that *T. castaneum* has evolved to respond to a variety of different cereal grain and fungal associated volatile compounds.

Insect odorant receptors have been shown to be highly tuned to the detection of host and environmental volatiles that are of acute biological relevance to the species (Hansson and Stensmyr, 2011). *T. castaneum* has presumably evolved to respond to certain wheat germ oil volatiles because they are indicators of nearby food sources such as wheat flour. As previously mentioned, despite fungi not being a primary food source for *T. castaneum*, it is possible that certain fungi produced volatiles could also indicate the presence of nearby food sources.

As many current lures contain wheat germ oil as a food-based attractant there is the potential to improve the effectiveness of these traps by identifying which specific compounds within wheat germ oil are the most attractive and then producing a more targeted lure using these compounds. There is also the potential to discover novel attractive compounds by testing the responses of *T. castaneum* to the volatiles produced by grain-associated fungi.

As beetles can be some of the most damaging pest species there is merit to focusing on improving the management of a pest beetle species such as *T. castaneum* as it is possible that any attractive volatiles identified will also be attractive to other related beetle pest species. In particular, other beetles in the same family as *T. castaneum* are bark beetles which can be significant pests of important tree species (Dawson, 1977) and if they share common olfactory responses then any findings in *T. castaneum* could have applications for the management of a diverse range of related beetle pests.

1.7. *Tribolium castaneum* as a laboratory model species

Tribolium castaneum has an established history as a laboratory model system, which provides numerous practical advantages to studying this species over other stored product insects. *Tribolium castaneum* was initially established as an ecological model for studying different aspects of population ecology and population genetics (Brown et al., 2009). Many of these studies focused on the differing life-history traits exhibited by *T. castaneum* and *T. confusum* (e.g. fecundity (Park and Frank, 1948), cannibalism rates (Park et al., 1965), media conditioning (Ghent, 1963)) and the resulting inter-species competition that can occur when they are cultured together (Park, 1957). The simple fact that this species is a model system provides the benefit that, unlike some other pest species, there are established protocols for the easy maintenance of *T. castaneum* cultures.

Tribolium castaneum is also being developed as a model system for studying genetics and developmental biology. This is relevant as it means that a variety of genetic techniques exist that could be used to study olfactory function in this species. RNA interference (RNAi) is one reverse genetic technique that is well established in *T. castaneum* and allows for the creation of loss of function phenotypes in the absence of the time-consuming process of generating mutants (Posnien et al., 2009; Kumar et al., 2018). This technique relies upon manipulating part of an organism's immune response to viruses, transposons and other foreign genetic material. This immune pathway allows potentially harmful messenger RNAs (mRNAs) to be selectively degraded, silencing the foreign gene. In this experimental technique double-stranded RNA (dsRNA) is synthesised with a sequence complementary to the gene of interest and injected into the beetles. When injected, this dsRNA is cleaved by the enzyme Dicer to produce small interfering RNAs (siRNAs). One strand of the 21-mer siRNA is then incorporated into a multiprotein RNA-induced silencing complex (RISC). RISC can then use the siRNA as a guide to find and cleave complementary mRNA of the target gene, thus targeting the RNA for degradation. The RNAi effect spreads systemically and knocks down gene expression throughout the entire body of the organism, as well as across generations (i.e. from injected mothers to their offspring). This is one specific advantage of *T. castaneum* because, although the systematic spread of RNAi

from cell to cell has been shown to occur in some organisms (e.g. the nematode *C. elegans*), it does not appear to occur in the other major insect laboratory model *D. melanogaster* (Tomoyasu et al., 2008), which has been a focus of studies on olfaction in insects. This technique has previously been used to knock-down the function of a specific co-receptor involved in odour perception in *T. castaneum* to abolish the behavioural responses to its aggregation pheromone (Engsontia et al., 2008).

The recent demonstration of CRISPR-mediated gene knockout in *T. castaneum* (Gilles et al., 2015) presents another emerging genetic technique to study the function of genes that may be relevant to pest behaviours in *T. castaneum.* It is also one of the few beetle species to have a published genome sequence (Tribolium Genome Sequencing Consortium, 2008), and is the only one that is a stored product insect, although some beetles with published genome sequences are pests of other resources such as pine trees (the mountain pine beetle *Dendroctonus ponderosae*) or coffee fruits (the coffee borer beetle *Hypothenemus hampei*). The availability of these techniques and resources makes *T. castaneum* an ideal model for identifying some of the genes responsible for pest behaviours in an important stored product insect.

1.8. Olfaction in insects

As *T. castaneum* is the only stored product pest with a published genome sequence (Tribolium Genome Sequencing Consortium, 2008) with extensively annotated olfactory genes (Engsontia et al., 2008) there is a unique opportunity to use chemical ecology and emerging reverse-chemical ecology techniques to help identify the compounds and receptors this stored product insect uses to locate the foods it infests. To understand how insights about the mechanisms of specific olfactory genes could lead to improved pest management in *T. casteneum*, it is first necessary to briefly describe the mechanisms of olfaction in insects.

In insects, molecules are detected by receptors within olfactory sensory neurones (OSNs) which are housed within specialised structures (sensilla) (Fig. 1.2) predominantly on the antennae, legs and maxillary palps (a structure

located on insect mouth parts) (Hansson and Stensmyr, 2011). Odorant receptors (ORs), sometimes called olfactory receptors, are the largest and most important group of receptors involved in olfaction in insects and are involved with detection of air-borne odorants. ORs are expressed on the dendritic membranes of OSNs. As the dendrites of sensory neurones are surrounded by sensillar lymph, and because the most common odorant molecules are hydrophobic, odorant binding proteins (OBPs) are needed to transport hydrophobic odorant molecules to ORs (Fan et al., 2011). Insect ORs share some common characteristics to vertebrate ORs but also have several key differences; they are both 7 trans-membrane G protein-coupled receptors (GPCRs), but insects have an inverted membrane topology compared to vertebrates with an intercellular N terminus (Benton et al., 2006).

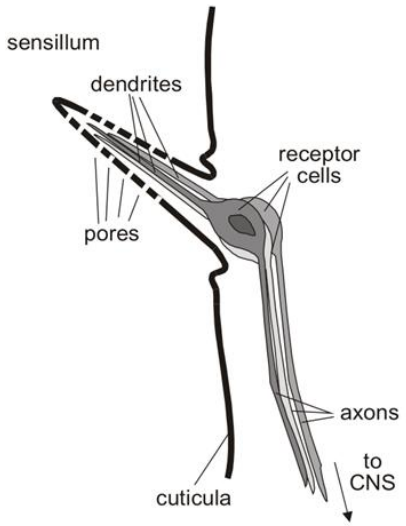

Figure 1.2. Diagram showing the arrangement of insect olfactory sensilla (Syntech, 2004).

ORs form a complex with an odorant co-receptor called Orco. This conserved odorant co-receptor is necessary for all OR-mediated olfactory function in insects and is known to be involved with the trafficking and localisation of ORs to the dendrites of OSNs and has roles in signal transduction (Stengl and Funk, 2013). The OR-Orco heteromeric complex appears to function as an odorant-gated cation channel (Fig. 1.3), but currently there is little knowledge of the specific mechanistic action of this complex (Stengl and Funk, 2013; Turner et al., 2014), although several possible mechanisms of signal transduction have been proposed (Stengl and Funk, 2013). Typically only one

type of OR and Orco are expressed in each OSN (Hansson and Stensmyr, 2011). The odorant co-receptor gene that encodes Orco was first discovered in *D. melanogaster* (Larsson et al., 2004), and orthologs of the gene have been found in over forty insect species that have had their ORs sequenced (Stengl and Funk, 2013). Knocking-down the function of Orco has been shown to severely impair olfactory function in a diverse range of species including: *D. melanogaster* (Larsson et al., 2004), the yellow fever mosquito *Aedes aegypti* (DeGennaro et al., 2013), a parasitoid wasp *Microplitis mediator* (Li et al., 2012), a cotton pest bug *Apolygus lucorum* (Zhou et al., 2014) and the striped flea beetle *Phyllotreta striolata* ((Zhao et al., 2011).

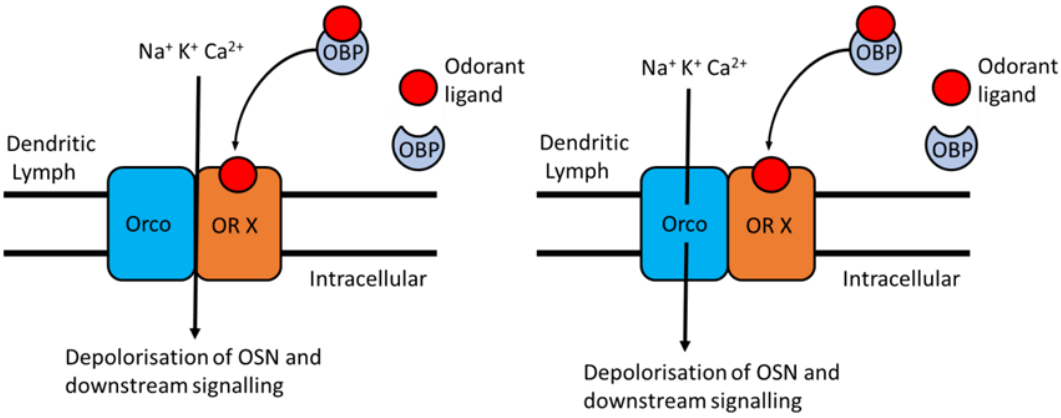

Figure 1.3. Diagram showing the two proposed mechanisms of how the Orco-OR complex interacts with OBPs to cause depolarisation of the OSN when activated by specific odorant ligands.

Adapted from (Stengl and Funk, 2013).

ORs can be both highly selective, responding only to a specific compound, or they can respond to a broad spectrum of compounds (Hansson and Stensmyr, 2011). Receptors that only respond to one compound are usually tuned to an odorant of acute biological relevance, such as pheromones or the volatiles of an important host plant (Wilson and Mainen, 2006; Hansson and Stensmyr, 2011). However, evidence from many herbivorous insects suggests that they locate their host plants by responding to specific blends of ubiquitous plant volatiles (Bruce and Pickett, 2011; Carrasco et al., 2015). It is unclear whether stored product insects use specific key compounds or blends of ubiquitous compounds to locate the food products they infest. It is also not clear whether different stored product insects have evolved similar receptors to respond to the same stored product odours. If this is the case then it is possible that any

volatiles identified as eliciting a strong attraction in one stored product pest may also be attractive to other pest species, and identification of these compounds could lead to the development of a novel multispecies lure.

1.9. Olfactory experiments in *Tribolium castaneum* and other insects

Several methods exist for testing the responses of insects such as *T. castaneum* to different odour sources. Electrophysiological methods can measure the depolarisation of insect antennae, a single sensory sensillar or individual ORs, in response to different odours. Electroantennography (EAG) can be used to measure the depolarisation of the whole insect antenna, and this can be used to test a range of volatile compounds from specific sources that have been separated by gas-chromatography (GC-EAG) (Syntech, 2004). Single sensillum electrophysiology can also be used to measure the action potentials generated by OSNs within single sensilla on an insect antenna (Olsson and Hansson, 2013). EAG has been previously used to demonstrate the responses of *T. confusum* to some of the compounds it secrets, including DMD and benzoquinones (Verheggen et al., 2007). EAG in combination with a simple behavioural bioassay has also been used to demonstrate that knocking down the *T. castaneum orco* gene (*Tc-or1*) with RNAi abolished the insect's behavioural response to DMD (Engsontia et al., 2008). During the research period of this thesis the first large-scale EAG screen using *T. castaneum* was published testing the antennal responses of *T. castaneum* to 94 VOCs from various sources (Balakrishnan et al., 2017).

ORs can also be tested using the *Xenopus* oocyte and *Drosophila* empty neuron expression systems to measure the activation of individual OR in response to different odorants. However, there are issues with these systems not expressing olfactory proteins specific to the test species, such as OBPs, that could lead to false negatives (B. Wang et al., 2016) and these techniques have not been previously used to express any *T. castaneum* olfactory receptors.

Although electrophysiological bioassays can provide a clear indication of the compounds an insect can actually physiologically detect, not all compounds

that an insect detects will elicit an attraction, or indeed any behavioural response at all. As such, electrophysiological experiments are often used as screening tools to identify promising compounds for further testing using more time consuming behavioural bioassays (Beck et al., 2012). A variety of different bioassays exist for testing the behavioural responses elicited by different odours in insects. Bioassays used for measuring the attraction of insects to different odours are referred to as olfactometers, and often allow the insect to choose between different odour sources, clearly establishing which odour the insect prefers. Different behavioural bioassays that have been used to test the responses of *T. castaneum* and other insect species are discussed in detail in Chapter 2.

1.10. Insect olfactory receptors and their potential importance in the development of pest management strategies

It has been suggested that sequenced genomes and transcriptomes from stored product insects could provide data on sensory genes that could lead to the development of more effective lures (Perkin et al., 2016). As olfactory proteins are responsible for sensory processing in insects, they could provide insight into the specific compounds that attract certain insects to stored products. It is possible that the gene sequences and structures of these olfactory proteins could help identify attractive compounds for use in pest lures, with ORs and OBPs having been specifically identified as proteins that could be used to identify new attractive semiochemicals (Venthur and Zhou, 2018). As species (or taxon)-specific expansions of OR lineages have been found in most insects studied, these lineages could be examples of species specific ecological adaptation and indicators of important semiochemicals (Andersson et al., 2013). However it should be noted that ORs within these species-specific expansions may not necessarily be adaptive as there is evidence that neutral processes, like genetic drift and gene duplication can contribute significantly to the diversity in insect ORs (Nei et al., 2008; Sánchez-Gracia et al., 2009; Eirín-López et al., 2012).

However, phylogenies of OR genes have been used to identify genes that may be related to ecological adaptation in other insect species. Twenty four

ORs of the lepidopteran pest species the African cotton leafworm *Spodoptera littoralis* were expressed transgenically using the *Drosophila* empty neuron system (Fouchier et al., 2017). Single-sensillum recordings were then taken from the antenna expressing each of these ORs when they were presented with a diverse array of 51 ecologically relevant volatiles. When the responses elicited by the different ORs to these compounds were compared against a maximum-likelihood phylogeny of the ORs of 8 lepidopteran species, patterns in the receptiveness of ORs across different lineages could be identified. Conserved clades were found be tuned towards aromatics whereas ORs from recently diverged groups responded most strongly to terpenes and short-chain acetates. As these recently diverged groups also showed a higher rate of evolution they were predicted to be involved in the adaptation to new ecological feeding niches in this species. Phylogenetic analysis of ORs in the pea aphid *Acyrthosiphon pisum* have also revealed a large expansion of species-specific ORs that show signs of positive selection and recent rapid expansion, and are predicted to be involved in host-plant selection in this pest species (Smadja et al., 2008). In both of these cases it was suggested that the clades of ORs identified could have implications for pest management as targets for further research (Smadja et al., 2008; Fouchier et al., 2017). This could be especially useful for identifying pest management related ORs in stored product insects, where the adaption to feeding primarily on human stored product has happened relatively recently on an evolutionary timescale (Dawson, 1977). ORs that are shared by multiple different species could also lead to the identification of semiochemicals that could be used in a multi-species stored product insect lure. For example, an OR identified in a field crop pest, the tobacco budworm *Heliothis virescens,* was found to be shared by five other noctuid moth species and it was suggested that targeting this gene could provide a way disrupt behaviours of the noctuid pest species without affecting other beneficial insect species, e.g. pollinators (Brigaud et al., 2009).

Expression patterns of OR genes have also been used to identify ORs of interest in terms of insect pest management. In the green plant bug, *A. lucorum*, a subset of OR genes were found to be highly expressed in the antenna, and as such, were predicted to play an important role in the detection

of host plant volatiles (An et al., 2016). This technique of using gene expression patterns to identify ORs of interest has been taken further in other pest insect species, leading to the deorphaning of key receptors. In the southern house mosquito *Culex quinquefasciatus* when seven of the ten most highly expressed ORs were expressed in *Xenopus* oocytes and their EAG responses were screened against a panel of odorants, a receptor that responds strongly to acetaldehyde was identified (Choo et al., 2018). This compound was then shown in behavioural bioassays to be an oviposition attractant for this species (Choo et al., 2018). Also in *A. pisum* an OR that was expressed only in the antenna was singled out for functional analysis and, when expressed in *Xenopus* oocytes, was found to respond strongly to eight plant volatiles (Zhang et al., 2019).

Computational 3-D modelling has also been used to predict compounds that have favourable binding energies with the OBPs of the pest fruit fly, *Bactrocera dorsalis*, and demonstrate a positive relationship between the predicted free binding energies of compounds and their attractiveness to this species in behavioural bioassays (Kamala et al., 2014). It is possible that in the future the same technique could be used to predict the ligands that will activate specific ORs.

Feeding insects dsRNA has been suggested as a method of targeted gene disruption for pest control of stored product insects (Perkin et al., 2016; Mamta and Rajam, 2017). Genes that are lethal when disrupted have been suggested as potential targets, such as those involved in: cuticle formation or moulting, hormone biosynthesis, digestion, metabolism or the immune system (Perkin et al., 2016). However, in addition to being used to identify new attractive semiochemicals the possibility of using olfactory proteins such as ORs and OBPs as pest control targets has been suggested (Venthur and Zhou, 2018). Disrupting the ORs involved with detecting food-associated odours could be effective as a management strategy if it prevents pest insects from locating stored products. RNAi disruption of pheromone ORs has also been suggested as a method of pest management in stored product pests (Perkin et al., 2016). Selective disruption of the olfactory receptors involved in pheromone detection could prevent male and female pests from locating each other,

reducing the chance of successful mating and helping to control the population of the pest species.

Targeted RNAi disruption of the *orco* orthologue of *P. striolata* has been suggested as a way of managing this agricultural pest (Zhao et al., 2011). However, as there is the possibility of off-target effects on other species, it is possible that useful insects such as pollinators could also be disrupted if conserved genes are targeted (Perkin et al., 2016; Mamta and Rajam, 2017). It has specifically been suggested that the *Orco* gene may be too highly conserved to target without off-target effects on other insect species and that species-specific olfactory receptors would make better targets (Andersson and Newcomb, 2017). However analysis of 59 *Orco* codons from six insect orders have shown that, although 48.9% of the sequence is conserved, there are also areas of high variation in the amino acid sequences (Soffan et al., 2018). Areas of positive selection were also identified within the Coleoptera, Diptera, Lepidoptera, and Psocodea orders (Soffan et al., 2018) so it may be possible to target a specific pest containing order.

Although RNAi is still under development as a commercial insect pest management strategy, dsRNA applied to leaves has been demonstrated to produce a variety of effects in coleopteran, lepidopteran and hemipteran agricultural pests which, depending on the gene targeted, caused increased mortality, growth and development impairment, reduced fecundity or increased susceptibility to insecticides (Palli, 2014; Mamta and Rajam, 2017). The emerging CRISPR technology has also been suggested as another method of targeted gene disruption for the control of stored product insects (Perkin et al., 2016).

However, the use of these techniques in stored product insects is limited by the absence of annotated ORs for most of these species. *Tribolium castaneum* is the only stored product insects with a published genome (Tribolium Genome Sequencing Consortium, 2008) and annotated ORs (Engsontia et al., 2008). However, so far none of its ORs have been deorphaned. Two hundred and fifty-nine odorant receptor genes that were predicted to be functional were identified in *T. castaneum* based on sequence similarity to the ORs of *D. melanogaster*, the mosquito *Anopheles gambiae;*

the domestic silk moth *Bombyx mori*, the European honey bee *Apis mellifera* and *Aedes aegypti* (Engsontia et al., 2008). In addition, 79 pseudo-genes, previously protein coding genes that can no longer be expressed due to mutations, were also identified based on the presence of in-frame stop codons, frame-shifting indels, or unacceptable intron splice sites (Engsontia et al., 2008). The number of receptors found is surprising, as *T. castaneum* appears to have more olfactory receptors than species with complex olfactory based social systems, with the honey bee, *A. mellifera*, only predicted to have 163 functional ORs (Robertson and Wanner, 2006). However, only 147 of the 259 identified ORs (including the *T. castaneum orco* orthologue, *Tc-or1*) could be amplified from cDNA in either adults or larvae. This suggests that many of the OR genes are not expressed, at least under laboratory conditions (Engsontia et al., 2008). This raises the idea that some ORs in *T. castaneum* may only be expressed under certain environmental conditions and this could have relevance to its pest management if they are not expressed in stored product warehouse conditions.

As the number of olfactory receptors in other stored product pests have not been quantified it is impossible to know if this large number of olfactory receptors is unique to *T. castaneum* or a feature common to stored product pests. Some comparisons have been made to the closely related bark beetle species, the European spruce bark beetle *Ips typographus* and the North American mountain pine beetle *Dendroctonus ponderosae,* which are predicted to have 43 and 49 OR genes respectively. Of these, only 11 and 27 ORs respectively were predicted to be full length protein coding genes, the others likely being pseudogenes owing to their much smaller size (Andersson et al., 2013). These are much lower than the number of ORs identified in *T. castaneum* and, as the other two beetle species feed on bark and *T. castaneum* ancestrally fed on rotting bark and other detritus (Dawson, 1977), it is possible that the expanded OR genes of *T. castaneum* are related to the specialisation of feeding primarily on stored products. If these ORs are related to the detection of stored products they could be important targets for further research into how *T. castaenum* locates the food-sources it infests. However, it should be noted that the *Curculionidae* family (to which *I. typographus* and *D. ponderosae* belong) and the Tenebrionidae family (to which *T. castaneum*

belong) diverged from a common ancestor ca. 230–240 Mya (Hunt et al., 2007), and *T. castaneum* is only thought to have been associated with anthropogenic stored products for a few thousand years (Dawson, 1977). As such, it is possible that not all the genes in the *T. castaneum* specific expansion are related to the relatively recent, in evolutionary terms, adaptation to feeding on anthropogenic food sources.

1.11. Strain and sex variation in *Tribolium castaneum* and other stored product insects

Several different strains have been used in *T. castaneum* olfactory and behavioural experiments with some experiments using established laboratory strains (Seifelnasr et al., 1982; Hawkin et al., 2011; Campbell, 2012; Stevenson et al., 2017; Balakrishnan et al., 2017), while others have used beetles recently cultured from wild populations (Trematerra et al., 2000; Ahmad, Ridley, et al., 2013; Ahmad, Daglish, et al., 2013; Campbell, 2013). Using an established laboratory strain may make the results of experiments more consistent and easier for other researchers to replicate or expand, However, if the research is intended to contribute towards applied approaches for managing a pest species, there may be disadvantages to using laboratory strains if their responses differ from beetles freshly cultured from wild populations.

Culturing insects under laboratory conditions can limit the maximum population size and prevent the influx of new genetic variation from outside the population. This can lead to a reduction in genetic diversity causing inbreeding depression even when insects are cultured at relatively large population sizes (Briscoe et al., 1992). If this loss of genetic diversity affects any genes related to olfactory perception or behaviour it could result in laboratory populations responding differently compared with populations in the wild. However, this may not be as important for stored product pests, as it has been suggested the degree of genetic variation in wild *T. castaneum* populations may also be quite low (Michalczyk et al., 2010). This is due to the way this pest species encounters frequent population bottlenecks in the wild, owing to pest control measures and its method of spreading via repeated

founder effects (i.e. genetic variation is lost when a new population is established by a very small number of individuals) (Michalczyk et al., 2010).

Laboratory adaptations can also occur when the conditions that strains are reared under exert significantly different selection pressures to those encountered in the wild, with rapid rates of laboratory adaptation being demonstrated in many insect species (Hoffmann and Ross, 2018). Of particular interest to this research is the possibility that a uniform environment, containing a single abundant food source, which *T. castaneum* is typically cultured in, could lead to a relaxation of the selection pressures of genes involved in locating food sources. The olfactory responses of insects have been shown to be susceptible to selective forces in artificial selection experiments, with individuals of a *D. melanogaster* population being artificially selected over 30 generations to exhibit a significantly stronger response to a food-associated volatile attractant (Brown et al., 2017). The same selected population also showed differential expression of 91 genes, demonstrating that this type of selection has the potential to effect a variety of different behaviours and metabolic processes in insects (Brown et al., 2017) and that olfactory responses can change rapidly under strong selective forces. This could result in different responses between recently caught wild populations and older laboratory cultured strains, and as such, any findings made using laboratory strains may not be applicable to natural insect populations. There is some evidence of differences between the olfactory responses of wild and laboratory strain *T. castaneum*, with freshly caught wild populations having been shown to be less attracted to traps baited with cereal oil and DMD compared to laboratory populations (Hawkin et al., 2011). Changes in the ability of other coleopteran species to utilise different food resources after laboratory rearing have already been demonstrated (Hoffmann and Ross, 2018). If significant differences are found between the responses of laboratory strains and wild *T. castaneum* populations this could have implications for future research in this area in *T. castaneum* and other insect species. There is also evidence that natural variation exists between the responses of different *T. castaneum* populations in the wild. When the attractivness of aggregation pheromone and an oil-based kairomone lure was tested using Y-tube olfactometer, wind tunnel, and petri dish bioassays, differences were

found in the responses of *T. castaneum* from different geographic areas (Gerken et al., 2018). There is also evidence of variance in the olfactory responses of other insects to food associated volatiles, with polymorphisms in wild *D. malanogaster* ORs demonstrated to be responsible for differences in attraction to fruit associated volatiles (Rollmann et al., 2010; Richgels and Rollmann, 2012). If this variation in the responses of wild populations is typical across insects this could limit how useful food-associated volatiles are for monitoring pest insect species.

If variation exists between the responses of male and female *T. castaneum* to food-associated volatiles this could also affect the usefulness of these volatiles in pest lures. However, there does not seem to be any evidence that the responses of *T. castaneum* to VOCs differ due to sex in a significant way. Behavioural bioassays comparing the responses of male and female *T. castaneum* to odours of freshly milled barley flour did not find their responses to be significantly different (Duehl et al., 2011) and, although it was not focused specifically on food-associated volatiles, a large EAG screen of 94 VOCs in *T. castaneum* also did not find any significant differences between the sexes (Balakrishnan et al., 2017). Although the EAG response a compound elicits does not correlate directly with the behavioural response it elicits, the lack of any differences over such a range of different volatile compounds suggests that the responses to environmental volatiles might not differ significantly due to sex in *T. castaneum.*

1.12. *Tribolium castaneum* strains used in this thesis

To test for the effects of laboratory adaptation and strain variation, and to identify the most responsive strain in behavioural bioassays, several different *T. castaneum* strains were used in this thesis. The established San Bernardino laboratory strain was used to represent a potentially inbred laboratory strain. Originating in San Bernardino, California, this strain has been cultured continuously in the laboratory since at least the 1970s. After having difficulties getting consistent and reproducible behavioural responses with this laboratory strain in many bioassays, the CTC12 strain and a Fera laboratory strain were used as alternative laboratory strains. The CTC12 strain

was a field caught Australian organophosphate-resistant strain (Champ and Campbell-Brown, 1970) that has since been cultured in the laboratory and was chosen as the main laboratory species in this thesis as it was the most responsive in behavioural bioassay, as well as having a well-documented origin. Two recently caught wild populations were also used, which were designated wild UK and wild Zim. The wild UK population was used in some initial experiments and were collected in 2014 from a Doves Farm mill in the UK. The wild Zim strain was cultured from a population found inside a shipment of infested grain from Zimbabwe in 2017, and was used in all subsequent experiments as, being more recently captured, it will have had less time to develop potential laboratory adaptations and should more closely mimic the responses of a typical wild population.

1.13. Project aims

The overall aim of the work reported in this thesis is to broaden our understanding of the mechanisms of olfaction in *T. castaneum*, with the long-term aim of improving how this important stored product insect is managed. As little is known about the attraction of *T. castaneum* to specific volatile compounds found within stored product environments, compounds from these sources could potentially be useful in an attractant lure for this species. VOCs from wheat germ oil and grain-associated fungi were identified from the literature as potential sources of attractive semiochemicals and were tested using electrophysiological and behavioural bioassays to identify if they elicited significant responses from *T. castanum.* As insects can differ in their responses to blends of compounds, as opposed to the same compounds encountered individually, the responses of *T. castaneum* were also tested to a variety of different blends of compounds to try and identify an optimal blend that could potentially be used in future traps.

As a variety of different behavioural bioassays have previously been used to test the responses of *T. castaneum* and other insects there is no consensus on which bioassay might be most suitable for testing the behavioural responses of *T. castaneum* to environmental compounds. To address this, the effectiveness of four different bioassays for testing the responses of *T.*

castaneum to environmental VOCs was evaluated. Different strains of *T. castaneum* were tested to identify if the responses to environmental volatile compounds vary between different wild populations and between wild populations and laboratory populations, as this has relevance to future pest management research in this species. The unique status of *T. castaneum* as both an important laboratory model and a significant stored product insect provides a unique opportunity to deorphan important ORs involved in the attraction of a stored product pest species to the food it infests. As *T. castaneum* is one of the few beetle pest species with fully annotated ORs and established protocols for reverse genetic techniques such as RNAi, the effects of knocking-down specific OR genes responsible for the attraction of *T. castaneum* to specific environmental volatiles could be tested in the future. However, as no *T. castaneum* OR has had its associated odorant identified yet, the olfactory co-receptor *orco* was knocked-down using RNAi and the effect was recorded using EAG and behavioural bioassays to demonstrate the feasibility of this methodology. This will provide a foundation for future research into *T. castaneum* olfaction by establishing a methodology for identifying the ligand of specific ORs.

1.14 Thesis overview

Chapter 2: The responses of different *T. castaneum* strains were tested against a range of known attractants using four different bioassays: a pitfall bioassay, a 2-choice arena bioassay, a 4-way olfactometer and a Y-tube olfactometer, to identify the effectiveness of different bioassays for testing the olfactory responses of *T. castaneum.*

Chapter 3: The antennal responses of two strains of *T. castaneum*: the laboratory strain (CTC12) and a wild population (wild Zim), were tested against a selection of volatile compounds found in wheat germ oil and produced by grain associated fungi using EAG. This was done as an efficient screening tool to identify what compounds elicited the largest responses from the antenna of the different strains.

Chapter 4: The wheat germ oil and fungal VOCs that elicited the largest EAG responses were tested (individually and as part of blends), using behavioural bioassays, against CTC 12 strain and wild Zim *T. castaneum* to identify if

environmental VOCs have the potential to be used as attractants for this species.

Chapter 5: A methodology for deorphaning specific OR in *T. castaneum* using RNAi knockdowns in combination with EAG and behavioural bioassays was demonstrated using *Tc-or1* (the *T. castaneum Orco orthologue)* as an example.

Chapter 6: The main findings of the thesis, and their implications for *T. castaneum* pest management, as well as suggestions for future work, are discussed here.

Chapter 2
Establishing an effective behavioural bioassay for assessing the attractiveness of different volatile organic compounds to *Tribolium castaneum*

2.1. Introduction

Across the field of entomology a wide variety of different bioassays have been designed to measure the responses of insects to different stimuli and conditions, and this is also true across research involving *Tribolium* species. The aim of the work reported in this chapter was to identify an effective bioassay for measuring the level of attraction of *T. castaneum* to different odour sources. To achieve this, different *T. castenauem* strains were tested against a range of known attractants and compounds of interest using a variety of common olfactometer bioassays. The results of these experiments informed the choice of bioassays used to test the responses of *T. castaneum* to wheat germ oil and fungal VOCs, as covered in later chapters of this thesis. The results of the experiments in this chapter also suggest that variation may exist in how different *T. castaneum* strains respond to environmental volatiles and this is also explored further in later chapters.

2.1.1. Different types of olfactometer

Bioassays designed to measure the responses of insects to olfactory stimuli are often referred to as olfactometers. These olfactometers typically consist of an enclosed arena in which the behaviours of insects, in response to different odour sources, can be recorded. The simplest of these bioassays consist of enclosed arenas in which the level of aggregation around an odour source by insects can be recorded. Video recording is often utilised to collect data on the movements of insects within these arenas, and this type of bioassay has been previously used to demonstrate the attraction of *T. castaneum* to a DMD lure in a petri dish arena (Duehl et al., 2011).

Other common arena based olfactometers are pitfall or trap bioassays. In these bioassays the odour source is placed at the bottom of a pit or inside a trap from which beetles can enter but not exit, resulting in any insects attracted to the odour source becoming captured. These bioassays may contain two or more different traps/pits in the same arena allowing the beetles to choose

between the odour sources in the different traps. An advantage of these bioassays is that once they are set up they do not need to be actively monitored, as the number of insects captured can be counted after the desired time interval. In these bioassays the responses of a large number of insects can be tested at once if many insets are placed in the same bioassay arena. However, the presence of other individuals can affect the behaviour of insects in the arena (Campbell, 2012). If the odour sources tested are not highly attractive it is possible that only a small number of insects will be captured, which may make it difficult to find a statistically significant difference compared to a control treatment.

Single tube olfactometers are another type of simple olfactometer where insects are placed inside a hollow tube with a different odour source at each end. The number of insects that move to different sides of the tube (and therefore towards different odour sources) can then be recorded. A bioassay of this type has previously been used to demonstrate the effect of RNAi knockdown of the olfactory co-receptor, Orco, on the attraction of *T. castaneum* to DMD (Engsontia et al., 2008).

Another commonly used type of olfactometer are airflow olfactometers, in which insects are placed downwind of directed airflow from an odour source, and their behaviours in response are recorded. The responses to odour sources can be stronger under directed airflow than they are under still air conditions (Campbell, 2012), which can make it easier to identify behavioural responses. However, this may be seen as a disadvantage from an applied pest management perspective as insect pests are likely to encounter lures under still-air conditions in stored-product warehouses. As such the results from airflow olfactometer may not reflect how attractive odour sources will be under the real-world conditions in which lures will be used. The most basic airflow olfactometers consist of simple wind tunnels in which the speed and direction of insect movements can be recorded in responses to airflow from different odour sources. More advanced airflow olfactometers can present insects with airflow from multiple different odour sources; in this way the insect can actively choose between moving towards airflow from different sources. Y or T-shaped olfactometers (mazes) are one example of this type of

olfactometer. They consist of a tube, into which beetles are inserted, that branches out in two directions (in a T or a Y-shape). Different odour sources are placed at the end of each of the two different branches and a vacuum pump is used to draw air from both odour sources down the branches towards the insect. This results in insects moving along the tube downwind of the airflow from both odour sources before reaching a branch point where the insect can choose which stream of air to move towards. However, as with all airflow olfactometers, care must be taken to ensure that there is no variation in the strength of the airflow from the different odour sources as this could influence the behaviours of the insects.

Although these bioassays can be time consuming to use because they are usually designed for single insects, every insect that moves through the bioassay will provide a data point via its choice between the two odour sources. Insects that do not make a choice within a set period of time can also be removed preventing non-responding beetles from reducing the statistical power of the experiments. Y-tube olfactometers have previously been used to demonstrate the attraction of *T. castaneum* to different types of infested wheat (Stevenson et al., 2017) and, in *T. confusum*, to test the attractiveness of different VOC blends (Wenda-Piesik et al., 2017).

Another type of airflow olfactometer is the four-way olfactometer (or Pettersson olfactometer) (Pettersson, 1970). This consists of a sealed area that is connected at four corners to different odour sources. A vacuum pump connected to a small hole in the centre of the arena draws air from each of the four different odour sources into the arena. If the four-way olfactometer is properly calibrated, four separate equally-sized odour fields will be established in the arena for each of the odour sources. The attraction of the insects to the different odour fields in the arena can then be measured by recording the amount of time they spend in each area, or by counting the number of individual visits they make to each zone (Ukeh and Umoetok, 2011). This bioassay can allow the responses caused by multiple different odour sources to be efficiently tested, and it has been used in *T. castaneum* to demonstrate a repellent effect of certain secondary plant compounds (Ukeh and Umoetok, 2011). Other variants of this olfactometer exist that allow for

insects to choose between up to six different odour sources simultaneously (Turlings et al., 2004).

Other variants of olfactometer exist as bioassays are modified to suit the physiology and behaviour of the insect being tested, however, the most commonly used olfactometers have been described in this section.

2.1.2. Previous olfactometer experiments in *Tribolium spp.*

In terms of identifying the most effective olfactometer bioassay for use with *T. castaneum* a variety of different bioassays have previously been used in experiments involving *T. castaneum* and *T. confusum,* including: simple arena bioassays (Ryan and O'Ceallachain, 1976; Seifelnasr et al., 1982; Levinson and Mori, 1983; Engsontia et al., 2008; Duehl et al., 2011; Campbell, 2012), pitfall bioassays (Phillips et al., 1993; Hawkin et al., 2011), trap bioassays (Ahmad, Ridley, et al., 2013), wind tunnel bioassays (Obeng-Ofori and Coaker, 1990; Christian Olsson et al., 2006; Verheggen et al., 2007), Y-tube olfactometers (Ahmad, Daglish, et al., 2012; Wenda-Piesik et al., 2017; Stevenson et al., 2017; Gerken et al., 2018) and four-way olfactometers (Ukeh and Umoetok, 2011). Making direct comparisons between the responses of *T. castaneum* across these different bioassays is difficult due to the variety of different conditions, insect strains, odour sources and odour concentrations that have been used across different experiments. However, the fact that each of these experiments managed to detect a significant behavioural response suggests that a wide variety of bioassays have the potential to be effective for use with *T. castaneum.*

A variety of environmental odour sources have been tested in behavioural bioassays involving *T. castaneum* including: plant volatile compounds (Ukeh and Umoetok, 2011; Wenda-Piesik et al., 2017), *Tribolium* derived volatile compounds (Christian Olsson et al., 2006; Verheggen et al., 2007), cereal grains and flours (Seifelnasr et al., 1982; Ahmad, Ridley, et al., 2013; Stevenson et al., 2017) and cotton seed associate fungi (Ahmad, Daglish, et al., 2013). This indicates that several different bioassays have the potential to be suitable for testing the responses of *T. castaneum* to different environmental VOCs such as those produced by grain associated fungi or found in wheat germ oil.

2.1.3. Olfactometer selection

The bioassays used in this chapter: a pitfall arena bioassay, a two-choice arena bioassay, a four-way olfactometer, and a Y-tube olfactometer (Figures 2.1-2.4), were selected based on them having previously detected significant behavioural responses from *T. casteneum*. These bioassays incorporate a wide variety of different parameters, most notably whether the beetles are tested individually (the 'four-way' and Y-tube olfactometers) or in groups (the pitfall and two-choice bioassays), and whether they encounter the volatiles by diffusion in still-air (the pitfall and two-choice bioassays) or through directed airflow (the 'four-way' and Y-tube olfactometers). The 4-way olfactometer had issues with the movement of the beetles inside the bioassay that prevented valid data from being recorded. However, the details of the bioassay design are presented in the methods, Section 2.2.5, to allow for specific issues with the experimental design to be explaineded in the discussion of this chapter.

2.1.4. Volatile selection

To identify a bioassay that can be used to efficiently test the responses of *T. castaneum* to different environmental VOCs the responses of *T. castaneum* were tested against a range of known attractive compounds, alongside wheat germ oil and fungal compounds. One of the most commonly used compounds when conducting olfactory behavioural bioassays in *T. castaneum* is the *Tribolium* aggregation pheromone, DMD. This pheromone is produced by male *T. castaneum* and *T. confusum* and is known to be attractive to both male and females of these species (Duehl et al., 2011). DMD has been shown to be attractive to *T. castaneum* in both still air conditions in an open arena bioassays (Duehl et al., 2011) and with airflow in Y-tube bioassays (Stevenson et al., 2017). As such DMD should elicit a strong positive response from *T. castaneum* in each of the different bioassays. Wheat germ oil has previously been shown to be attractive to *T. castaneum* in still air conditions (Seifelnasr et al., 1982) and should mimic the odour of a common food source for this species. Many *Tribolium* specific and multispecies lures also use food-associated odours, such as wheat germ oil, in combination with DMD and other insect pheromones. The additive effect of combining pheromone and food odours has previously been demonstrated in *T. castaneum* using two-choice pitfall bioassays (Phillips et al., 1993). The "pantry patrol" commercial

lure, produced by Insects Limited (16950 Westfield park road, Westfield, IN, 46074, USA) was also tested. This lure contains wheat germ oil and different stored-product insect pheromones, including DMD. Bioassays that showed promise after testing the responses of *T. castaneum* to some of these known attractants were then tested using a small number of fungal associated VOCs, prior to more systematic testing of individual wheat germ oil and fungal VOCs, as described in later chapters.

2.1.5. Strain variation

The possibility of between-strain variation in the responses of *Tribolium spp*. to commercial pheromone lures has previously been suggested (Hawkin et al., 2011; Campbell, 2012). However, the effects are unclear, as no significant differences between the responses of laboratory strain and freshly caught wild *T. castaneum* to pheromone and food oil traps was demonstrated by Campbell et al. (2012), while Hawkin et al. (2011) did find a significant difference in the responses of wild and laboratory strain beetles to DMD and food oil. Hawkin et al. (2011) suggested that the difference they report may not be due to a difference in how attractive the different odours are to different stains, but rather due to a difference in motility/activity between the strains, leading to more laboratory beetles being captured by traps in one treatment group. A recent study has also identified variation in the responses of different *T. castaneum* strains to a commercial lure (Gerken et al., 2018). As such, some of the bioassays in this chapter compared the responses of different *T. castaneum* strains. However, more extensive comparisons between the responses of different *T. castaneum* stains to selected wheat germ oil and fungal produced VOCs were conducted in later chapters.

2.2. Methods

2.2.1. *Tribolium castaneum* husbandry

The established San Bernardino, CTC12 & Fera laboratory strains and a wild UK *T. castaneum* population were used in the experiments described in this chapter. Cultures were incubated at 30°C in containers of 200 g of whole grain flour (Doves Farm Organic Strong Wholemeal Bread Flour) with the addition of 10 g yeast powder as an additional protein source (Holland & Barrett

Debittered Brewer's Yeast Powder) and 1 g of 0.3 g/kg antimicrobial agent Fumagillin to inhibit fungal growth in the cultures. All beetles used in the experiments were aged between four to eight weeks post-emergence to ensure all insects were sexually mature and mated to reduce the variance in their responses to DMD aggregation pheromone (Duehl et al., 2011). The sexes of the beetles used in the different bioassay experiments were controlled, and the sexes of the beetles used are stated in the methods section for each of the bioassays.

2.2.2. Odour sources

A variety of known and suspected *T. castaneum* attractants were used in the bioassays in this chapter. Synthetic DMD (4,8-Dimethyldecanal), the *Tribolium* spp. aggregation pheromone was used as an odour source in each of the bioassays. The wheat germ oil used as an odour source in the pitfall bioassays was 100% pressed *Triticum vulgare* (common wheat). The "pantry patrol" commercial lure, produced by Insects Limited (16950 Westfield park road, Westfield, IN, 46074, USA) was also used in the two-choice arena bioassays. The 14 fungal compounds used across the two-choice arena and Y-tube olfactometer bioassays were identified from a review article listing volatiles produced by common fungi grown on cereal and grain substrates (Magan and Evans, 2000). All compounds were obtained from commercial suppliers (see appendix A for details). Compounds were diluted to working concentrations using either pentane or hexane and details of the solvent used, and concentrations tested are stated in the methods section of each bioassay. Pentane, an established solvent for olfactory experiments in insects (Beck et al., 2012), was used to dilute almost all of the odour sources and as a negative control in those experiments. Hexane was used to dilute the VOCs and as a negative control in the final Y-tube olfactometer bioassay in Section 2.3. This was due to this experiment being conducted after the EAG experiments in Chapter 3 which identified hexane as eliciting significantly lower responses from *T. castaneum* antenna than pentane did.

2.2.3. Pitfall bioassay

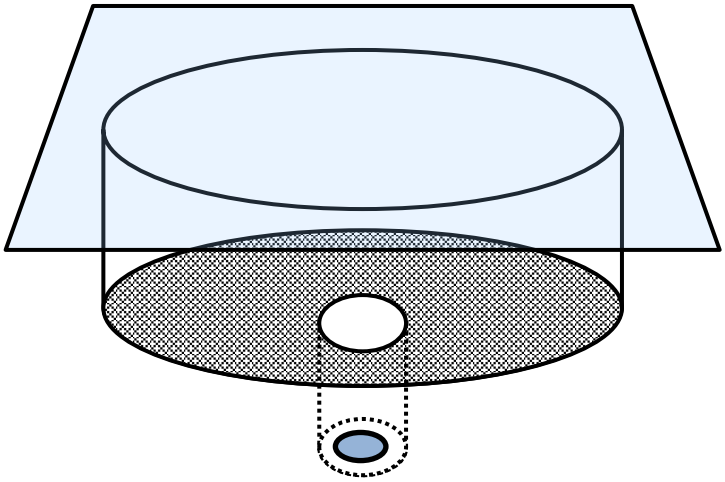

Figure 2.1. 3D schematic diagram of the pitfall bioassay arena.

The cross-hatched area represents the area encircled by the steel ring of the arena. The collection vial below the arena is shown as a dotted cylinder with the odour source represented by the blue circle. The glass sheet enclosing the arena is shown in light blue.

The pitfall bioassay arena (Fig. 2.1) consisted of a 4 cm high, 20 cm diameter stainless steel ring covered with a glass sheet. The floor of the arena was covered with a Whatman's paper disk (that was changed between experiments) with a 2.5 cm circular hole in the middle. A 7.5 cm tall glass collection vial was glued underneath this hole using a modified PVAc (polyvinyl acetate) emulsion wood adhesive. In each experiment, an odour source was added to a Whatman's paper disk and placed at the bottom of the collection vial. In experiments where DMD was used, 5 µl of 200 ng/µl DMD diluted in pentane was added to the paper disk. When wheat germ oil was tested, 5 µl of undiluted wheat germ oil was added the paper disk. When DMD and wheat germ oil were tested together, 5 µl of 200 ng/µl DMD in pentane and 5 µl of wheat germ oil were applied to the same paper disk. In all experiments 5 µl of pentane was added to paper disks as a negative control. Groups of twenty beetles (10 male and 10 females) were used in each replicate, with this number being chosen to give a large sample size without having so many beetles in the arena that the area around the pitfall trap became overcrowded. The number of beetles in each collection vial was then counted after two hours. The responses index (RI) for each compound was calculated using the formula RI = [(T − C)/Total] × 100, where T is the number

responding to the treatment, C is the number responding to the associated control, and Total is the number of insects released in each arena. An RI of 100 % indicates that all the beetles were captured in the treatment pitfall traps and none of them were captured in the control traps, with an RI of -100 % meaning the opposite. This is an established methodology for measuring the responses of insects in choice bioassays (Phillips et al., 1993; Mahroof and Phillips, 2007; Germinara et al., 2008; Richgels and Rollmann, 2012; Phoonan et al., 2014). All trials were conducted in complete darkness in a CT room at 20 °C between 10 AM and 5 PM

2.2.4. Two-choice arena bioassay

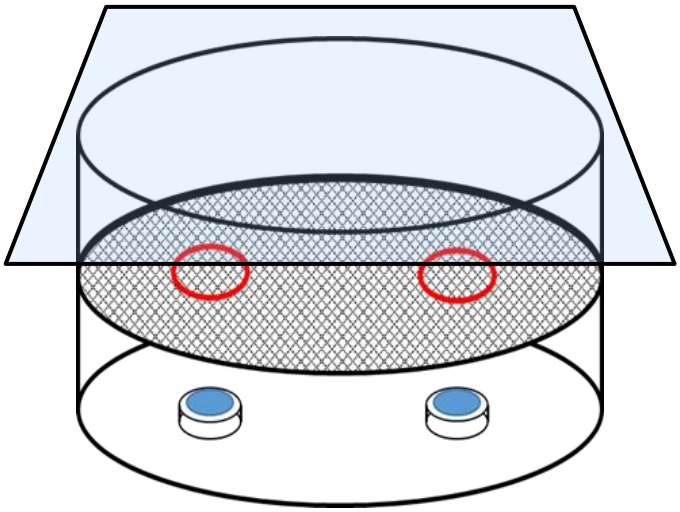

Figure 2.2. 3D schematic diagram of the two-choice arena bioassay.

The crosshatched area represents the mesh floor of bioassay arena. Above and below this mesh are the two steel rings that enclose the arena. The odour sources are shown in blue on raised platforms beneath the mesh. The red circles indicate the area above each odour source where the beetles were counted. The glass sheet enclosing the arena is shown in light blue.

The two-choice arena bioassay (Fig. 2.2) consisted of two, 4 cm high, 20 cm diameter stainless steel rings, placed on top of each other. The two rings were separated by a mesh gauze, which formed the floor of the arena. Two 4 cm diameter circles, centred at points 5 cm from the edge of the steel rings, were drawn at opposite sides of the arena. Odour sources were added to Whatman's paper disks and placed on raised platforms beneath each ring on the gauze, to allow the volatiles to diffuse through the gauze and into the

arena. In each experiment 5 µl of a test odour source, diluted to a given concentration in pentane, was added to one of the paper disks, and 5 µl of pentane was added to the other as a negative control. Groups of twenty beetles (10 male and 10 female) were placed inside and the arena was covered with a glass sheet. The movements of the beetles were recorded with a LUMIX® DMC-F5 digital camera for 10 minutes. The footage was reviewed and the number of beetles inside each of the gauze circles was counted at 30s intervals. The data from the resulting 20 time points were totalled to give an index of the amount of time beetles spent around the two different odour sources. As this measure was calculated from multiple observations of the same individuals the same RI calculated for the pitfall bioassays could not be used. A simpler index T – C was used instead (where T is the total number of times beetles were observed around to the test odour source and C is the total for the control). This index has also been previously used as a way to present the results of behavioural choice bioassays (Stevenson et al., 2017). All trials were conducted in a CT room under red light at 20 °C between 10 AM and 5 PM and the arena was cleaned with ethanol between each trial.

2.2.5. Four-way olfactometer

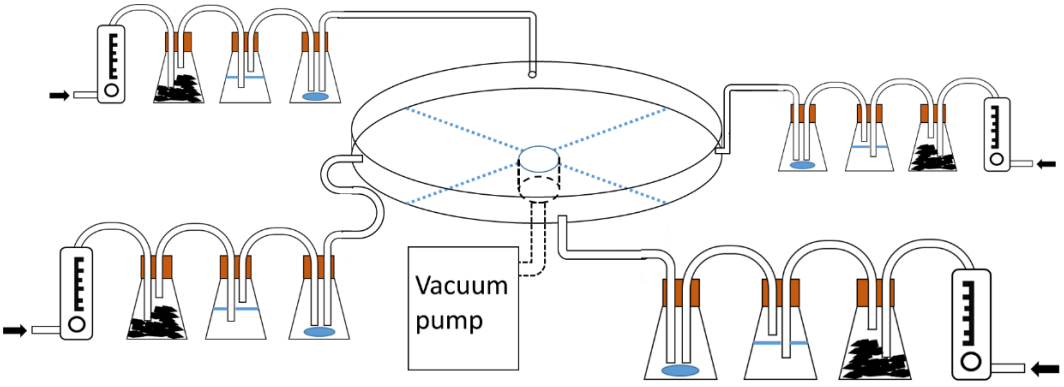

Figure 2.3. 3D schematic diagram of the four-way olfactometer.

The bioassay arena is represented by a circle in the centre divided by a blue dotted lines into the different zones around each odour source. A vacuum pump is shown connected to the centre of the arena. Each of the four corners of the bioassay arena are connected to a series of vials containing, in the following order: an odour source, distilled water and activated charcoal. The final vial in each series of vial is connected to an airflow metre, with the direction of the airflow through the olfactometer set-up shown with arrows.

The four-way olfactometer (Fig. 2.3) consisted of a circular central chamber at the centre of which was a hole connected to a vacuum pump. Equally spaced around the edge of the central chamber were four holes. These were each connected by PTFE (polytetrafluroethylene) tubing to three vials: the first containing a Whatman paper disk to which a given test compound was added, the second containing distilled water and the third containing activated charcoal. In each experiment 5 µl of a test volatile diluted to 200 ng/µl in pentane, or 5 µl of pentane as a negative control, were added to the paper disks. The final vial in each series of vials was connected to an airflow meter. This allowed air to enter each corner of the olfactometer at a controlled rate of 0.2 L/min. The central chamber was divided into four equally sized segments, each one centralised around one of the holes in the side of the arena. The arena was sealed by an acrylic sheet that covered the central chamber, which was secured in place with clamps. Beetles were intended to be placed into the tube connecting the vacuum pump to the 4-way olfactometer arena allowing them to climb into the centre of bioassay arena. However the beetles were unable to climb up the wall of the tube. This was essential to ensure the beetles entered the arena in the presence of airflow from all four odour sources and prevented the bioassay from being usable. The design of this bioassay is presented here to allow issues with this bioassay to be discussed in Section 2.4.3.

2.2.6. Y-tube olfactometer

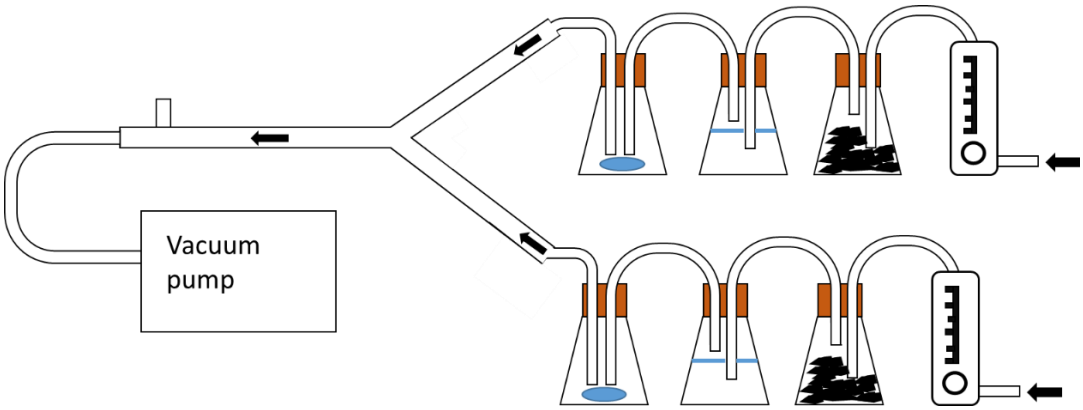

Figure 2.4. Schematic diagram of the Y-tube olfactometer.

The Y-shaped glass tube is shown attached to a vacuum pump at one end. The other two ends are each connected to a series of vials containing, in the following order: an odour source, distilled water and activated charcoal. The final vial in each series of vials is connected to an airflow metre, with the direction of the airflow through the olfactometer set up shown with arrows.

The Y-tube olfactometer apparatus (Fig. 2.4) consisted of a 20 cm long, 6 cm diameter, glass cylinder that branches in the middle to form a two-armed (Y-shaped) glass tube. The Y-tube was connected by PTFE tubing to a vacuum pump, which drew air through each of the two Y-arms. Each arm was in turn connected by PTFE tubing to three vials: the first containing a Whatman's paper disks to which an odour source was added, the second containing distilled water and the third containing activated charcoal. In each experiment 5 µl of a test volatile diluted to 200 ng/µl in pentane was added to one of the paper disks and 5 µl of pentane was added to the other as a negative control. The last vial in each series of vials was connected to an air-flow meter that allowed air to enter each arm of the olfactometer at a controlled rate of 0.2 L/min. The Y-tube olfactometer had a sealable hole on the main stem that allowed a beetle to be inserted while the vacuum pump was running. A single beetle was inserted into the Y-tube through this hole for each trial, and its movements were observed for five minutes. Once a beetle had walked 2 cm down one of the two branches of the Y-tube it was recorded as having chosen that arm of the olfactometer. If no choice was made within five minutes the beetle was deemed to be non-responsive and was discarded. The odorants connected to each arm of the olfactometer were switched every 10 trials to prevent the direction of the arms from potentially biasing the choices of the

beetles. The number of beetles that went down each arm of the olfactometer, independent of the odorant at each end, was also recorded, and no significant bias for one arm of the olfactometer over the other was shown in any of the experiments. Although the olfactometer was cleaned with ethanol between trials, only females were used as other researchers have suggested that the aggregation pheromone produced by males within the olfactometer could influence the behaviour of beetles used in subsequent trails (Ahmad, Daglish, et al., 2012). This was not a concern in the pitfall arena and two-choice bioassays described in this chapter as they had a paper or mesh flooring that could be easily replaced between trials, preventing a build-up of beetle produced odours in the bioassay arena. All trials were conducted in a 20 °C CT room under red light between 10 AM and 5 PM.

2.2.7. Statistics

For the still-air arena bioassays (pitfall bioassay and two-choice arena) the differences between the experimental treatments and their associated control treatments were tested using Mann–Whitney U tests. Non-parametric tests were used as the results from each bioassay were count data skewed by a large number of observations where zero or very few beetles were recorded. Many of the treatments also had small sample sizes that made testing the data for deviations from a normal distribution difficult.

As the data from both of these bioassays were analysed using multiple comparisons between treatment pairs there is an increased risk of type 1 errors, which could result in false positive results. The importance of controlling the false discovery rate in complex olfactometer experiments has been previously raised (Ricard and Davison, 2007). Bonferroni corrections have previously been applied when analysing pitfall data (Work et al., 2002; Hyvärinen et al., 2006; McCravy and Willand, 2007; Reddy et al., 2018) and the Benjamini-Hochberg procedure, recommended for use in ecological research involving repeated testing (García, 2003), has been used to adjust for conducting multiple comparisons in other choice bioassays involving insects (Peters, 2011; Kergunteuil et al., 2015; Lenschow et al., 2018). However, it should be noted that not every study that compared a large number of treatments using pitfall bioassays adjusted for multiple

comparisons (Pierce et al., 1990; Germinara et al., 2008). To address this issue, the P values of the Mann–Whitney U tests reported in this chapter have been adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). This methodology was chosen as it retains more statistical power than the Bonferroni procedure and has been recommended as a suitable methodology for many studies of ecology and evolution (Pike, 2011).

χ^2 goodness-of-fit tests were used to identify differences between the attraction to the experimental and control odour sources in the Y-tube olfactometer experiments. In each case the expected frequency of beetles responding to each odour source was 20 (i.e. an equal number of beetles responding to each of the odour sources). Previous research using Y-tube olfactometers have corrected the P values obtained from χ^2 test (von Hoermann et al., 2012) and paired-sample t-tests (Najar-Rodriguez et al., 2010) using the Benjamin-Hochberg procedure. Other researchers have applied the more conservative Bonferroni correction to adjust the P values of χ^2 tests (DeVries et al., 2019) and goodness-of-fit G-test (Gadino et al., 2012) of Y-tube olfactometer data. However, many previous papers tested the responses of insects in Y-tube olfactometers to many odour sources using multiple χ^2 goodness-of-fit tests without accounting for the inflated chance of type 1 errors (Ahmad, Daglish, et al., 2012; Piesik and Wenda-Piesik, 2015; Y. Wang et al., 2016; Wenda-Piesik et al., 2017; Yang et al., 2017; Cao et al., 2018; Giunti et al., 2018; Gerken et al., 2018). As before, the Benjamini-Hochberg procedure was used to adjust the P values of the χ^2 goodness-of-fit tests reported in this chapter.

All statistics were performed using SPSS 24 software.

2.3. Results

2.3.1. Pitfall bioassay

The RIs, and the results of Benjamini-Hochberg corrected Mann-Whitney U tests for each of the pitfall bioassay experiments, are shown in Table 2.1. The distributions of the data from these experiments are also shown as boxplots in Figures 2.5 to 2.8.

Table 2.1. The RIs (shown in bold) observed for four different *T. castaneum* strains tested for their attraction to DMD, wheat germ oil, and/or a combination of the two volatile sources, in pitfall bioassays.

The results of Benjamini-Hochberg corrected Mann–Whitney U tests comparing each of the test volatiles to its associated pentane negative control are also shown. Significantly different responses are indicated with *.

***T. castaneum* strain**	**Volatile(s)**		
	DMD	**Wheat germ oil**	**DMD and wheat germ oil**
San Bernardino	**-0.125%** Z = -0.774 ($n_1 = n_2 = 4$) P=0.627	-	-
Fera Laboratory	**-1%** Z = -0.899 ($n_1 = n_2 = 4$) P = 0.617	**5.667%** Z = -1.253 ($n_1 = n_2 =15$) P = 0.525	**15%** Z = -1.697 ($n_1 = n_2 = 8$) P = 0.45
CTC12	**10.5%** Z = -1.304 ($n_1 = n_2 = 10$) P = 0.525	**3.75%** Z = -0.897 ($n_1 = n_2 = 20$) P = 0.617	**22.25%*** Z = -3.491 ($n_1 = n_2 = 20$) P = 0.005
Wild UK	**0%** Z = -0.235 ($n_1 = n_2 = 10$) P = 0.938	**-1%** Z = -0.078 ($n_1 = n_2 = 10$) P = 0.938	**1.5%** Z = -0.194 ($n_1 = n_2 = 10$) P = 0.938

Figure 2.5 shows the responses of the San Bernardino strain to DMD and a pentane control. DMD was not found to be attractive, with the average response to DMD being slightly lower that the response to the pentane control. A very small number of beetles were captured overall by both the DMD and pentane baited pitfall traps.

Figure 2.6 shows the responses of the Fera laboratory strain to three different odour sources (wheat germ oil, DMD and a combination of wheat germ oil and DMD). None of the odour sources were found to be significantly more attractive than the pentane control. Although the combination of DMD and wheat germ oil gave a much larger RI (Table 2.1) than the constituent odours tested separately, the attraction was still not significantly different from the pentane control.

Similar results were observed when using the CTC12 strain: the combination of DMD and wheat germ oil was found to give a much stronger RI (Table 2.1) than when the two individual odour sources were tested alone (Fig. 2.7). However, in this strain the combination of DMD and wheat germ oil was found to be significantly more attractive than the pentane control.

When the same three odour sources were tested in the wild UK strain the RI to each of the compounds tested was negligible (Table 2.1), and none of the compounds tested were found to give a significantly different response to the pentane control (Fig 2.8).

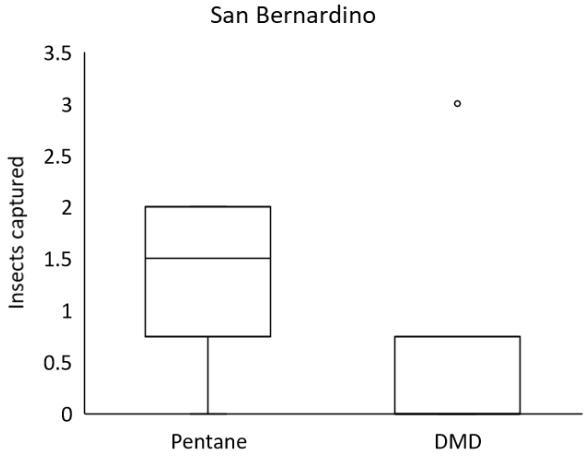

Figure 2.5. Boxplots showing the responses of San Bernardino strain *T. castaneum* to pitfall traps baited with either pentane or DMD.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicted with dots. Four replicate groups containing 20 beetles each (10 male and 10 female) were used for each odour source. No significant differences were found between the responses to DMD and the pentane control (see Table 2.1 for full statistics).

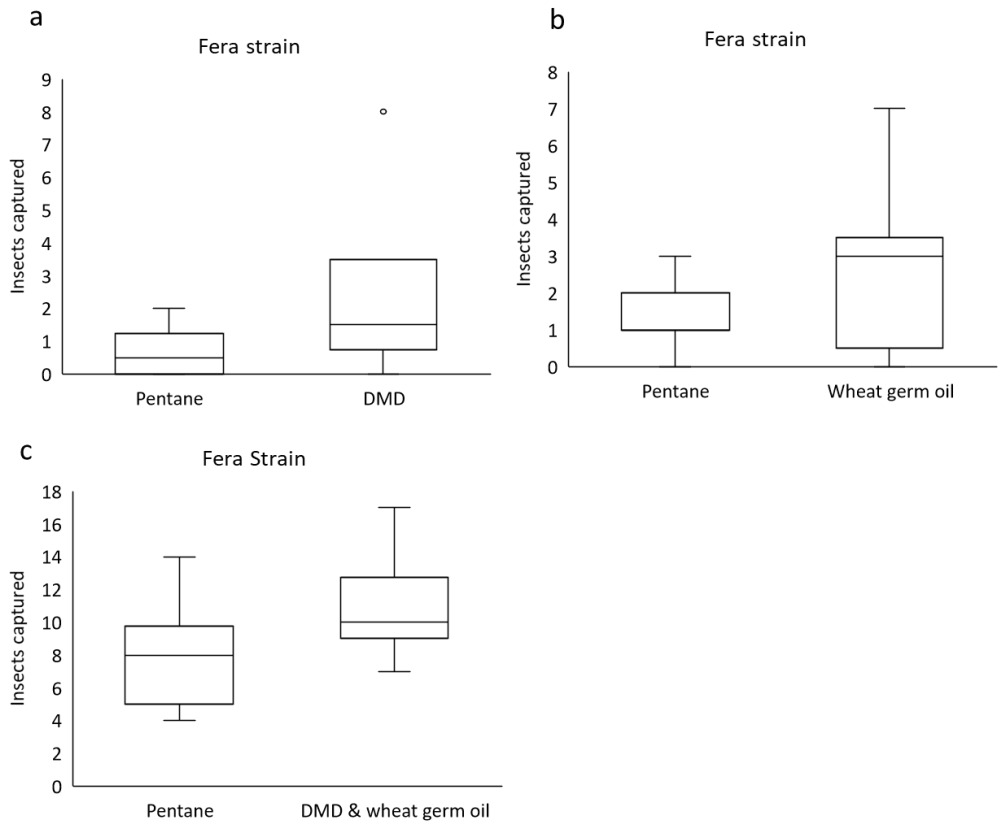

Figure 2.6. Boxplots showing the responses of Fera laboratory strain *T. castaneum* to pitfall traps baited with pentane and either: (a) DMD, (b) wheat germ oil, or (c) a combination of DMD and wheat germ oil.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Fifteen replicate groups containing 20 beetles each (10 male and 10 female) were used in (b), four groups were used in (a) and eight were used in (c). No significant differences were found between the distributions of the control and test volatiles in any of the experiments (see Table 2.1 for full statistics).

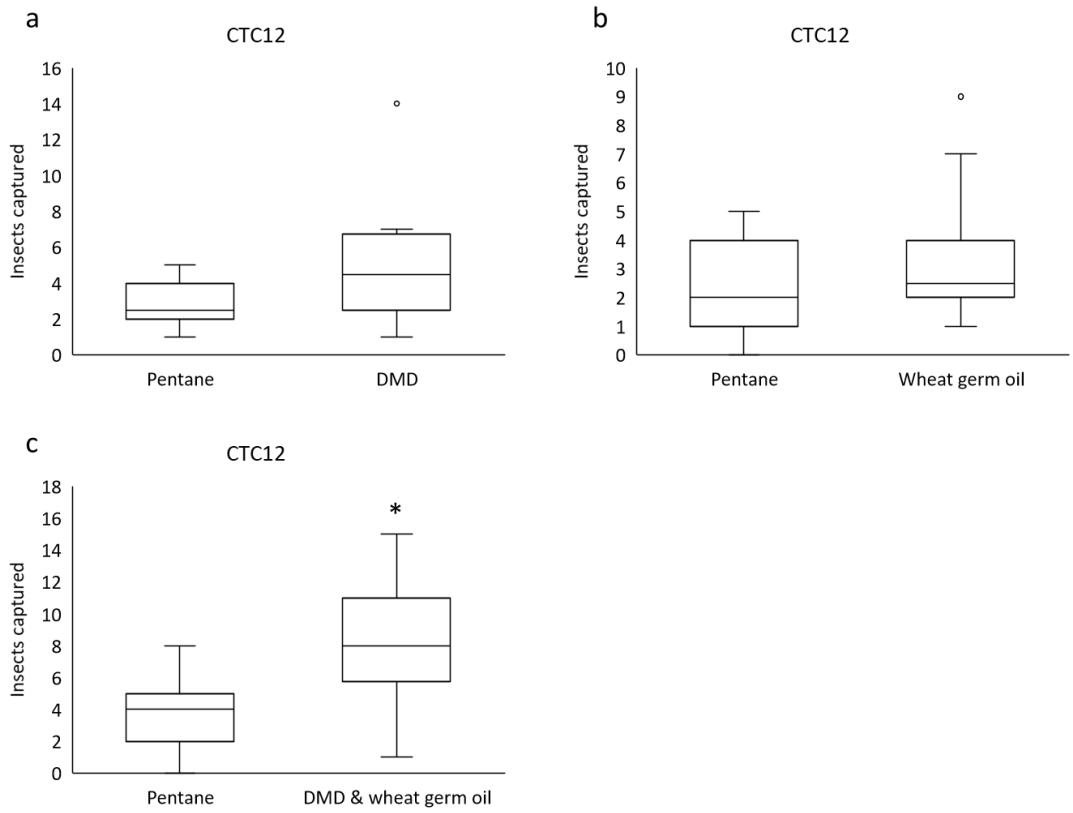

Figure 2.7. Boxplots showing the responses of CTC12 strain *T. castaneum* to pitfall traps baited with pentane and either: (a) DMD, (b) wheat germ oil, or (c) a combination of DMD and wheat germ oil.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Twenty replicate groups containing 20 beetles each (10 male and 10 female) were used for each odour source in (b) and (c), 10 groups were used in (a). Significant differences were found between the distributions of the control and test volatiles in (c) ($P = 0.005$) (see Table 2.1 for full statistics).

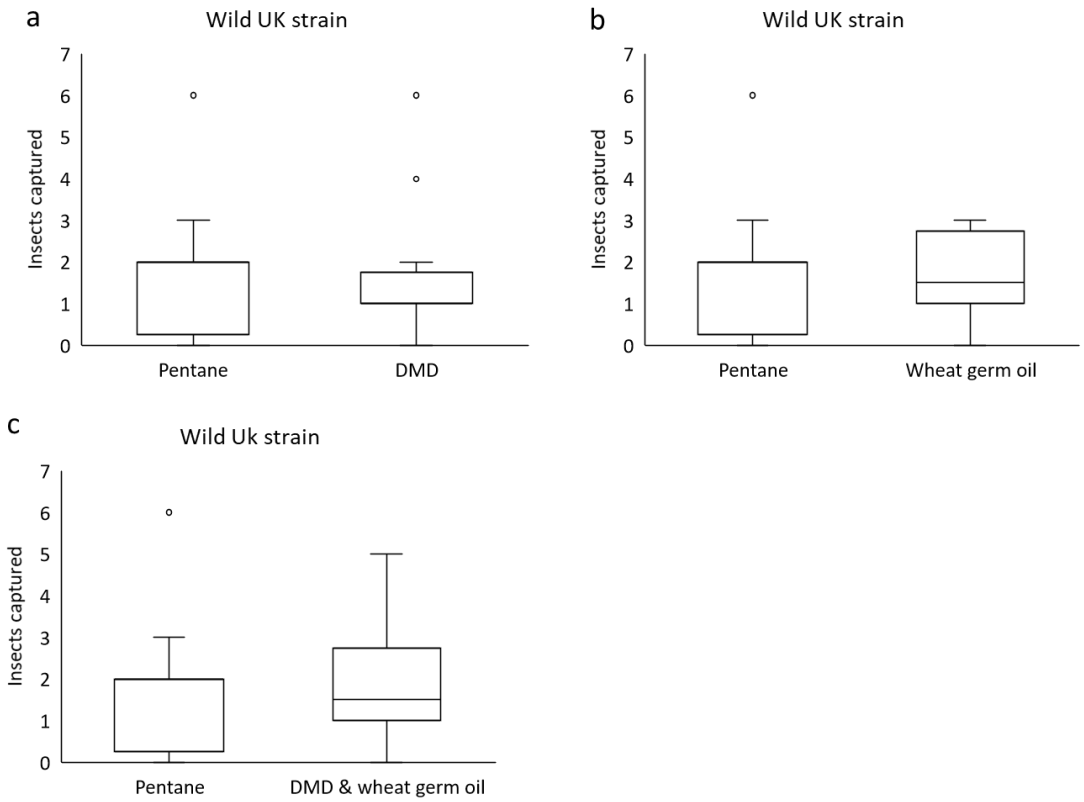

Figure 2.8. Boxplots showing the responses of wild UK strain *T. castaneum* to pitfall traps baited with pentane and either: (a) DMD (b) wheat germ oil, or (c) a combination of DMD and wheat germ oil.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetles each (10 male and 10 female) were used for each odour source in each experiment. No significant differences were found between the distributions of the control and test volatiles in any of the experiments (see Table 2.1 for full statistics).

2.3.2. Two-choice arena

The RIs, and the results of Benjamini-Hochberg corrected Mann-Whitney U tests for each of the two-choice arena bioassays, are shown in Table 2.2. The distributions of the data from these experiments are also shown as boxplots in Figures 2.9 to 2.13.

Table 2.2. The RIs (shown in bold) observed for CTC12 strain *T. castaneum* when tested for their attraction to a range of volatiles at different concentrations in two-choice arena bioassays.

Also shown are the results of Benjamini-Hochberg corrected Mann–Whitney U tests comparing the response to each of the test volatiles with its associated pentane negative control. Significantly different responses are indicated with *.

Volatile tested	Concentration				
	Undiluted	**20 µg/µl**	**2 µg/µl**	**200 ng/µl**	**20 ng/µl**
Wheat germ oil	**25** Z = -1.714 ($n_1 = n_2 =$ 10) P = 0.173	-	-	-	-
DMD	**-75 *** Z = -3.171 ($n_1 = n_2 =$ 10) P = 0.017	**-35** Z = -1.636 ($n_1 = n_2 =$ 10) P = 0.173	-	**-30** Z = -1.373 ($n_1 = n_2 =$ 10) P = 0.241	**11** Z = -0.766 ($n_1 = n_2 =$ 10) P = 0.545
Lure	**162 *** Z = -2.917 ($n_1 = n_2$ =10) P = 0.017	**36** Z = -2.372 ($n_1 = n_2$ =10) P = 0.058	**128 *** Z = -3.106 ($n_1 = n_2 =$ 10) P = 0.017	**14** Z = -0.418 ($n_1 = n_2 =$ 10) P = 0.676	**-19** Z = -1.674 ($n_1 = n_2 =$ 10) P = 0.173
1-Octen-3-ol	**-8** Z = -1.674 ($n_1 = n_2$ =10) P = 0.173	**-25** Z = -1.526 ($n_1 = n_2 =$ 10) P = 0.196	**13** Z = -0.916 ($n_1 = n_2 =$ 10) P = 0.470	**-13** Z = -0.745 ($n_1 = n_2 =$ 10) P = 0.545	-
3-Methyl-1-butanol	-	**9** Z = -1.648 ($n_1 = n_2$ =10) P = 0.602	-	-	-
3-Octanone	-	**68 *** Z = -2.920 ($n_1 = n_2 =$ 10) P = 0.017	-	-	-
Ethyl acetate	-	**-18** Z = -1.648 ($n_1 = n_2 =$ 10) P = 0 .173	-	-	-

When the responses of CTC12 *T. castaneum* were tested against wheat germ oil, although wheat germ oil gave a positive RI, it was not significantly more attractive than the pentane control (Fig. 2.9).

The response of the CTC12 strain to DMD was tested at four different concentrations with attraction to DMD appearing to decrease as its concentration increased (Fig. 2.10). However, only the highest concentration of DMD appeared to be significantly repulsive.

The response of the CTC12 strain to the commercial lure was tested at five different concentrations (Fig. 2.11), and there appeared to be a general trend of increased attraction as the concentration of the lure was increased, with the undiluted and 2 µg/µl concentration found to be significantly more attractive than the pentane control. However the 20 µg/µl concentration was not found to be significantly more attractive than the pentane control and gave a lower response than the weaker 2 µg/µl dilution.

The responses of CTC12 beetles to four different fungal compounds (1-octen-3-ol, 3-methyl-butanol, 3-octanone and ethyl ethanoate) was also tested in a two-choice area. 1-octen-3-ol was also tested at four concentrations and no clear pattern was found across the concentrations tested, with none of them found to give a significantly different response compared to the pentane control odour source (Fig. 2.12). The other fungal compounds were tested at a 20 µg/µl concentration, and only 3-octanone was found to significantly more attractive than the pentane control odour source (Fig. 2.13).

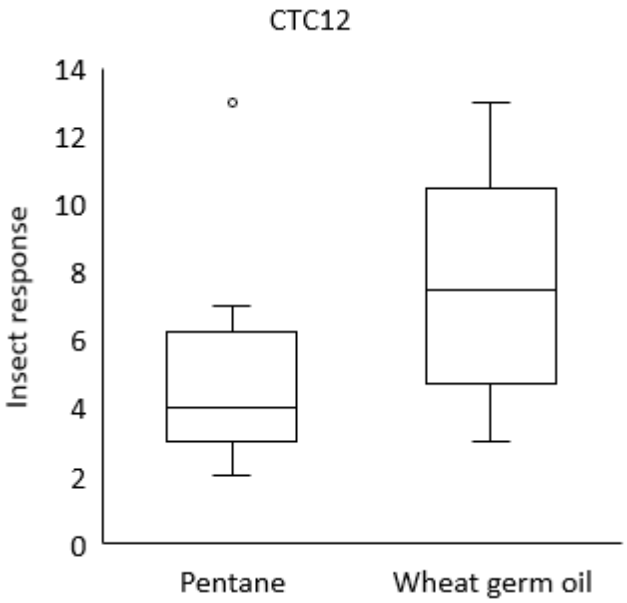

Figure 2.9. Boxplots showing the amount of time CTC12 strain *T. castaneum* spent around two different odour sources, pentane or wheat germ oil.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetles each (10 male and 10 female) were used. No significant differences were found between the distributions of the two groups (see Table 2.2 for full statistics).

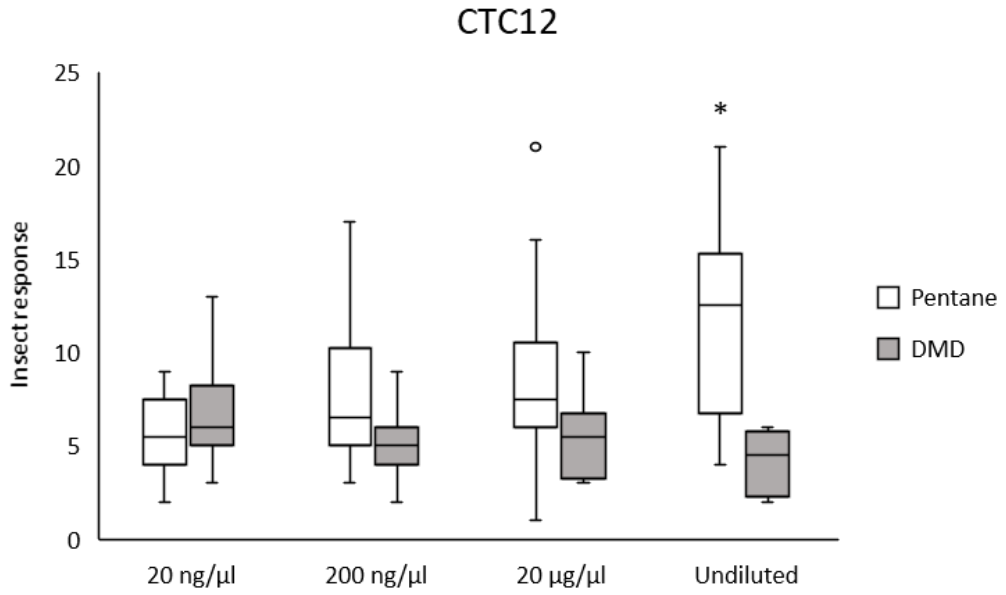

Figure 2.10. Boxplots showing of the amount of time CTC12 strain *T. castaneum* spent around two different odour sources, pentane (control) and DMD (diluted in pentane) used at four different concentrations: 20 ng/µl, 200 ng/µl, 20 µg/µl and undiluted.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetles each (10 male and 10 female) were used. Undiluted DMD was found to be significantly less attractive than the pentane control (see Table 2.2 for full statistics).

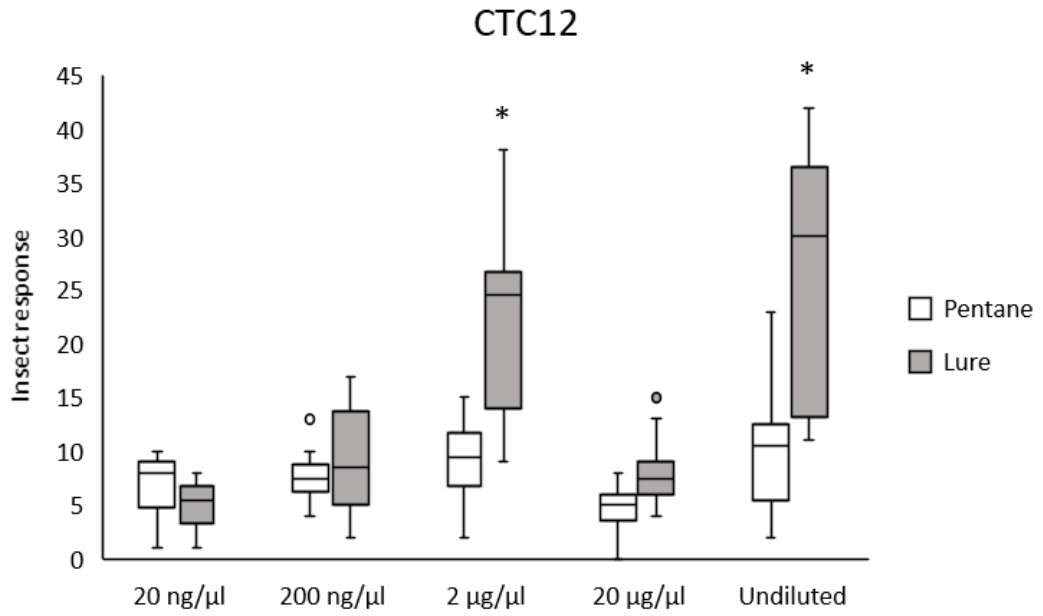

Figure 2.11. Boxplots showing the amount of time CTC12 strain *T. castaneum* spent around two different odour sources; pentane (controls), and a commercial lure (diluted in pentane) used at five different concentrations: 20 ng/µl, 200 ng/µl, 2 µg/µl, 20 µg/µl and undiluted.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetles each (10 male and 10 female) were used. The lure was found to be significantly more attractive than the pentane control when undiluted and at a 2 µg/µl concentration (see Table 2.2 for full statistics).

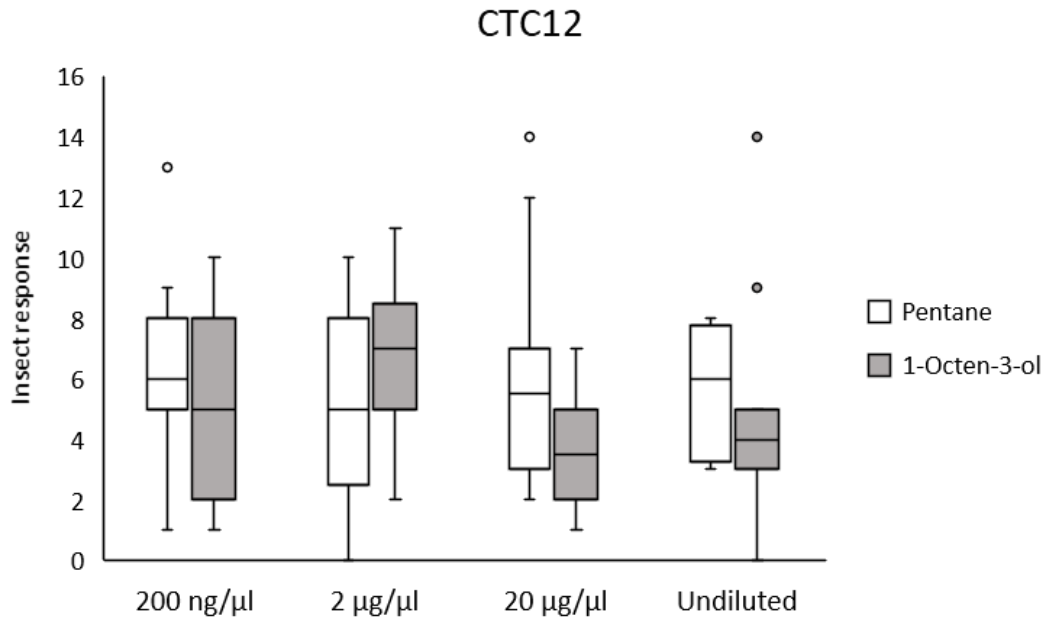

Figure 2.12. Boxplots showing the amount of time CTC12 strain *T. castaneum* spent around two different odour sources; pentane (controls), and 1-octen-3-ol (diluted in pentane) used at four different concentrations: 200 ng/µl, 2 µg/µl, 20 µg/µl and undiluted.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetles each (10 male and 10 female) were used. No significant differences were found between the responses to the different 1-octen-3-ol concentrations and the pentane controls (see Table 2.2 for full statistics).

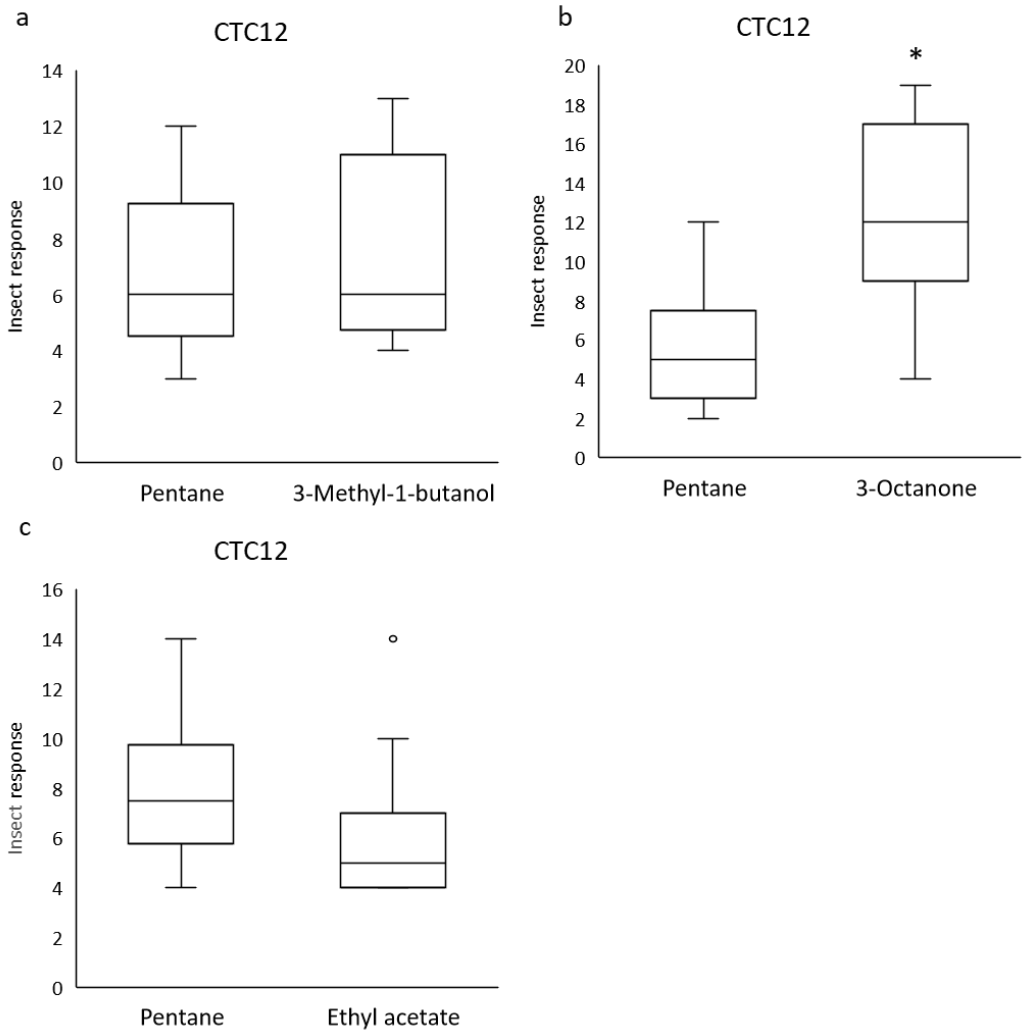

Figure 2.13. Boxplots showing the amount of time CTC12 strain *T. castaneum* spent around two different odour sources; pentane (controls), and one of three different fungal volatiles: (a) 3-methyl-1-butanol, (b) 3-octanone, and (c) ethyl acetate.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetles each (10 male and 10 female) were used. Only 3-octanone was found to be significantly more attractive than the pentane control (see Table 2.2 for full statistics).

2.3.3. Y-tube olfactometer

The responses of two different *T. castaneum* strains, San Bernardino and CTC12, were initially tested against DMD and a pentane control in the Y-tube olfactometer (Fig. 2.14). Only the CTC12 strain was found to be significantly more attracted to DMD than the pentane control.

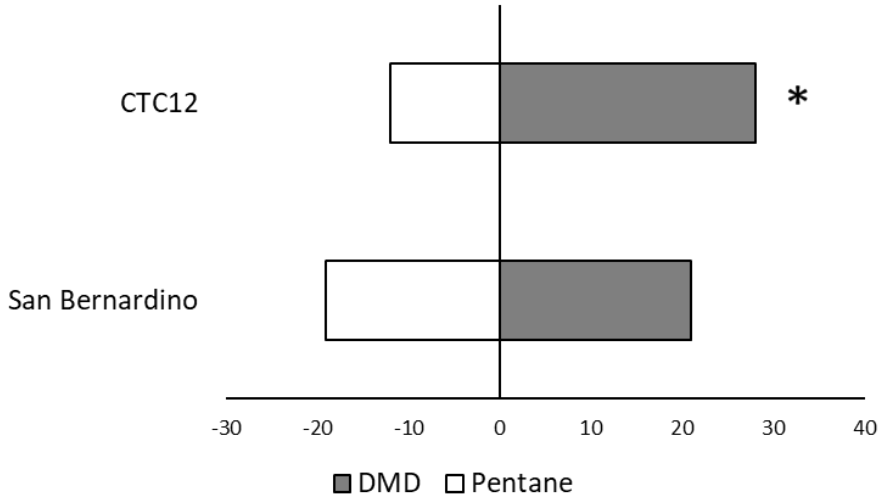

Figure 2.14. The responses of 40 female San Bernardino and CTC12 strain *T. castaneum* to DMD and pentane in a Y-tube olfactometer bioassay.

CTC12 ($\chi^2 = 6.4$, df = 1 P = 0.011), San Bernardino ($\chi^2 = 0.1$ df = 1 P = 0.752). Groups with statistically significant χ^2 goodness-of-fit values are indicated with *.

After identifying the CTC12 strain as being more responsive to DMD, the responses of CTC12 beetles were tested against 13 different fungal compounds (Fig. 2.15 and Table 2.3). Three of the compounds, 1-octanol, 3-methyl-1-butanol, and 3-octanone were found to be significantly more attractive than the pentane control.

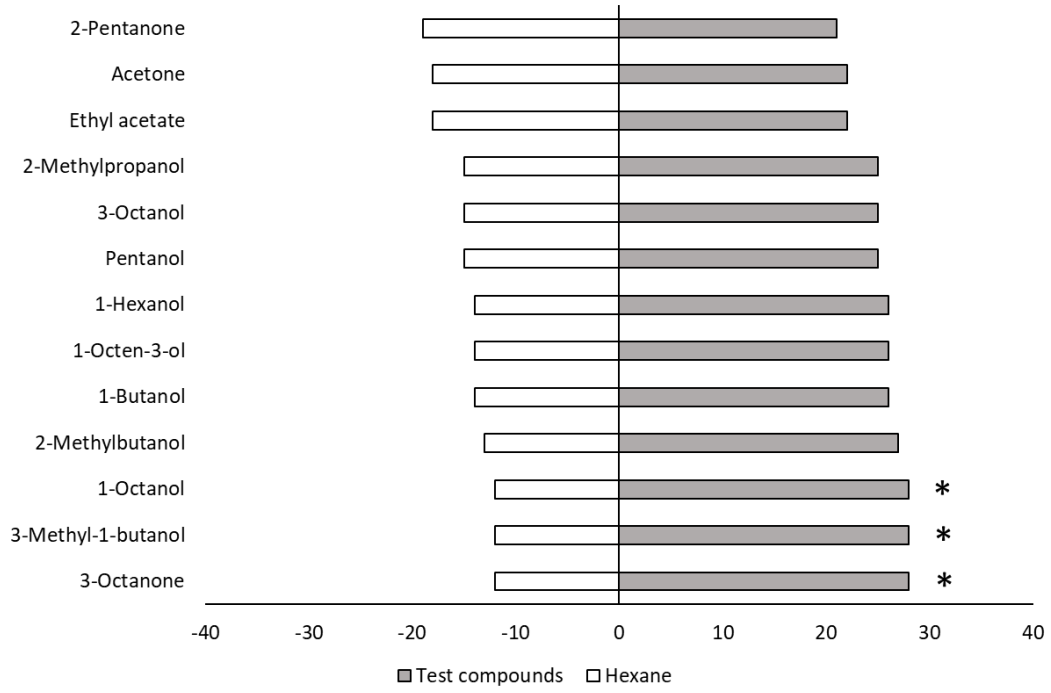

Figure 2.15. The responses of 40 female San Bernardino and CTC12 strain *T. castaneum* to 13 different fungal volatiles or hexane in a Y-tube olfactometer bioassay.

Groups with statistically significant χ^2 goodness of fit values are indicated with * (see Table 2.3 for full statistics).

Table 2.3. The number of *T. castaneum* attracted to different volatiles in Y-tube olfactometer bioassays.

The key statistics from Benjamini-Hochberg adjusted χ^2 goodness-of-fit tests are also presented, comparing the observed number of insects that were attracted to either the test volatile or pentane control arm of the olfactometer to the expected frequency of 20 for each.

Volatile	Beetles attracted	N	χ^2	df	P
2-Pentanone	21	40	0.752	1	0.752
Acetone	22	40	0.527	1	0.571
Ethyl acetate	22	40	0.527	1	0.571
2-Methlypropanol	25	40	0.114	1	0.148
3-Octanol	25	40	0.114	1	0.148
Pentanol	25	40	0.114	1	0.148
1-Hexanol	26	40	0.058	1	0.108
1-Octen-3-ol	26	40	0.058	1	0.108
1-Butanol	26	40	0.058	1	0.108
2-Methylbutanol	27	40	0.027	1	0.088
1-Octanol	28	40	0.011	1	0.0476
3-Methyl-1-butanol	28	40	0.011	1	0.0476
3-Octanone	28	40	0.011	1	0.0476

2.4. Discussion

Based on the results of the experiments in this chapter several conclusions can be drawn regarding the usefulness of different bioassays for testing the behavioural responses of *T. castaneum.* Some initial observations can also be made regarding the attractiveness of fungal volatiles and the effects of inter-strain variation in *T. castaneum* and these ideas are explored more extensively in later chapters.

2.4.1. Pitfall bioassay

The first pitfall bioassays experiments in this chapter tested the responses of San Bernardino strain *T. castaneum* to DMD. This strain was not significantly attracted to DMD and was found to be largely inactive in the olfactometer as the beetles spent the majority of the time remaining around the edge of arena. Due to the lack of movement in the bioassay from this strain they were not used in further pitfall bioassay experiments. This was followed up with experiments testing the responses of Fera laboratory, CTC12 and wild UK laboratory strain beetles to DMD, wheat germ oil, and a combination of the

two odour sources. Across all of these treatment groups only the attraction of the CTC12 strain to a combination of DMD and wheat germ oil was found to be significantly different than the response to the pentane control. Most other treatments gave a very low response index, although these strains were found to be more motile in the arena than the San Bernardino strain.

Among all the strains tested in the pitfall bioassay, DMD on its own was not found to be significantly attractive to any of them. This lack of response from any of the strains is surprising given that the use of pheromone baited pitfall traps is an established method of *Tribolium* pest management (Mullen, 1992; Campbell, 2012), and the same dosage of DMD had previously been shown to be attractive to *T. confusum* walking in a wind tunnel olfactometer (Verheggen et al., 2007). However, as much of the data on the responses of *T. castaneum* to pheromone lures comes from traps set in mills and warehouse environments (Mullen, 1992; Hawkin et al., 2011; Semeao et al., 2013), it is possible that this does not reflect the responses of *T. castaneum* in smaller, more enclosed bioassays, possibly resulting in a lower capture rate seen here.

Wheat germ oil on its own was also not found to be attractive to any of the strains tested. This was also surprising as the odour of wheat germ oil is associated with a major food source for this species. When the responses of other stored-product insect species were tested against food odours in a similar pitfall bioassay they were found to give much stronger responses. For example, the cigarette beetle *Lasioderma serricorne* showed mean RIs as high as 90% to host food materials, including cereal grains and spices, with the response to cracked whole wheat specifically giving an RI of about 40% (Phoonan et al., 2014). *Lasioderma serricorne* was also found to have a response index of about 40% to wheat germ oil in another similar pitfall bioassay experiment (Mahroof and Phillips, 2007). When the responses of the sawtoothed grain beetle *Oryzaephilus surinamensis* were tested against individual volatiles from rolled oats certain volatiles gave an RI as high as 50% (Mikolajczak et al., 1984) and individual cereal volatiles gave a response as high as 60% when tested in pitfall bioassays using the grain weevil *Sitophilus granarius* (Germinara et al., 2008).

Across each of the *T. castanuem* strains tested in this study, wheat germ oil and DMD in combination were found to be the most attractive odour source. However, it was only significantly more attractive than the pentane control for the CTC12 strain. Wheat germ oil and DMD in combination being more attractive than either of the compounds when tested alone is not surprising. A synergistic effect of combining food and pheromone odours has already been demonstrated in *T. castaneum* and other stored product insects, as combinations of pheromone and food volatiles they were shown to be more attractive together than either was alone when tested against *T. castaneum* and *S. oryzae* in two-choice pitfall bioassays (Phillips et al., 1993). However, unlike in this study, wheat germ oil on its own was also found to be significantly attractive.

The results of these experiments also provide early evidence that variation may exist between the behavioural responses of different *T. castaneum* strains. For example, the CTC12 strain appeared to be more responsive than the other strains, being the only strain that was significantly attracted to the combination of wheat germ oil and DMD. The responses of the wild UK strain were also striking, with the strain giving a negligible response to all of the compounds tested. This contrasts with the elevated response to the DMD and wheat germ oil combination shown by the other strains. However, as the wild UK strain did not respond to any of the volatiles tested it is possible that the lack of response may be due to motility differences in this strain and not due to a lack of attraction to the odours, as this strain appeared largely immobile in the bioassay arena. As such, after a more active recently caught wild population from Zimbabwe was sourced, the UK wild population was no longer used.

Overall this bioassay appeared to be rather ineffective at detecting attraction to odour sources by *T. castaneum*. This bioassay did not identify a significant attraction to DMD, and the only significant response found (the attraction of CTC12 beetles to a combination of DMD and wheat germ oil) was relatively small compared to the responses of other stored product insects in similar bioassays reported in earlier studies. As the bioassays tested groups of

beetles it also required more animal husbandry than some of the other bioassays.

2.4.2. Two-choice arena

The two-choice arena was used to test the responses of CTC12 strain *T. castaneum* to several known attractants and ecologically relevant odour sources. As only the CTC12 strain gave a significant behavioural response in the pitfall bioassay, only this strain was tested using the two-choice arena. As this bioassay was only monitored for ten minutes it was time-efficient to test the majority of the compounds across a range of concentrations. Two compounds that had previously been tested using this strain in the pitfall bioassay - wheat germ oil and DMD - were also tested in the two-choice arena. As the design of this bioassay was similar to the pitfall bioassay in many ways, the responses to these compounds was expected to be similar across both bioassays. In both bioassays wheat germ oil was found to elicit a positive response index but not to a degree that it was significantly more attractive than the pentane control. DMD was tested at a series of concentrations and the response was found to decrease as the concentration was increased. At the highest concentration of DMD there was evidence of repulsion, with beetles spending significantly more time around the pentane control odour source. Other experiments using *T. castaneum* have shown that the responses to host volatiles such as DMD and benzoquinones decrease at higher concentrations (Verheggen et al., 2007). However, it is surprising that in the two-choice arena no significant attraction was found to DMD at any concentration, as DMD is commonly used as an attractant in *Tribolium* lures. A commercial *Tribolium* lure was also tested at five different concentrations, and there appeared to be a general trend for increasing attraction with concentration. The trend was not perfect however, as although the undiluted and 2 µg/µl concentration was attractive, the 20 µg/µl concentration was not found to be significantly attractive. It is possible that this deviation from the trend is merely due to random variation in the behavioural responses of individuals, and that if the experiment was repeated this concentration would be found to be significantly attractive.

After demonstrating that this bioassay could detect significant behavioural responses by *T. castaneum* to some known attractants, the responses of CTC12 beetles were tested against four different fungal compounds: 1-octen-3-ol, 3-methyl-1-butanol, 3-octanone and ethyl acetate. These initial experiments were conducted to identify if this bioassay could detect significant responses to these odour sources, before systematically testing the responses of *T. castaneum* to individual fungal and wheat germ oil volatiles, in later chapters. Three of the fungal volatiles tested - 3-octanone, 1-octen-3-ol and 3-methylbutanol - had previously been shown to be attractive to bark beetles in two-choice pitfall olfactometers (Pierce et al., 1991). The response to 1-octen-3-ol was tested at a range of concentrations and there was no clear pattern across the responses, with none of them found to be significantly different to the pentane control response. The other three fungal compounds were tested at one concentration and 3-octanone was found to be significantly more attractive than the pentane control. This indicates that this bioassay can detect significant attraction by *T. castaneum* in response to exposure to individual environmental VOCs.

Overall this bioassay appeared to be capable of detecting significant behavioural responses to known attractants, such as a commercial lure and individual fungal VOCs. However, it did not detect a significant attraction to DMD at any of the concentrations tested. This bioassay was also less time consuming than the pitfall bioassays as significant differences could be found after only observing the insects for ten minutes, in contrast to the pitfall bioassays where data was collected after two hours.

2.4.3. Four-way olfactometer

Although attempts were made to test the responses of *T. castaneum* beetles to DMD and fungal volatiles in the four-way olfactometer, the results of these experiments are not reported due to technical issues related to how beetles were introduced into the bioassay arena. The beetles were found to be unable to enter the arena through the entrance at the centre of the arena. Mesh was placed around the entrance to the chamber in an attempt to help facilitate beetle movement into the olfactometer chamber but this did not help. This meant that beetles could not enter the arena in the presence of airflow from

all of the odour sources. As such, it is possible that the beetles would not encounter airflow from each of the odour sources and this would bias their behaviours inside the bioassay. A four-way olfactometer had been previously used to demonstrate a repulsive effect of certain plant secondary compounds in *T. castaneum*, however it is not clear how beetles entered the olfactometer in this experiment (Ukeh and Umoetok, 2011). As a result of this issue valid experiments using this bioassay could not be conducted.

2.4.4. Y-tube olfactometer

Initial experiments using the Y-tube olfactometer tested the responses of two *T. castaneum* strains, San Bernardino and CTC12. Female *T. castaneum* have previously been shown to be strongly attracted to DMD in Y-tube olfactometer bioassays (Stevenson et al., 2017; Gerken et al., 2018), however these studies tested different *T. castaneum* strains to the ones used in this thesis. In the Y-tube experiments described in this chapter not all the strains tested were found to be attracted to DMD. The San Bernardino strain was not found to be significantly attracted to DMD in the Y-tube olfactometer. When the responses of the San Bernardino strain were tested against the same DMD concentration what was tested in the pitfall bioassays it was also not found to be significantly attractive. The responses of the CTC12 strain to DMD in the Y-tube olfactometer were remarkably different, with DMD being found to be significantly attractive. This contrasts with the previous bioassays in this chapter where the same concentration of DMD was found to be no more attractive than the pentane control. This could indicate that the Y-tube olfactometer is more efficient at detecting behavioural responses in *T. castaneum*. It could also indicate that the beetles change their behaviours based upon the bioassay, as *T. castaneum* has previously been shown to respond differently to the same odours depending on the bioassay used (Gerken et al., 2018).

The movements of the two strains within the olfactometer were also noticeably different. San Bernardino beetles had significant problems navigating the olfactometer, frequently becoming stuck on their backs after falling over while attempting to climb up the glass walls of the olfactometer. This happened much less frequently with the CTC12 strain. This is similar to the problems

with the San Bernardino strain being less mobile in the pitfall bioassays. It is possible that this behaviour is due to DMD being less attractive to the San Bernardino strain, resulting in the beetles being less motivated to move through the olfactometer and more likely to try to crawl up the walls of the olfactometer to try and escape. Other authors have described the need to line the bottom of Y-tube olfactometers with a thin layer of coarse sand to facilitate movement (Campbell and Hagstrum, 2002; Ahmad, Daglish, et al., 2012). This suggests that other researchers also had difficulty getting *Tribolium* beetles to move the desired way in olfactometers bioassays.

After demonstrating that the CTC12 strain responded to DMD in the Y-tube olfactometer, the responses of this strain were also tested to a range of fungal volatiles. Although fungal odours have been demonstrated to be attractive to *T. castaneum* in Y-tube olfactometer bioassays (Ahmad, Daglish, et al., 2012) and field trials (Ahmad, Daglish, et al., 2013) their responses to specific volatile compounds from these sources have not been tested individually. Of the compounds tested, three of them - 1-octanol, 3-methyl-1-butanol, and 3-octanone - were found to be significantly attractive. Two of the fungal volatiles – 3-methyl-1-butanol and 3-octanone - had been previously tested in this chapter using the two-choice bioassay. 3-octanone was found to be significantly attractive in both of the bioassays, however 3-methyl-1-butanol was only found to be significantly attractive in the Y-tube olfactometer. This presents more evidence that the responses of *T. castaneum* to different odour sources can vary depending on the bioassay used to test them and suggests that the Y-tube olfactometer may be more able to detect behavioural responses exhibited by *T. castaneum*. As previously noted, the compounds 1-octen-3-ol and 3-octenone have previously been tested in two-choice pitfall olfactometers using other stored-product insects, and both were found to be attractive (Pierce et al., 1991). However, in the Y-tube olfactometer experiments in this study only 3-octenone, not 1-octen-3-ol, was found to significantly attractive to *T. castaneum*.

Overall the Y-tube olfactometer experiments demonstrate that this bioassay is capable of detecting significant attractions to both known attractants (such as DMD) and volatiles of interest to this study (such as fungal volatiles). As

40 replicate trials were sufficient to detect statistically significant responses to odour sources, and each trial took no more than five minutes to conduct, these bioassays were less time consuming than some of the other bioassays used in this chapter. As the Y-tube olfactometer tested individual beetles, rather than groups of beetles, it also required less beetles than the other bioassays, reducing the amount of time spent on insect husbandry. Testing individual beetles may also be advantageous, as the beetles will not be influenced by other individuals in the same olfactometer. However, as it has been suggested that this bioassay may only be suitable for testing the responses of female beetles (which do not produce aggregation pheromone) (Ahmad, Daglish, et al., 2012), only female beetles were used in the Y-tube olfactometer in this study. If differences exist between the responses of male and female *T. castaneum* to environmental volatiles it may be necessary to develop a different bioassay for testing the responses of males, and to enable a fair comparison between the responses of males and females.

2.4.5. Conclusion

After using four different olfactometers to test the responses of several *T. castaneum* strains to a variety of known *Tribolium* attractants and VOCs of interest, the Y-tube olfactometer bioassay was identified as being the most effective and efficient bioassay. The three other behavioural bioassays either did not identify a significant behavioural response to known attractive compounds or had clear issues with their design that prevented the collection of useful data. Although the pitfall bioassay and the two-choice arena identified a significant attraction to some of the known attractants, only the Y-tube olfactometer identified a significant attraction to the commonly used *Tribolium* attractant, DMD. The Y-tube olfactometer also appeared to be more able to detect significant responses to fungal VOCs as, when the responses of CTC12 strain beetles were tested against 3-octanone and 3-methyl-butanol using both the Y-tube olfactometer and two-choice bioassay, only in the Y-tube olfactometer were both compounds found to be significantly attractive. This suggests that the Y-tube olfactometer is the bioassay most able to detect behavioural responses to these compounds and is therefore the most useful for identifying new *T. castaneum* attractants. There are also practical advantages to using the Y-tube olfactometer over other bioassays, regarding

the amount of time needed to conduct these experiments and the reduced number of beetles required.

However, it should be noted that the responses of *T. castaneum* were not tested against fungal VOCs using the pitfall bioassay or four-way olfactometer. As significant deficiencies were found with the responses of beetles in initial experiments using these bioassays, research effort was instead focused on the other olfactometers. It is therefore possible that either of these olfactometers could also be capable of detecting significant responses to environmental VOCs. As all the bioassays tested in this chapter have been shown by other researchers to be capable of detecting significant behavioural responses from *T. castaneum* it is also possible any of the olfactometers could have been more effective under modified conditions.

The experiments conducted in this chapter also allow for some initial inferences to be made regarding how *T. castaneum* may locate food sources in stored-product environments. The fact that several fungal VOCs were found to be significantly attractive to *T. castaneum* suggests that they are important cues that could be used to locate rotten or damaged grains in stored-product warehouses. As only a few of the compounds tested were found to be attractive to *T. castaneum* this suggests that they may locate food sources by responding to only a small number of key food-source associated VOCs.

Comparing the responses of different strains in the pitfall and Y-tube olfactometer bioassays provides early evidence that inter-strain variation in the behavioural responses of *T. castaneum* may exist. In both experiments, significant attraction was only observed in the CTC12 strain. Other researchers have also presented recent evidence that the responses of *T. castaneum* to commercial lures can vary across different strains (Gerken et al., 2018). The diminished responses shown by the wild UK strain in pitfall bioassays also suggests that there may be inter-strain variation in the responses of *T. castaneum* to important olfactory cues. If *T. castaneum* behavioural responses to other odour sources also vary by strain this could have pest management implications, as the effectiveness of a lure will be severely limited if it is not attractive to some wild *T. castaneum* populations.

Having identified an olfactometer and strain that appears to be suitable for testing the responses of *T. castaneum* to environmental VOCs, more systematic testing of the responses of *T. castaneum* to wheat germ oil and fungal VOCs was conducted. The results of these experiments are reported in Chapters 3 and 4. The aim of these experiments was to identify novel attractive volatile blends that could be incorporated into a pest management lure. The strains and bioassays used in this chapter also informed the behavioural experiments conducted in Chapter 5, testing the effects of knocking-down expression of the important olfactory co-receptor Orco using RNAi.

Chapter 3
The antennal responses of *Tribolium castaneum* to common environmental volatile organic compounds

3.1 Introduction

As *T. castaneum* is a secondary, or external-feeding, stored product pest (Dawson, 1977; Phillips and Throne, 2010), feeding primarily on milled, broken or rotten grain kernels, volatile organic compounds (VOCs) from these sources could be used by the insect as indicators that grains are in a suitable condition to be fed upon. VOCs found within the internal wheat germ could indicate the nearby presence of milled or broken grain kernels, whereas the VOCs produced by grain-associated fungi could indicate nearby rotten grains. If *T. castaneum* has evolved to be attracted to these odours they could prove useful in developing an improved lure to capture *T. castaneum* and other secondary stored-product pests. *Tribolium castaneum* is known to be attracted to the odours of wheat kernels (Seifelnasr et al., 1982), wheat germ oil (Phillips et al., 1993) and grain-associated fungi (Ahmad, Daglish, et al., 2012; Ahmad, Ridley, et al., 2013; Ahmad, Daglish, et al., 2013). However, little research has been conducted to identify which specific compounds from these sources are responsible for this attraction, with one previous experiment testing the responses of *T. castaneum* to three grain volatiles not finding any of them to be significantly attractive (Phillips et al., 1993).

To determine if any wheat germ oil or fungal associated volatiles have the potential to be attractive to *T. castaneum*, a diverse range of compounds representing some of the most abundant compounds from these sources (see Table 3.1 and Section 3.2.2 for more details) were tested using electroantennography (EAG). EAG is an established electrophysiological technique for recording the response of an insect's antenna to different odour sources (Syntech, 2004; Beck et al., 2012; Olsson and Hansson, 2013). EAG measures the change in microvoltage across an insect antenna, caused by the depolarisation of olfactory neurones in response to olfactory receptor activation, allowing the electrophysiological response elicited by a specific odour source to be quantified (Syntech, 2004; Olsson and Hansson, 2013). EAG can be performed using intact live insects or freshly excised antennae.

If the specimen preparation is of a high quality, EAG can be used to record responses for extended periods of time, allowing the responses of the same antenna to be tested against a large number of different compounds (Syntech, 2004).

As a result of this ability to test a large number of compounds with relative ease, EAG has been developed as a method of pre-screening the responses of insects to a range of compounds before conducting more time consuming behavioural experiments on compounds of interest (Beck et al., 2012). Using EAG allows the easy identification of the most bioactive components of a given odour source, and therefore the compounds most likely to induce behavioural responses (Beck et al., 2012). The strength of the EAG response is determined by the level of activation of the olfactory receptors. As such, EAG testing does not directly determine how an insect will behaviourally respond to a given compound, rather the size of the depolarisation gives an indication of how receptive the antenna is to different compounds. As insect olfactory receptors are known to be tuned towards ecologically relevant odorants (Hansson and Stensmyr, 2011; Andersson et al., 2015), compounds that elicit large antennal depolarisations are likely to represent important compounds for this species, and therefore, are likely to elicit behavioural responses. However, without behavioural bioassays it is impossible to know what type of behaviour a particular volatile will elicit (e.g. attractive or repulsive), or indeed if it will elicit any behaviour at all.

The principles underpinning EAG were first described by Schneider (1957), and since then EAG has become an important technique in the study of olfaction across a range of insects, including: aphids (Fan et al., 2015), flies (Larsson et al., 2004; Martin et al., 2011), wasps (Germinara et al., 2009; Li et al., 2012), moths (Merlin et al., 2007) and beetles (Collins et al., 2007; Guarino et al., 2013; Zhang et al., 2016). However, EAG is a relatively underused technique in *Tribolium* species. EAG has been used to quantify the electrophysiological responses of *T. confusum* to a small number of *Tribolium* secondary metabolites using excised antennae (Verheggen et al., 2007), and the first large scale EAG screen involving *T. castaneum* was conducted recently testing the antennal responses to 94 VOCs that had previously been presented to other stored-product pests (Balakrishnan et al., 2017). This large

EAG screen tested a diverse range of volatiles rather than focusing on VOCs associated with a particular odour source. However, several compounds that are found within wheat germ oil, or produced by grain associate fungi, were coincidentally tested and found to elicit large antennal depolarisations. This suggests that compounds from these sources may elicit behavioural responses from *T. castaneum*, however none of these compounds have yet been tested using behavioural bioassays.

As the research presented in this chapter is intended to contribute towards identifying compounds that could improve lures used for *T. castaneum*, it is important to ensure that the responses of beetles tested are representative of wild populations. A variety of insects have already been shown to rapidly undergo various behavioural and physiological changes after being cultured in laboratory conditions (Hoffmann and Ross, 2018). Wild *T. castaneum* specifically have been shown to have increased fecundity and an ability to utilise different food resources, when compared to laboratory populations (Ahmad, Walter, et al., 2012). In laboratory cultured insect populations the relaxation of selection pressures, inbreeding depression and genetic drift could affect how they respond to food-associated olfactory cues. Behavioural bioassays testing the response of eight different *T. castaneum* strains to kairomone and pheromone lures have already shown some evidence that variation can exist in the responses of *T. castaneum* to food-associated volatiles (Gerken et al., 2018). As such, commonly used *T. castaneum* laboratory strains may not respond the same way as wild populations, which impacts on the use of laboratory strains for research related to pest management. Because of this, the responses of both established laboratory strain and recently caught wild *T. castaneum* were tested in this chapter.

It is important to ensure that any compounds that are intended to be used in *T. castaneum* lures are attractive to both sexes, otherwise the effectiveness of the lure will be reduced. As the volatiles tested in this chapter are believed to be used primarily for food location, these cues are likely to be important to both sexes. If there appear to be no differences between the responses of males and females, then only testing the responses of one sex would significantly reduce the number of experiments needed.

To identify the volatiles produced by wheat germ oil and grain-associated fungi that are most likely to be attractive to *T. castaneum*, EAG was used to record antennal responses to a range of volatile compounds from these sources. A number of small EAG screens were initially conducted to test the responses of both male and female CTC12 strain *T. castaneum* to a small number of fungal or wheat germ oil associated volatiles and other environmental odour sources. After performing these initial experiments, a much larger EAG screen was performed to test the responses of female CTC12 and wild Zim strain *T. castaneum* to 66 of the most abundant volatiles found in wheat germ oil or produced by grain-associated fungi.

3.2. Methods

3.2.1. *Tribolium castaneum* husbandry

CTC12 and wild Zim strain *T. castaneum* were used in the experiments in this chapter. Cultures were incubated at 30°C in containers of 200 g of whole grain flour (Doves Farm Organic Strong Wholemeal Bread Flour) with the addition of 10 g yeast powder as an additional protein source (Holland & Barrett Debittered Brewer's Yeast Powder) and 1 g of 0.3 g/kg antimicrobial agent Fumagillin to inhibit fungal growth in the cultures. All beetles used in the experiments were aged between 4 and 8 weeks post-emergence to ensure all insects were sexually mature and mated to reduce the variance in their responses to DMD aggregation pheromone (Duehl et al., 2011).

3.2.2. Odour sources

Pentane solvent extractions of adult *T. castaneum* beetles, whole grain flour (Doves Farm Organic Strong Wholemeal Bread Flour) and the same wheat flour that had been heavily conditioned by *T. castaneum* infestation were performed. 1 g of clean flour, 1 g of conditioned flour or 0.04 g of male *T. castaneum* were immersed in 3 ml of pentane for one hour. The solids were removed from each mixture and the liquid was evaporated under a stream of nitrogen gas for a few seconds to concentrate the extracts. Synthetic DMD (4,8-Dimethyldecanal), the *Tribolium* spp. aggregation pheromone, was used as a positive control as it is known to be attractive and elicit strong antennal depolarisations in *T. castaneum* (Levinson and Mori, 1983). The wheat germ

oil used in these experiments was 100% pressed *Triticum vulgare* (common wheat). Sixty-six VOCs present in wheat germ oil and/or produced by grain-associated fungi were used in the electroantennography assays (Table 3.1). The 39 wheat germ oil volatiles used in these experiments had been previously identified through headspace-solid phase microextraction of a sample of wheat germ oil (Niu et al., 2013). The 33 fungal compounds were identified from a review article listing volatiles produced by common fungi grown on cereal and grain substrates (Magan and Evans, 2000). Six compounds were identified as being associated with both wheat germ oil and grain-associated fungi (Niu et al., 2013; Magan and Evans, 2000). All compounds were obtained from commercial suppliers (see appendix A for details). In the preliminary studies (Figures 3.2-3.5) odorants were diluted to working concentrations of 20% vol/vol using pentane, an established solvent for use in insect olfactory behavioural experiments (Beck et al., 2012). For the large-scale EAG screen hexane was instead used to dilute the test compounds to working concentrations of 20% vol/vol. This was done to allow for clearer comparisons with the results of published EAG experiments that had used hexane as a solvent (Verheggen et al., 2007; Guarino et al., 2013).

Table 3.1. The environmental VOCs tested using EAG, and whether they were identified as being found in wheat germ oil, produced by grain-associated fungi or both.

Wheat germ oil	Fungal	Both
1-Heptene	1-Butanol	1-Hexanol
1-Octene	1-Octanol	1-Octen-3-ol
1-Penten-3-one	1-Pentanol	3-Methyl-1-butanol
2-Heptanone	1-Phenylethanol	Ethanol
2-Methyl-2-butene	2,2,4-Trimethylhexane	Hexanal
2-Pentylfuran	2-Butanol	Nonanal
4-Allylanisol	2-Methyl-1-propanol	
5-Methyl-3-heptanone	2-Methyl-2-butanol	
Amyl acetate	2-Methylacetophone	
Ethyl benzene	2-Methylfuran	
Ethyl hexanoate	2-Nonanone	
Ethyl octanoate	2-Pentanone	
Hexane	2-Propanol	
Iovaleraldehyde	3-Methylanisol	
Limonene	3-Octanol	
Nonane	3-Octanone	
Octanal	Acetone	
Octanoic acid	Benzaldehyde	
p-Anisaldehyde	Butyl acetate	
Pentane	Damascenone	
Toluene	Dimethyl benzene	
trans,trans-2,4-Decadienal	Ethyl ethanoate	
trans-2-Decenal	Naphthalene	
trans-2-Heptenal	Octyl acetate	
trans-2-Octenal	Styrene	
trans-2-Octene	trans-2-Hexen-1-al	
trans-2-Pentanal	trans-2-Octen-1-ol	
trans-3-Octene		
trans-5-Decene		
trans-Cinnamaldehyde		
trans-trans-2,4,-Heptandinal		
Tridecane		
Undecane		

3.2.3 Electroantennography

The EAG protocol used was adapted from the Syntech electroantennography manual (Syntech, 2004). Live beetles were restrained on a glass slide to allow EAG recordings to be taken. Double sided sticky tape was applied to a glass slide onto which the head and antenna of the beetles were pressed. A small drop of cyanoacrylate glue was also used to secure the head of the beetles (with care taken to avoid getting any glue on the antennae of the beetles) and

this was sufficient to prevent movement of the beetles antenna. The bodies of the beetles were also restrained with a further piece of tape across their bodies, securing them to the glass slide. Small holes were pierced into the tip of one of the beetle's antennae and through one of the eyes with an electrolytically sharpened tungsten wire. This allowed glass capillary electrodes filled with Ringer's solution, in contact with silver wire, to be inserted into the holes. Filtered air continuously flowed over the restrained beetle and the test odorants were delivered by an air-puff from a Syntech stimulus controller. When triggered the stimulus controller delivered a one second puff of air to the end of a Pasteur pipette pointed at the head of the restrained beetle. Strips of Whatman filter paper with 5 µl of a 20% vol/vol dilution in solvent of each volatile compound were inserted into this pipette to present the beetles with the different odour sources used in the experiments. In each experiment the order in which compounds were presented was randomised for each biological replicate. Live insect preparations were used as they produce a stable EAG signal for much longer than excised insect antenna (Syntech, 2004). This allowed for a large number of compounds to be tested against the same individual over several hours. However, steps were taken to account for drifting of the EAG signal that can occur in lengthy EAG experiments as the antenna dries out and its resistance changes (Syntech, 2004). In the large EAG screen, after every 10 volatiles the responses of the beetles were tested against DMD and the responses of the preceding 10 volatiles were normalised against this DMD response. This normalisation was unnecessary for the shorter preliminary EAG experiments as the experiments were quick enough that drift of the EAG signal did not occur. The EAG potential was recorded on a computer using a signal amplifier, IDAC convertor (Fig. 3.1), and EAG 2000 software.

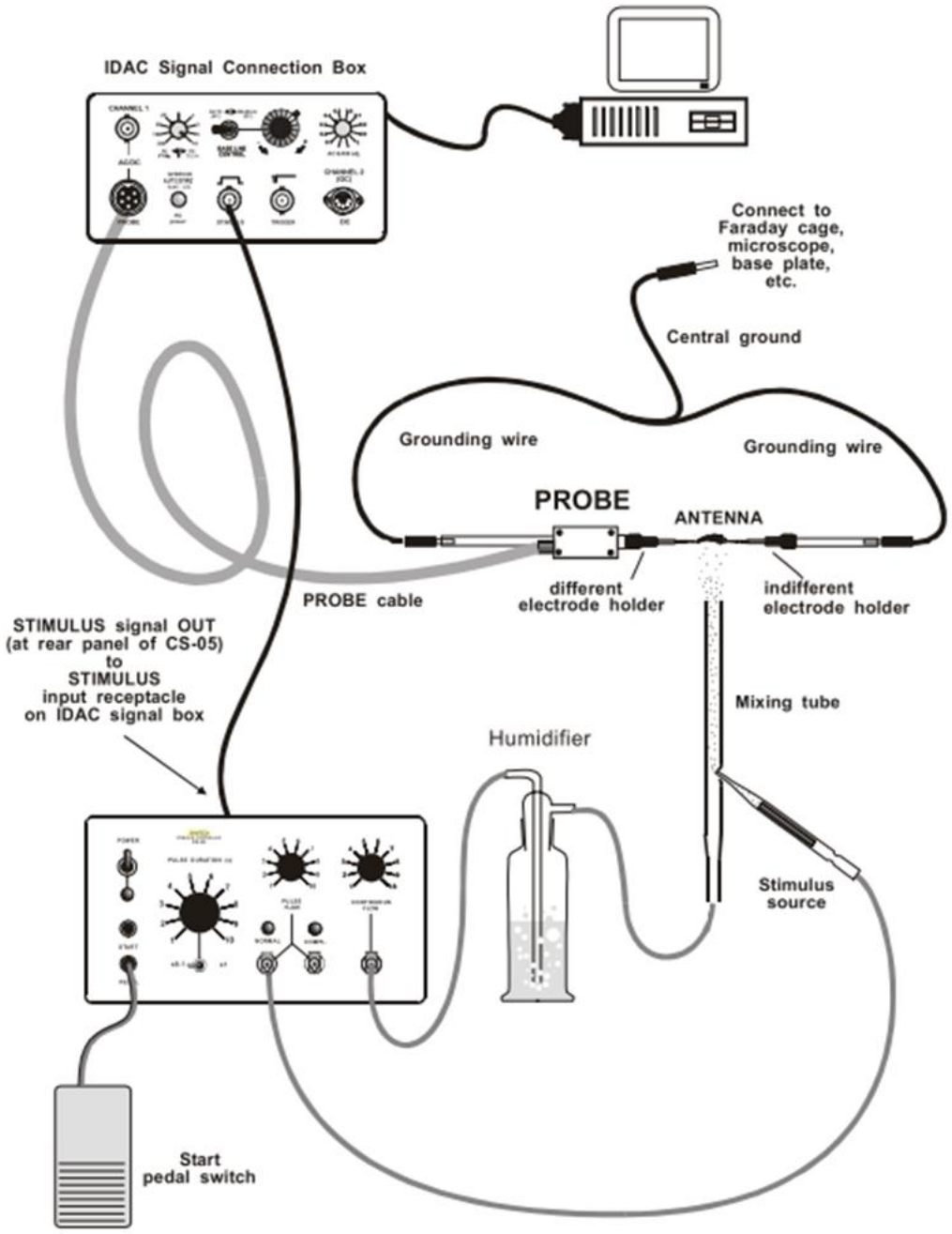

Figure 3.1. A schematic of the EAG equipment used in this chapter (Syntech, 2004).

3.2.4 Statistical analysis

As pseudoreplication is a known issue in electrophysiological experiments (Lazic, 2010), care was taken to address this issue in the statistical analysis. Pseudoreplication can occur in EAG experiments when multiple recordings taken from the same individual are analysed as if they are statistically independent (Lazic, 2010). To account for this the results of the EAG

experiments were analysed with either a repeated measures ANOVA or a two-way mixed ANOVA. In experiments where only one strain or sex of beetle was analysed, a repeated measures ANOVA was used to account for the non-independence of presenting multiple volatiles to the same individual. In experiments where two different sexes or strains of beetle were tested against the same volatiles a two-way mixed ANOVA was used. In these experiments the volatile presented was the within-subject factor and the between subject factor was the sex or strain to which each beetle belonged. This allowed for the effects of both the volatile presented and strain/sex to be analysed while controlling for the non-independence of observations taken from the same individual. The effects of an interaction between the volatile presented and the different sex or strain of the beetles were also tested. In both the repeated measures ANOVA and two-way mixed ANOVA the degrees of freedom were adjusted using Greenhouse–Geisser estimates to account for a lack of sphericity.

In experiments where the volatiles presented was found to have a significant effect on the EAG response, pairwise planned contrasts were performed for each insect group. Paired *t*-tests between the control solvent and each of the volatiles tested, were used to identify which volatiles gave a significantly different response to the control. Paired tests were necessary as different volatiles tested against the same individual are non-independent observations.

In each case a square root transformation was performed on the EAG data to help meet these tests assumptions regarding having normally distributed and heterogeneous residuals. The data from one of the small initial experiments did not meet the assumptions of normality and heterogeneity even after square root transformation and so could not be analysed with an ANOVA. This data set was instead analysed with two separate non-parametric Friedman tests, one for each sex. Where significant differences were found this was followed up with pairwise planned contrasts between each test volatile and the control solvent, using Wilcoxon signed-rank tests.

To correct for the increased risk of type 1 errors associated with performing multiple pairwise statistical tests on the same dataset, the P values of all

paired *t*-tests and Wilcoxon signed-rank tests were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

All statistics were performed using IBM SPSS Statistics 24 software.

3.3. Results

3.3.1. Responses of male and female CTC12 strain *Tribolium castaneum* to a range of ecologically relevant odour sources

The average antennal depolarisations elicited by nine male and ten female CTC12 strain *T. castaneum* to a variety of ecologically relevant odour sources are shown in Fig. 3.2. These included solvent extracts of environmental odour sources and known *T. castaneum* attractants. Separate Friedmans test analyses in each sex revealed a significant difference across the responses to the volatiles in each case (Table 3.2). After identifying these differences, pairwise Wilcoxon signed rank tests were performed to identify which volatiles gave a significantly larger response than the pentane control volatile (Tables 3.3 and 3.4 and indicated on Fig. 3.2).

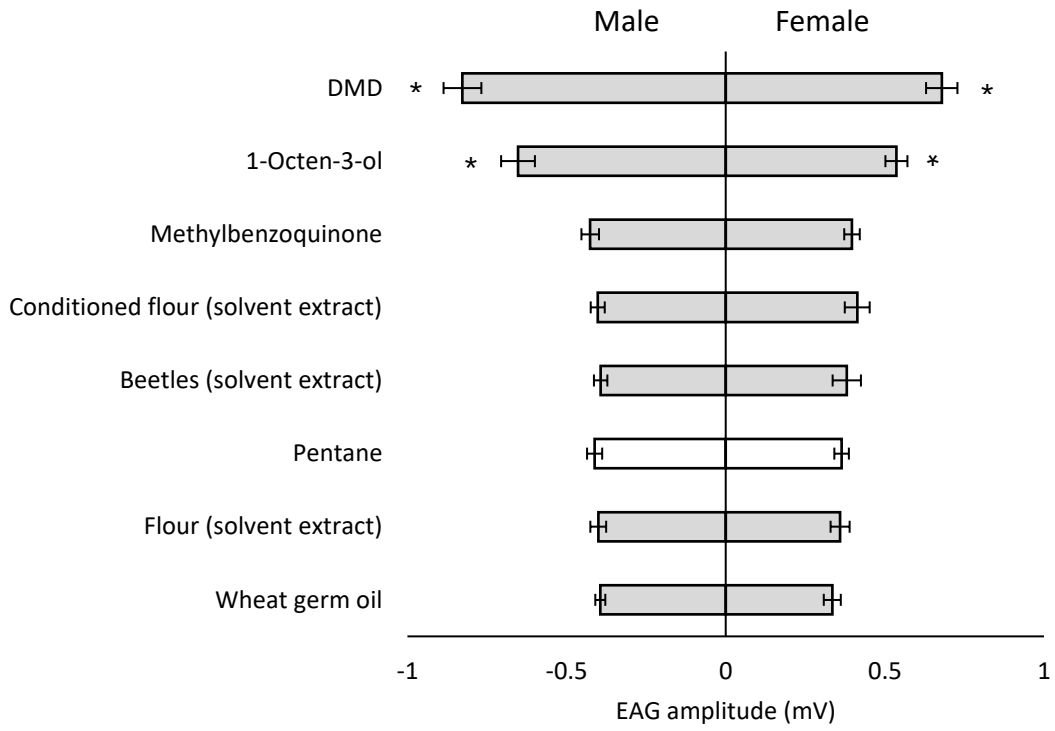

Figure 3.2. EAG responses of nine male and ten female CTC12 *T. castaneum* to a variety of ecologically relevant odour sources and solvent extracts (grey) and a pentane control (white).

Horizontal columns represent the average depolarisation across the individuals tested, and the error bars represent the standard error of the mean. Odour sources that elicited a significantly different EAG response compared to the pentane control are indicated with asterisks: * P<0.05, ** P<0.01, *** P<0.001 (full statistics in Tables 3.3 and 3.4).

Table 3.2. The results of two separate Friedman tests comparing the effect of volatile in different *T. castaneum* sexes.

Statistically significant P values are highlighted in bold.

Variable	**Sex**	**df**	**χ^2**	**P**
Volatile	Male	7	36.094	**<0.001**
Volatile	Female	7	41.267	**<0.001**

Table 3.3. The results of different pairwise Wilcoxon signed rank tests comparing the responses elicited by different volatiles to the response to a pentane control, across nine male CTC12 *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedure and statistically significant adjusted P values are highlighted in bold ($P<0.05$).

Volatile	Z	df	Adjusted P
DMD	-2.666	8	**0.028**
1-Octen-3-ol	-2.666	8	**0.028**
Methylbenzoquinone	-0.889	8	0.5145
Conditioned flour (solvent extract)	-0.77	8	0.5145
Beetles (solvent extract)	-0.77	8	0.515
Flour (solvent extract)	-0.415	8	0.678
Wheat germ oil	-1.125	8	0.515

Table 3.4. The results of different pairwise Wilcoxon signed rank tests comparing the responses elicited by different volatiles to the response to a pentane control across ten female CTC12 *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedure and statistically significant adjusted P values are highlighted in bold (P<0.05).

Volatile	Z	df	Adjusted P
DMD	-2.666	9	**0.028**
1-Octen-3-ol	-2.666	9	**0.028**
Methylbenzoquinone	-1.718	9	0.151
Beetles (solvent extract)	-0.59	9	0.953
Conditioned flour (solvent extract)	-1.718	9	0.151
Flour (solvent extract)	-0.296	9	0.895
Wheat germ oil	-1.4	9	0.225

3.3.2. Responses of male and female CTC12 strain *Tribolium castaneum* to grain-associated fungal volatiles

The average antennal depolarisations elicited by ten male and ten female CTC12 strain *T. castaneum* to common fungal volatiles are shown in Fig. 3.3. A two-way mixed ANOVA revealed the volatile presented had a significant effect on antennal depolarisation, however, sex, or an interaction between volatile and sex, were not found to have a significant effect on the EAG response (Table 3.5). After identifying these differences pairwise paired *t*-tests were performed to identify which volatiles elicited a significantly larger response than the pentane control (Tables 3.6 and 3.7 and indicated on Fig. 3.3).

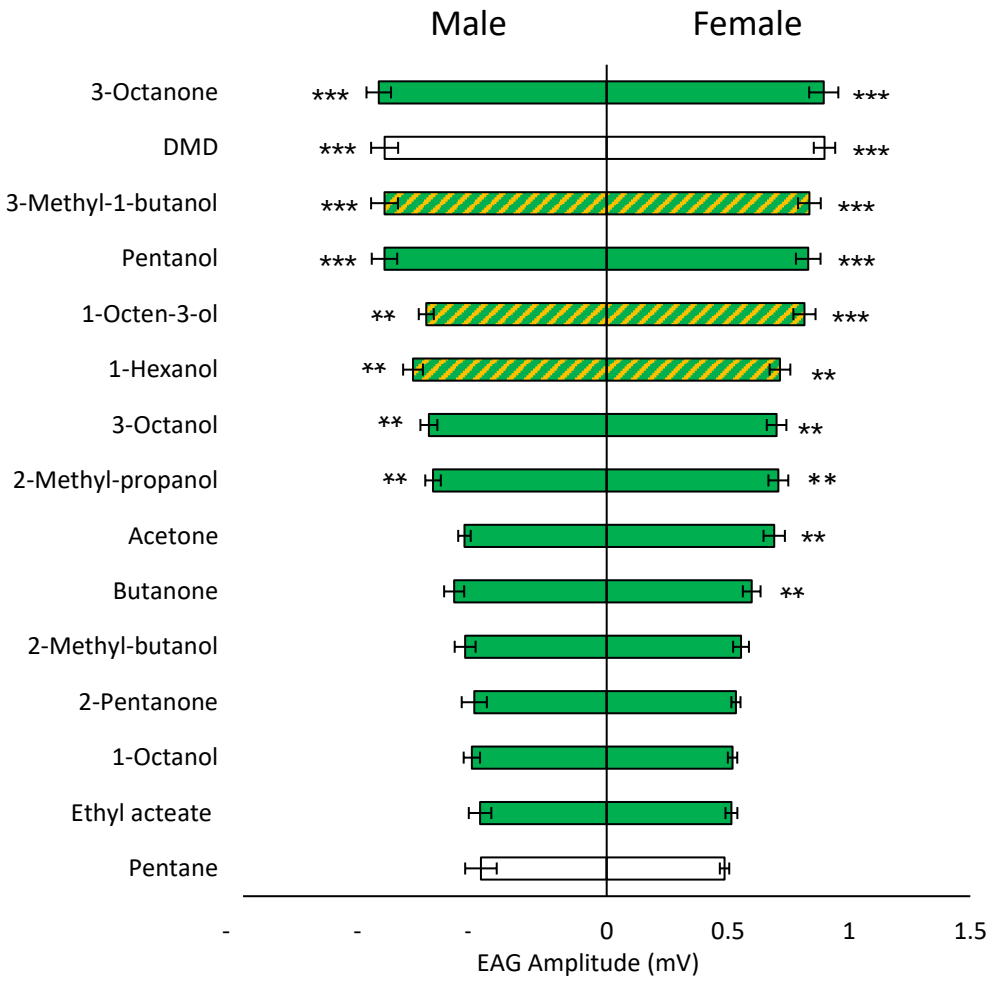

Figure 3.3. Average EAG responses of ten male and ten female CTC12 *T. castaneum* to common fungal volatiles (green), fungal and wheat germ oil associated volatiles (striped) and control odour sources DMD and pentane (white).

Horizontal columns represent the average depolarisation across ten individuals, and the error bars represent the standard error of the mean. Odour sources that elicited a significantly different EAG response compared to the pentane control are indicated with asterisks: * P<0.05, ** P<0.01, *** P<0.001 (full statistics in Tables 3.6 and 3.7).

Table 3.5. The results of a two-way mixed ANOVA investigating the effects of sex and volatile presented on the EAG responses of CTC12 *T. castaneum*.

Greenhouse–Geisser adjusted degrees of freedom were used to calculate P values and the original degrees of freedom are shown in brackets. Significantly significant P values are highlighted in bold (P<0.05).

Variable	**df**	**MS**	**F**	**P**
Volatile	(14) 3.278	1.916	53.459	**<0.001**
Sex	1	0.036	0.23	0.638
Volatile × Sex	(14) 3.278	0.013	1.599	0.196

Table 3.6. The results of different pairwise paired *t*-tests comparing the responses elicited by different volatiles to the response to a pentane control across ten male CTC12 *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedure and statistically significant P values are highlighted in bold (P<0.05).

Volatile	***t***	**df**	**Adjusted P**
3-Octanone	-6.572	9	**<0.001**
DMD	-12.538	9	**<0.001**
3-Methyl-1-butanol	-6.494	9	**<0.001**
Pentanol	-7.303	9	**<0.001**
1-Octen-3-ol	-4.129	9	**0.005**
1-Hexanol	-4.836	9	**0.003**
3-Octanol	-4.423	9	**0.004**
2-Methyl-propanol	-3.995	9	**0.005**
Acetone	-1.299	9	0.288
Butanone	-1.887	9	0.143
2-Methyl-butanol	-1.303	9	0.288
2-Pentanone	-0.712	9	0.532
1-Octanol	-0.858	9	0.482
Ethyl acetate	-0.083	9	0.935

Table 3.7. The results of different pairwise paired *t*-tests comparing the responses elicited by different volatiles to the response to a pentane control across ten female CTC12 *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedure and statistically significant P values are highlighted in bold (P<0.05).

Volatile	***t***	**df**	**Adjusted P**
3-Octanone	-8.559	9	**<0.001**
DMD	-12.792	9	**<0.001**
3-Methyl-1-butanol	-10.685	9	**<0.001**
Pentanol	-9.264	9	**<0.001**
1-Octen-3-ol	-9.061	9	**<0.001**
1-Hexanol	-7.093	9	**0.001**
3-Octanol	-7.175	9	**0.001**
2-Methyl-propanol	-6.909	9	**0.001**
Acetone	-5.588	9	**0.005**
Butanone	-3.877	9	**0.005**
2-Methyl-butanol	-2.177	9	0.067
2-Pentanone	-2.362	9	0.054
1-Octanol	-1.671	9	0.139
Ethyl acetate	-1.546	9	0.156

3.3.3. Responses of male CTC12 strain *Tribolium castaneum* to wheat germ oil volatiles

The average antennal depolarisations elicited by nine male CTC12 strain *T. castaneum* to wheat germ oil volatiles are shown in Fig. 3.4. A repeated measures ANOVA revealed the volatile presented had a significant effect on antennal depolarisation (Table 3.8). After identifying this significant effect, pairwise paired *t*-tests were performed to identify which volatiles elicited significantly larger responses than the pentane control volatile (Table 3.9 and indicated in Fig. 3.4).

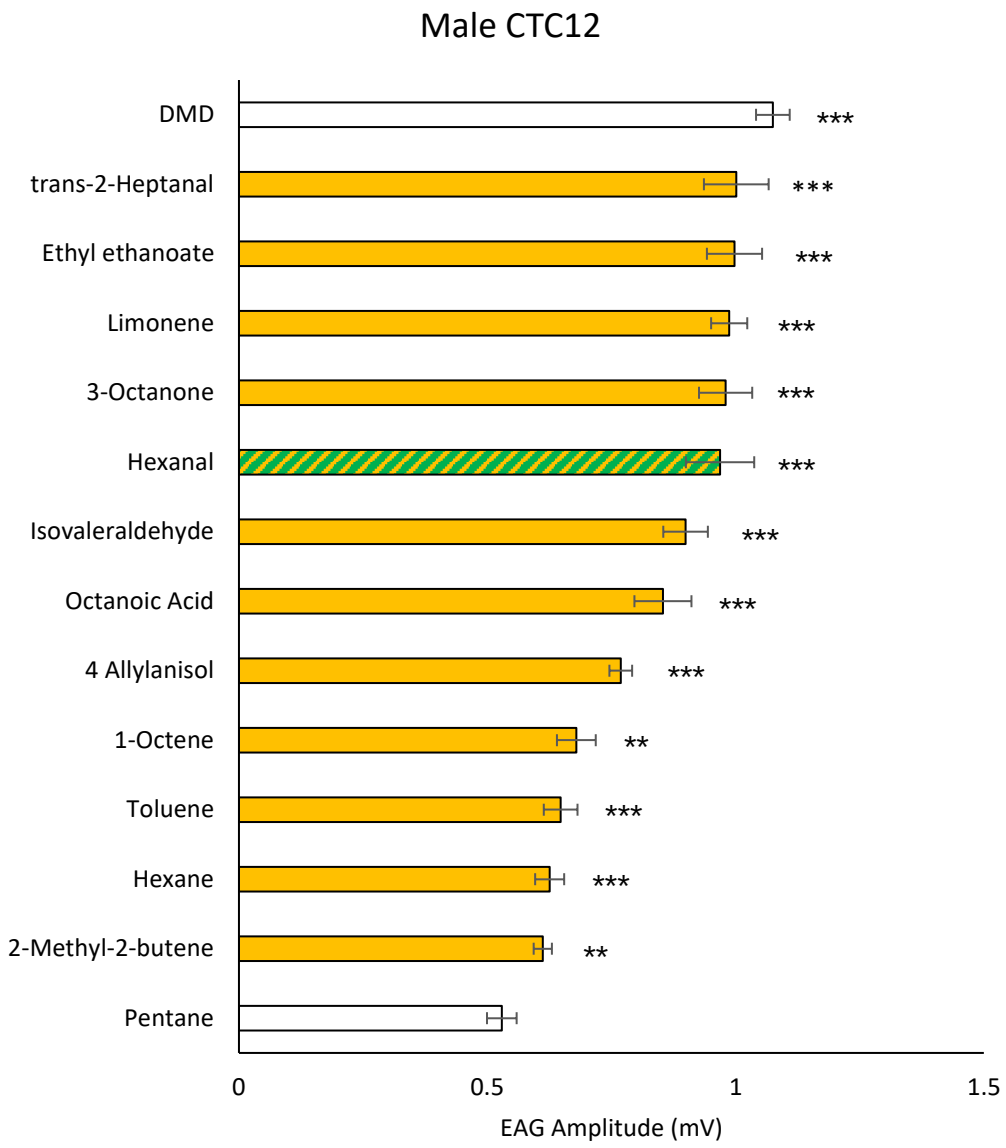

Figure 3.4. Average EAG responses of nine male CTC12 *T. castaneum* to common wheat germ oil volatiles (yellow), volatiles of wheat germ oil and fungi (striped) and control odour sources DMD and pentane (white).

Horizontal columns represent the average depolarisation across nine individuals, and the error bars represent the standard error of the mean. Odour sources that elicited a significantly different EAG response compared to the pentane control are indicated with asterisks: * P<0.05, ** P<0.01, *** P<0.001 (full statistics in table 3.9).

Table 3.8. The results of a repeated measures ANOVA investigating the effect of volatile presented on the EAG responses of male CTC12 *T. castaneum*.

Greenhouse–Geisser adjusted degrees of freedom were used to calculate P values and the original degrees of freedom are shown in brackets. Statically significant P values are highlighted in bold (P<0.05).

Variable	df	MS	F	P
Volatile	(13) 3.361	1.149	30.461	**<0.001**

Table 3.9. The results of different pairwise paired *t*-tests comparing the responses elicited by different volatiles to the response to a pentane control across nine male CTC12 *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedure and statistically significant P values are highlighted in bold (P<0.05).

Volatile	T	df	Adjusted P
DMD	-24.824	8	**<0.001**
trans-2-Heptanal	-8.794	8	**<0.001**
Ethyl ethanoate	-7.680	8	**<0.001**
Limonene	-15.458	8	**<0.001**
3-Octanone	-10.108	8	**<0.001**
Hexanal	-6.932	8	**<0.001**
Isovaleraldehyde	-8.528	8	**<0.001**
Octanoic acid	-9.888	8	**<0.001**
4-Allylanisol	-8.528	8	**<0.001**
1-Octene	-3.902	8	**0.005**
Toluene	-8.170	8	**<0.001**
Hexane	-10.419	8	**<0.001**
2-Methyl-2-butene	-3.563	8	**0.007**

3.3.4. Responses of female CTC12 and wild Zim strain *Tribolium castaneum* to wheat germ oil and grain-associated fungal volatiles

The average antennal depolarisations elicited by eight female CTC12 strain *T. castaneum* and eight female wild Zim *T. castaneum* in response to 66 wheat germ oil and/or fungal-associated VOCs are shown in Fig. 3.5. A two-way mixed ANOVA revealed the volatile presented had a significant effect on antennal depolarisation, however, strain, and an interaction between volatile and strain, were not found to have a significant effect on the EAG response (Table 3.10). After identifying these differences, pairwise paired *t*-tests were performed to identify which volatiles elicited significantly larger responses than the hexane control (Tables 3.11 and 3.12 and indicated on Fig. 3.5).

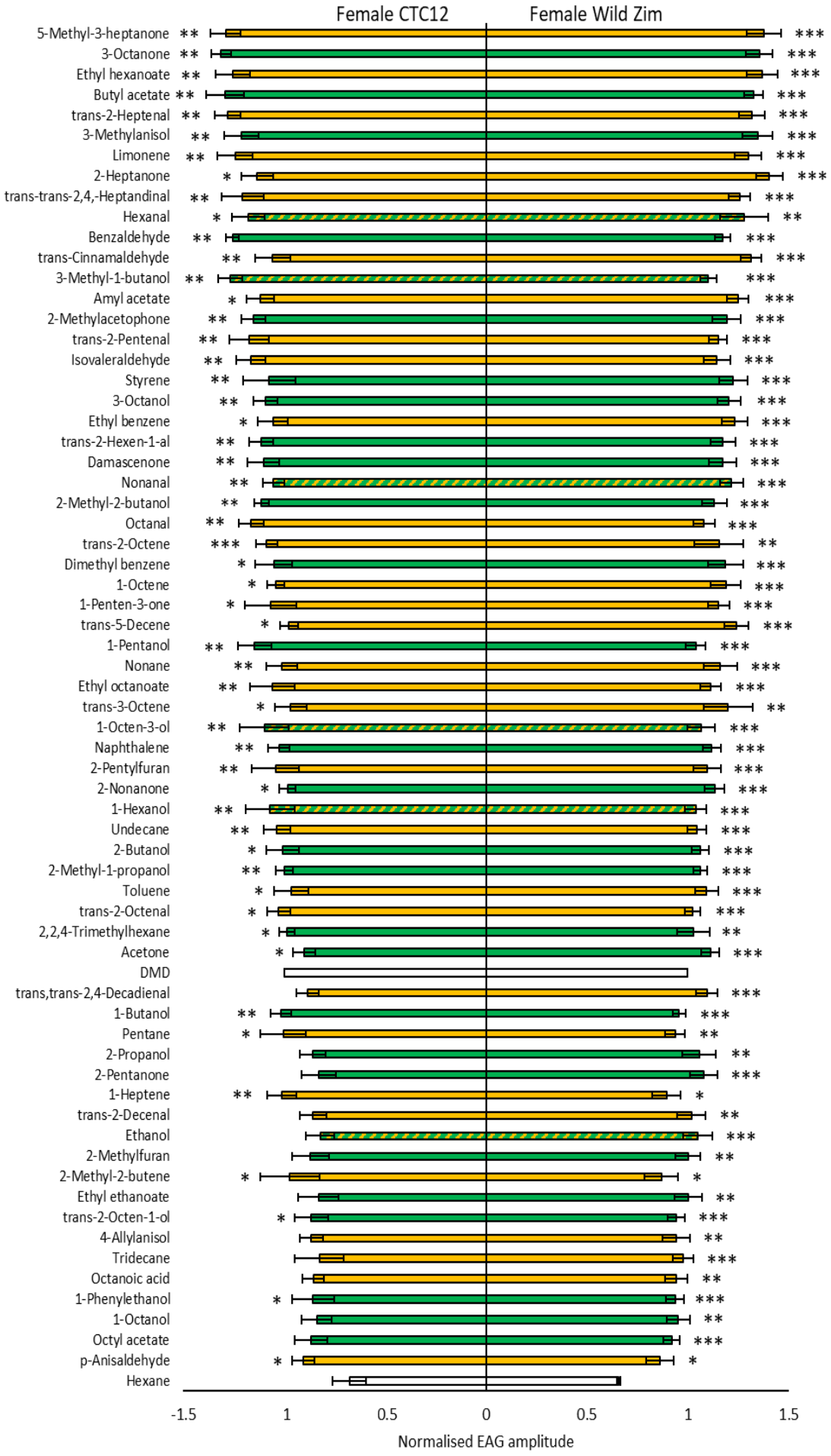
Female CTC12
Female Wild Zim
5-Methyl-3-heptanone
3-Octanone
Ethyl hexanoate
Butyl acetate
trans-2-Heptenal
3-Methylanisol
Limonene
2-Heptanone
trans-trans-2,4,-Heptandinal
Hexanal
Benzaldehyde
trans-Cinnamaldehyde
3-Methyl-1-butanol
Amyl acetate
2-Methylacetophone
trans-2-Pentenal
Isovaleraldehyde
Styrene
3-Octanol
Ethyl benzene
trans-2-Hexen-1-al
Damascenone
Nonanal
2-Methyl-2-butanol
Octanal
trans-2-Octene
Dimethyl benzene
1-Octene
1-Penten-3-one
trans-5-Decene
1-Pentanol
Nonane
Ethyl octanoate
trans-3-Octene
1-Octen-3-ol
Naphthalene
2-Pentylfuran
2-Nonanone
1-Hexanol
Undecane
2-Butanol
2-Methyl-1-propanol
Toluene
trans-2-Octenal
2,2,4-Trimethylhexane
Acetone
DMD
trans,trans-2,4-Decadienal
1-Butanol
Pentane
2-Propanol
2-Pentanone
1-Heptene
trans-2-Decenal
Ethanol
2-Methylfuran
2-Methyl-2-butene
Ethyl ethanoate
trans-2-Octen-1-ol
4-Allylanisol
Tridecane
Octanoic acid
1-Phenylethanol
1-Octanol
Octyl acetate
p-Anisaldehyde
Hexane
-1.5
1
0.5
0
0.5
1
1.5
Normalised EAG amplitude

Figure 3.5. Average EAG responses of 8 female CTC12 strain and 8 female wild Zim *T. castaneum*, to 66 volatile organic compounds found in wheat germ oil and/or produced by grain associated fungi, normalised against the response to the *Tribolium* aggregation pheromone DMD.

Horizontal columns, arranged by descending EAG response, represent the average depolarisation across eight individuals, and the error bars represent the standard error of the mean. Yellow columns represent volatile compounds found in wheat germ oil, green bars represent compounds identified as being products of grain-associated fungi, striped columns represent compounds identified as being associated with both sources, and white columns represent the two control compounds: DMD (positive control) and hexane (negative control). Odour sources that elicited a significantly different EAG response compared to the hexane control are indicated with asterisks: * P<0.05, ** P<0.01, *** P<0.001 (full statistics in Table 3.11 and 3.12).

Table 3.10. The results of a two-way mixed ANOVA investigating the effect of strain and volatile presented on the EAG responses of CTC12 *T. castaneum*.

Greenhouse–Geisser adjusted degrees of freedom were used to calculate P values and the original degrees of freedom are shown in brackets. Statistically significant P values are highlighted in bold (P<0.05).

Variable	df	MS	F	P
Volatile	(65) 9.483	2.018	10.127	**<0.001**
Strain	1	1.581	1.724	0.210
Volatile × Strain	(65) 9.483	0.279	1.400	0.191

Table 3.11. The results of different pairwise paired *t*-tests comparing the responses elicited by different volatiles to the response to a hexane control across eight female CTC12 *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedures and statistically significant P values are highlighted in bold (P<0.05).

Volatile	*t*	df	Adjusted P
5-Methyl-3-heptanone	-5.879	7	**0.003**
3-Octanone	-6.245	7	**0.003**
Ethyl hexanoate	-6.012	7	**0.003**
Butyl acetate	-4.951	7	**0.004**
trans-2-Heptenal	-7.405	7	**0.002**
3-Methylanisol	-8.747	7	**0.002**
Limonene	-7.144	7	**0.002**
2-Heptanone	-3.301	7	**0.018**
trans-trans-2,4,-Heptandinal	-4.533	7	**0.006**
Hexanal	-3.692	7	**0.014**
Benzaldehyde	-8.185	7	**0.002**
trans-Cinnamaldehyde	-4.24	7	**0.008**
3-Methyl-1-butanol	-7.62	7	**0.002**
Amyl acetate	-3.597	7	**0.015**
2-Methylacetophone	-5.859	7	**0.003**
trans-2-Pentenal	-6.693	7	**0.002**
Isovaleraldehyde	-5.959	7	**0.003**
Styrene	-5.633	7	**0.003**
3-Octanol	-7.15	7	**0.002**
Ethyl benzene	-3.758	7	**0.014**
trans-2-Hexen-1-al	-5.009	7	**0.004**
Damascenone	-7.558	7	**0.002**

Nonanal	-10.231	7	**0.001**
2-Methyl-2-butanol	-5.663	7	**0.003**
Octanal	-5.033	7	**0.004**
trans-2-Octene	-4.018	7	**<0.001**
Dimethyl benzene	-3.298	7	**0.018**
1-Octene	-3.332	7	**0.018**
1-Penten-3-one	-3.254	7	**0.019**
trans-5-Decene	-3.347	7	**0.018**
1-Pentanol	-5.437	7	**0.003**
Nonane	-4.536	7	**0.006**
Ethyl octanoate	-6.058	7	**0.003**
trans-3-Octene	-3.126	7	**0.022**
1-Octen-3-ol	-5.014	7	**0.004**
Naphthalene	-4.759	7	**0.005**
2-Pentylfuran	-6.172	7	**0.003**
2-Nonanone	-3.468	7	**0.016**
1-Hexanol	-5.204	7	**0.004**
Undecane	-5.677	7	**0.003**
2-Butanol	-3.703	7	**0.014**
2-Methyl-1-propanol	-4.724	7	**0.005**
Toluene	-2.763	7	**0.035**
trans-2-Octenal	-3.555	7	**0.015**
2,2,4-Trimethylhexane	-3.488	7	**0.016**
Acetone	-3.664	7	**0.014**
trans,trans-2,4-Decadienal	-2.245	7	0.069
1-Butanol	-4.583	7	**0.006**
Pentane	-2.704	7	**0.037**

2-Propanol	-2.086	7	0.083
2-Pentanone	-1.567	7	0.164
1-Heptene	-4.397	7	**0.007**
trans-2-Decenal	-2.179	7	0.075
Ethanol	-1.941	7	0.1
2-Methylfuran	-2.296	7	0.065
2-Methyl-2-butene	-3.575	7	**0.015**
Ethyl ethanoate	-1.587	7	0.162
trans-2-Octen-1-ol	-3.37	7	**0.018**
4-Allylanisol	-2.413	7	0.056
Tridecane	-1.446	7	0.192
Octanoic acid	-2.09	7	0.083
1-Phenylethanol	-3.045	7	**0.024**
1-Octanol	-1.956	7	0.099
Octyl acetate	-1.929	7	0.1
p-Anisaldehyde	-2.83	7	**0.032**

Table 3.12. The results of different pairwise paired t-tests comparing the responses elicited by different volatiles to the response to a hexane control across eight female wild Zim *T. castaneum*.

The P values were adjusted using the Benjamini-Hochberg procedure and statistically significant P values are highlighted in bold (P<0.05).

Volatile	*t*	df	Adjusted P
5-Methyl-3-heptanone	-8	7	**<0.001**
3-Octanone	-9.611	7	**<0.001**
Ethyl hexanoate	-8.935	7	**<0.001**
Butyl acetate	-13.048	7	**<0.001**
trans-2-Heptenal	-10.659	7	**<0.001**
3-Methylanisol	-8.607	7	**<0.001**
Limonene	-8.909	7	**<0.001**
2-Heptanone	-11.75	7	**<0.001**
trans-trans-2,4,-Heptandinal	-10.724	7	**<0.001**
Hexanal	-5.246	7	**0.001**
Benzaldehyde	-12.29	7	**<0.001**
trans-Cinnamaldehyde	-12.038	7	**<0.001**
3-Methyl-1-butanol	-10.544	7	**<0.001**
Amyl acetate	-11.906	7	**<0.001**
2-Methylacetophone	-7.23	7	**<0.001**
trans-2-Pentenal	-12.535	7	**<0.001**
Iovaleraldehyde	-7.227	7	**<0.001**
Styrene	-7.694	7	**<0.001**
3-Octanol	-9.586	7	**<0.001**
Ethyl benzene	-8.197	7	**<0.001**
trans-2-Hexen-1-al	-7.764	7	**<0.001**
Damascenone	-7.684	7	**<0.001**
Nonanal	-8.851	7	**<0.001**

2-Methyl-2-butanol	-7.785	7	**<0.001**
Octanal	-7.305	7	**<0.001**
trans-2-Octene	-4.047	7	**0.005**
Dimethyl benzene	-5.659	7	**<0.001**
1-Octene	-6.903	7	**<0.001**
1-Penten-3-one	-8.408	7	**<0.001**
trans-5-Decene	-9.817	7	**<0.001**
1-Pentanol	-6.899	7	**<0.001**
Nonane	-6.048	7	**<0.001**
Ethyl octanoate	-8.017	7	**<0.001**
trans-3-Octene	-4.458	7	**0.003**
1-Octen-3-ol	-6.538	7	**<0.001**
Naphthalene	-9.694	7	**<0.001**
2-Pentylfuran	-6.327	7	**<0.001**
2-Nonanone	-9.797	7	**<0.001**
1-Hexanol	-6.407	7	**<0.001**
Undecane	-8.857	7	**<0.001**
2-Butanol	-8.726	7	**<0.001**
2-Methyl-1-propanol	-11.652	7	**<0.001**
Toluene	-7.111	7	**<0.001**
trans-2-Octenal	-7.756	7	**<0.001**
2,2,4-Trimethylhexane	-4.54	7	**0.003**
Acetone	-10.192	7	**<0.001**
trans,trans-2,4-Decadienal	-8.424	7	**<0.001**
1-Butanol	-9.103	7	**<0.001**
Pentane	-5.571	7	**0.001**
2-Propanol	-4.752	7	**0.002**

2-Pentanone	-6.42	7	**<0.001**
1-Heptene	-3.086	7	**0.0182**
trans-2-Decenal	-5.033	7	**0.002**
Ethanol	-5.726	7	**<0.001**
2-Methylfuran	-5.434	7	**0.001**
2-Methyl-2-butene	-2.476	7	**0.042**
Ethyl ethanoate	-4.715	7	**0.002**
trans-2-Octen-1-ol	-6.013	7	**<0.001**
4-Allylanisol	-4.235	7	**0.004**
Tridecane	-6.503	7	**<0.001**
Octanoic acid	-4.956	7	**0.002**
1-Phenylethanol	-5.786	7	**<0.001**
1-Octanol	-5.105	7	**0.002**
Octyl acetate	-6.402	7	**<0.001**
p-Anisaldehyde	-2.904	7	**0.023**

3.4. Discussion

3.4.1. EAG responses of *Tribolium castaneum* to ecologically relevant odour sources and solvent extracts

Before focusing on individual wheat germ oil and fungal associated VOCs, the responses of male and female CTC12 strain *T. castaneum* were tested against a range of ecologically relevant volatiles and solvent extracts of odour sources (Fig 3.2). This was intended to identify odour sources that elicit strong EAG responses from *T. castaneum* to collect preliminary data on their responses to environmental odour sources. Compounds found to give strong responses could also be used as reference compounds in later EAG experiments. The volatile presented were found to have a significant effect on the degree of antennal depolarisation. However, only a few of the odour sources tested in this initial screen were found to elicit significant antennal depolarisations.

Of the compounds tested in the initial EAG experiments, DMD was found to give the strongest responses. This is unsurprising as DMD is the *T. castaneum* aggregation pheromone and has been previously shown to elicit significant antennal depolarisations in *T. castaneum* and *T. confusum* EAG experiments (Levinson and Mori, 1983; Verheggen et al., 2007). DMD has been used as a reference compound in other *T. castaneum* EAG experiments (Balakrishnan et al., 2017). In this preliminary small EAG screen, and across most of the subsequent experiments, the average depolarisation in response to DMD was recorded between around 0.7 to 0.9 mV, which is similar to the magnitude of response *T. confusum* exhibited to DMD in a previous EAG screen (Verheggen et al., 2007). Owing to it eliciting the strongest responses, DMD was used as a positive reference compound in the later EAG experiments in this chapter.

Another *Tribolium* secreted compound, methylbenzoquinone, was also tested in this initial screen. However, this compound did not elicit a significant EAG response, with the response being almost identical to the response elicited by the pentane control. Methylbenzoquinone was expected to elicit a significant response as it had been found to elicit a significant response in previous *Tribolium* EAG experiments (Verheggen et al., 2007). However, in that study a much larger dose was needed to elicit a response comparable to the DMD response, and it is possible that the dose used here was not sufficient to produce a significant response.

Solvent extracts from a range of ecologically relevant sources including: clean wheat flour, wheat flour that had been heavily conditioned by *T. castaneum* infestation, and *T. castaneum* bodies, were also tested in the preliminary EAG screen. However, none of these extracts were found to elicit significant antennal depolarisations, contrasting with the findings of other researchers. Diethyl ether solvent extracts of wheat flour and whole kernels equivalent to 1g have been previously shown to be significantly attractive to *T. castaneum* in an arena bioassay (Seifelnasr et al., 1982). *T. castaneum* has also been shown to be attractive to *T. castaneum* infested flour using a Y-tube olfactometer bioassay (Stevenson et al., 2017), but was repelled by heavily conditioned flour in a walking bioassay (Duehl et al., 2011). The solvent extracts prepared from *T. castaneum* beetle bodies should contain DMD,

benzoquinones and other compounds that have previously been shown to elicit significant EAG responses from *Tribolium* (Verheggen et al., 2007). As all these odour sources have been previously shown to elicit behavioural responses it is surprising that no significant EAG responses were found here. However, as none of the previously mentioned experiments used pentane as a solvent for the odour sources they tested, it is possible that the lack of responses to these odour sources in the thesis could be due to the secreted compounds not being soluble in pentane. It is also possible that the compound were not tested at a sufficiently high concentration to elicit a behavioural response.

Wheat germ oil also did not elicit significant antennal depolarisations from CTC12 strain *T. castaneum*. As the scent of wheat can be attractive to *Tribolium* species (Seifelnasr et al., 1982) and wheat germ oil is a common component in commercial *Tribolium* lures, it is unlikely that *T. castaneum* antennae cannot detect any of the compounds comprising wheat germ oil. The lack of response in this EAG experiment could be due to wheat germ oil not releasing volatiles at a sufficient rate to induce a significant depolarisation. This idea is supported by many of the constituent compounds of wheat germ oil being found to elicit significant EAG responses when tested individually in later EAG screens.

1-Octen-3-ol, a compound known to be attractive to other pest insect species (de Bruyne and Baker, 2008), was also tested and found to elicit significant EAG responses from *T. castaneum*. In both sexes the responses to 1-octen-3-ol was found to be about 80% as large as the response to DMD. This is still a relatively large response and suggests that 1-octen-3-ol could be an important chemical signal for this species. As 1-octen-3-ol is both fungal produced and found within wheat germ oil, this indicates that compounds from these sources have potential as *T. castaneum* attractants, even if wheat germ oil on its own did not elicit a significant response in these experiments.

The final compound tested in the preliminary EAG screen, pentane, gave consistently low responses across the experiments. It was important to demonstrate that pentane did not elicit significant EAG responses as pentane

was used as a solvent and reference compound in many of the subsequent EAG experiments.

This initial screen was conducted in both male and female CTC12 strain *T. castaneum*. As the data from the preliminary screen did not meet the assumptions of a mixed model ANOVA, even after data transformation, the effect of sex could not be directly tested. However, the responses of each sex were similar in that, in each sex, only DMD and 1-octen-3-ol were found to elicit a significant response. This suggests that the ability of male and female *T. castaneum* to detect odours from these sources was similar.

3.4.2. EAG responses to fungal and wheat germ oil derived volatile organic compounds

Before conducting large-scale EAG experiments testing the responses of *T. castaneum* to wheat germ oil and fungal associated compounds, smaller experiments were conducted on a subset of these compounds (Figures 3.3 & 3.4). This was done to identify if compounds from these sources were likely to elicit significant EAG responses, before committing to a larger more comprehensive EAG screen. In each case these smaller EAG experiments identified a significant effect of the volatile presented, with many compounds eliciting significantly stronger responses than the pentane control.

When the responses of male and female CTC12 *T. castaneum* were tested against 13 fungal volatiles, several of the compounds were found to elicit responses of a similar magnitude to the DMD response (Fig. 3.3). This suggests that certain fungal compounds could be important signals for this species. The sex of the beetles was not found to have a significant effect on their responses to these compounds. Considering that fungal volatiles could indicate the presence of rotten grains, a food source of this species, it was expected that males and females would respond similarly to volatile compounds from these sources. No significant sex differences were previously found when the responses of *T. castaneum* was tested against volatile organic compounds from a variety of different sources (Balakrishnan et al., 2017). This is important from an applied perspective, as compounds will only be useful in lures for stored-product pests if they are attractive to both male and female insects.

With respect to the absolute EAG readings in mV, a similar pattern of responses was found when the responses of male beetles were tested against 12 wheat germ oil volatiles (Fig. 3.4). A range of EAG responses was observed, with a few of the compounds eliciting responses similar to the response to DMD. In this case, however, all of the volatiles were found to give a significantly different response from the pentane control. Again, this suggests that *T. castaneum* may have evolved to respond to certain volatiles from these sources. Due to time constraints, only the responses of male *T. castaneum* were tested in this EAG screen so comparisons between the responses of males and females to these compounds cannot be made. However, as no significant sex differences were found when the responses of *T. castaneum* were tested against food source associated fungal volatiles, it is likely that the responses of male and females to these food associated wheat germ volatiles would be similar.

The significant EAG responses identified in these smaller screens justified conducting a much larger EAG screen of fungal and wheat germ oil volatiles. After demonstrating in the earlier preliminary EAG screens that the responses of males and females were so similar across a variety of different compounds, only one sex was tested in the large EAG screen. Instead, this screen focused on testing the variation between an established laboratory and a recently caught wild *T. castaneum* population. The results of the large EAG screen revealed a significant effect of the volatile presented in both of the strains tested (Fig. 3.5). In both strains a spectrum of responses was seen with some compounds eliciting very strong antennal responses while others elicited much smaller depolarisations. This indicates that there is a degree of discrimination in the responses of *T. castaneum* antennae to different VOCs from these sources. There was no clear pattern between the responses of the beetles and whether the compounds were identified as being fungal or wheat germ oil associated, with individual compounds from each group eliciting both large and small EAG responses. However, due to the very small responses to the hexane control solvent, nearly all the volatiles tested in the CTC 12 strain, and all of the compounds in the wild Zim strain, were found to elicit significantly larger EAG responses compared to the hexane response. There was also no clear class of chemicals that elicited the strongest responses, an observation

that has previously been made when the responses of *T. castaenum* were tested to a range of volatile organic compounds from a variety of sources using EAG (Balakrishnan et al., 2017). In both of the strains tested, over a third of the compounds elicited EAG responses larger than the response to DMD. This indicates that these volatiles are either eliciting antennal responses stronger than the response to the *Tribolium* aggregation pheromone or that the responses to DMD in these EAG experiments is lower than it is under natural conditions. However, the response to DMD recorded here was similar to the EAG depolarisation to DMD recorded by *T. confusum* in previous EAG experiments (Verheggen et al., 2007). The compounds that elicited the largest average EAG responses across the antennas of both strains were: 5-methyl-3-heptanone, 3-octanone, butyl acetate, ethyl hexanoate, 2-heptanone, trans-2-heptenal, 3-methylanisole, limonene, hexanal and trans-trans-2,4-heptandinal, and the responses of *T. castaneum* to these volatiles were tested behaviourally in Chapter 4. The discovery that certain wheat germ oil and fungal derived volatiles elicit very large antennal depolarisations supports the idea that fungal volatiles may be used by *T. castaneum* to locate food sources such as rotten grains in stored-product warehouses and in its historic ecological niche (Dawson, 1977).

Several insects are known to have olfactory sensory neurones (OSNs) that are tuned to respond to a range of ubiquitously produced plant volatiles (Bruce and Pickett, 2011). It has been proposed that this method of detection, using specific blends of ubiquitously produced plant volatiles, is how phytophagous insects detect their host plants (Bruce et al., 2005). Typically insects are more attracted to specific combinations of plant volatiles, as opposed to individual compounds (Bruce and Pickett, 2011), with the simultaneous detection of multiple different volatile compounds from the same source required for a behavioural response. If stored-product insects such as *T. castaneum* detect their food sources in the same way, this could explain why a large number of wheat germ and fungal volatiles elicited moderate to strong EAG responses. Highly specific OSNs also exist for detecting compounds of key biological importance, such as pheromone compounds. However, such OSNs have also been shown to detect key environmental compounds related to locating food sources (Hansson and Stensmyr, 2011; Bruce and Pickett, 2011). It is

therefore possible that some of the individual volatiles that elicited large antennal responses in the EAG experiments in this chapter represent key volatiles involved in the detection of suitable food sources.

The large EAG screen testing the responses of *T. castaneum* to fungal and wheat germ oil VOCs was conducted in two different strains to allow for between-strain variation to be examined. One of the strains tested was CTC12, a strain captured from Australia that has been cultured in laboratory conditions since the 1970s. The other strain was a wild caught population originating in a grain shipment from Zimbabwe in 2017. These particular strains were chosen to identify differences between the responses of potentially inbred established laboratory population and wild populations recently caught from their typical environment. In the large EAG screen the strain of the beetle was not found to have a significant effect on their responses to these volatiles. This suggests that there is little variation in the response of the two strains to these compounds. This could indicate that prolonged laboratory culturing does not affect the responses of *T. castaneum* to environmental volatiles. However, it is possible that the behaviour of the two strains to these volatiles could be different even if their ability to detect the volatiles is similar. However, as only two strains have been tested it is possible that variation could exist between other strains, especially if the two strains tested here do not represent typical laboratory cultured or wild strains.

As the vast majority of the compounds tested in this EAG screen have not been previously tested in *Tribolium spp.,* these results reveal important information about how *T. castaneum* antennae respond to ecologically relevant odours. This has potential implications for identifying attractive compounds for use in a *Tribolium* lure. An EAG experiment using a similar methodology was published shortly after the completion of the large EAG screen in this chapter (Balakrishnan et al., 2017), demonstrating the active research interest in identifying compounds that elicit strong responses from *T. castaneum*. This paper tested 94 VOCs that had previously been shown to elicit significant EAG or behavioural responses from other stored product insects, rather than focusing specifically on fungal or wheat germ oil associated volatiles, as in this thesis. As a result, the majority of the compounds tested in this chapter were unique, with only 20 of the compounds

also tested in Balakrishnan et al., 2017. The responses across the VOCs tested in this study were similar to the pattern of responses seen across the EAG experiments conducted in this thesis. The compounds tested elicited a range of responses, with some of the compounds eliciting negligible EAG responses while other compounds elicited very large antennal depolorisations. It is difficult to draw direct comparisons between the responses of insects in both studies because, in both cases, the data were normalised and presented as a percentage of a reference response. The responses to the few compounds tested in both studies were generally similar, however, the responses to certain compounds differed between the two studies. The beetles tested in Balakrishnan et al. (2017) appeared to show larger responses to limonene and naphthalene and smaller responses to 2-heptanone and 2-pentanone, compared to their responses in this thesis. As Balakrishnan et al. (2017) tested the responses of San Bernardino strain *T. castaneum* it is possible that these differences could be due to variation between the responses of different *T. castaneum* strains. This could be caused by the different geographic origin of this strain (San Bernardino, California, USA) or due to the San Bernardino strain being cultured more intensively, as it is a more commonly used laboratory strain (possibly resulting in laboratory adaptation).

To reduce the number of EAG experiments that needed to be conducted, the responses to wheat germ oil and fungal volatile compounds were tested at only one concentration. Previous *T. castaneum* EAG experiments have shown that, although most compounds elicited higher EAG responses at higher concentrations, increasing the concentration did not generally change which compounds elicited the largest responses (Balakrishnan et al., 2017). Compounds were tested at a relatively high concentration to increase the likelihood of identifying compounds that produce strong antennal responses, or do not elicit responses even at high concentrations. This was done to identify volatiles that were most likely to be attractive to *T. castaneum* when tested at different concentrations in later behavioural bioassays (see Chapter 4). As such, although the results of this experiment have the potential to be very useful in identifying potentially attractive compounds for use in a *Tribolium* lure, they may not represent the degree of antennal depolarisation

these compounds would elicit if encountered at natural environmental concentrations.

It should be noted that the list of fungal volatiles tested in this chapter is far from comprehensive, as many other volatile compounds will also be produced by different fungi. However, by focusing specifically on the volatiles produced by grain spoilage fungi grown on grain substrates it is likely that the most prevalent and ecologically relevant compounds will have been tested. It should also be noted that certain compounds identified from the literature were either not available to purchase from commercial suppliers or were prohibitively expensive. However, enough compounds were tested to draw general conclusions about the attractiveness of wheat germ oil and grain associated fungi to *T. castaneum*.

3.4.3. Conclusion

Overall, these results demonstrate the electrophysiological responses of *T. castaneum* antennae to important volatile compounds found in stored-product environments. Wheat (Seifelnasr et al., 1982) and fungal odours (Ahmad, Daglish, et al., 2012) have been previously shown to be attractive to *T. castaneum*, but very few of the specific volatiles from these sources that could be responsible for this attraction have been identified. The results of the large EAG screen have identified many compounds that, for the first time, have been demonstrated to elicit large depolarisations in *T. castaneum* antennae. This makes the compounds strong candidates for being attractive to *T. castaneum*. The evidence that wild and laboratory strains showed a highly similar pattern of responses suggests that being cultured under laboratory conditions has not affected the responses of *T. castaneum* to these volatiles. Although only a few of the fungal compounds were tested in both sexes, the high degree of similarity in their responses suggests that the responses of both sexes are similar. This is important if these volatile compounds are to be used as part of a *Tribolium* lure.

Although these experiments identified that many compounds elicit strong responses from olfactory receptors in *T. castaneum* antennae, this in itself is not evidence that these compounds will be attractive to *T. castaenum*. It merely indicates that the insect’s antenna responds strongly to the

compounds, and although this is a strong indication that they could potentially elicit a behavioural response, it does not indicate what the behaviour will be. It is possible that a large antennal depolarisation could in fact indicate that a compound is strongly repulsive, or it could induce a different behavioural response e.g. it could stimulate oviposition or arrest the beetle's movement, or it could elicit no behavioural response at all. Therefore, in Chapter 4, the responses of *T. castaneum* to some of these volatiles were tested using some of the behavioural bioassays evaluated in Chapter 2 of the thesis to identify whether they are attractive or not.

Chapter 4
Determining the attractiveness of common environmental volatile organic compounds to *Tribolium castaneum*

4.1. Introduction

In the previous chapter, EAG was used to test the antennal responses of *T. castaneum* to a variety of VOCs found in wheat germ oil or produced by grain-associated fungi and several of these compounds were found to elicit large antennal depolarisations. As these volatiles are associated with *T. castaneum* food sources it is possible that these compounds are used for food location in this species. Although this EAG data provides evidence of strong odorant receptor activation in response to these volatile compounds, this by itself does not indicate that a compound will be attractive. It is possible that certain compounds that elicit strong EAG responses could elicit a different behaviour, such as repulsion, or they may not elicit any behavioural response at all (Beck et al., 2012). Evidence from other herbivorous insects has demonstrated that plant based cues can induce a variety of different behaviours such as feeding, mating and oviposition (Schoonhoven et al., 2005). Whether a compound will be attractive or not can ultimately only be shown with a bioassay that directly records the behaviour of an insect.

In Chapter 2, several different behavioural bioassays were tested and the Y-tube olfactometer was identified as being capable of identifying the attraction of *T. castaneum* to environmental odour sources. Y-tube olfactometers have also previously been used to measure the responses of *T. castaneum* and *T. confusum* to a range of ecologically relevant odours such as: infested flours and pest lures (Stevenson et al., 2017), blends of plant VOCs (Wenda-Piesik et al., 2017), and ethanol extracts prepared from seeds and fungi (Ahmad, Daglish, et al., 2012). For these reasons Y-tube olfactometer bioassays were used as the main method for testing whether *T. castaneum* is attracted to particular fungal or wheat germ oil VOCs.

The behavioural responses of *T. castaneum* were tested against both individual VOCs from these sources and to different blends of these compounds. It was important to test the responses of blends as these compounds would not be detected in isolation in their natural environment and

the responses of insects can differ dramatically between individual compounds and blends of multiple compounds (Bruce and Pickett, 2011). Although the activation of certain receptors in response to single compounds can elicit behavioural responses from insects, e.g. pheromones (Sakurai et al., 2004), the responses to plant-based volatiles typically require the activation of multiple ORs by different compounds (Bruce and Pickett, 2011). It has been proposed that rather than detecting a small number of highly specific compounds, insects primarily locate the plants they feed on through the detection of specific ratios of ubiquitous plant volatiles (Bruce et al., 2005). It is well established that compounds encountered as part of a specific blend can elicit stronger responses than individual compounds alone, with compounds that are unattractive singularly becoming attractive when detected among other compounds in a blend (Bruce and Pickett, 2011). However, the effects of compound blends can be complex, with not all individually attractive volatiles contributing towards a synergistic effect. Only certain blends at specific ratios may be attractive due to the existence of both redundant and highly specific elements (Bruce and Pickett, 2011). Preferences for host plants among herbivorous insects has also been demonstrated to be highly plastic, with current environmental factors and the previous experiences of the organism affecting their responses to plant VOCs (Carrasco et al., 2015).

To identify if wheat germ oil and fungal associated VOCs are attractive to *T. castaneum*, and thus have the potential to be developed into lures for this pest species, the responses of *T. casaneum* were tested against a range of these compounds using a Y-tube olfactometer. The compounds were initially tested individually in two *T. castaneum* strains, and several of the compounds were found to be significantly attractive to both strains. The responses of the beetles were then tested against different blends of these VOCs and, after removing redundant elements, a blend containing three of the volatiles was identified as being particularly attractive. This blend could form the basis of a novel lure for this pest species and preliminary pitfall bioassay experiments were conducted to determine how attractive this lure might be under typical stored-product environment conditions.

4.2. Methods

4.2.1. *Tribolium* husbandry

Two *T. castaneum* strains were used in this chapter: CTC12 and wild Zim (see Chapter 1 Section 1.12 for more details). Cultures were incubated at 30°C in containers of 200 g of whole grain flour (Doves Farm Organic Strong Wholemeal Bread Flour) with the addition of 10 g yeast powder as an additional protein source (Holland & Barrett Debittered Brewer's Yeast Powder) and 1g of 0.3 g/kg antimicrobial agent Fumagillin to inhibit fungal growth in the cultures. All beetles used in these experiments were aged between four to eight weeks post-emergence to ensure all insects were sexually mature and mated to reduce the variance in their responses to the DMD aggregation pheromone (Duehl et al., 2011). The sex of the beetles used in each bioassay is stated in its associated methods section and different individual beetles were used for each bioassay treatment.

4.2.2. Y-tube olfactometer

The Y-tube olfactometer methodology is described in detail in the methods section of Chapter 2 (Section 2.2.6). Forty beetles were tested for each odour source and different individuals were used for each trial. Female CTC12 *T. castaneum* were use in the Y-tube olfactometer as the VOCs tested were chosen based on EAG experiments conducted using female CTC12 *T. castaneum.* It is also believed that male beetles could leave pheromone trails that may bias the responses of proceeding beetles (Ahmad, Daglish, et al., 2012), even though the Y-tube olfactometer was cleaned with ethanol between trials. Five microlitres of a given amount of each odour source was applied to paper disks in each experiment.

4.2.3. Pitfall bioassays

The design of the pitfall bioassays is described in detail in the methods section of Chapter 2 (Section 2.2.3). Twenty beetles (ten male and ten female) were used in each trial and ten replicates were conducted for each odour source. Different individuals were used for each trial. Five µl of a given odour source was applied to a paper disk or inserted into a slow release plastic capsule in each experiment. The plastic capsules were formed from short pieces of polyethylene (PE) capillary tubing into which an odour source was pipetted.

The ends of the tubing were then sealed by heating the ends until the plastic melted.

4.2.4. Odour sources

The VOCs tested in this chapter are listed in Table 4.1. Most of these VOCs had been identified from the literature as being found within wheat germ oil (Niu et al., 2013) or produced by grain associated fungi (Magan and Evans, 2000). The VOCs tested in this chapter include the compounds that elicited the ten largest and ten smallest average EAG responses when tested in two *T. castaneum* strains in Chapter 3. All compounds were obtained from commercial suppliers (see Appendix A for more details). Some of the VOCs were combined to produce a number of different blends, listed in Table 4.2, which were also tested. The 10-compound blend contained the 10 compounds that elicited the highest EAG responses in Chapter 3. The 5-compound blend (1) contained only the VOC that were found to be attractive to both strains and the 5-compound blend (2) contained the remaining VOCs. The wheat germ oil and fungal blends contained only the VOCs found within wheat germ oil or produced by grain associated fungi, respectively. The 3-compound blend was a minimal attractive blend formulated from the results of experiments conducted in this chapter. In every blend equal proportions of each compound were used. The *Tribolium* aggregation pheromone DMD (4,8-dimethyldecanal) was used to compare the effectiveness of some of the blends to a known *T. castaneum* attractant. Hexane was used as a solvent to dilute the individual compounds and blends to working concentrations and as a negative control odour source. In each experiment the concentrations of the odour sources used are stated in the associated figure legend.

Table 4.1. A list of the compounds tested in this chapter.

Compounds		
5-Methyl-3-heptanone	Hexanal	Octanoic acid
3-Octanone	trans,trans-2,4-Heptandinal	trans-2-Octen-1-ol
Butyl acetate	2-Methyl-2-butene	Octyl acetate
Ethyl hexanoate	Ethyl ethanoate	1-Phenylethanol
2-Heptanone	Tridecane	p-Anisaldehyde
trans-2-Heptenal	4-Allylanisol	Hexane
3-Methylanisol	1-Octanol	DMD (4,8-Dimethyldecanal)
Limonene		

Table 4.2. A list of the names of the blends tested and their constituent VOCs.

Blend name	**Composition**
10-Compound blend	5-Methyl-3-heptanone, 3-Octanone, Butyl acetate, Ethyl hexanoate, 2-Heptanone, trans-2-Heptenal, 3-Methylanisol, Limonene, Hexanal, trans,trans-2,4-Heptandinal
5-Compound blend (1)	3-Octanone, Butyl acetate, 2-Heptanone, 3-Methylanisol, Hexanal
5-Compound blend (2)	5-Methyl-3-heptanone, Ethyl hexanoate, trans-2-Heptenal, Limonene, trans,trans-2,4,-Heptandinal
Wheat germ oil blend	trans,trans-2,4-Heptandinal, Limonene, trans-2-Heptenal, 2-Heptanone, Ethyl hexanoate, 5-Methyl-3-heptanone
Fungal blend	3-Octanone, 3-Methylanisol, Butyl acetate
3-Compound blend	2-Heptanone, 3-Methylanisol, Hexanal

4.2.5. Statistical analysis

χ^2 goodness-of-fit tests were used to identify differences between the number of beetles attracted to the test odour source and the hexane negative control in each Y-tube olfactometer experiment. In each case the expected frequency of beetles responding to each odour source was 20 (an equal number of beetles responding to the test odour source and the negative control). As the data for each experiment were analysed using multiple χ^2 goodness of fit tests (one for each odour source) there was an increased risk of type 1 errors, which could result in false positives. To account for this, the P values in each experiment were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Binomial logistic regressions were also performed in cases where compounds were tested at multiple concentrations or against multiple strains to allow for comparisons to be made between different experimental treatments. Binomial logistic regressions were also used to identify if any of the blends were significantly more attractive than any of their constituent volatiles.

A Spearman's rank correlation coefficient was used to test the correlation between the EAG responses a compound elicited and its attractiveness in behavioural bioassays. As only the compounds that gave the largest and smallest EAG responses were tested, a non-parametric test was used, as the data were not normally distributed.

Mann-Whitney U tests were used to analyse the difference between test odour sources and their associated hexane control treatments in the pitfall bioassay experiments. The count data collected by the pitfall bioassays were skewed by a large number of observations where zero beetles were captured. Therefore, a non-parametric test was used to analyse these data.

All statistical analysis was performed using SPSS 24 software.

4.3. Results

4.3.1. The attractiveness of four VOCs at different concentrations

Before conducting more systematic testing of the compounds that were previously found to elicit the largest EAG responses in Chapter 3, a small

number of these compounds were tested at different concentrations to identify the concentrations at which they were most attractive. The responses of CTC12 and wild Zim strain *T. castaneum* to 3-octanone, hexanal, 3-methylanisol and butyl acetate in a Y-tube olfactometer are shown in Fig. 4.1. Multiple χ^2 goodness of fit tests were performed to identify odour sources that attracted significantly more beetles than the hexane negative control. The results of these tests are presented in Table 4.3 and significant responses are also indicated on Fig. 4.1. Both strains were found to be significantly attracted to each of the compounds presented at a 1 µg dosage. The responses to these compounds at higher concentrations differed slightly across the two strains, with higher concentrations generally remaining attractive to the wild Zim strain but becoming unattractive to the CTC12 strain. At a 10 µg dosage all the compounds tested were significantly attractive to wild Zim beetles, while none were significantly attractive to the CTC12 strain. At a 100 µg dosage all the compounds were significantly attractive to the wild Zim strain apart from butyl acetate, whereas for the CTC12 strain none of the compounds were attractive, with 3-octanone even being significantly repulsive at this concentration.

The factors affecting the attraction of *T. castaneum* were examined using a binomial logistic regression and the results are summarised in Table 4.4. The results confirm that the concentration at which the compounds were tested had a significant effect on their attractiveness. However, the strain of the beetles and the volatiles presented were not found to have a significant effect on the responses of the beetles and none of the interaction terms were found to have a significant effect on the responses of the beetles.

As the attractiveness of these compounds was significantly affected by the concentration they were tested at, the remaining volatiles that elicited large EAG responses were tested at a 1 µg dose. This concentration was chosen as it was the only concentration at which all compounds tested were found to be attractive to both strains.

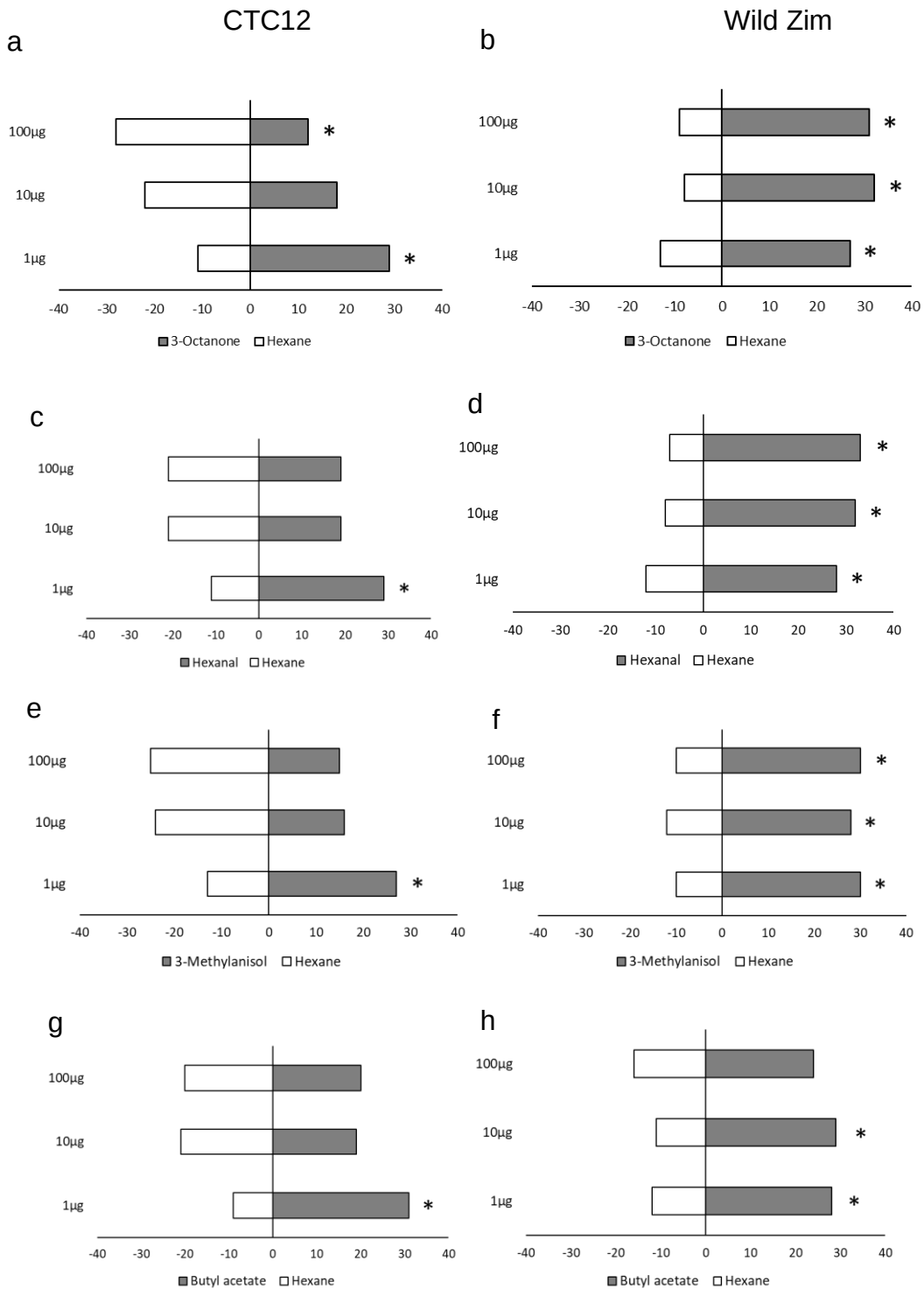

Figure 4.1. The responses of female CTC12 and wild Zim strain *T. castaneum* in a Y-tube olfactometer bioassay, to four different volatile compounds.

All compounds were tested at three different doses: 1 µg (5µl of 200ng/µl), 10 µg (5µl of 2µg/µl), and 100 µg (5µl of 20µg/µl). The strains and compounds tested were: a. CTC12, 3-octanone; b. wild Zim, 3-octanone; c. CTC12, hexanal; d. wild Zim, hexanal; e. CTC12, 3-methylanisol; f. wild Zim, 3-methylanisol; g. CTC12, butyl acetate; h. wild Zim, butyl acetate. The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the test volatile and the white bars indicate the number that chose the arm containing the hexane negative control. Statically significant differences ($P<0.05$) between the test compounds and their associated hexane negative controls are indicated with * (full statistics are presented in Table 4.3).

Table 4.3. The responses of female CTC12 and wild Zim strain *T. castaneum* to four different volatile compounds in Y-tube olfactometer bioassays.

All compounds were tested at three different doses: 1µg 10µg, 100µg.The results of χ^2 goodness-of-fit tests are presented, comparing the observed number of individuals that were attracted to the test volatile or the hexane negative control arm of the olfactometer to the expected frequency of 20. The P values were adjusted using the Benjamini-Hochberg procedure and significantly different P values ($P<0.05$) are highlighted in bold.

Volatile	Strain	Dose	Beetles attracted	N	χ^2	df	Adjusted P
3-Octanone	CTC12	100 µg	12	40	6.4	1	**0.02**
		10 µg	18	40	0.4	1	0.633
		1 µg	29	40	8.1	1	**0.011**
	Wild Zim	100 µg	31	40	12.1	1	**0.002**
		10 µg	32	40	14.4	1	**<0.001**
		1 µg	27	40	4.9	1	**0.040**
Hexanal	CTC12	100 µg	19	40	0.1	1	0.784
		10 µg	19	40	0.1	1	0.784
		1 µg	29	40	8.1	1	**0.011**
	Wild Zim	100 µg	33	40	16.9	1	**<0.001**
		10 µg	32	40	14.4	1	**<0.001**
		1 µg	28	40	6.4	1	**0.02**
3-Methylanisol	CTC12	100 µg	15	40	2.5	1	0.161
		10 µg	16	40	1.6	1	0.26
		1 µg	27	40	4.9	1	**0.04**
	Wild Zim	100 µg	30	40	10	1	**0.005**
		10 µg	28	40	6.4	1	**0.02**
		1 µg	30	40	10	1	**0.005**
Butyl acetate	CTC12	100 µg	20	40	0	1	1
		10 µg	19	40	0.1	1	0.784
		1 µg	31	40	12.1	1	**0.002**
	Wild Zim	100 µg	24	40	1.6	1	0.26
		10 µg	29	40	8.1	1	**0.011**
		1 µg	28	40	6.4	1	**0.02**

Table 4.4. The results of a binomial logistic regression comparing the effect of volatile presented, volatile concentration, beetle strain and interactions between these factors on the number of T. castaneum attracted in Y-tube olfactometer bioassays.

Statistically significant effects (P<0.05) are highlighted in bold.

Variable	Wald χ^2	df	P
Volatile	0.996	3	0.802
Strain	0.547	1	0.460
Concentration	8.527	2	**0.014**
Strain * volatile	1.285	3	0.733
Concentration * volatile	2.046	6	0.915
Concentration * strain	3.352	2	0.187
Concentration * strain * volatile	3.055	6	0.802

4.3.2. The attractiveness of the 10 VOCs that elicited the largest EAG responses

After testing the responses of four of the ten compounds that elicited the largest EAG responses from *T. castaneum* in Section 4.3.1, the remaining compounds were then tested. The responses of female CTC12 and wild Zim *T. castaneum* to these compounds in a Y-tube olfactometer are shown in Fig. 4.2. As the responses of *T. castaneum* to 3-octanone, hexanal, 3-methylanisol and butyl acetate were tested in the previous section, the same data is presented in this section to allow for comparisons to be made across all the compounds that gave the largest EAG responses. Multiple χ^2 goodness of fit tests were performed to identify VOCs that attracted significantly more beetles than the hexane negative control. The results of these tests are presented in Table 4.5 and significant responses are indicated in Fig. 4.2. Despite all these compounds being shown to elicit large EAG responses in Chapter 3, not all of these compounds were found to be significantly attractive at the concentration tested. Overall, the responses across the two strains to these 10 compounds were similar. Five of the VOCs: hexanal, 3-methylanisol, 2-heptanone, butyl

acetate and 3-octanone were found to be attractive to both strains, while limonene, trans-2-heptanone, and ethyl hexanoate were not attractive to either strain. Trans-trans-2,4,-heptandinal and 5-methyl-3-heptanone were only attractive to the CTC12 strain. A binomial logistic regression (Table 4.6) comparing the effects of different factors on the number of beetles attracted revealed that, neither the volatile presented, the strain of the beetles or an interaction between these factors had a significant effect on the number of *T. castaneum* attracted.

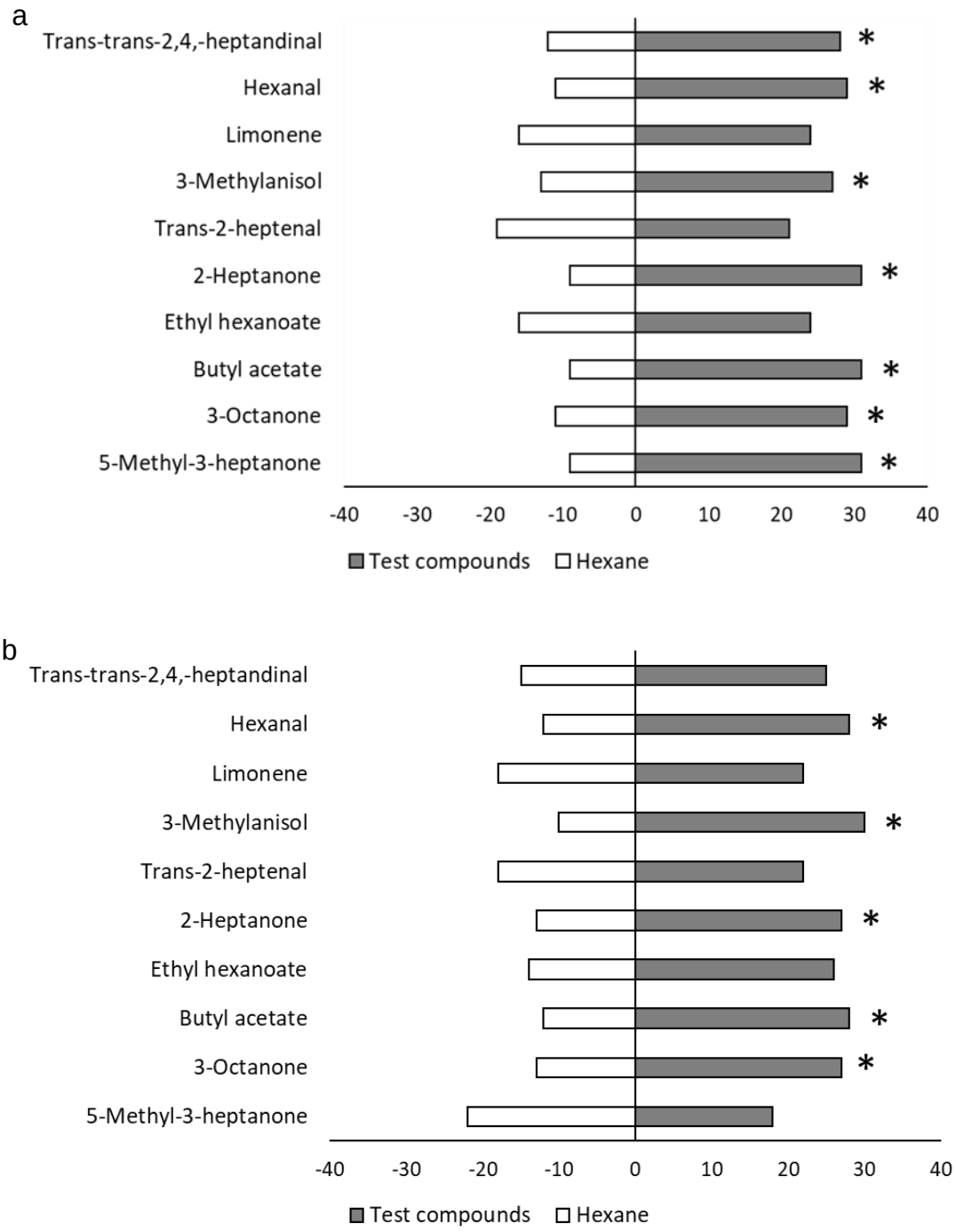

Figure 4.2. The responses of female (a) CTC12 and (b) wild Zim *T. castaneum* in a Y-tube olfactometer to the 10 VOCs that elicited the largest EAG depolarisations in Chapter 3.

The grey bars indicate the number of beetles (out of 40 individuals) that chose the Y-tube arm containing the test volatile and the white bars indicate the number that chose the arm containing the hexane negative control. All compounds were tested at a 1 µg dosage (5µl of 200ng/µl). Statically significant ($P<0.05$) differences between test compounds and the associated hexane negative control are indicated with * (full statistics are presented in Table 4.5).

Table 4.5. The responses of female CTC12 and wild Zim strain *T. castaneum* to 10 different volatile compounds in Y-tube olfactometer bioassays.

The results of χ^2 goodness-of-fit tests are also presented, comparing the observed number of individuals that were attracted to either the test volatile or hexane negative control arm of the olfactometer to the expected frequency of 20 for each. The P values were adjusted using the Benjamini-Hochberg procedure and significantly different P values ($P<0.05$) are highlighted in bold.

Volatile	Strain	Beetles attracted	N	χ^2	df	Adjusted P
5-Methyl-3-heptanone	CTC12	31	40	12.1	1	**0.003**
	Wild Zim	18	40	0.4	1	0.555
3-Octanone	CTC12	29	40	8.1	1	**0.015**
	Wild Zim	27	40	4.9	1	**0.045**
Butyl acetate	CTC12	31	40	12.1	1	**0.003**
	Wild Zim	28	40	6.4	1	**0.025**
Ethyl hexanoate	CTC12	24	40	1.6	1	0.257
	Wild Zim	26	40	3.6	1	0.089
2-Heptanone	CTC12	31	40	12.1	1	**0.003**
	Wild Zim	27	40	4.9	1	**0.045**
trans-2-Heptenal	CTC12	21	40	0.1	1	0.752
	Wild Zim	22	40	0.4	1	0.555
3-Methylanisol	CTC12	27	40	4.9	1	**0.045**
	Wild Zim	30	40	10	1	**0.008**
Limonene	CTC12	24	40	1.6	1	0.257
	Wild Zim	22	40	0.4	1	0.555
Hexanal	CTC12	29	40	8.1	1	**0.014**
	Wild Zim	28	40	6.4	1	**0.025**
trans-trans-2,4-Heptandinal	CTC12	28	40	6.4	1	**0.025**
	Wild Zim	25	40	2.5	1	0.163

Table 4.6. The results of a binomial logistic regression comparing the effect of volatile presented, beetle strain and an interaction between these factors on the number of *T. castaneum* attracted in a Y-tube olfactometer bioassay.

Significantly different P values (P<0.05) are highlighted in bold.

Variable	Wald χ^2	df	P
Volatile	12.283	9	0.198
Strain	0.05	1	0.823
Strain * volatile	9.362	9	0.405

4.3.3. The attractiveness of the 10 VOCs that elicited the smallest EAG responses

To help demonstrate the relationship between the magnitude of EAG depolarisation a compound produces and its attractiveness, the responses of CTC12 *T. castaneum* were also tested against the 10 compounds that were shown in Chapter 3 to elicit the smallest EAG responses. The responses of female CTC12 *T. castaneum* to these VOCs in Y-tube olfactometer bioassays are shown in Fig. 4.3. Multiple χ^2 goodness of fit tests were performed to identify odour sources that attracted significantly more beetles than the hexane negative control. The results of these tests are presented in Table 4.7 and significant responses are indicated on Fig. 4.3. None of the compounds tested were found to be significantly attractive.

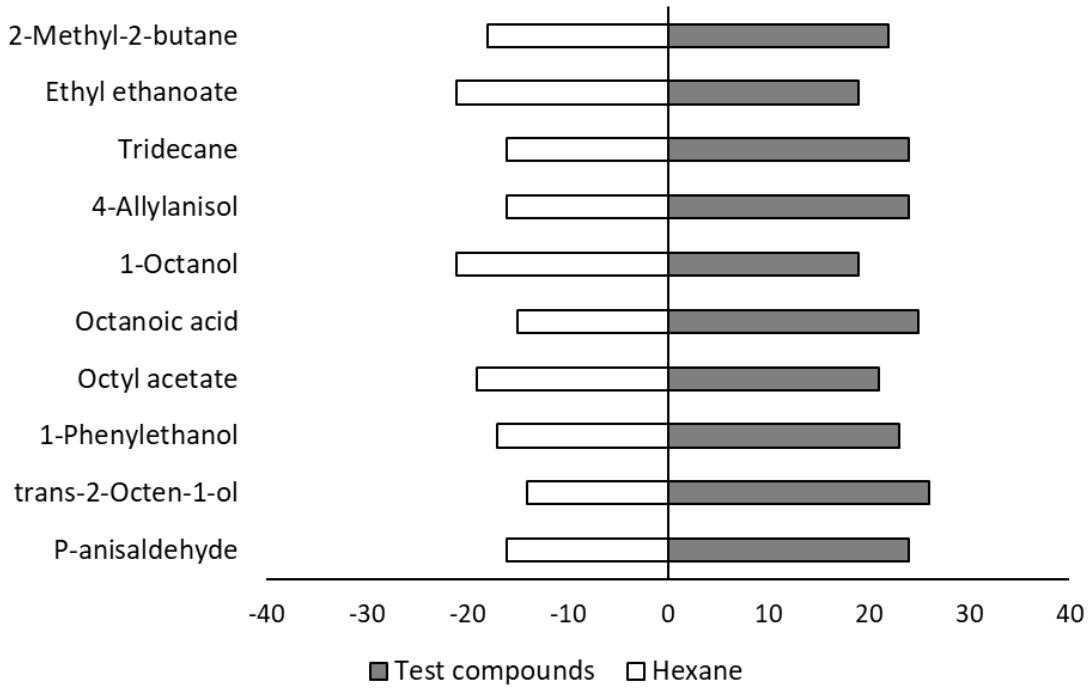

Figure 4.3. The responses of female CTC12 strain *T. castaneum*, in a Y-tube olfactometer bioassay, to the 10 volatile compounds that were found to elicit the weakest EAG depolarisations in Chapter 3.

The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the test volatile, and the white bars indicate the number that chose the arm containing the hexane negative control. All compounds were tested at a 1 µg dosage (5µl of 200ng/µl). No statistically significant differences ($P<0.05$) were found between the response to any of the test compounds and their associated hexane negative controls (full statistics in Table 4.7).

Table 4.7. The responses of female CTC12 strain *T. castaneum* to 10 different volatiles in Y-tube olfactometer bioassays.

The results of χ^2 goodness-of-fit tests are also presented, comparing the observed number of insects that were attracted to either the test volatile or hexane control arm of the olfactometer to the expected frequency of 20 for each. The P values were adjusted using the Benjamini-Hochberg procedures and significantly different P values ($P<0.05$) are highlighted in bold.

Volatile	Beetles attracted	N	χ^2	df	Adjusted P
2-Methyl-2-butene	22	40	0.4	1	0.752
Ethyl ethanoate	19	40	0.1	1	0.752
Tridecane	24	40	1.6	1	0.412
4-Allylanisol	24	40	1.6	1	0.412
1-Octanol	19	40	0.1	1	0.752
Octanoic acid	25	40	2.5	1	0.412
trans-2-Octen-1-ol	26	40	3.6	1	0.412
Octyl acetate	21	40	0.1	1	0.752
1-Phenylethanol	23	40	0.9	1	0.571
p-Anisaldehyde	24	40	1.6	1	0.412

4.3.4. The correlation between the EAG depolarisation elicited by selected compounds and their attractiveness to *Tribolium castaneum*

The relationship between EAG depolarisation and behavioural attraction across the compounds that were shown to give the largest and smallest EAG responses in *T. castaneum*, is shown in Fig. 4.4. A spearman's rank correlation coefficient identified a significant correlation between the size of EAG depolarisation a VOC elicited and its attractiveness ($r_s(18) = 0.551$, p 0.012) across the 20 compounds.

Figure 4.4. The attractiveness of 20 compounds, in a Y-tube olfactometer, plotted against the EAG response the same compound elicited tested using female CTC12 *T. castaneum*.

The VOCs that elicited the 10 largest and 10 smallest average depolarisations in *T. castaneum* are listed in Table 4.1 (minus hexane and DMD). The data on the attractiveness of the VOCs in a Y-tube olfactometer are shown in Tables 4.5 and 4.7 and the data on the EAG depolarisations these compounds elicited are shown in Chapter 3 Section 3.3.4.

4.3.5. The attractiveness of different VOC blends

After identifying that many of the compounds tested in Section 4.3.2. were significantly attractive, the responses of *T. castaneum* to different blends of these compounds were tested. The composition of each of these blends is detailed in Table 4.2. The responses of CTC12 and wild Zim *T. castaneum* to these blends are show in Fig. 4.5. Multiple χ^2 goodness of fit tests were performed to identify odour sources that attracted significantly more beetles than the hexane negative control. The results of these tests are presented in Table 4.8 and significant responses are indicated on Fig. 4.5. Overall, the responses across both strains are similar, with the 10-compound blend, 5-compound blend (1) and wheat germ oil blend eliciting significant responses from both strains, while the response to the 5-compound blend (2) was not significantly different to the hexane negative control in either strain. The response to the fungal blend was significantly different from the hexane negative control in the wild Zim strain, but not in the CTC12 strain. In the

CTC12 strain the 10-compound blend and 5-compound blend (1) attracted more beetles than any of the volatile compounds did when tested on their own. This was not the case with the wild strain, where 3-methylanisol by itself elicited as large a response as any of the blends did. Table 4.9 shows the results of a binomial logistic regression comparing the effects of different factors on the number of beetles attracted. The blend presented was found to have a significant effect on the behavioural responses, however neither the strain of the beetles tested or an interaction between the beetle strain and the blend presented was found to be have a significant effect.

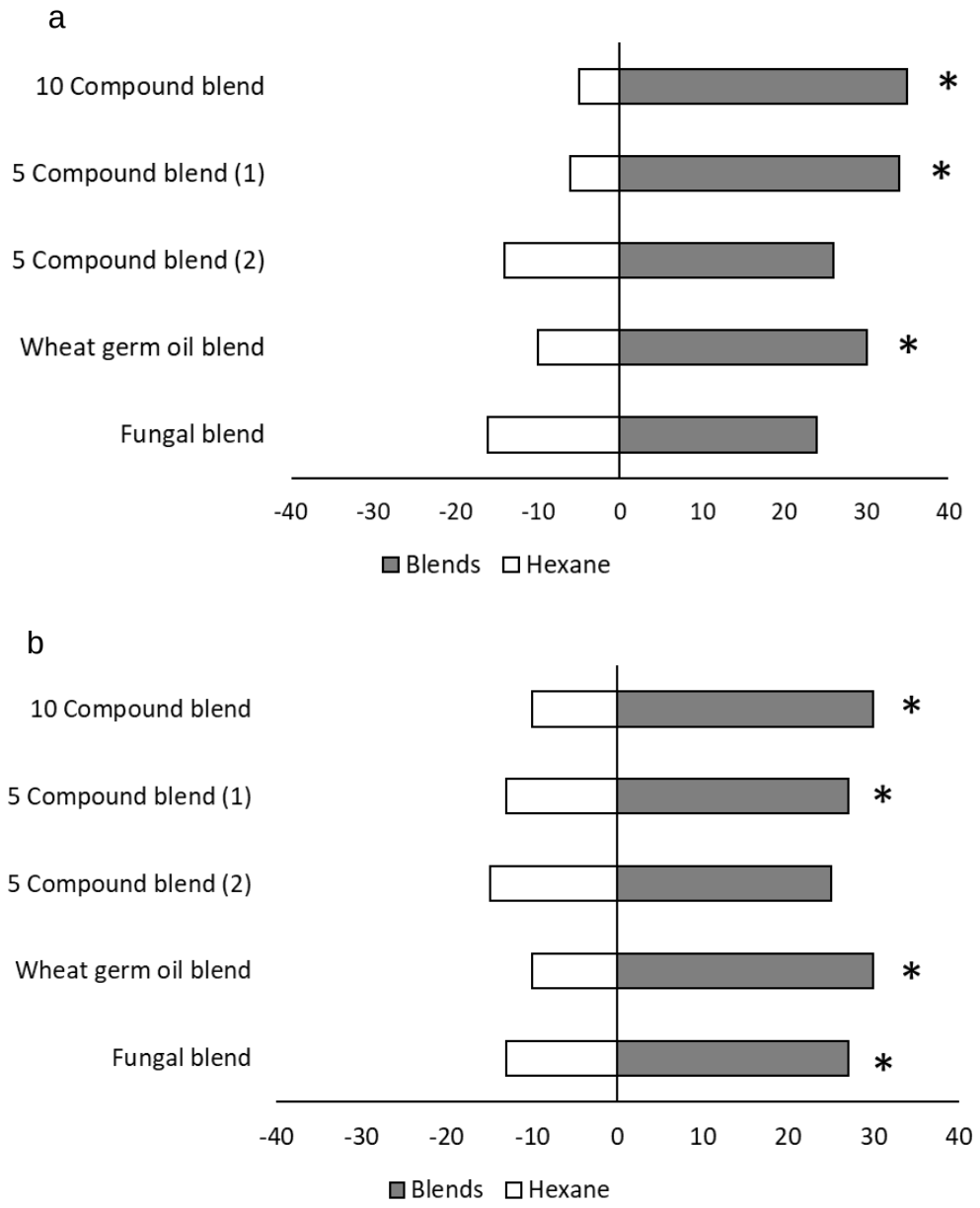

Figure 4.5. The responses of (a) CTC12 and (b) wild Zim strain female *T. castaneum* in a Y-tube olfactometer bioassay, to five blends containing different combinations of the compounds found to elicit the largest EAG responses in Chapter 3.

10-compound blend: 3-octanone, ethyl hexanoate, limonene, 2-heptanal, 5-methyl-3-heptanone, butyl acetate, 3-methylanisol, trans-trans-2,4-heptandinal, 2-heptanone, hexanal; **5-compound blend (1):** hexanal, 3-methylanisol, 2-heptanone, butyl acetate, 3-octanone; **5-compound blend (2):** trans-trans-2,4-heptandinal, limonene, trans-2-heptanal, ethyl hexanoate, 5-methyl-3-heptanone; **wheat germ oil blend:** trans-trans-2,4-heptandinal, limonene, trans-2-heptanal, 2-heptanone, ethyl hexanoate, and 5-methyl-3-heptanone; **fungal blend:** 3-methylanisol, butyl acetate and 3-octanone. The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the test volatile, and the white bars indicate the number that chose the arm containing the hexane negative control. All odour sources were tested at a 1 µg dosage (5µl of 200ng/µl). Statistically significant ($P<0.05$) differences between test compounds and the hexane negative control are indicated with * (full statistics in Table 4.8).

Table 4.8. The number of beetles attracted to different VOC blends in Y-tube olfactometer bioassays.

The results of χ^2 goodness-of-fit tests are also presented, comparing the observed number of insects that were attracted to either the test blend or hexane negative control arm of the olfactometer to the expected frequency of 20 for each. The P values were adjusted using the Benjamini-Hochberg procedures and significantly different P ($P<0.05$) values are highlighted in bold.

Volatile	Strain	Beetles attracted	N	χ^2	df	Adjusted P
10 Compound blend	CTC12	35	40	22.5	1	**<0.001**
	Wild Zim	30	40	10	1	**0.003**
5 Compound blend (1)	CTC12	34	40	19.6	1	**<0.001**
	Wild Zim	27	40	4.9	1	**0.038**
5 Compound blend (2)	CTC12	26	40	3.6	1	0.072
	Wild Zim	25	40	2.5	1	0.126
Wheat germ oil blend	CTC12	30	40	10	1	**0.003**
	Wild Zim	30	40	10	1	**0.003**
Fungal blend	CTC12	24	40	1.600	1	0.206
	Wild Zim	27	40	4.9	1	**0.038**

Table 4.9. The results of a binomial logistic regression comparing the effect of volatile presented, beetle strain and an interaction between these factors on the number of *T. castaneum* attracted in a Y-tube olfactometer bioassay.

Significantly different P values (P<0.05) are highlighted in bold.

Variable	Wald χ^2	df	P
Blend	12.299	4	**0.015**
Strain	0.054	1	0.816
Strain * Volatile	5.093	4	0.278

For each blend the elicited a significantly attractive response, binomial linear regressions were used to identify whether the blends were significantly more attractive than their constituent volatiles. The results for the CTC12 and wild Zim strains are shown in Table 4.10 and 4.11 respectively. Data on the responses of the beetles to individual volatiles was previously presented in Fig. 4.2 and Table 4.5. In both strains only the 10-compound blend and the wheat germ oil blend were found to be significantly more attractive than some of their constituent compounds. However, in all cases, individual compounds that were not significantly less attractive than the blend could be identified.

Table 4.10. The results of a binomial logistic regression comparing the attractiveness of VOC blends with each of their constituent VOCs on their own using female CTC12 *T. castaneum.*

Significantly different P values (P<0.05) are highlighted in bold.

Blend	Volatile	Wald X^2	df	P
10-Compound blend	5-Methyl-3-heptanone	1.352	1	0.245
	3-Octanone	2.694	1	0.101
	Butyl acetate	1.352	1	0.245
	Ethyl hexanoate	**7.132**	**1**	**0.008**
	2-Heptanone	1.352	1	0.245
	trans-2-Heptenal	**10.361**	**1**	**0.001**
	3-Methylanisol	**4.310**	**1**	**0.038**
	Limonene	**7.132**	**1**	**0.008**
	Hexanal	2.694	1	0.101
	trans-trans-2,4,-Heptandinal	3.472	1	0.062
5-Compound blend (1)	3-Octanone	1.821	1	0.177
	Butyl acetate	0.730	1	0.393
	2-Heptanone	0.730	1	0.393
	3-Methylanisol	3.249	1	0.071
	Hexanal	1.821	1	0.177
WGO blend	5-Methyl-3-heptanone	0.492	1	0.483
	Ethyl hexanoate	2.023	1	0.155
	2-Heptanone	0.069	1	0.793
	Trans-2-heptenal	**4.269**	**1**	**0.039**
	Limonene	2.023	1	0.155

	Trans-trans-2,4,-heptandinal	0.250	1	0.617

Table 4.11. The results of a binomial logistic regression comparing the attractiveness of VOC blends with each of their constituent VOCs on their own, using female wild Zim *T. castaneum.*

Significantly different P values (P<0.05) are highlighted in bold.

Blend	Volatile	Wald χ^2	df	P
10 Compound blends	**5-Methyl-3-heptanone**	**7.204**	**1**	**0.007**
	3-Octanone	0.547	1	0.460
	Butyl acetate	0.250	1	0.617
	Ethyl hexanoate	0.946	1	0.331
	2-Heptanone	0.547	1	0.460
	Trans-2-heptenal	3.441	1	0.064
	3-Methylanisol	0.000	1	1.000
	Limonene	3.441	1	0.064
	Hexanal	0.250	1	0.617
	Trans-trans-2,4,-heptandinal	1.440	1	0.230
5 Compound blend (1)	**3-Octanone**	0.000	0.477	1
	Butyl acetate	0.116	0.483	0.809
	2-Heptanone	0.000	0.477	1
	3-Methylanisol	0.368	0.497	0.460
	Hexanal	0.116	0.483	0.809
WGO blend	**5-Methyl-3-heptanone**	**7.204**	**1**	**0.007**
	Ethyl hexanoate	0.946	1	0.331
	2-Heptanone	0.547	1	0.460
	Trans-2-heptenal	3.441	1	0.064
	Limonene	3.441	1	0.064

	Trans-trans-2,4,-heptandinal	1.440	1	0.230
Fungal blend	3-Octanone	0.000	1	1.000
	Butyl acetate	0.058	1	0.809
	3-Methylanisol	0.547	1	0.460

After identifying a number of significantly attractive blends the most attractive of these (the 10 compound blend) was tested across a range of concentrations. The responses of female CTC12 and wild Zim strain *T. castaneum* to the 10 compound blend at four concentrations is shown in Fig. 4.6. Multiple χ^2 goodness of fit tests were performed to identify odour sources that attracted significantly more beetles than the hexane control. The results of these tests are presented in Table 4.12 and significant responses are indicated in Fig. 4.6. In both strains the 1 µg and the 10 µg dosage gave a statistically larger response than the hexane control, with the 10µg dose eliciting a larger response in the wild Zim strain and the 1 µg dosage eliciting a larger response in the CTC12 strain. The 100 ng dosage was only attractive to the CTC12 strain and the 10 ng dosage was not significantly attractive to either strain. Table 4.13 shows the results of a binomial logistic regression comparing the effects of different factors on the number of beetles attracted. All of the factors tested, volatile concentration, beetle strain, and the interaction between these factors were found to have a significant effect on the responses of the beetles.

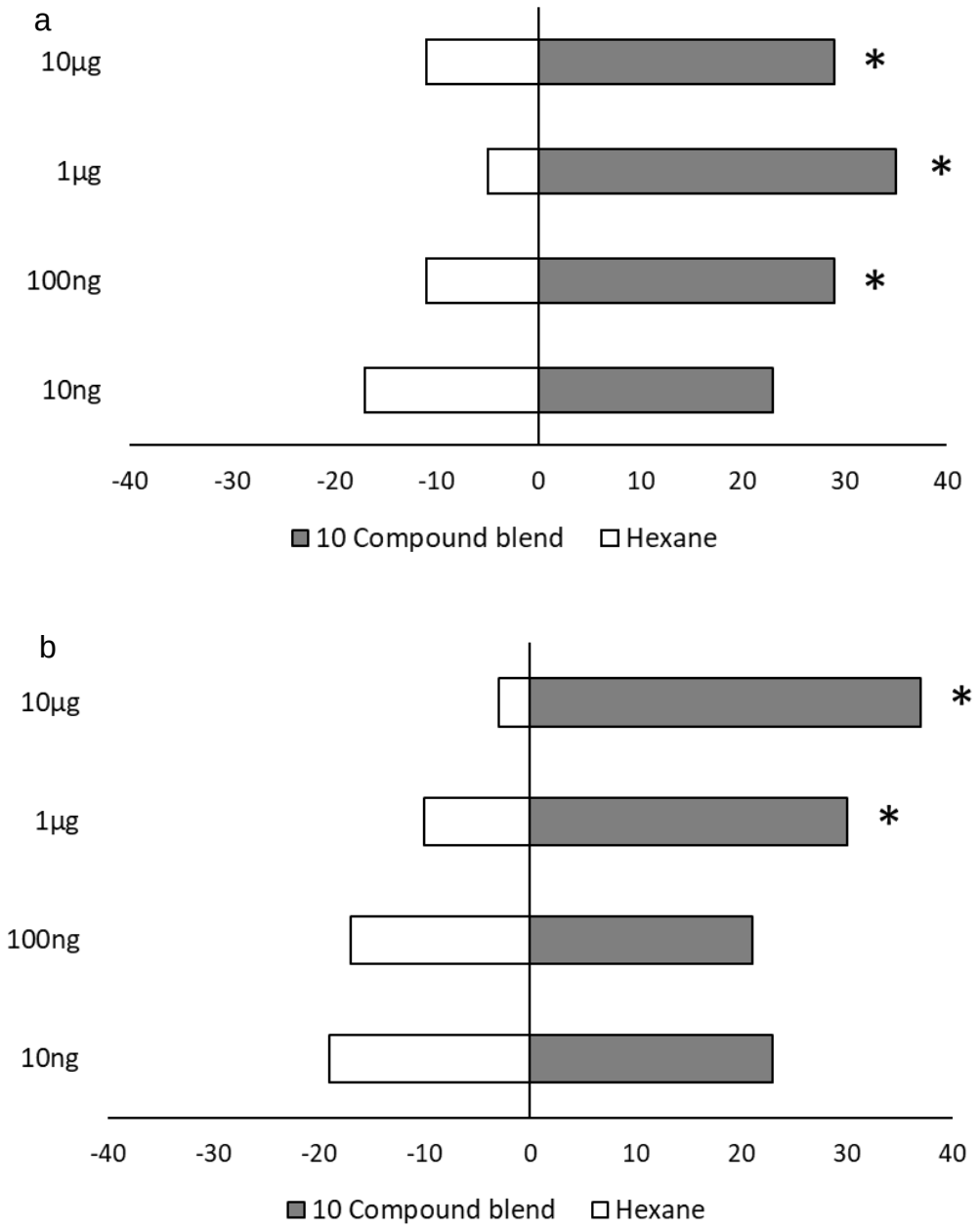

Figure 4.6. The responses of female (a) CTC12 and (b) wild Zim strain *T. castaneum*, in a Y-tube olfactometer bioassay, to a VOC blend containing 10 compounds (3-octanone, ethyl hexanoate, limonene, 2-heptanal, 5-methyl-3-heptanone, butyl acetate, 3-methylanisol, trans-trans-2,4-heptandinal, 2-heptanone, hexanal).

All blend were tested at four different doses: 10 ng (5µl of 2ng/µl), 100ng (5µl of 20ng/µl), 1 µg (5µl of 200ng/µl) and 10 µg (5µl of 2µg/µl). The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the test volatile, and the white bars indicate the number that chose the arm containing the hexane negative control. Statistically significant ($P<0.05$) differences between test compounds and the hexane negative control are indicated with * (full statistics in Table 4.12).

Table 4.12. The responses of female CTC12 and wild Zim strain *T. castaneum* to a VOC blend at four different concentrations in a Y-tube olfactometer bioassay.

The results of χ^2 goodness-of-fit tests are also presented, comparing the observed number of insects that were attracted to either the test volatile or hexane negative control arm of the olfactometer to the expected frequency of 20. The P values were adjusted using the Benjamini-Hochberg procedures and significantly different P values (P<0.05) are highlighted in bold.

Volatile	Dose	Strain	Beetles attracted	N	χ^2	df	Adjusted P
10-Compound blend	10 ng	CTC12	23	40	0.9	1	0.392
		Wild Zim	23	40	0.9	1	0.392
	100 ng	CTC12	29	40	8.1	1	**0.007**
		Wild Zim	21	40	0.1	1	0.752
	1 ug	CTC12	35	40	22.5	1	**<0.001**
		Wild Zim	30	40	10	1	**0.004**
	10 ug	CTC12	29	40	8.1	1	**0.007**
		Wild Zim	37	40	28.9	1	**<0.001**

Table 4.13. The results of a binomial logistic regression comparing the effect of volatile concentration, beetle strain and an interaction between these factors on the number of *T. castaneum* attracted in Y-tube olfactometer bioassays.

Significant effects (P<0.05) are highlighted in bold.

Variable	Wald χ^2	df	P
Concentration	15.328	3	**0.002**
Strain	4.901	1	**0.027**
Concentration * Strain	9.59	3	**0.022**

4.3.6. Optimising the VOC blend

In both strains the 10-compound blend was found to be the most attractive. However, the 5-compound blend (1), which contained half the number of compounds was found to be almost as attractive to both strains. To identify the compounds that contributed most to blend attractiveness individual compounds were removed from the 5-compound blend (1), before testing its

attractiveness. The responses of CTC12 strain beetles to the 5-compound blend (1) when different compounds were removed are shown in Fig. 4.7. Multiple χ^2 goodness of fit tests were performed to identify odour sources that attracted significantly more beetles than the hexane control. The results of these tests are presented in Table 4.14 and significant responses are indicated in Fig. 4.7. The removal of 2-heptanone or hexanal from the blend was found to abolish its attractiveness. In contrast, the removal of 3-methylanisol, butyl acetate or 3-octanone did not render the blend unattractive, although the removal of 3-methylanisol was found to moderately decrease the attractiveness. A binomial linear regression was used to compare the attractiveness of the 5-compound blend to each of the blends with VOCs removed (Table 4.15). Only the blends with 2-heptanone or hexanal removed were found to significantly less attractiveness than the original blend.

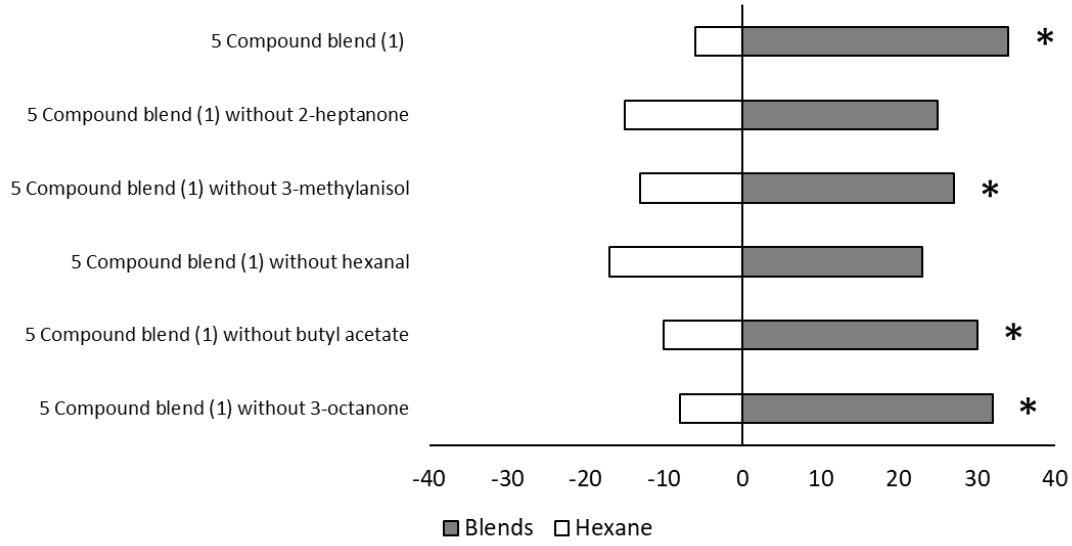

Figure 4.7. The responses of female CTC12 *T. castaneum* to five VOC blends in a Y-tube olfactometer bioassay.

Four of the blends are based on the 5 compound blend (1) (detailed in Table 4.2) but with a different compound removed for each blend (2-heptanone, 3-methylanisol, hexanal, butyl acetate or 3-octanone, respectively). The responses of CTC12 *T. castaneum* to the 5-compound blend (1) from Fig 4.5a are also presented to allow for comparisons with the original blend. The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the test volatile, and the white bars indicate the number that chose the arm containing the hexane negative control. All odour sources were tested at a 1 µg dosage (5µl of 200ng/µl). Statically significant ($P<0.05$) differences between test compounds and the hexane negative control are indicated with * (full statistics in Table 4.12).

Table 4.14. The responses of female CTC12 *T. castaneum* to four VOC blends in a Y-tube olfactometer bioassay.

The results of χ^2 goodness-of-fit tests are also presented, comparing the observed number of insects that were attracted to either the test volatile or hexane negative control arm of the olfactometer to the expected frequency of 20. The P values were adjusted using the Benjamini-Hochberg procedures and significantly different P values ($P<0.05$) are highlighted in bold.

Volatile	Beetles attracted	N	χ^2	df	Adjusted P
5-Compound blend (1) without 3-octanone	32	40	14.4	1	**<0.001**
5-Compound blend (1) without butyl acetate	30	40	10	1	**0.004**
5-Compound blend (1) without hexanal	23	40	0.9	1	0.343
5-Compound blend (1) without 3-methylanisol	27	40	4.9	1	**0.045**
5-Compound blend (1) without 2-heptanone	25	40	2.5	1	0.142

Table 4.15. The results of a binomial logistic regression comparing the attractiveness of the 5-compound blend (1) with different compounds removed to the original 5-compound blend in female CTC12 *T. castaneum.*

Significantly different P values (P<0.05) are highlighted in bold.

VOCs	Wald χ^2	df	P
5-Compound blend (1) without 2-heptanone	**4.947**	**1**	**0.026**
5-Compound blend (1) without 3-methylanisol	3.249	1	0.071
5-Compound blend (1) without hexanal	**6.876**	**1**	**0.009**
5-Compound blend (1) without butyl acetate	1.228	1	0.268
5-Compound blend (1) without 3-octanone	0.344	1	0.557

Based on the results in Fig. 4.7 and Table 4.15, which demonstrated that removing 2-heptanone, 3-methylanisol and hexanal from the 5-compound blend (1) either abolished completely or severely reduced its attractiveness, these compounds were identified as contributing most significantly to the attractiveness of this blend. The attractiveness of a new 3-compound blend containing just these compounds was tested in female CTC12 and wild Zim strain *T. castaneum* using a Y-tube olfactometer bioassay. To allow comparisons to be made with a known attractant, the responses of *T. castaneum* were also tested against DMD. To identify if DMD and this VOC blend have a synergistic effect when combined, the responses of *T. castaneum* were also tested against a combination of DMD and the 3-compound blend. The results of these experiments are shown in Fig. 4.8. Multiple χ^2 goodness of fit tests were performed to identify odour sources that attracted significantly more beetles than the hexane control. The results of these tests are presented in Table 4.16 and significant responses are indicated on Fig. 4.8. Each of the odour sources tested were found to be

attractive to both *T. castaneum* strains. The 3-compound blend gave a similar response in both strains, while DMD gave a lower response in the CTC12 strain and the combination of DMD and VOC blend gave a lower response in wild Zim. However, a binomial logistic regression comparing the effects of different factors on the number of beetles attracted revealed that neither the volatile presented, the strain of the beetles tested or an interaction between these factors had a significant effect on the attraction of *T. castaneum* (Table 4.17). A binomial linear regression was also used to identify if the 3-compound blend was significantly more attractive than any of its constituent VOCs. The results are shown in Table 4.18 and reveal that the blend was not significantly more attractive than any of its constituent VOCs were on their own.

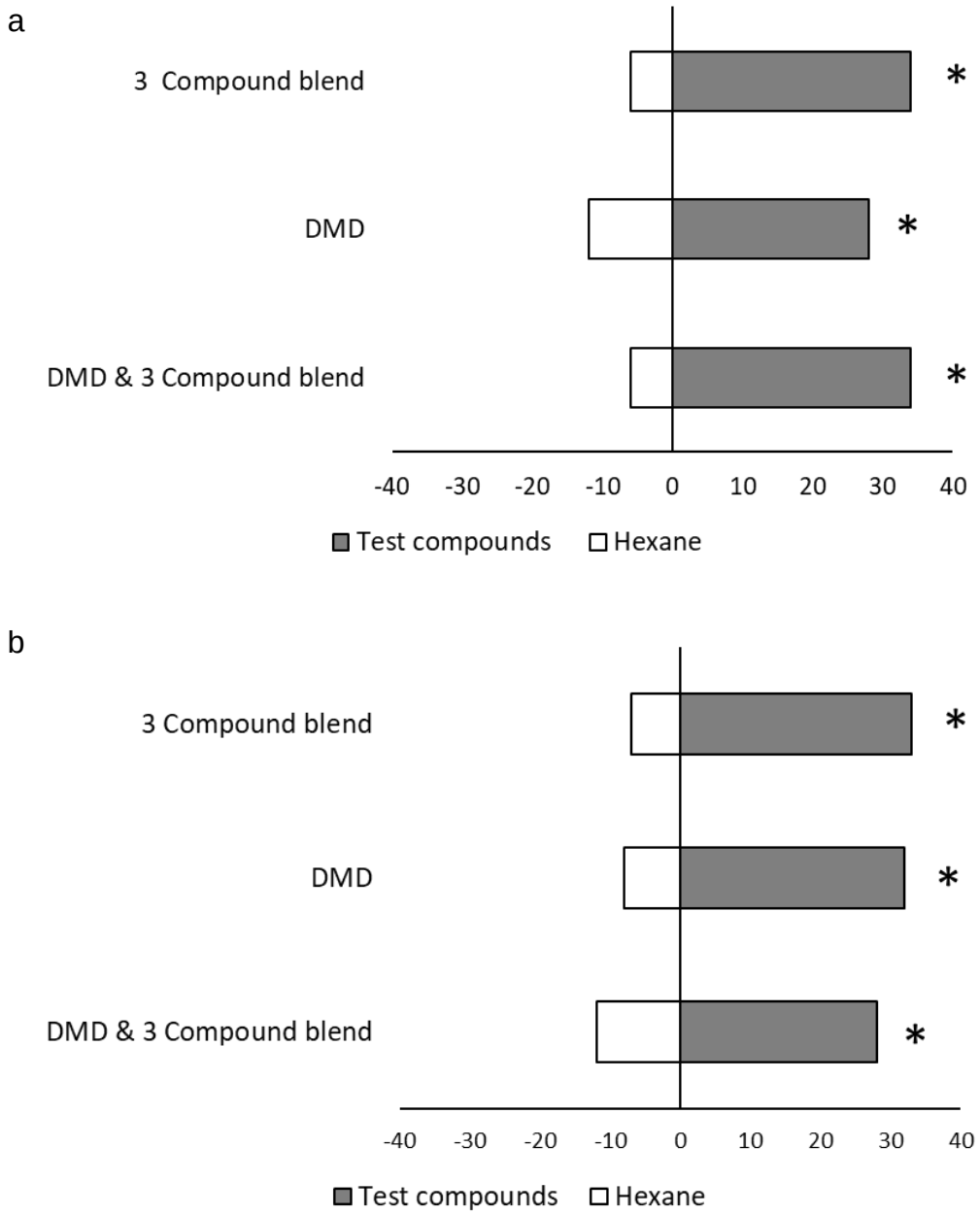

Figure 4.8. The responses of female (a) CTC12 or (b) wild Zim *T. castaneum* to a blend of three volatile compounds (hexanal, 3-methylanisol, and heptanone), DMD (the *Tribolium* aggregation pheromone) or both together.

All odour sources were tested at a 1 µg dose (5µl of 200ng/µl). The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the test volatile, and the white bars indicate the number that chose the arm containing the hexane negative control. Statically significant ($P<0.05$) differences between test compounds and the hexane negative control solvent are indicated with * (full statistics in Table 4.16).

Table 4.16. The responses of female CTC12 and wild Zim strain *T. castaneum* to three different odour sources.

The results of χ^2 goodness-of-fit tests are also presented, comparing the observed number of insects that were attracted to either the test volatile or hexane negative control arm of the olfactometer to the expected frequency of 20. The P values were adjusted using the Benjamini-Hochberg procedures and significantly different P values (P<0.05) are highlighted in bold.

Odour source	Strain	Beetles attracted	N	χ^2	df	Adjusted P
3-Compound blend	CTC12	34	40	19.6	1	**<0.001**
	Wild Zim	33	40	16.9	1	**<0.001**
DMD	CTC12	28	40	6.4	1	**0.011**
	Wild Zim	32	40	14.4	1	**<0.001**
DMD & 3-Compound blend	CTC12	34	40	19.6	1	**<0.001**
	Wild Zim	28	40	6.4	1	**0.011**

Table 4.17. The results of a binomial logistic regression comparing the effect of volatile presented, beetle strain and an interaction between these factors on the number of *T. castaneum* attracted in a Y-tube olfactometer bioassay.

Significantly different P values (P<0.05) are highlighted in bold.

Variable	Wald χ^2	df	P
Volatile	3.627	2	0.163
Strain	1.055	1	0.304
Strain * Volatile	3.452	2	0.178

Table 4.18. The results of a binomial logistic regression comparing the attractiveness of the 3-compound blend with its constituent VOCs in female CTC12 *T. castaneum*.

Significantly different P values (P<0.05) are highlighted in bold.

VOC	Wald χ^2	df	P
Hexanal	1.821	1	0.177
3-Methylanisol	3.249	1	0.071
2-Heptanone	0.730	1	0.393

4.3.7. The attractiveness of a three-compound blend in pitfall bioassays

In order to determine whether these blends have the potential to be used as lures in traps to capture *T. castaneum* in stored product environments, the responses of the beetles were tested using a pitfall bioassay. A bioassay that allows insects to move around more freely than the Y-tube olfactometer and allows volatiles to diffuse in still-air. The pitfall bioassay was used to test the responses of CTC12 and wild Zim *T. castaneum* to the 3-compound blend or hexane added to paper disks (Fig. 4.9) and sealed inside slow release plastic capsules (Fig. 4.10). Table 4.19 shows the response index and the results of Mann-Whitney U tests comparing the responses of beetles to the 3-compound lure to the associated hexane negative control and significant responses are indicated on Figures 4.9 and 4.10. When the 3-compound blend was applied to paper disks it was not found to be significantly more attractive than the hexane negative control in either strain. When the 3-compound blend was inserted into a slow release plastic capsule it was found to be significantly attractive to the CTC12 strain but not to the wild Zim strain. However, the capture rate was still relatively low across all of the treatment groups.

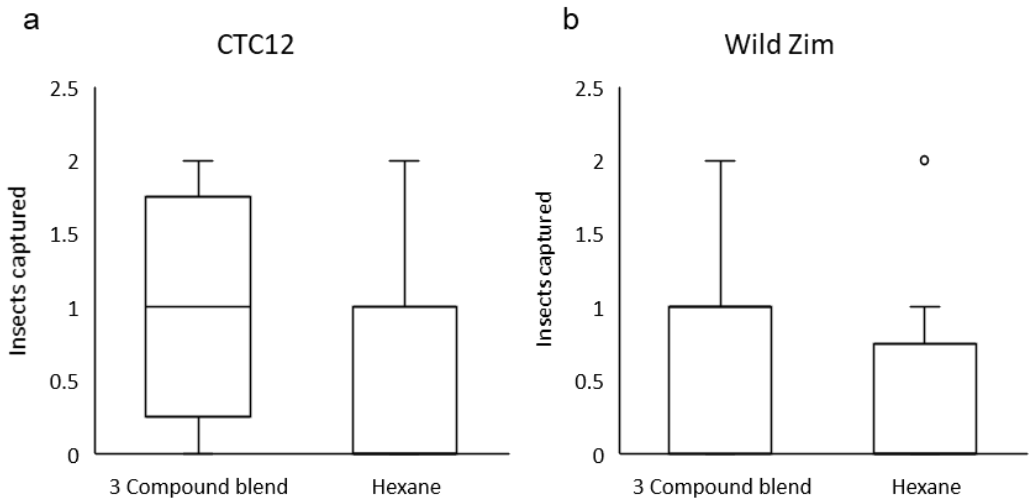

Figure 4.9. Boxplots representing the responses of (a) CTC12 or (b) wild Zim *T. castaneum* to pitfall traps baited with a 3-compound blend or a hexane negative control.

The horizontal line in the centre of the boxplots represents the median value with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (point falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetle each (10 male and 10 female) were used in each trial. All compounds were tested at a 1µg dosage (5µl of 200ng/µl). No statistically significant differences ($P<0.05$) were found between the distributions of beetles responding to the 3-compound blend or the hexane negative control in either of the experiments (see Table 4.19 for full statistics).

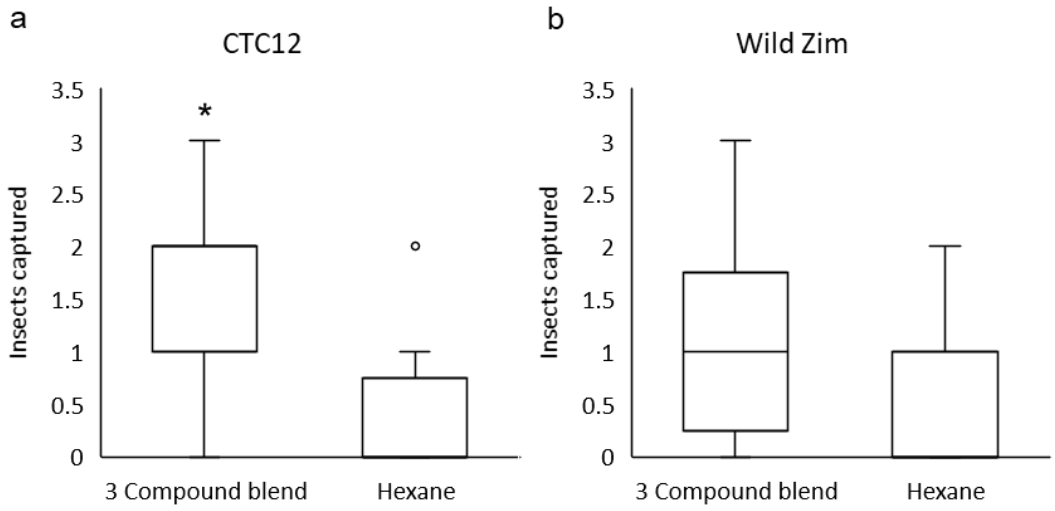

Fig 4.10. Boxplots representing the responses of (a) CTC12 or (b) wild Zim *T. castaneum* to pitfall traps baited with a 3-compound blend or a hexane negative control inside slow release plastic capsules.

The horizontal line in the centre of the boxplots represents the median value with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (point falling outside the whiskers) are indicated with dots. Ten replicate groups containing 20 beetle each (10 male and 10 female) were used in each trial. All compounds were tested at a 1µg dosage (5µl of 200ng/µl). Significant differences between the distributions of beetles responding to the test odour source and the hexane negative control are indicated in bold (see Table 4.19 for full statistics).

Table 4.19. The response index, shown in bold of two different *T. castaneum* strains when the 3-compound blend was added to either a paper disk or inserted into a slow release plastic capsule.

Also shown are the results of Benjamini-Hochberg corrected Mann–Whitney U tests comparing the response to each of the test volatiles with its associated hexane negative control. Significantly attractive treatments (P<0.05) are indicated in bold.

		Strain	
		CTC12	**Wild Zim**
Release method	**Paper disk**	**0.02** Z = -1.426 (n1 = n2 = 10) P = 0.227	**0.015** Z = -1.264 (n1 = n2 = 10) P = 0.302
	Slow release capsule	**0.055** Z = -2.489 (n1 = n2 = 10) **P = 0.018**	**0.02** Z = -1.463 (n1 = n2 = 10) P = 0.196

4.4. Discussion

The results of the experiments in this chapter have identified several wheat germ oil and fungal VOCs, and blends of these compounds, that appear to be highly attractive to both an established laboratory strain of *T. castaenum*, and a recently caught wild population. Some differences between the responses of wild and laboratory strain *T. casteneum* to common environmental VOCs were also identified. Both these findings have important implications for the future management of this important stored-product pest.

4.4.1 The effects of concentration on the attractiveness of VOCs to *Tribolium castaneum*

Before conducting systematic experiments to identify the most attractive fungal and wheat germ oil volatiles, the responses of CTC12 and wild Zim strain *T. castaneum* were tested against four individual VOCs at a variety of concentrations (Fig. 4.1). These compounds had been previously shown to elicit large antennal depolarisations in EAG experiments (see Chapter 3) and they represent a variety of different compound classes. The compounds were tested across a range of concentrations as the attractiveness of volatile compound, such as those produced by plants, are known to differ in their attractiveness to insects depending on their concentration, with some attractive compounds becoming repulsive at higher concentrations (Huetteroth and Waddell, 2011).

A binomial logistic regression (Table 4.4) revealed that the concentration that compounds were tested at had a significant effect on the responses across both beetle strains. Individual χ^2 tests demonstrated that all compounds were significantly attractive at the 1 µg dose (Fig. 4.1). At higher concentrations each of the compounds were only attractive to the wild Zim strain, with higher concentrations being found to be either unattractive or actively repulsive for the CTC12 strain. However, despite these apparent inter-strain differences the logistic regression did not identify strain or an interaction with strain as having a significant effect on the responses of these beetles. The results from this experiment demonstrate that the responses to these volatiles differed significantly across the range of concentrations tested. The 1 µg dosage was the only concentration at which all VOCs were significantly attractive to both

strains. As such, this concentration was chosen to test the remaining wheat germ oil and fungal VOCs.

Concentration was also found to have a significant effect on the responses of *T. castaneum* to a volatile blend (Table 4.13). However, in this case the strain and an interaction between strain and concentration were also found to have a significant effect on the responses of the beetles. In this case the CTC12 strain was found to be attracted to 100 ng, 1 µg and 10 µg doses while only 1 µg and 10 µg dosages were found to be attractive to the wild Zim strain. In the CTC12 strain the 1 µg dosage was found to be the most attractive while 10 µg was found to be most attractive to the wild Zim strain.

These results suggest that the responses of *T. castaneum* to individual food-associated VOCs do not appear to differ between strains. However, the responses of beetles to blends containing some of these same VOCs were found to vary significantly between the two strains tested. As the wild Zim strain was cultured from a recently caught wild population, while the CTC12 strain has been cultured for much longer, it is possible that laboratory adaptation could be responsible for the differences in their responses. The results from Fig. 4.6 and Table 4.13 suggests that wild populations may be less able to respond to volatile blends at low concentrations. As freshly caught wild *T. castaneum* populations have previously been shown to be less attracted than laboratory strains to traps baited with cereal oil and DMD (Hawkin et al., 2011), if wild populations do not respond as well to environmental volatiles at low concentrations this could explain their reduced responses to food oil based traps.

4.4.2 The responses of *Tribolium castaneum* to individual VOCs

Due to time constraints it was not feasible to record the behaviour responses of *T. castaneum* to all of the VOCs compound tested using EAG in Chapter 2. Instead, the 10 compounds that gave the strongest average EAG responses were tested against CTC12 and wild Zim strain *T. castaneum* in Y-tube olfactometers and the 10 that gave the weakest EAG responses were tested in the CTC12 strain. Of the 10 compounds that gave the largest EAG responses, five of the compounds were found to be attractive to both strains and two were found to be attractive to only the CTC12 strain. The similarity of

the responses across the two strains was demonstrated by a logistic regression analysis showing that the strain of the beetles did not significantly affect their responses. The compound presented was also not found to have a significant effect but, as all of the compounds tested were known to elicit similar antennal responses, it was unsurprising that they should also elicit similar behavioural responses. However, despite the volatile presented not being found to have a significant effect on the responses of the beetles, not all of the compounds that elicited large EAG responses were found to elicit a significantly attractive response. However, it is still possible that these compounds could contribute to the attractiveness of a blend containing other compounds from these sources. In contrast, none of the 10-compounds that gave the smallest EAG depolarisations were found to be significantly attractive. This demonstrates a general trend, across the food-associated VOCs tested, of compounds that elicit strong EAG depolarisations being more likely to be attractive than compounds that elicited weaker EAG responses. This is also supported by the results of a Spearman's rank correlation across all 20 of these compounds (the compounds that elicited the 10 largest and 10 smallest average EAG responses). The results revealed a significant correlation between the size of the EAG depolarisation a compound elicited and its attractiveness in a Y-tube olfactometer, in CTC12 females. However, as only the compounds that elicited the largest and smallest EAG responses were tested it is unclear if this trend applies across the full spectrum of responses.

Significantly attractive compounds were identified as belonging to a variety of different chemical groups and were associated with both wheat germ oil and fungi. These results suggest that the chemical group that a particular volatile belongs to cannot predict how attractive it will be. This trend was also observed across the EAG experiments in Chapter 3, and has also been reported in other EAG experiments testing the responses of *T. castaneum* to common VOCs (Balakrishnan et al., 2017).

4.4.3. Identifying attractive VOC blends

After identifying that many of the VOCs that elicited strong depolarisations were significantly attractive to *T. castaneum* when presented in isolation, the

responses of *T. castaneum* were tested against blends of these compounds. Two *T. castaneum* strains were tested against different blends of the 10 VOCs that gave the largest EAG responses (Fig. 4.5). The 10-compound blend, 5-compound blend (1) and wheat germ oil blend were found to be attractive to the CTC12 strain. The same blends were significantly attractive to the wild Zim strain but in addition the fungal blend was also found to be attractive. The composition of the different blends was found to have a significant effect on the responses of beetles (Table 4.9), demonstrating that some of the blends were more attractive than others. However, despite some apparent differences between the responses of the two strains to these blends, strain or an interaction between beetle strain and the volatile presented was not found to significantly influence their responses. This suggests that different strains of *T. castaneum* may respond similarly to food associated VOC blends.

In both strains the 10-compound blend was found to elicit the strongest responses of the different blends tested. However, other blends composed of different subsets of these 10 volatiles were also tested and several of them were found to act as attractants. This demonstrates that not all of the compounds contained within the 10-compound blend are necessary to elicit a significant behavioural attraction. This is not unexpected as redundancy in the number of compounds needed to elicit an active response to a plant derived blend appears to be a common component of insect olfaction (Bruce and Pickett, 2011).

When the 10-compound blend was separated into blends that contained only VOCs that were attractive to both strains (the 5-compound blend (1)) and compounds that were not attractive to both strains (the 5-compound blend (2)), only the blend containing the attractive VOCs was significantly attractive to *T. castaneum*. This demonstrates that, in this case, food based VOCs that were unattractive when encountered on their own did not become attractive when combined as a blend. However, it is possible that other blend compositions could be more attractive.

The volatiles from the 10-compound blend were also separated into two sub-blends, one containing only the compounds identified as being wheat germ oil associated, the other containing only fungal associated compounds. The

wheat germ oil blend was found to be attractive to both strains tested, but the fungal blend was only attractive to the wild Zim strain. In none of the strains were the responses to the wheat germ oil or fungal blends stronger than the response to the 10-compound blend, suggesting that combinations of both fungal and wheat germ oil compounds produce the most attractive blends.

As the 10-compound blend elicited a stronger response than any of the individual compounds tested in the CTC12 strain, and almost every compound in the wild Zim strain, a binomial linear regression was used to identify if any of the blends were significantly more attractive than any of their constituent VOCs. However, for each strain, individual VOCs that were as attractive as the blend could be identified. This means that although some of the blends attracted more beetles than any of the individual compounds did on their own, this is no evidence to suggest that the blends were significantly more attractive than some individual VOCs.

These experiments do however demonstrate that not all of the compounds in the 10-compound blend are required to attract *T. castaneum*, as blends containing half the number of compounds were still found to be significantly attractive. To identify the compounds that contribute the most to the attractiveness of these blends, the effects of removing volatiles from one of the blends was tested in the CTC12 strain. A similar methodology of removing sequential compounds from a blend had previously been used to identify redundant elements in a *Lobesia botrana* (grapevine moth) lure (Tasin et al., 2007).

After subtracting different compound from the 5-compound blend (1) it was identified that removing 3-octanone and butyl acetate from the blend had little effect on the blend's attractiveness. The compounds 2-heptanone, 3-methylanisol and hexanal were each found to either cause a large decrease in the attractiveness of the blend, or completely abolish its attraction, when removed. Indeed, the blends with 2-hepanone and hexanal removed were found to be significantly less attractive than the original 5-compound blend. This indicates that these compounds are not redundant and that they contributed significantly to the attractiveness of the original blend. This demonstrates that individual compounds do not contribute equally to the

overall attractiveness of the blend and that certain compounds appear to be essential when determining if a blend is attractive or not. Due to time constraints only the responses of the CTC12 strain were tested. As such the results may not be the same for other *T. castaneum* strains and, in particular, wild populations. However, no significant differences were previously found between the responses of the two different strains when the compounds in the blend were tested individually.

4.4.4 The attractiveness of the 3-compound blend in Y-tube olfactometer bioassays

After identifying 2-heptanone, 3-methylanisol and hexanal as the three compounds that contributed most significantly to the attractiveness of the 5-compound blend (1), the responses of CTC12 and wild Zim *T. castaneum* were tested against a blend containing just these compounds.

This 3 compound blend was found to be highly attractive to both strains in a Y-tube olfactometer. For comparison, the attractiveness of this blend was also tested alongside and in combination with DMD. DMD on its own and DMD combined with the 3-compound blend was also found to be significantly attractive. Binomial logistic regression analysis revealed that neither the volatile presented, the strain or an interaction between the two factors had a significant effect on the number of *T. castaneum* attracted. This demonstrates that, at the concentration tested, the 3-compound blend is as attractive as the DMD aggregation pheromone. These results also demonstrate that combining DMD and the blend together did not have a significant additive effect on its attractiveness to *T. castaneum*. As a blend of similar attractiveness to the *T. castaneum* aggregation pheromone could be made from just, 2-heptanone, 3-methylanisol and hexanal, it is likely that these compounds are some of the most important compounds responsible for the attraction of *T. castaneum* to the stored-grains. For both strains, the 3-compound blend was more attractive in the Y-tube olfactometer than any of its constituent VOCs were on their own. However, a binomial logistic regression revealed that the blend was not significantly more attractive than any of its constituent VOCs were in isolation. As such, there is insufficient evidence to suggest that these compounds have a synergistic effect when combined together in a blend. However, as the

responses of only 40 individual beetles were treated in the Y-tube olfactometer it is possible that the bioassays lack statistical power to distinguish between the relative attractiveness of different highly attractive odour sources.

As 2-heptanone, 3-methylanisol and hexanal were highly attractive to *T. castaneum* both individually and when combined in a blend, it is likely that these compounds have the potential to be incorporated into a highly attractive *T. castaneum* lure. The attractiveness of this VOC blend is a novel discovery in *T. castaneum*, but the responses to some of its constituent compounds have been tested in other insect species. Hexanal has been previously shown to be attractive to the stored product pests *Oryzaephilus surinamensis* and *O. mercator* in two-choice pitfall bioassays (Pierce et al., 1990). Heptanone has also been demonstrated to elicit large EAG responses in several different insect species including *Theocolax elegans* (a wasp that parasitises stored-product pests) (Germinara et al., 2009), *Drosophila melanogaster* (Bruyne et al., 2001), and the malaria mosquito *Anopheles gambiae* (Carey et al., 2010), but does not appear to have been tested behaviourally in any of these species. This would suggest that there is the possibility that other insects that feed on fungi, or fungi associated food sources could also be attracted to the VOCs that make up the 3-compound blend. If this is the case, these compounds could potentially be developed into a multi-species stored-product pest lure. However, 3-methylanisol has been shown to reduce the attraction of the pine weevil *Hylobius abietisits* to its host plant, Scots pine (Azeem et al., 2013). This could suggest that this compound may only be attractive to insects that have adapted to feed in stored-product warehouses or that the attraction to this compound could be unique to *T. castaneum.* Further study is needed to confirm whether these compounds could function as lures for stored-product insects other than *T. castaneum*.

4.4.5. The attractiveness of the 3-compound blend in pitfall bioassays

Although the attractiveness of the 3-compound blend has been demonstrated in the Y-tube olfactometer, if these compounds are to be used in a commercial lure it is important that they are also attractive in more open environments and under still-air conditions. To this end, the attraction of CTC12 *T. castaneum* to

the 3-compound blend was also tested using a pitfall bioassay. However, the blend was not found to be significantly attractive when added to paper disks in a pitfall arena bioassay. As the responses to other odour sources in this bioassay were also found to be low in previous experiments (see Chapter 2), the lack of responses could be due to an issue with the bioassay itself and may not reflect the true attractiveness of the blend. As the bioassay arena was sealed it is possible that volatiles are released too quickly from the paper disks, preventing insects from locating the attractive odour source because a gradient of airborne odorants is not formed between the beetles and the odour source.

To address this potential issue the 3-compound blend was also inserted into a plastic capsule, instead of being added to paper disks, to allow for the volatiles to diffuse out more slowly. This was found to slightly increase the responses of both strains, with the CTC12 strain now being significantly more attracted to the blend than the hexane control. However, although the responses were slightly stronger, the overall capture rate was still relatively low, with on average less than two beetles being captured in two hours. These results suggest that, although this blend appears to be highly attractive in certain behavioural bioassays, further research is needed to develop a formulation or release mechanism that will be effective under the conditions in which a lure would be used.

4.4.6. Conclusion

Overall, the results of this chapter clearly demonstrate that certain compounds that are found within wheat germ oil or produced by grain-associated fungi can be highly attractive to *T. castaneum*. This supports the idea that *T. castaneum* may have evolved to respond to fungal volatiles as a way to find pre-anthropogenic sources of rotten grains or detritus. It could have retained these same receptive ORs during the evolutionary transition to seeking food within stored-product environments. It is also possible that these fungal detecting ORs are novel adaptations that emerged after *T. castaneum* began feeding on stored products and are unrelated to its previous ecological niche.

These results also demonstrate that *T. castaneum* respond differently to different blends of these compounds with the compounds 2-heptanone, 3-

methylanisol and hexanal being identified as contributing substantially to the attractiveness of these blends. The degree of behavioural attraction observed in the Y-tube olfactometer bioassays to these compounds, both individually and in blends, indicates that they have the potential to be developed into a pest management lure for *T. castaneum,* However, the low capture-rate when the 3-compound blend was tested in pitfall bioassays could indicate that further research is needed to develop a release method suitable for use in stored-product environments. Further research testing the effectiveness of this lure in conditions closer to these found in grain warehouses would be necessary to identify if the issue is with the lure (e.g. its release rate) or with the bioassay (e.g. it does not establish a gradient of volatiles between the beetles and the odour source).

The results of comparisons between the responses of established laboratory strains and freshly caught wild populations demonstrate that being cultured under laboratory condition might not significantly affect the responses of *T. castaneum* to common environmental odours, as in most experiments the strain of the beetles was not found to significantly influence their responses. However, the responses of the laboratory cultured and wild strains were found to differ when the 10-compound blend was tested at different concentrations. This could suggest that being cultured under laboratory conditions affects how sensitive *T. castaneum* is to food-associated odours. This observation could have wider implications on the use of inbred laboratory lines in applied pest management research. However, as only two different strains were tested in this study, it is not clear how broad this effect is. As such it still remains unclear whether the results of behavioural experiments using laboratory *Tribolium* strains are truly applicable to wild populations.

Chapter 5
Demonstrating the effects of RNAi knockdown of olfactory receptor co-receptor (*Tc-or1*) on olfaction in *Tribolium castaneum*

5.1. Introduction

Olfaction is a key sense that insects use to identify food sources (de Bruyne and Baker, 2008; Hansson and Stensmyr, 2011). As such, information about the olfactory receptors (ORs) that pest insects use to locate stored products could have pest management implications. However, excluding the Orco orthologue, *Tc-or1*, all of *T. castaneum* ORs are orphan receptors (receptors where the specific ligand the receptor binds has not been identified). If some of the specific ORs mediating the attraction of *T. castaneum* to stored products were identified, this could allow genomic information to be used to develop new methods of pest management. Genetic information from pest species ORs could also provide new insights into the adaptation of some insects to feeding on stored products.

One potential avenue of research would be the use of sequence data from ORs known to bind particular stored product-associated volatiles to identify novel attractive compounds for use in insect pest lures. Once the compound that an OR binds has been identified the gene sequence could be used to predict other ORs that bind similar compounds. Sequence data from a OR known to bind a particular stored product-associated volatile could also be used to identify the targets of receptors with a similar sequence in other pest species. In this way the OR sequences of one stored-product insect could be used to identify candidate stored product volatile receptors in another pest species. This could be used to identify compounds that have the potential to be attractive to multiple different species and could lead to the development of a highly attractive multispecies lure for stored product insects. Although 3D crystal structures do not yet exist for insect ORs (Venthur and Zhou, 2018), in the future it may be possible to predict what ligand an OR will bind based on its gene sequence alone. This would enable novel attractive compounds to be identified using just the sequence data of an OR.

Identifying the specific ORs that detect important stored product volatiles could also lead to the development of novel stored product pest management strategies. It has been suggested that disrupting olfactory receptors could prevent pest species from locating food sources or mates (Zhou et al., 2014; Perkin et al., 2016). However, it has been suggested that disrupting the highly conserved *orco* gene could lead to off-target effects in beneficial insects (Andersson and Newcomb, 2017). As such, species specific olfactory genes have been suggested as alternative targets to disrupt (Andersson and Newcomb, 2017). Species specific ORs such as pheromone receptors or ORs involved with locating a specific host food source would be less likely to have off-target effects on other insect species.

Studying the ORs of a stored product insect also provides an opportunity to research the effects of evolutionary forces on olfactory genes. As the adaptation of *T. castaneum* to feeding on human stored products happened relatively recently on an evolutionary timescale, genes related to locating stored products are likely to have undergone recent positive selection. The ORs of stored product insects could therefore provide an important model system for studying the effects of rapid evolution and adaptation.

However, all of the above approaches rely on having specific information about the ligands that bind to individual ORs. Currently, very little is known about the ligands of specific ORs in *T. castaneum,* however the existence of a published genome sequence (Tribolium Genome Sequencing Consortium, 2008) and annotated olfactory receptors (Engsontia et al., 2008) means there is great potential to develop approaches to identify complementary ligands and receptors in this species. Several *in vitro* methods have been proposed for deorphaning ORs in different insect species including the *Xenopus* oocyte expression system (Lu et al., 2007) and the *Drosophila* empty neurone system (Hallem and Carlson, 2006). The *Xenopus* oocyte system allows for specific insect ORs to be expressed in *Xenopus laevis* oocytes and for the activation of these receptors to be measured. However, this system has been criticised because it uses a non-insect model system which lacks many endogenous insect proteins involved in olfaction, such as odorant binding proteins (OBPs) (Ueira-Vieira et al., 2014). The *Drosophila* empty neurone system can be used

to express ORs in *Drosophila* neurones that have been mutated to not otherwise express ORs. Although this system has the advantage of expressing other insect olfactory proteins, there is no guarantee that olfactory proteins in *Drosophila* will function in the same way as they do in other insect species. For this reason, it has been suggested that this system should only be used for closely related insect species i.e. other dipteran spp. (Ueira-Vieira et al., 2014). However, when using either of these bioassays, although the systems may be able to provide information on the ligands that a specific OR can detect, identifying if the activation of a given receptor elicits an attractive behavioural response will still require behavioural bioassays using the original insect species. The bioassays and experimental techniques used previously in this thesis, in combination with RNAi, provide a potential method of deorphaning *T. castaneum* ORs and identifying the behavioural response they elicit without the need to express them in other species.

RNAi in combination with behavioural bioassays could be used to identify the VOCs that a specific OR detects. ORs of interest, identified from comparisons with the OR sequences of other species, could be knocked-down using RNAi to produce beetles not expressing a specific OR. As *T. castaneum* is also a laboratory model species, the effects of RNAi have been well studied (Tomoyasu et al., 2008; Posnien et al., 2009), providing an advantage when conducting genetic functional analysis over other stored-product insects. An EAG screen against individual compounds could then be used to identify compounds that elicit a response in control beetles but that have impaired antennal depolarisations in RNAi knockdown beetles. This would identify the specific ligand that an olfactory receptor binds by demonstrating a decreased response when the expression of that gene is knocked down using RNAi. The results of the EAG experiments in Chapter 3, and the results of other EAG experiments using *T. castaneum* (Balakrishnan et al., 2017), demonstrate that it is possible to test a large number of volatile compounds at once in *T. castaneum*. These studies also provide examples of compounds that elicit large EAG responses but have not had their associated OR identified, that could potentially be tested in the future. After identifying an OR that elicits a reduced response to a particular compound when knocked-down with RNAi the effect of the same knock-down could be tested behaviourally. If the

attraction is abolished this would be strong evidence that a given OR is responsible for the reception of this compound. Some of the bioassays tested in Chapter 2 should be suitable for testing the effects of OR RNAi knock-down on the attraction to a particular compound. Identifying ORs that respond to stored-product odours would open up new avenues of OR related research previously discussed in this section.

As no other ORs have been deorphaned in *T. castaneum*, the effects of knocking down the *Tribolium orco* orthologue (*Tc-or1*) were tested in this chapter using EAG, pitfall bioassay and Y-tube olfactometer bioassays. As the *Tc-or1* co-receptor is needed for other ORs to function, knocking down *Tc-or1* should impair the function of all ORs in the same way that knocking down any other OR gene would affect a single specific receptor. The effect of knocking-down *Tc-or1* on the responses of *T. castaneum* to DMD has already been demonstrated using a simple behavioural bioassay (Engsontia et al., 2008). This chapter will expand on this work by demonstrating that in future behavioural bioassays in combination with RNAi knockdown of individual ORs could be used to identify the VOCs that a specific OR responds to.

5.2. Methods

5.2.1. *Tribolium castaneum* husbandry

San Bernardino and CTC12 strain *T. castaneum* were used in the experiments in this chapter. Cultures were incubated at 30°C in containers of 200 g of whole grain flour (Doves Farm Organic Strong Wholemeal Bread Flour) with the addition of 10 g yeast powder as an additional protein source (Holland & Barrett Debittered Brewer's Yeast Powder) and 1g of 0.3 g/kg antimicrobial agent Fumagillin to inhibit fungal growth in the cultures. All beetles used in the experiments were aged between four and eight weeks post-emergence to ensure all insects were sexually mature and mated to reduce the variance in their responses to DMD aggregation pheromone (Duehl et al., 2011). The sex of the beetles used in each bioassay is stated in its associated methods section. Different individual beetles were used for each individual bioassay treatment except for in Section 5.3.3. were the same beetles were tested at three different time periods to identify the duration of the knockdown effect.

5.2.2. *Tc-or1* cDNA template synthesis

PCR primers were designed (Appendix B), with reference to the previously identified *Tc-or1* gene sequence (Engsontia et al., 2008) and the published *T. castaneum* genome sequence (Tribolium Genome Sequencing Consortium, 2008). The PrimerQuest program was used to help optimise primer selection and avoid overlaps with other *T. castaneum* gene sequences. Total RNA was prepared from San Bernardino strain *T. castaneum* using a Qiagen RNeasy mini kit and an Invitrogen cloned AMV first-strand cDNA synthesis kit was used to prepare cDNA from this total RNA. The *Tc-or1* gene was then amplified by PCR using this cDNA. The identity of the PCR product was confirmed by visualising on an agarose gel (Fig. 5.1). This PCR product was then excised from an agarose gel using the QIAGEN gel extraction kit.

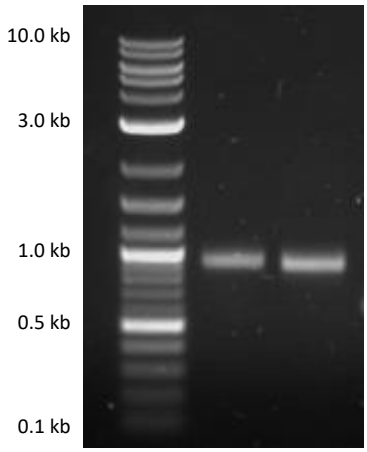

Figure 5.1. An agarose gel showing two successfully PCR amplified *Tc-or1* gene fragments with a size of 903bp next to a 10kb ladder.

5.2.3. *Tc-or1* molecular cloning

Tc-or1 DNA was amplified by molecular cloning to produce enough cDNA template for later RNA synthesis. First, the *Tc-or1* PCR DNA fragment was ligated into the pGEM-T Easy vector (Fig. 5.2) and transformed into chemically competent cells (DH5 alpha). A bacterial culture grown from a single *Tc-or1* positive colony, and the QIAGEN midiprep kit was used to generate a large amount of *Tc-or1* template plasmid for double stranded RNA (dsRNA) synthesis.

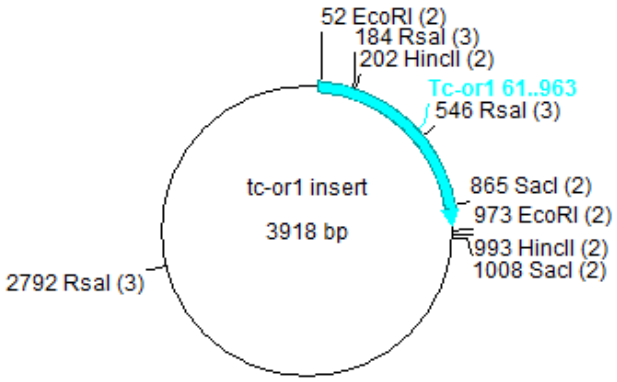

Figure 5.2. A diagram showing the pGem-T easy vector plasmid with the *Tc-or1* insert and the restriction sites used to confirm its identity.

To confirm that the *Tc-or1* plasmid had been successfully cloned, amplified and extracted, a diagnostic restriction digest was performed. Four restriction enzymes: Hinc2, Rsa1, Sac1 and EcoR1 were chosen to produce a distinctive banding pattern (Fig. 5.3) unique to a pGEM-T easy plasmid containing the *Tc-or1* 903bp fragment (Fig. 5.2). Hinc2, Rsa1 and Sac2 were chosen as they gave a very distinctive pattern of small and large fragments that would change size depending on the orientation of the insert within the plasmid (5' to 3' or 3' to 5'). EcoR1 was chosen as it cuts the vector twice, approximately 10 base pairs each side of the insert site and so produces fragments approximately the same size as the insert and vector. The insert size was 903bp and the fragment sizes produced were of the expected size (Fig. 5.3). The fact that all the fragments from the other digests were the expected sizes for the reverse (3' to 5') orientation, shown in Table 5.1, confirms that the *Tc-or1* gene had been successfully cloned. The identity of this gene was also confirmed via sequencing.

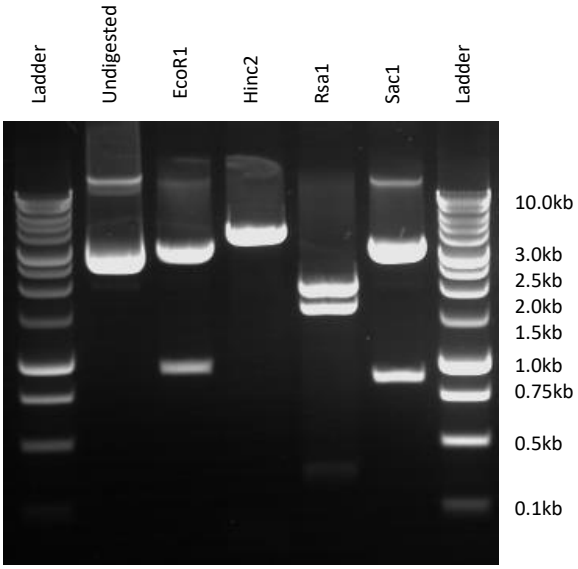

Figure 5.3. An agarose gel showing the fragments produced from the different diagnostic restriction digestions of the *Tc-or1* containing pGEM-Teasy vector.

Lanes 1 and 7 contain a 10 kb ladder. Lane 2 contains the undigested vector. Lane 3 was digested with EcoR1. Lane 4 was digested with Hinc2. Lane 5 was digested with Rsa1. Lane 6 was digested with Sac1.

Table 5.1. The cut sites of different restriction enzymes within the *Tc-or1* containing pGEM-T easy vector and the potential fragment that could be produced depending on the orientation of the *Tc-or1* gene fragment within the vector (forward or reverse orientation).

The actual fragments produced are indicated with bold text. The Hinc2 176 bp band is assumed to have run off the gel due to its small size.

Restriction enzyme	**Cut site(s) in vector**	**Cut site(s) in insert**	**Possible fragment sizes**
Ecor1	52, 70	-	**923, 2997**
Hinc2	92	144	Forward 791, 3129 **Reverse** 176**, 3744**
Rsa1	1890	125, 487	Forward 1312, 362, 2246 **Reverse 1601, 362, 1955**
Sac1	109	809	Forward 143, 3777 **Reverse 858, 3062**

5.2.4. RNA synthesis

The *Tc-or1* containing pGEM-Teasy plasmid was linearized with restriction enzymes Sac2 and Spe1, which have restriction sites flanking either side of the insert. Sense and anti-sense single stranded RNA (ssRNA) was then synthesised from the linearized *Tc-or1* DNA samples using the Sp6 and T7 Megascript Kits (Ambion) respectively, following kit instructions. The concentration of each ssRNA sample was calculated using a Nanodrop200 spectrophotometer. To produce *Tc-or1* double stranded (dsRNA) an equal concentration of sense and anti-sense ssRNA was mixed together, annealed via heating to 95°C and allowing the mixture to cool slowly. This dsRNA was measured at a concentration of 0.760 µg/µl. This is slightly lower than the recommended concentrated for microinjection into adult *Tribolium* of 1-5 µg/µl (Posnien et al., 2009), but as a concentration of 1 µg/µl achieved a strong

effect in previous *Tc-or1* knock-downs (Engsontia et al., 2008) a strong effect should still be seen with this concentration of dsRNA.

5.2.5. RNAi microinjection

Across all of the RNAi experiments, beetles were injected using a pulled glass capillary needle following an established *Tribolium* microinjection procedure (Posnien et al., 2009). However, the beetles were injected in the abdomen underneath the wing elytra, just below their flight muscles (taking care to inject away from the midline to avoid the ventral nervous system of the beetles), rather than into their genitalia, as this method of injection was found to be easier. The choice of injection site should not affect the efficiency of RNAi (Posnien et al., 2009). Each beetle was injected until the body cavity was filled with fluid and the head was observed to move away from the thorax, and the thorax from the abdomen, due to the increased pressure. By injecting insects until the body cavity was filled each beetle received dosages approximately proportional to their body size, helping to standardise the effect across different individuals. Water was injected into the beetles as a negative control to demonstrate that the physical process of micro-injection did not affect the responses of the beetles. RNA encoding a fragment of GFP was used as a control in some experiments to demonstrate that non-specific RNAi injection does not affect the responses of the beetles. Beetles that survived microinjection were allowed to recover on whole grain flour for at least 48 hours prior to bioassay testing in all experiments.

5.2.6. Electroantennography

The EAG methodology is described in detail in the methods section of Chapter 3 (Section 3.2.3). Twelve different female CTC12 *T. castaneum* were tested in each treatment. In each trial 5 µl of an odour source was applied to a paper disk and presented to the insects. The odour sources were either pure pentane or a 1 µg (5µl of 200ng/µl) dosage of an odour source diluted in pentane.

5.2.7. Pitfall bioassay

The designs of the pitfall bioassays are described in detail in the methods section of Chapter 2 (Section 2.2.3). Twenty beetles (ten male and ten female) were used in each trial and 15 replicates were conducted for each treatment.

Different individuals were used in each trial. In each trial the odour source was 5 µl of pure wheat germ oil applied to a paper disk.

5.2.8. Y-tube olfactometer

The Y-tube olfactometer methodology is described in detail in the methods section of Chapter 2 (Section 2.2.6). Forty beetles were tested for each odour and the same individuals were tested against each odour source at each time period. Only female beetles were use in the Y-tube olfactometer as it is believed that male beetles could leave pheromone trails that may bias the responses of proceeding beetles (Ahmad, Daglish, et al., 2012). A 1 µg dose (5µl of 200ng/µl) of the 10-compound blend, diluted in hexane, was used as an attractant and pure hexane was used as a negative control odour source. Five µl of each odour source were applied to paper disks in each experiment.

5.2.9. Odour sources

A variety of compounds established as being attractive to *T. castaneum* in the previous chapters were used as odour sources in these experiments. As the different bioassays were conducted at different time periods throughout the research, not all of them used the same odour sources. Each bioassay was tested against the most attractive food-associated odour source identified at the time the experiment was conducted. DMD, 1-octen-3-ol and the negative control solvent pentane were used in the EAG experiments. Wheat germ oil was used in the pitfall bioassays. The 10-compound blend and the negative control solvent hexane was used in the Y-tube olfactometer bioassay. The 10-compound blend was previously tested in Chapter 4 and is composed of: 5-methyl-3-heptanone, 3-octanone, butyl acetate, ethyl hexanoate, 2-heptanone, trans-2-heptenal, 3-methylanisol, limonene, hexanal and trans-trans-2,4-heptandinal in equal proportions.

5.2.10. Statistical analysis

The results of the EAG experiments were analysed using a two-way mixed model ANOVA. After identifying that both the within-subject factor and between-subject factor were significant, further statistical analysis was performed. Multiple independent *t*-tests were performed to identify which volatiles elicited significantly different responses between the two beetle treatments. Multiple paired *t*-tests were also performed to identify compounds

in each treatment that gave a significantly difference response to the pentane control. In both cases the P values were adjusted using the Benjamini-Hochberg procedure to correct for the increased risk of type 1 errors associated with multiple testing (Benjamini and Hochberg, 1995).

The results of the pitfall bioassay experiments were analysed with a Kruskal–Wallis test. After identifying that the treatment of the beetles had a significant effect on their responses to wheat germ oil, multiple Mann-Whitney U tests were used to compare the responses of each of the treatments to those of uninjected beetles.

Multiple χ^2 goodness-of-fit tests were used to identify differences between the number of beetles attracted to the test odour source and the hexane negative control in each Y-tube olfactometer experiment. In each case the expected frequency of beetles responding to each odour source was 20 (an equal number of beetles responding to the test odour source and the negative control). To account for the increased risk of type 1 errors associated with making multiple comparisons, the P values in each experiment were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). A binomial logistic regression was also performed to identify if the various treatments of the beetles or the time since injection significantly affected their responses to the volatiles tested.

All statistics were performed using SPSS 24 software.

5.3. Results

5.3.1. EAG responses of *Tc-or1* RNAi *Tribolium castaneum*

The EAG responses of CTC12 strain *T. castaneum*, injected with either *Tcor1* dsRNA or a H_2O sham injection, to various odour sources are shown in Fig. 5.4. A two-way mixed ANOVA revealed that the volatile presented and the beetle treatment both had a significant effect on the size of the EAG depolarisation (Table 5.2). Paired *t*-tests revealed that in both beetle treatment groups the response to DMD and 1-octen-3-ol was significantly larger than the response to the associated pentane negative control (Table 5.3). Independent *t*-tests were used to compare the responses of the different treatments to the same odour sources (Table 5.4 and indicate on Fig. 5.4). The results revealed

that the response to DMD and 1-octen-3-ol were significantly reduced in *Tcor1* RNAi beetles, but there was no difference between the responses of the two beetle treatments to the pentane negative control.

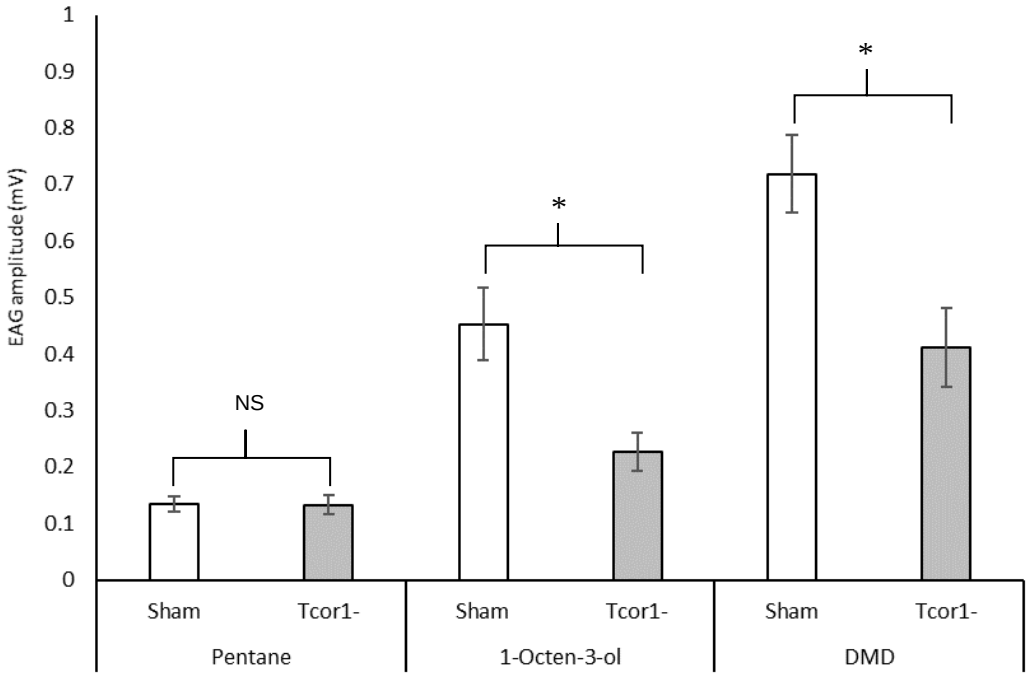

Figure 5.4. The average EAG responses of female CTC12 strain *Tc-or1* RNAi and sham injected control *T. castaneum* (injected with H_2O) to DMD and 1-octen-3-ol and a pentane negative control.

Columns represent the average depolarisation across 12 individuals, and the error bars represent the standard error. Volatiles that gave statistically significantly (P<0.05) different responses between responses between the two groups are indicated with * (see Table 5.4 for full statistics).

Table 5.2. The results of a two-way mixed ANOVA investigating the effects of volatile presented, the beetle treatment and an interaction between these factors on the EAG responses of CTC12 *T. castaneum*.

Significantly different P values (P<0.05) are highlighted in bold.

Variable	df	MS	F	P
Volatile	2	0.817	102.760	**<0.001**
Treatment	1	0.344	9.986	**0.005**
Volatile*Treatment	2	0.083	10.463	**<0.001**

Table 5.3. The results of multiple paired *t*-tests comparing the response of *T. castaneum* to a pentane negative control and different odour sources.

The responses of both *Tc-or1* RNAi and sham injected CTC12 *T. castaneum* were tested. The P values were adjusted using the Benjamini-Hochberg procedure and significantly different P values (P<0.05) are highlighted in bold.

Treatment	Volatiles	*t*	df	P
Sham injection	Pentane vs DMD	-15.312	11	**<0.001**
	Pentane vs 1-octen-3-ol	-8.097	11	**<0.001**
Tcor1-	Pentane vs DMD	-6.019	11	**<0.001**
	Pentane vs 1-octen-3-ol	-4.913	11	**<0.001**

Table 5.4. The results of independent *t*-tests comparing the responses of *Tc-or1* RNAi and sham injected CTC12 *T. castaneum* to the same odour sources.

The P values were adjusted using the Benjamini-Hochberg procedure and significantly different P values (P<0.05) are highlighted in bold and indicated on Fig. 5.4.

Volatile	*t*	df	P
Pentane	0.115	22	0.909
DMD	3.317	22	**0.003**
1-Octen-3-ol	3.446	22	**0.002**

5.3.2. Responses of *Tc-or1* RNAi *Tribolium castaneum* in pitfall bioassays

The responses of *Tc-or1* RNAi beetles and control beetles in pitfall bioassays with either wheat germ oil or a blank disk are shown in Fig. 5.5. The treatment of the beetles was found to significantly affect their attraction to wheat germ oil (Kruskal–Wallis test, $\chi^2 = 11.487$, df = 2, P=0.003). When the responses of uninjected beetles were compared to the other beetle treatments, *Tc-or1* RNAi beetles were found to be significantly less attracted (Mann-Whitney U test, U=37.5 n=15 P=0.001) while the responses of sham injected control beetles were not significantly different (Mann-Whitney U test, U=94.5 n=15 P=0.447).

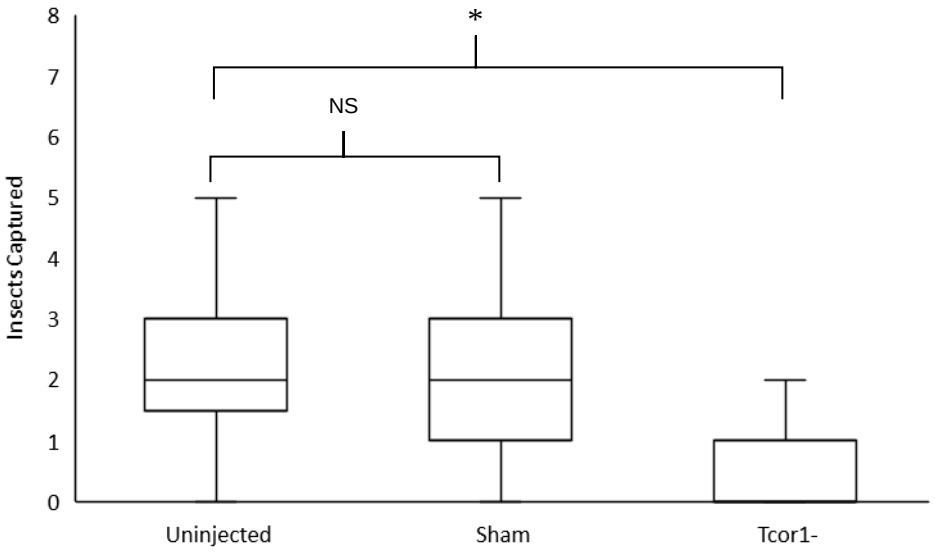

Figure 5.5. The average number of *Tc-or1* RNAi, sham injected, and uninjected CTC12 *T. castaneum* that fell into pitfall traps baited with wheat germ oil over two hours.

The horizontal line in the centre of the boxplots represents the median value, with the box representing the interquartile range and the whisker lines are 1.5 times the interquartile range. Outliers (points falling outside the whiskers) are indicted with dots. 15 groups of 20 beetles (10 male and 10 female) were used for each treatment group/odour source. Statistically significant differences ($P<0.05$) between different treatments and the response of uninjected beetles are indicated with *. NS indicates no significant differences.

5.3.3. Responses of *Tc-or1* RNAi *Tribolium castaneum* in Y-tube olfactometer bioassays

The responses of *Tc-or1* RNAi *T. castaneum* and two negative control treatments were also tested against an attractive VOC blend (the 10-compound blend tested in Chapter 4) in a Y-tube olfactometer bioassay at different times post-injection (Fig. 5.6). Multiple χ^2 goodness of fit tests were used to identify beetle groups that were significantly more attracted to the VOC blend than the hexane control (Table 5.5) and significantly attractive responses ($P<0.05$) are indicated on Fig. 5.6. At each time period the GFP and H_2O injected beetles were found to be significantly attracted to the odour source, while the *Tc-or1* RNAi beetles were not found to be significantly attracted at any time period. A binomial logistic regression confirmed that the treatment of the beetles had a significant effect on their responses and that *Tc-or1* RNAi beetles were significantly less attracted to the blend than the other two beetle treatments (Table 5.6). The binomial logistic regression also revealed that the responses of the beetles did not significantly differ over the time period tested.

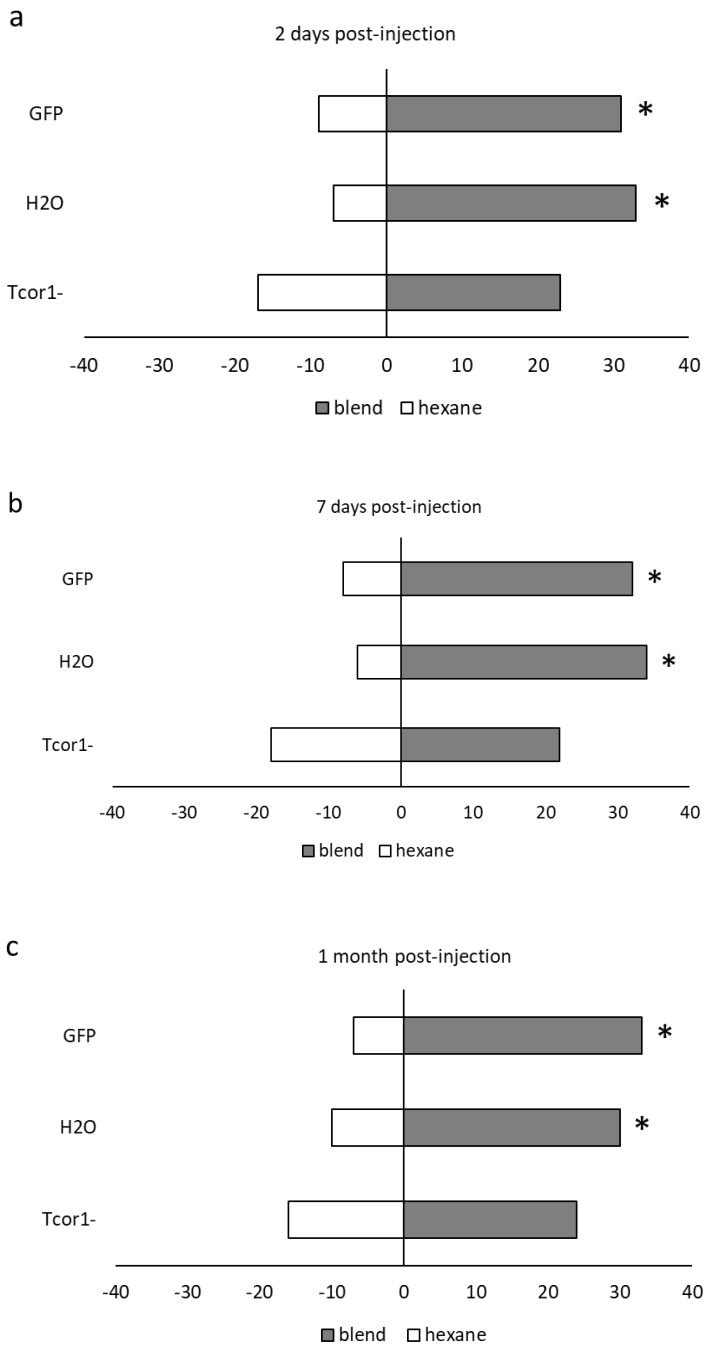

Figure 5.6. The attraction of female CTC12 *Tc-or1* RNAi *T. castaneum* and RNAi control *T. castaneum* (GFP or H_2O injected beetles) to a blend of attractive VOCs at three different time points post-injection (a = two days, b = seven days and c = one month).

The grey bars indicate the number of beetles (out of 40 individuals tested) that chose the Y-tube arm containing the attractive blend, and the white bars indicate the number that chose the arm containing the hexane negative control. Statistically significant ($P<0.05$) differences between test compounds and the hexane control solvent are indicated with * (see table 5.5 for full statistics).

Table 5.5. The responses of female CTC12 *T. castaneum* with different injection treatments to the 10-compound blend in Y-tube olfactometer bioassays.

Each treatment group was tested at three different time periods post-injection. The results of χ^2 goodness-of-fit tests are presented, comparing the observed number of individuals that were attracted to the volatile blend or the hexane negative control arm of the olfactometer to the expected frequency of 20. The P values were adjusted using the Benjamini-Hochberg procedure and significantly different P values (P<0.05) are highlighted in bold.

Time	Treatment	Beetles attracted	N	χ^2	df	Adjusted P values
2 days post-injection	*Tc-or1-*	23	40	0.9	1	0.386
	H2O	33	40	16.9	1	**<0.001**
	GFP	31	40	12.1	1	**0.009**
7 days post-injection	*Tc-or1-*	22	40	0.4	1	0.527
	H2O	34	40	19.6	1	**<0.001**
	GFP	32	40	14.4	1	**<0.001**
1 month post-injection	*Tc-or1-*	24	40	1.6	1	0.265
	H2O	30	40	10	1	**0.002**
	GFP	33	40	16.9	1	**<0.001**

Table 5.6. The results of a binomial logistic regression identifying the effect of beetle treatment and time post injection on the responses of CTC12 *T. castaneum*.

Significantly different P values (P<0.05) are highlighted in bold. Treatment (1) and (2) indicates, respectively, the responses of GFP and H2O injected beetles compared to the *Tcor1-* reference group. Time (1) and (2) indicates, respectively, the responses of 7 days and 1 month post-injection beetles compared to the 2 day post-injection reference group.

Variables	Wald χ^2	df	P
Treatment	20.374	2	**<0.001**
Treatment(1)	14.709	1	**<0.001**
Treatment(2)	13.636	1	**<0.001**
Time	0.030	2	0.985
Time(1)	0.022	1	0.881
Time(2)	0.000	1	1.000

5.4. Discussion

5.4.1. The effect of *Tc-or1* RNAi on *Tribolium castaneum* EAG responses

The results of the EAG experiment identified that knocking down the *T. castaneum* orco (*Tc-or1*) gene significantly reduced the antennal responses of *T. castaneum* to DMD and a key food-associated volatile. This demonstrates that the decrease in antennal depolarisation caused by knocking down an OR can be detected using EAG. This suggests that EAG could be used as part of a strategy for deorphaning *T. castaneum* ORs, possibly as a screening tool before conducting more time-consuming behavioural bioassays on beetles that have had specific ORs knocked-down. The effect of knocking down OR genes on EAG responses has not previously been tested in *T. castaneum*, but the reduction in EAG response is similar to the reduction seen for other species in which the *orco* co-receptor gene has been knocked down, such as the gypsy moth *Lymantria dispar* (Lin et al., 2015) and the Chinese White pine beetle *Dendroctonus armandi* (Zhang et al., 2016). However, knocking down the *orco* gene of the plant bug *Apolygus lucorum* produced a larger reductions in EAG response, of up to 80%, to certain compounds (Zhou et al., 2014).

5.4.2. The effect of *Tc-or1* RNAi on *Tribolium castaneum* responses in a pitfall bioassay

When the effect of knocking down *Tc-or1* was tested using a pitfall bioassay a significant reduction in the attraction to wheat germ oil was identified. The response to wheat germ oil in these beetles was reduced to a capture rate of less than one beetle in two hours, suggesting that their ability to detect wheat germ oil had been severely impaired. The results also demonstrate that the micro-injection procedure does not appear to affect the movements of beetles in this behavioural bioassay as the responses of sham injected control beetles were not significantly different to the uninjected control beetles. However, although a significant reduction in the responses of RNAi beetles could be identified using this bioassay the overall response rate in the pitfall bioassays was relatively low, as also observed with the pitfall bioassays in Chapter 2. As such the Y-tube olfactometer, which could detect much stronger behavioural

responses from the beetles, appears to be a more suitable bioassay for detecting the effects of knocking down ORs in *T. castaneum*.

5.4.3. The effect of *Tcor1* RNAi on *Tribolium castaneum* responses in Y-tube olfactometer bioassays and the duration of RNAi knock-down effects

The results of Y-tube olfactometer bioassays clearly demonstrate that the *Tc-or1* RNAi knockdown abolished the attraction of *T. castaneum* to a highly attractive VOC blend. The additional control of injecting beetles with GFP dsRNA demonstrates that the injection of dsRNA itself did not influence the behaviours of the beetles, and provides further validation of the suitability of this bioassay. The results also indicate that the effects of RNAi knockdowns in *T. castaneum* can last for an extended period of time, more than a month in this instance. The duration of the effect of RNAi knockdowns for other genes are known in *T. castaneum* (Posnien et al., 2009), but this is the first demonstration of how long the effects of RNAi knockdowns of OR genes can last for in this species. This information is useful not just for research into the specificity of ORs in *T. castaneum*; it could potentially also have direct implications for potential pest management strategies in this species. The use of RNAi as a method of pest management has been suggest, either by producing transgenic plants that produce specific dsRNA or by directly spraying dsRNA onto crops (Mamta and Rajam, 2017). Although these dsRNAs are typically targeted at genes that, when knocked down, produce a lethal effect, targeting genes that abolish the attraction the pest towards stored products could also be a useful management strategy as food-associated odours are a key cue for host detection and orientation in insects (Reisenman et al., 2016). These results suggest that RNAi could be used for long lasting disruption of OR genes as a form of *T. castaneum* pest management.

5.4.4. Conclusion

Overall, the results of the experiments in this chapter demonstrate that RNAi knock-down of specific ORs in combination with EAG and behavioural bioassays could potentially be used in future to deorphan *T. castaneum* ORs. Each experiment demonstrated that knocking down *Tc-or1* resulted in a significantly reduced response to the attractant used. However, as previously

observed, these results suggest that as the responses of beetles in the pitfall bioassays were so small, the Y-tube olfactometer is a better bioassay for testing the effects of knocking down *T. castaneum* ORs.

In these experiments sham injection with H_2O was used to control for the physical effects of microinjecting the beetles, and microinjection with non-specific GFP dsRNA was used to control for behavioural changes that could be potentially triggered by the RNAi response. When performing RNAi knockdowns, real-time reverse transcription PCR (qRT-PCR) can be performed to quantify the exact reduction in gene expression the RNAi mechanism produces and verify that the correct gene has been disrupted. As such the exact degree *Tc-or1* gene expression was reduced cannot be stated, only that it was reduced enough to abolish the behavioural responses of *T. castaneum* to several known attractants. The possibility of off-target effects also cannot be completely ruled out. If the use of RNAi is to be developed into a major *T. castaneum* management tool it would be necessary to verify the efficiency of the RNAi knockdown of ORs with qRT-PCR or a similar methodology.

Now that it has been demonstrated that this methodology can be used to show the behavioural effects of knocking down *Tc-or1*, this research could be followed up with other OR research. One possibility would be to use RNAi against other ORs of interest identified from gene sequence homology with other stored product insects. Overall, the results of the experiments in this chapter demonstrate that EAG and behavioural bioassays in combination with RNAi have the potential to be used to deorphan the ORs involved in the attraction of *T. castaneum* to stored products. The evidence that the effects of RNAi knockdown of *T. castaneum* ORs can last for at least a month suggests that RNAi also has the potential to be used for direct pest management in this species.

Chapter 6
General Discussion

6.1 Overview

This thesis reports several new discoveries regarding olfaction in *T. castaneum* which are listed below. Many of these findings will have practical implications for the pest management of this species and other stored-product insects.

This research has tested the olfactory response of *T. castaneum* to 66 volatiles associated with wheat germ oil and grain-associated fungi using EAG. This is the first large-scale EAG screen focusing on testing the response of *T. castaneum* to specific food-associated volatiles and has revealed that their antenna appear to be tuned to detect a wide range of volatiles from these sources. When a selection of the compounds that elicited large antennal responses were tested in behavioural bioassays, several novel attractive compounds were identified. Several blends comprised of different combinations of these compounds were also found to be highly attractive. However, none of these blends were found to be significantly more attractive than some of the constituent compounds on their own. Many of these compounds were not previously known to be significantly attractive to *T. castaneum* and therefore have potential to be developed into novel *T. castaneum* lures. The fact that so many fungal associated VOCs were found to be attractive suggests that *T. castaneum* may have evolved to use fungal VOCs to locate stored products. This suggests that fungal-produced VOCs could be a potentially overlooked source of attractive compounds that could be incorporated into a stored product insect lure. However, although certain wheat germ oil and fungal VOCs were identified as being highly attractive in a Y-tube olfactometer bioassay, the strong attraction was not observed in bioassays without directed airflow between the odour source and the beetles, such as pitfall traps. This suggests that more research is needed to develop a method of releasing these VOCs that will work in stored product environments.

The responses of *T. castaneum* to food-associated volatiles were tested in two strains: an established laboratory strain (CTC12) and a recently cultured

wild population (wild Zim), to identify if there was any evidence that laboratory adaptation had influenced their responses to odours from these sources. No significant differences were found in the EAG responses of the two strains to the 66 food-associated VOCs. There were also no significant behavioural differences when the attraction of the two strains were tested against 10 compounds that gave large antennal responses or to different blends of these compounds. However, the responses of the two strains were found to differ when one of these blends was tested at different concentrations. This suggests that laboratory adaptation has not affected the compounds that *T. castaneum* can detect, or their responses to these compounds, however it provides some limited evidence that their sensitivity to these compounds may have been affected. However, as only two strains were tested these findings may not be representative of other *T. castaneum* strains and further testing is necessary.

As there was previously no consensus on the optimal bioassays for testing the attraction of *T. castaneum* to different odour sources, the effectiveness of several behavioural bioassays was compared in this thesis. The Y-tube olfactometer was identified as the most effective bioassay and insights about the effectiveness of the different bioassays could inform future research with this species. The EAG and Y-tube/pitfall bioassays used in this thesis also have potential as methods for deorphaning ORs in *T. castaneum* when used in combination with RNAi knockdowns of specific ORs. This methodology was validated by knocking-down the *T. castaneum Orco* gene, *Tc-or1*, and demonstrating that the effects of the OR knockdown could be detected by each of the bioassays. The observation that the effects of olfactory receptor knockdowns can persist for up to a month also suggests that the targeted disruption of OR genes could be used as a method of direct pest management in *T. castaneum*.

6.2. The responses of *Tribolium castaneum* to food-associated VOCs

One of the main issues preventing the development of more effective *T. castaneum* lures is a lack of knowledge regarding which specific compounds

are attractive to this pest species. To address this issue one of the aims of the thesis was to identify some of the specific VOCs that attract *T. castaneum* to stored products. In Chapter 3, EAG was used to screen the antennal responses of *T. castaneum* to 66 wheat germ oil or fungal-associated VOCs. These experiments identified that many compounds found in wheat germ oil or produced by grain-associated fungi elicit large antennal depolarisations in the two strains tested (Fig. 3.5). Most of the compounds tested were found to elicit responses stronger than the commonly used *Tribolium* attractant, DMD. This suggests that *T. castaneum* ORs are tuned to detect a wide range of wheat germ oil and fungal-associated VOCs. The discovery that such a large-number of wheat germ oil and fungal VOCs produce large antennal depolarisations suggests that these VOCs could be important cues for locating food-sources in this species. As *T. castaneum* has previously been shown to be attracted to wheat germ oil (Seifelnasr et al., 1982) and fungal odours (Ahmad, Daglish, et al., 2012) it is not unexpected that compounds from these sources would elicit large antennal responses. However, this is the first study to specifically test individual VOCs from these sources in an attempt to identify the compounds responsible for causing this attraction.

The large number of compounds that elicited significant antennal depolarisations, combined with these compounds possessing a variety of different functional groups, suggests that *T. castaneum* uses a variety of different VOCs to locate food sources within the stored-product environment. The idea that insects might locate food sources by responding to a unique combination of ubiquitously produced compounds has been suggested by other researchers (Bruce et al., 2005) and phytophagous insects are known to locate host plants in this way (Bruce and Pickett, 2011). The fact that many fungal volatiles elicited large depolarisations is evidence that *T. castaneum* antennae are tuned to detect a wide range of different fungal-produced volatiles. This sensitivity to fungal compounds supports the idea that *T. castaneum* may have fed on decaying tree bark before adapting to feed on human stored-products (Dawson, 1977). It is possible that the same fungal receptors that *T. castaneum* once used to detect fungi on rotten tree bark could have been retained to locate grain-associated fungi in the stored-product environment as the same VOCs could be present in both sources. As

T. castaneum is a secondary stored-product insect that cannot attack undamaged grains, the volatile compounds produced by grain-associated fungi could indicate the presence of grains that are in a suitable condition for *T. castaneum* to infest. This reveals a potential new source of attractive volatiles that could be incorporated into lures for *T. castaneum*. It is possible that the same fungal and wheat germ oil VOCs could also be attractive to other stored-product pests, and if so, these VOCs could lead to the development of a multi-species stored-product pest lure.

When the ten compounds that elicited the largest EAG responses were tested against *T. castaneum* in behavioural bioassays in Chapter 4 many of them were found to result in significant attraction (Fig. 4.2), supporting the idea that these volatiles could be used for food location. The ten compounds were a combination of fungal and wheat germ-associated compounds, demonstrating that *T. castaneum* is attracted to both fungal and wheat germ oil associated compounds. The response of *T. castaneum* was also tested against the ten compounds that gave the lowest EAG responses, but none of these compounds were found to result in significant attraction (Fig. 4.3). This indicates there is a degree of discrimination in the responses of *T. castaneum* to food associated volatiles and demonstrates that not all the volatiles associated with food sources are attractive. Together the EAG screen and behavioural bioassays reveal a strong correlation between the size of antennal depolorisation that a compound elicited and how attractive it is. However, not all compounds that elicited large EAG responses were found to be behaviourally attractive, highlighting that EAG data on its own cannot be used to determine whether a compound will be attractive or not. This could be due to the compound not eliciting a behavioural response or it could induce a behaviour that the bioassay cannot detect i.e. they may be repulsive, arresting or oviposition stimulating.

The responses of *T. castaneum* were also tested against different blends of the VOCs that elicited the largest EAG responses and several of the blends were identified as being significantly attractive (Fig. 4.5). By removing individual compounds from an attractive 5-compound blend hexanal, 3-methylanisol, and heptanone were identified as contributing the most to the attractiveness of this blend to *T. castaneum* (Fig. 4.7). These compounds

were also found to be highly attractive when tested in a blend together (Fig. 4.8). These three compounds are therefore strong candidates to be developed into a lure to attract *T. castaneum*, and possibly other stored-product pests. However, across all blends tested, none of the blends were significantly more attractive than their individual constituent VOCs (Tables 4.10 and 4.11). Therefore, although these blends were significantly attractive there is no evidence that the particular compounds tested had a synergistic effect when presented together.

Although the observation that *T. castaneum* antennae exhibit strong responses to a wide variety of different food-associated VOCs suggests that they may detect food sources by responding to a number of different volatiles, the results of the behavioural bioassays demonstrate that individual compounds from these sources are sufficient to attract them. This suggests that *T. castaneum* ORs may also locate food sources by responding to specific compounds of key ecological importance. This has been suggested to occur in other insects, for example the malaria mosquito *Anopheles gambiae*, has several receptors specifically tuned to respond strongly to a single VOC produced by human skin microflora (Carey et al., 2010).

To identify how *T. castaneum* may respond to these VOCs in real-world conditions, one of the blends that was found to be highly attractive in the Y-tube olfactometer bioassays was tested in a pitfall bioassay. This bioassay does not have directed airflow and has a large arena that allows the beetles to move more freely and this should more closely mimic how these odours are encountered in stored-product warehouses. However, the blend of hexanal, 3-methylanisol, and heptanone was not significantly attractive to either the CTC12 or wild Zim strain when applied to a paper disk (Fig. 4.9). When the blend was inserted into a slow release plastic capsule it was found to become significantly attractive to the CTC12 strain, although the overall capture rate after two hours was still relatively low (Fig. 4.10). This suggests that the release rate of these volatiles will be an important factor in determining their attractiveness in real world conditions. As this bioassay also had low capture rates when used to test other known *T. castaneum* attractants, this suggests that there may be issues with the bioassay itself. It is therefore possible that the number of beetles captured in this bioassay may not reflect the true

attractiveness of the odour source. In either case, further research is needed to identify if these compounds are significantly attractive under the conditions found in a typical stored-product warehouse.

6.3. Inter-strain and inter-sex variation in the responses of *Tribolium castaneum* to food-associated VOCs

In addition to being an important pest species, *T. castaneum* is also a model organism, particularly in the field of developmental biology, and has been extensively cultured in the laboratory (Brown et al., 2009). As *T. castaneum* are usually cultured in an environment of abundant food, it is possible that this could have caused laboratory adaptation, resulting in evolutionary changes such as relaxed selection of genes related for food location. If this is the case it could mean that the behavioural responses recorded by laboratory strains may not be representative of wild populations. To address this issue one of the aims of the thesis was to identify if the responses of *T. castaneum* to food-associated VOC appear to have been influenced by laboratory adaptation. The responses of *T. castaneum* to common wheat germ oil and fungal associated compounds were tested primarily using two different strains: wild Zim and CTC12. The wild Zim strain had been recently cultured from a wild population while the CTC12 strain has been continuously cultured in laboratories for decades.

No significant differences were found between the antennal responses of the two strains to 66 food-associated VOCs (Table 3.10). The lack of differences between the responses of the two strains suggests that laboratory adaptation has not influenced the ability of *T. castaneum* ORs to detect these odours. When the attraction of these two strains were tested against the VOCs that elicited the ten largest antennal depolarisations no significant differences were found between the responses of the two strains (Fig. 4.2). No significant differences were also found between the responses of the two strains to different blends of these VOCs (Fig 4.5). However, when a 10-compound blend was tested at different concentrations the responses of the two strains were found to differ significantly with concentration, with the wild Zim strain being more attracted to the blend at higher concentrations (Table 4.13). This

could indicate that the two strains differ in their sensitivity to odours from these sources. However, the responses of the two strains did not significantly differ when four individual VOCs were tested at different concentrations (Table 4.4). As such, it is unclear to what degree the responses of these strains of *T. castaneum* differ with concentration. These results may indicate that, although the compounds that *T. castaneum* are attracted to have not changed, their sensitivity to food associated volatiles might have been affected.

However, it should be noted that the data supporting the possibility of laboratory adaptation comes from laboratory and wild populations originally captured in different geographic regions: Australia in the case of the CTC12 strain and Zimbabwe in the case of the wild Zim strain. As it has been demonstrated that *T. castaneum* from different geographic areas can differ in their responses to food-associated odours (Gerken et al., 2018) it is possible that the differences observed between the two strains could be due to underlying geographic difference between the strains. Further research would be needed to demonstrate that the differences observed were not caused by geographic variation.

Although most of the data on the differences between *T. castaneum* strains comes from just two strains (CTC12 and wild Zim), a small number of behavioural experiments were also conducted using a Fera laboratory strain and the San Bernardino strain *T. castaneum*. However, these strains were found to be unresponsive in behavioural bioassays, with the San Bernardino strain, in particular, observed to be inactive in many of the bioassays. This apparent inactivity could be due to differences in their mobility, and not due to differences in their attraction to the odour sources, as these strains appeared to have difficulty navigating the bioassay arena. As a more responsive strain was subsequently found, the causes of these differences were not explored further.

Differences between the responses of male and female *T. castaneum* were not extensively tested as the EAG responses of male and female *T. castaneum* had been previously tested to a large range of VOCs and were not found to significantly differ (Balakrishnan et al., 2017). Replicating the experiments in each sex would have also doubled the amount of time it would

have taken to conduct the experiments and meant that less compounds could be tested. However, the EAG responses of male and female *T. castaneum* were tested against a small number of environmental odour sources in Chapter 3 and no significant differences were found between their responses (Fig. 3.2). Although this provides some evidence that the responses of male and female *T. castaneum* to food-associated odour sources do not differ, more extensive research would be needed to confirm this.

6.4. Assessing the usefulness of different *Tribolium castaneum* behavioural bioassays

Several different behavioural bioassays have been used by different researchers to test the attraction of *T. castaneum* to different odour sources (see Chapter 2). However, it is not readily apparent which bioassay is the most effective for studying the responses of *T. castaneum* to food-associated VOCs. To address this issue the thesis aimed to identify the most efficient bioassay for testing the attraction of *T. castaneum* to different odour sources. This information supported the choice of bioassays used in the thesis to test the responses of *T. castaneum* to food-associated VOCs and could also inform the bioassays choices of other researchers in the future.

The bioassays compared in this thesis represent some of the most commonly used behavioural bioassays for testing *T. castaneum* and other insects. Of the bioassays tested in Chapter 3, the Y-tube olfactometer was found to be capable of detecting strong attractions to food-associated VOCs and was the only bioassay that could detect a significant behavioural response to the known *T. castaneum* attractant, DMD. The other bioassays that were tested (a 4-way olfactometer, pitfall bioassay and 2-choice arena bioassay) either had significant issues regarding the movement of beetles inside the bioassay arenas or failed to detect a response from known *T. castaneum* attractants. The 4-way olfactometer could not be used as *T. castaneum* appears to be a poor climber, which prevented the beetles from entering the 4-way olfactometer through an inlet pipe containing airflow from each odour source in the olfactometer. There were also similar motility issues when the San Bernardino strain was tested using the Y-tube olfactometer, as these beetles

had trouble navigating the olfactometer and frequently fell onto their backs and were unable to right themselves. CTC12 and wild Zim strain beetles did not have this issue when tested in the Y-tube olfactometer. Other researchers have successfully used the 4-way olfactometer to study *T. castaneum* behavioural responses, however it is unclear how these beetles entered the olfactometer chamber in these studies (Ukeh and Umoetok, 2011). It is therefore possible that the 4-way olfactometer could be used if it was modified in such a way that the beetles can enter the arena in the presence of airflow from all directions without needing to climb. As the 4-way olfactometer is similar in many ways to the Y-tube olfactometer, but allows many compounds to be tested at once, it is possible that with modification the 4-way olfactometer could be used in future to more effectively test the relative attractiveness of different volatiles or volatile blends.

The absence of strong attraction in any of the still-air olfactometers suggests that the responses of *T. castaneum* to environmental VOCs are weaker under still-air conditions. Although the Y-tube olfactometer appears to be effective at determining the response a compound elicits when there is an active airflow carrying the volatile(s), if this does not reflect the attractiveness of these compounds under natural conditions then different methodologies may be needed to determine how effective these compounds could be in a *T. castaneum* lure. It is possible that the pitfall bioassays and the two-choice arena bioassays could be useful for testing the responses of *T. castaenum* under still air conditions if their capture rates could be improved. Possible modifications to these bioassays could involve rounding the edges of the arena and the pitfall holes, as this may prevent beetles from huddling around the edges of the arenas and avoiding the pitfall trap in the centre. It is also possible that the responses of *T. castaneum* in olfactometer bioassay arenas are fundamentally different to its behaviour when they encounter these compounds under natural conditions. If that is the case, then ultimately only experiments conducted in real or simulated warehouse environments will be able to predict the behaviours of beetles to odour sources in this setting.

6.5. RNAi knockdown of *Tc-or1* in *Tribolium castaneum*

As *T. castaneum* has a sequenced genome with annotated ORs, there is the opportunity to identify the VOCs these receptors detect and develop new pest management technologies using this information. However current methodologies for deorphaning ORs, such as the *Drosophila* empty neurone system and the *Xenopus* oocyte system, involve expressing *T. castaneum* ORs in other species and therefore cannot identify the behavioural responses an OR will elicit in *T. castaneum*.

To address this issue, the final aim of this thesis was to identify if behavioural bioassays could be used alongside RNAi as a method of deorphaning *T. castaneum* ORs. The potential of this methodology was demonstrated by using RNAi to knockdown the only *T. castaneum* OR that has been functionally identified, the *T. castaneum Orco* orthologue *Tc-or1*. As *Tc-or1* encodes an essential olfactory co-receptor, knocking-down *Tcor1* should disrupt the function of all other ORs. Knocking down this gene was shown to significantly reduce the EAG responses of *T. castaneum* to DMD and 1-octen-3-ol (Fig. 5.4). The knockdown also abolished the attraction of beetles to wheat germ oil in pitfall bioassays (Fig. 5.5) and the attraction to a blend of hexanal, 3-methylanisol, and heptanone in a Y-tube olfactometer bioassays (Fig. 5.6). This demonstrates that these bioassays could be used to detect the effects of RNAi knockdown of *T. castaneum* ORs. However, although this demonstrates that these bioassays are capable of detecting the effects of knocking-down ORs in *T. castaneum,* further molecular verification, using qRT-PCR or a Northern blot would be necessary to verify that the correct OR gene has been disrupted.

Now that these bioassays have been demonstrated to be able to detect the behavioural effects of knocking down ORs they could be used as a screening tool to deorphan *T. castaneum* ORs. EAG could be used as a tool to rapidly screen the responses of *T. castaneum* that have had specific ORs knocked down with RNAi. Compounds that elicit impaired EAG responses compared to uninjected beetles could then be tested using a Y-tube olfactometer or pitfall bioassay to identify if the attraction to these compounds has been abolished.

This would provide strong evidence that the attraction to that compound is mediated by the OR that had been knocked-down with RNAi.

These experiments also reveal that the effects on *T. castaneum* olfaction can last for up to a month without a significant reduction of the knockdown effect (Fig. 5.6). As the knockdown duration of OR genes in *T. castaneum* was not previously known this finding is important and opens up new avenues of pest management in *T. castaneum*. This finding suggests that the long-lasting targeted disruption of *T. castaneum* ORs could be used as a method of species-specific pest management by preventing *T. castaneum* from being able to locate stored products.

6.6. Future work

There are several ways in which the research in this thesis could be continued, and some novel avenues of research that have been revealed by the findings, which are described below.

6.6.1. *Tribolium castaneum* lure development

Firstly, although the results from the Y-tube olfactometer experiments in Chapter 4 clearly demonstrated that certain wheat germ oil and fungal associated compounds can be highly attractive to *T. castaneum*, further research is needed to develop these VOCs into a usable commercial lure. The results of the pitfall bioassays in Chapter 4 demonstrated that inserting the blend into a slow release plastic capsule increased its attractiveness, suggesting that altering the release rate of the blend could increase its attractiveness. As insects rely on moving towards odour gradients to locate food sources (Visser, 1986), it is possible that a slower release rate establishes a clearer gradient of VOCs. However, the overall capture rate of the CTC12 was still relatively low. It may be that different release mechanisms are needed to improve the effectiveness of the VOCs before they can be used as *T. castaneum* lures. Several such methods exist including, trapping the lure within a solid matrix such as a rubber septum or polyethylene vials, or a liquid matrix such as an aqueous paraffin emulsion (Heuskin et al., 2011). These techniques could be used to see if changing the release rate of the VOCs can improve their attractiveness under real word conditions. Ultimately,

experiments in simulated or real warehouse conditions may be the only way to determine how attractive these VOCs are in real world conditions. Once a lure containing these VOC has been determined to be attractive under real world conditions, its attractiveness needs to be compared to existing *T. castaneum* lures to determine if it is more attractive than existing commercial lures, or if it can be incorporated to enhance their attractiveness.

Further research could also be conducted into how the composition of food-associated VOC blends effects their attractiveness to *T. castaneum.* Although significantly attractive blends of food-associated volatiles were identified in this study, none of them were found to be significantly more attractive than their constituent VOCs. However, it is possible that different blends of these compounds could behave synergistically and be significantly more attractive than some of their constituent VOCs. It is also possible that blends with different ratios of compounds could give different responses. For simplicity, all of the blends tested contained an equal ratio of components, but there are examples of the ratio of a compound within a blend affecting its attractiveness (Bruce et al., 2005; Bruce and Pickett, 2011). Changing the ratios of different blends could lead to the discovery of new attractive blends or improve the attractiveness of combinations that have already been tested. Although, in this thesis, no instances were identified where compounds that were unattractive on their own became attractive as part of a blend, this has been demonstrated in other insects (Bruce and Pickett, 2011).

As only the compounds that elicited the 10 largest and 10 smallest EAG responses were tested behaviourally in Chapter 4, there are many compounds that elicited moderate to large EAG responses but were not tested behaviourally in this thesis. These compounds have the potential to be highly attractive to *T. castaneum*, and it would be worthwhile for these compounds to be tested behaviourally.

One of the potential advantages of using environmental compounds in a stored product pest lure, as opposed to species specific attractants such as sex pheromones, is the increased likelihood that the lure will be attractive to multiple different pest species. As such, after demonstrating that a lure containing wheat germ oil and fungal volatiles is attractive to *T. castaneum*

under real-world conditions, this research could be expanded to test the attractiveness of these compounds to other stored product insect species. As cereal grains are a common food source of many different pests, wheat germ oil VOCs could be attractive to a variety of different stored product insects. However, it is possible that common primary stored product insects including beetles such as, the larger grain borer *Prostephanus truncatus*, the lesser grain borer *Rhyzopertha dominica*, the grainweevil *Sitophilus granaries*, the maize weevil *S. zeamais* and the rice weevil *S. oryzae* and moths such as the tropical warehouse moth *Ephestia cautella* and the Angoumois grain moth *Sitotroga cerealella* may be less attracted to fungal compounds than secondary stored product pest such as *T. castaneum*. As primary stored-product pests are capable of feeding on intact grains, they may not be as reliant on detecting fungal volatiles for locating damaged and rotten grains and may instead only respond to wheat germ oil associated VOCs. As such, further research using other pest species could be conducted to determine if the different compounds and blend that have been demonstrated to be attractive to *T. castaneum* could be used to develop a multispecies lure for stored-product insects.

6.6.2 Variation in the responses of *Tribolium castaneum* to food-associated VOCs

As the differences between laboratory cultured strains and wild populations were tested using only two strains it would be useful to expand the research to test other strains to identify if these observations hold true for other *T. castaneum* strains. As the CTC12 and wild Zim strain were from different geographic regions it is possible that this could have influenced their responses to the volatiles tested. Testing strains from additional geographic regions would help to identify if this variation has affected the responses of the beetles. Testing inbred laboratory and wild populations that were originally captured from the same geographic region would help to remove the effect of geographic variation when studying the effects of laboratory adaptation. As strains from different geographic areas have been demonstrated to differ in their responses to food-based lures (Gerken et al., 2018), it would be useful to identify the degree to which the responses of food-associated VOCs differs by geographic area, as this would affect the usability to any lure developed

from these odour sources. It may also be possible to demonstrate the effects of laboratory adaptation by capturing and deliberately inbreeding a wild *T. castaneum* population to see if electrophysiological and behavioural changes in responses to food-associated VOCs can be induced. As the same type of laboratory adaptation could also occur in other pest species that are cultured in the laboratory under similar conditions to *T. castaneum,* this research would have implications for other pest species that are also commonly cultured in laboratories.

As olfactory responses in insects can show plasticity under certain conditions, such as starvation (Reisenman, 2014) or even different times of day (Merlin et al., 2007), efforts were taken to control these conditions in the experiments in this thesis. However, it would also be interesting to see if the responses to the compounds tested in this thesis are affected by these conditions. If the responses to these compounds are not consistent this could have negative implications for its usage as a pest lure.

6.6.3 *Tribolium castaneum* olfactory receptors

Now that it has been demonstrated that EAG and behavioural bioassays can be used to identify the effects of knocking-down ORs in *T. castaneum,* other ORs could be targeted to deorphan the receptor and identify the ligands they detect. Specific OR genes of interest could be identified based on sequence homology with OR genes in other species that have been identified as responding to food-associated VOCs. For example the *AgOR8* gene in *Anopheles gambiae* encodes an OR that responds strongly to the common fungal compound 1-octen-3-ol (Lu et al., 2007). It is possible that the sequence information from this gene could be used to locate *T. castaneum* ORs that may also respond to this potentially important food-associated compound. Other beetle species that have had their olfactory receptor genes sequenced and annotated could also provide information on the identity of ORs involved in locating food sources in the stored product environment. The bark beetles *Ips typographus* and *Dendroctonus ponderosae* have had their olfactory receptors sequenced, and when a dendrogram of the olfactory receptors of these two beetle species and *T. castaneum* was constructed, a *T. castaneum* specific OR expansion was identified, as well as several genes

that appeared to have a higher degree of sequence similarity to bark beetle ORs (Andersson et al., 2013). As *I. typographus* and *D. ponderosae* are not stored-product pests, having diverged from a common ancestor shared with *T. castaneum* ca. 230–240 Mya (Hunt et al., 2007) it is possible that these *T. castaneum* specific genes could be related to the adaptation of *T. castaneum* to feeding on stored products. As the other two beetles feed on dead and rotten bark, it could be that the ORs that are conserved between *T. castaneum* and these species represent the putative ancestral ORs that *T. castaneum* has retained to respond to fungal odours in the stored product environment. However, as some *T. castaneum* ORs have similar amino acid sequences it is possible that there may be redundancy between the ORs that *T. castaneum* uses to detect specific environmental VOCs. This may make detecting the effects of knocking down a single receptor difficult, as the remaining OR(s) could be sufficient to elicit a respond to the compound.

Although the results of the *Tc-or1* experiments have demonstrated that the EAG and the Y-tube olfactometer are capable of detecting physiological and behavioural changes associated with OR knockdowns, further molecular verification would be necessary to confirm that the effect observed was due to successfully knocking down the *Tc-or1* gene. A Northern blot or qRT-PCR could be used to verify the actual reduction in *Tc-or1* gene expression (mRNA levels) caused by the *Tc-or1* dsRNA microinjection and verify that the result seen are not due to an off-target effect.

6.6.4 Summary

Overall the research presented in this thesis reports several new findings about *T. castaneum* that have important pest management applications and could lead to the development of new management strategies for *T. castaneum* and other stored product insects.

This research has demonstrated that both fungal and grain-associated volatiles appear to be used for food-source location by *T. castaneum*. This important discovery opens up new areas of research in *T. castaneum* and other stored product insects that could lead to the development of a multispecies stored product pest lure. Several individual compounds found in wheat germ oil or produced by grain-associated fungi, as well as blends of

these compounds, were tested in behavioural bioassays and found to be significantly attractive. The results also suggest that there is scope to improve the attractiveness of the attractive blends identified by altering the blend composition or volatile release method. If these compounds can be shown to be effective in attracting stored product insects to traps in stored product environments, they could be used to improve the effectiveness of lures used to monitor stored product insects, increasing the effectiveness of pest management strategies. This research also identified several other compounds that elicited large EAG responses but that were not tested behaviourally. These compounds are also candidates for being attractive to *T. castaneum* and other stored product insects.

The research in this thesis also suggests that the responses of established laboratory strain and recently caught wild *T. castaneum* do not significantly differ to food-associated compounds. However, there was some evidence that they may differ in their sensitivity to some of these compounds. This has relevance to the use of these compounds for *T. castaneum* pest management and other strains could be tested to see how prevalent these potential inter-strain differences are.

This research has also assessed different bioassays for testing the responses of *T. castaneum* to ecologically relevant odour sources and identified the Y-tube olfactometer as the most effective, which could inform the choice of olfactometer in future *T. castaneum* research. However, it is possible that modifying the experimental design could alter the effectiveness of the different bioassays.

It has also been demonstrated that the behavioural changes elicited by the RNAi knockdown of OR genes can be detected by some the bioassays used in this thesis. This opens up a new method of olfactory receptor research in *T. castaneum* and could lead to more ORs having their odorant ligands identified. The fact that the effect of OR gene knockdowns can last for up to a month also opens up the possibility of direct pest management by gene disruption in *T. castaneum*.

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List of Abbreviations

ANOVA	Analysis of variance
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CT Room	Controlled temperature room
DMD	4,8-Dimethyldecanal (*Tribolium* aggregation pheromone)
dsRNA	Double-strand RNA
EAG	Electroantennography
EBQ	Ethyl-1,4-benzoquinone
Fera	Food and Environment Research Agency
GC-EAG	Gas-chromatography-electroantennography
GPCR	G protein-coupled receptor
IPM	Integrated pest management
MBQ	Methyl-1,4-benzoquinone
Mer	Repeat unit
mRNA	Messenger RNA
Mya	Million years ago
OBP	Odorant binding protein
OR	Odorant receptor
ORCO	Odorant receptor co-receptor
OSN	Olfactory sensory neurone
PCR	Polymerase chain reaction
PTFE	Polytetrafluoroethylene
PVAc	Polyvinyl acetate

qRT-PCR	Real-time reverse transcription PCR
RI	Response index
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	Small interfering RNA
SPI	Stored product insect
Tc-or1	*Tribolium castaneum* odorant receptor one
VOC	Volatile organic compound
Wild Zim	Wild Zimbabwean (*Tribolium castaneum* strain)

Appendix A
Additional information on the VOCs used in this thesis

A.1 Additional information on the source and purity of VOCs used in this thesis.

Compound	CAS Number	Purity	Product code	Supplier	Notes
5-Methyl-3-heptanone	541-85-5	94.0%	8.18663	Sigma-Aldrich	
Trans-2-heptenal	8829-55-5	97.0%	324140	Sigma-Aldrich	
Ethyl hexanoate	123-66-0	≥99%	148962	Sigma-Aldrich	
Limonene	5989-27-5	97.0%	183164	Sigma-Aldrich	
Trans,trans-2,4,-heptandinal	4313-03-5	≥88%	W316407	Sigma-Aldrich	
2-Heptanone	110-43-0	≥98%	W254401	Sigma-Aldrich	
Trans-2-pentenal	1576-87-0	≥95%	W321818	Sigma-Aldrich	
Isovaleraldehyde	590-86-3	97.0%	146455	Sigma-Aldrich	
Octanal	124-13-0	99.0%	05608	Sigma-Aldrich	
Amyl acetate	628-63-7	≥99%	W504009	Sigma-Aldrich	
Trans-2-octene	13389-42-9	97.0%	111236	Sigma-Aldrich	
1-Penten-3-one	1629-58-9	97.0%	E51309	Sigma-Aldrich	Contains 0.1% BHT as stabilizer
Ethylbenzene	100-41-4	99.8%	296848	Sigma-Aldrich	
1-Octene	111-66-0	98.0%	04806	Sigma-Aldrich	
Trans-Cinnamaldehyde	14371-10-9	99.0%	C80687	Sigma-Aldrich	
Ethyl octanoate	106-32-1	≥99%	112321	Sigma-Aldrich	
Pentane	109-66-0	≥99%	236705	Sigma-Aldrich	Anhydrous
2-Pentylfuran	3777-69-3	≥98%	W331708	Sigma-Aldrich	
Trans-2-octenal	2548-87-0	≥95%	W321508	Sigma-Aldrich	Stabilized

Undecane	1120-21-4	≥99%	U407	Sigma-Aldrich	
1-Heptene	592-76-7	97.0%	262269	Sigma-Aldrich	
Nonane	111-84-2	≥99%	296821	Sigma-Aldrich	Anhydrous
Trans-5-decene	7433-56-9	99.0%	110485	Sigma-Aldrich	
Toluene	108-88-3	99.8%	244511	Sigma-Aldrich	
Trans-3-octene	14919-01-8	98.0%	111244	Sigma-Aldrich	
2-Methyl-2-butene	513-35-9	95.0%	86262	Sigma-Aldrich	
P-anisaldehyde	123-11-5	98.0%	A88107	Sigma-Aldrich	
Trans,trans-2,4-d Decadienal	25152-84-5	85.0%	180513	Sigma-Aldrich	
Trans-2-decenal	3913-81-3	≥95%	30658	Sigma-Aldrich	
4-Allylanisol	140-67-0	98.0%	A29208	Sigma-Aldrich	
Octanoic acid	124-07-2	≥98%	03907	Sigma-Aldrich	
Tridecane	629-50-5	≥99%	T57401	Sigma-Aldrich	
Hexane	110-54-3	97.0%	34859	Sigma-Aldrich	
3-Octanone	136-91-3	≥98%	136913	Sigma-Aldrich	
Butyl acetate	123-86-4	≥99%	287725	Sigma-Aldrich	Anhydrous
Benzaldehyde	100-52-7	≥99.5%	418009	Sigma-Aldrich	Purified by redistilation
3-Methylanisol	100-84-5	99.0%	149160	Sigma-Aldrich	
2-Methylacetophenone	577-16-2	98.0%	M26593	Sigma-Aldrich	
1-Pentanol	71-41-0	≥99%	398268	Sigma-Aldrich	
Trans-2-hexen-1-al	6728-26-3	98.0%	132659	Sigma-Aldrich	
2-Methyl-2-butanol	75-85-4	99.0%	152463	Sigma-Aldrich	
Damascenone	23696-85-7	98.0%	41163	Sigma-Aldrich	
3-Octanol	589-98-0	99.0%	297887	Sigma-Aldrich	Anhydrous

Dimethylbenzene	1330-20-7	≥75%	214736	Sigma-Aldrich	Mixture of isomeric ortho-, meta- and para-forms of dimethylbenzene
Styrene	100-42-5	≥99%	S4972	Sigma-Aldrich	Contains 4-Tert-butylcatechol as stabilizer
2-Butanol	78-92-2	99.5%	294810	Sigma-Aldrich	Anhydrous
Naphthalene	91-20-3	99.0%	147141	Sigma-Aldrich	
1-Butanol	71-36-3	99.8%	281549	Sigma-Aldrich	Anhydrous
2-Methyl-1-propanol	78-83-1	99.5%	294829	Sigma-Aldrich	Anhydrous
2,2,4-Trimethylhexane	16747-26-5	≥98%	92470	Sigma-Aldrich	
2-Nonanone	821-55-6	≥99%	108731	Sigma-Aldrich	
Acetone	67-64-01	≥99.9%	270725	Sigma-Aldrich	
2-Methylfuran	534-22-5	99.0%	M46845	Sigma-Aldrich	Contains 200 - 400 BHT as Stabilizer
Octyl acetate	112-14-1	≥99%	05500	Sigma-Aldrich	
2-Pentanone	107-87-9	≥98%	W284203	Sigma-Aldrich	
1-Phenylethanol	98-85-1	98.0%	P13800	Sigma-Aldrich	
2-Propanol	67-63-0	99.9%	34863	Sigma-Aldrich	
Trans-2-octen-1-ol	18409-17-1	97.0%	547115	Sigma-Aldrich	
1-Octanol	111-87-5	≥99%	297887	Sigma-Aldrich	Anhydrous
Ethyl ethanoate	141-78-6	99.8%	270989	Sigma-Aldrich	Anhydrous
3-Methyl-1-butanol	123-51-3	≥99%	309435	Sigma-Aldrich	Anhydrous
Hexanal	66-25-1	98.0%	115606	Sigma-Aldrich	

1-Octen-3-ol	3391-86-4	98.0%	O5284	Sigma-Aldrich	
1-Hexanol	111-27-3	≥99%	471402	Sigma-Aldrich	Anhydrous
Nonanal	124-19-6	95.0%	PC1002 207886	Sigma-Aldrich	
Ethanol	64-17-5	99.8%	24103	Sigma-Aldrich	

Appendix B
Tc-or1 primer sequences

B.1 *Tc-or1* primer sequences

TcOR1 primers (5’-3’)

Forward: GGGCACTTCATGCTCAACTA

Reverse: CCTGACCATCAACTCCTGTTT