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**Larval performance, morphological,
behavioural and electrophysiological
studies on *Tenebrio molitor* L.
(Coleoptera: Tenebrionidae)**

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Abstract

The Yellow Mealworm Beetle (YMB) *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is a cosmopolitan stored product pest with infestations usually originating in poorly stored animal feed and stored grain; damages and subsequent economic losses, due to infestations of *T. molitor* are mainly due to their feeding activity. Moreover *T. molitor* larvae represents one of the most popular and common insect used as feed and food. Their larvae have the ability to recycle organic waste and food by-products of low-quality into high-quality feed and food, rich in energy, protein and fat. However, with the growing understanding of the role of bio-economy within Europe and the need to apply circularity in the use of natural resources, recognizing that the waste can represent a new starting material for other industrial processes is now a must. In this sense, as suggested by the EU community, a circular economy should be applied improving economic benefits, contributing to innovation and growth, encouraging sustainability and competitiveness in the long term. According to the concept of circular economy, insects represent good candidates since they can be cultured through environmental-friendly, cost-effective farming processes, on by-products/wastes. Producing edible insects on diets composed of organic by-products could increase sustainability. The aim of FAO, and of the several other initiatives established recently, is to reduce the amount of losses and wastes of food along the value chain in relation to the prospects for feeding a

population of nine billion people by 2050. The possibility to use food wastes, residues and by-products as substrate for the recovery of valuable nutrients is becoming quite popular using in particular insects. In this optic of a circular economy, *T. molitor* larvae represent a perfect candidate. In particular, the thesis explores the possibility to use *T. molitor* larvae to reduce food wastes generated by the industry. This possibility has been investigated considering different field of the entomological research including studies of insect morphology, insect rearing on different artificial diets and evaluation of their performances, characterization of Volatile Organic Compounds (VOCs) emitted from food substrates and evaluation of the electrophysiological and behavioral response of larvae and adult insects to the main VOCs identified. *T. molitor* growth performances were monitored under four different diets composed of organic food by-products, originating from mill and olive oil production: organic wheatmeal (WH), wheatmeal:olive-pomace 3:1 (M1), wheatmeal:olive-pomace 1:1 (M2), wheatmeal:olive-pomace 1:3 (M3). For each diet tested, there were analyzed: i) the larval growth performances from eclosion to the last molt; ii) the number of instars; iii) the duration of larval stage; iv) the weight of last instar larvae and pupae; v) the overall survival rate. The results showed that, diet affected mealworm development such that diets with higher percentage of wheatmeal appear favourable, with respect to shortening larval development time, reducing mortality and increasing weight gain. However, it was demonstrated that *T. molitor* larvae can be

grown successfully on diets composed up to 25% of organic olive pomace replacement. The evaluation of the insect's performances on different artificial diets provides immediately exploitable results for disposal of olive pomace. Moreover, these results highlight the importance of diets respect to larval growth performances in the set-up of a rearing systems for mass production. Regarding the morphological aspects, in particular it was investigated the external morphology and distribution pattern of the sensory structures, called sensilla, on mouthpart palps of *T. molitor* larvae using scanning electron microscopy. Based on the morphology of the features of the sensilla, we classified them into nine types: sensilla trichoidea, types 1 and 2, sensilla digitiformia, sensilla chaetica, sensilla coeloconica and sensilla basiconica types 1, 2, 3 and 4. A total of 23 and 13 sensilla on maxillary and labial palps were found, respectively. The knowledge about the type, number and distribution of sensilla on the mouthparts palps of *T. molitor* larvae will provide morphological basis for future ultrastructural, electrophysiological and behavioural studies. Behavioral responses of *T. molitor* larvae to VOCs emitted by flour (FR), wheatmeal (WH), mixture of wheatmeal and pomace (1:1) (M) and olive pomace (OP) was investigated using four and two-way choice. The larval behavior was analysed using a video tracking software. The results of this study showed that *T. molitor* larvae were significantly attracted to volatiles emitted both by WH and M. The behavioral responses of adults to VOCs emitted by wheatmeal were also investigated in a two-choice pitfall

olfactometer. Five doses, ranging from 1 g to 200 g of wheatmeal were tested. The data showed that adults of the yellow mealworm beetle have the ability to respond behaviorally to wheatmeal volatiles and that responses may change as a function of concentration of these volatiles. All doses elicited positive responses to beetles. However, females showed higher Response Index (RI) to higher doses of wheatmeal respect to males which were attracted at the same level to the different doses. Moreover a preliminary electroantennography bioassay (EAG) on 14 different VOCs extracted from wheatmeal on this adult beetle is presented. EAG tests showed that the antennal olfactory systems of *T. molitor* are able to perceive several VOCs emitted by wheatmeal. In particular the largest EAG amplitudes were recorded for pentanal, exanal, (E)-2-hexanal, (E,E)-2-4-nonadienal, (E,E)-2,4-hexadienal, pentanol and hexanol. An understanding of how the beetle respond to such volatiles could be useful for the development of effective integrated pest management strategies. The results from the behavioral studies on larvae and adults of *T. molitor* will represent also the starting point for the identification of interspecific semiochemicals to be used for the development of innovative and sustainable control strategies of this pest. In conclusion, the results of this thesis will contribute to clarify important aspects related to the morphology, physiology and behavior of an economically important pests.

INTRODUCTION

Background

Early detection of pests in stored cereals is essential for successful grain protection because it will give an indication of when control measures are required and whether they have been effective. It will also enable control strategies to be used that reduce the need for persistent pesticides with the associated risk of contaminating food and the environment. Various beetle traps have been developed for use in grain stores but these detect only a small proportion of the total population present (Wakefield and Cogan, 1999). In the past, work on trapping and monitoring of beetles in stores has favoured the development of lures based on pheromones produced by the beetles themselves. These lures have the disadvantage of usually attracting only one species to each attractant and are not always particularly effective under practical grain storage conditions. Ideally a lure should be inexpensive to produce with an adequate shelf life and be long lasting in use; it should attract as many different species of pest as possible. Food baits, whilst often capable of attracting a range of species, may themselves become a refuge for feeding and breeding pests if the bait is not fully contained and may contaminate the stored cereal. Insect behavior in grain stores is affected by the interaction of many different physical, chemical and biotic factors in their environment. These include light intensity, temperature, relative humidity, food availability, grain

moisture content, size and variety, grain store design and construction materials, disturbance caused by insect density and the presence of other arthropod species including predators and parasitoids, vertebrates and disease micro-organisms. Human activities can have profound effects on insect behaviour, as a result of grain handling, drying, cooling and cleaning, as well as the use of pest management measures. Chemicals that mediate interactions between organisms are called semiochemicals, and many of these are known to affect behaviour (Law and Regnier, 1971). These include oviposition deterrents, sex and aggregation pheromones, produced by the insects themselves, and chemicals associated with their food supply including fungal volatiles. Chemicals deliberately applied to the grain for pest control can also affect insect behaviour. In order to understand the behaviour of insects in bulks of stored grain it is important to consider the grain store environment itself together with the origins of the pest species. The environment of a grain store presents insects with a number of challenges. In evolutionary terms this is a new habitat, with the successful insects being either opportunists capable of some adaptation over a comparatively short time-span or organisms that were pre-adapted to man-made storage conditions. To some extent the store provides protection from the extremes of outside temperature and relative humidity fluctuations, both diurnal and seasonal, with minimal changes occurring at the centre of large bulks. However, the increasingly common practice of cooling and aerating stored grain means that to survive the winter,

beetles must be able to tolerate low temperatures for long periods or else seek out pockets of warmer grain. The successful pest must also be capable of surviving in a habitat usually lacking free water. Furthermore, under good storage conditions, particularly in the developed world, the food supply itself has a low moisture content and is of an intermittent nature. When full, the grain store provides vast amounts of readily available food but on farms it is often left empty for long periods between harvests, with only limited supplies for the resident pest population in the form of residues hidden in the fabric of the building. Thus, the beetle must be able to seek out small quantities of food scattered over a wide area. The ability to fly will be important for survival in some species. In addition, grain harvesting, drying and handling prior to storage will all affect the quality of the grain and its suitability for each potential pest. The ability to utilise different species of grain is also an advantage for a prospective pest. All these factors have an important bearing on the type of behaviour required by insects for successful utilisation of the storage environment. For example, they must be able to find sexual partners, oviposition sites and food when they are in short supply and spread over a wide area. Most stores are dark or dimly lit, and within the grain bulk there is a complete absence of light. Thus, beetles exploiting this habitat must be capable of behaviour that does not rely on visual cues or diurnal changes in light intensity. Cryptic behaviour will also confer an advantage by reducing the chances of contact

with parasites, predators and other control agents including pesticides.

Origins of beetles in grain stores. Based on evidence from archaeological deposits (Solomon, 1965) and ancient hieroglyphic records (Levinson and Levinson, 1998), beetle pests have been associated with the storage of grain by humans since at least the 25th century BC. It is thought that humans first began cultivating and storing cereals around 8000 BC. Before this, storage beetles may have evolved to exploit natural accumulations of seeds in dry, sheltered habitats; these included those blown into caves, cracks and crevices by the wind or found in the nests and food hoards of other animals, including birds, rodents and social insects (Linsley, 1944). These situations would have provided conditions not too different from those in which humans stored grain, thus making it easier for the beetles to move into the new habitat. However, the evolution of grain beetles to their present state may not have relied entirely on continued ties to dried seeds. For example, even in modern times some storage beetles have been found under the bark of dead trees or in haystacks, manure heaps and mouldy straw bales where they are probably feeding on fungi (Woodroffe, 1962; Howe, 1991). Today, beetles can invade stored grain by several routes. A few species can infest the standing crop in the field before harvest (Chesnut, 1972) but this is rare for grain pests in regions of temperate climate where the majority of infestations commence after harvest. If the store has been inadequately cleaned, pockets of insects

hidden in the storage structure may have been left behind from the previous harvest, ready to infest the new intake of grain (Coombs and Freeman, 1955; Cox *et al.*, 1990). Several species may invade the nests of birds, rodents and ants located in the fabric of the store (Woodroffe, 1953; Khare and Agrawal, 1964). Some beetles may crawl from outside, invading particularly from neighbouring stores containing infested commodities, but also sometimes from rubbish heaps, natural habitats and alternative hosts, perhaps attracted by the smell of the new grain (Southwood and Johnson, 1957; Howe, 1965; Barrer, 1983; Throne and Cline, 1994; Delobel and Grenier, 1993). Alternatively, beetles may gain access passively when other commodities are brought in from infested commercial stores or by transportation on vehicles, farm equipment, sacks and other packaging materials or even on clothing. Little quantitative data are available on the relative importance of these routes but recent studies in the UK and USA have highlighted the importance of residual infestations in commercial grain stores (Prickett and Muggleton, 1991; Dowdy and McGaughey, 1998).

Movement and distribution of stored product pests. Once in contact with the new grain, the beetles will move to locations within the bulk, dependent on the preferences of the particular species concerned. Beetle distribution is typically non-uniform and is dependent on factors such as moisture and temperature gradients, the presence of contaminants, damaged grains, grain dust, and other

arthropods including natural enemies. For example, *Cryptolestes ferrugineus* stephens, particularly at densities of less than five insects/kg, has been found moving from the top of grain masses down towards the bottom of bulks and aggregating in pockets of damp or damaged grain (Watters, 1969; Smith, 1978; Loschiavo, 1983; White and Loschiavo, 1986), as well as migrating to warmer portions of the grain mass as temperatures fall in winter (Flinn and Hagstrum, 1998). Recently, different types of surface and buried traps were used to monitor the distribution of *Sitophilus granarius* Linnaeus throughout a 100 t floor-store of wheat under natural storage conditions at various times of the year (Wakefield and Cogan, 1999). More detailed information of this type on insect distribution could assist in choosing the most effective trap numbers and locations. In *S. granarius*, there is a dispersal phase in the first few days after emergence but feeding activity remains constant throughout life; running activity increases shortly after emergence and then decreases again slowly as the beetle ages (Stein, 1994). Higher temperatures increase locomotory activity in beetles generally. Southwood (1962) has suggested that dispersal may be an adaptation to life in an unstable or unpredictable habitat; if the habitat is starting to become unfavourable there will be an advantage to insects that commence dispersion before conditions deteriorate too far, thus improving their chances of finding more suitable conditions. It has been found that in the flour beetle, *Tribolium castaneum* (Herbst), adults that exhibit high levels of dispersal behaviour, particularly at high population

densities, typically show greater flying ability, develop faster and lay more eggs than adults that show low levels of this behaviour, thereby emphasising the importance of dispersal as a survival and colonisation mechanism (Lavie and Ritte, 1978; Zirkle *et al.*, 1988). Studies involving reciprocal crosses suggested that dispersal behaviour is determined by a dominant allele at a single, sexlinked locus, with males tending to disperse more readily than females (Ritte and Lavie, 1977). The effects of grain temperature and moisture content on the dispersion behaviour of the beetles *C. ferrugineus*, *S. granarius* and *Oryzaephilus Surinamensis* (Linnaeus) in a grain bulk have been the subject of laboratory studies by Surtees (1965). He suggested that random movement causes disturbance within the population and leads to dispersion, while aggregation occurs where individual movement is most restricted in response to environmental factors and where disturbance is consequently at a minimum. He found that *O. surinamensis* accumulated in the warmer, damper parts of an experimental grain bulk while *S. granarius* tended to move towards the top and sides of the bulk. More recently, *C. ferrugineus* adults have been shown to move into and remain in the warmest areas of grain-filled cylinders with temperature gradients in the range 20–42°C (Flinn and Hagstrum, 1998). The presence of other species usually slows down the increase in numbers of most species (Keever, 1983). When adults of *T. castaneum* are jolted or lose contact with the substrate, predator defence death-feigning behaviour, also known as thanatosis, occurs with the beetle remaining

motionless for up to 30 s (Prohammer and Wade, 1981). Dense populations of species such as *T. castaneum* release benzoquinones onto the grain, causing the beetles to migrate from the crowded area (Faustini and Burkholder, 1987). Disturbance, whether due to other insects, movement of the grain or grain aeration, often results in upward movement in stored product beetles (Cogan, 1990). Collection and isolation of volatiles released by insects during upward mass migrations may yield new semiochemicals with potential for use as attractants or repellents. Insects release chemicals in a defensive response to attack by their natural enemies such as parasitoid wasps (Quicke, 1997) and predators (Bradshaw, 1985). In some insects, for example aphids, these chemicals are known to act as alarm pheromones (Nault and Phelan, 1984). Additionally, some predators themselves produce behaviour-modifying chemicals such as the terpenes secreted by the anthocorid bug *Xylocoris flavipes* (Reuter), a predator of many storage insects including *T. castaneum* and *O. surinamensis* (Press *et al.*, 1975; Arbogast, 1976; Phillips *et al.*, 1995). Although little information has been published for stored grain pests, it may be worth considering the collection of volatiles produced during interactions between storage beetles and their predators and parasitoids. The volatiles could be isolated and then tested for their repellency or attractivity to storage pests and potential as grain protectants. Stored grain beetles typically move away from bright light and tend to be more active at dusk (Richards, 1951; Smereka and Hodson, 1959; Surtees, 1963). *S. granarius* is negatively phototaxic, apart from a

short period a few days after emergence when it is positively phototactic (Stein, 1994). Cryptic species such as *C. ferrugineus* prefer to hide away from the light, typically seeking out refuges providing close physical contact with the insect's body on all sides (Cox *et al.*, 1989; Cox and Parish, 1991). This preference can affect the efficacy of traps; for example, commercial sticky trap catches of *T. castaneum* were found to be lower when food and shelter were available elsewhere (Stejskal, 1995). Refuge-seeking behaviour in laboratory arenas and behaviour in relation to pitfall traps has been studied in some species using time-lapse video recordings and marking techniques (Obeng-Ofori, 1993; Wakefield, 1995). Laboratory tests demonstrated the presence of a circadian rhythm influencing the foraging behaviour of *O. surinamensis* in light-dark cycles; adults placed in an arena containing food and an empty refuge showed a cyclic pattern of activity which was reduced when food was placed inside the refuge (Bell and Kerlake, 1986; Bell, 1991). *Thyphaea stercorea* L. and *Ahasverus advena* L. adults are attracted to ultra-violet light (Stolzenberg and Wohlgemuth, 1992). Movement and distribution within grain bulks are also affected by the aggregation and other pheromones produced by beetles, as well as possible oviposition deterrents in *S. granarius* which reduces egg density on individual grains (Stubbs and Abood, 1983; Cox *et al.*, 2000). Aggregations of beetles within the grain bulk can lead to local temperature increases when insect activity produces metabolic heat faster than the heat can escape. The development of these "hot spots" may

attract more beetles which in turn causes further grain deterioration by moisture migration and mould growth (Howe, 1962; Freeman, 1974). Collection of volatiles from these “hot spots” may be worth considering as a way of finding potential novel attractants for beetles. If the grain “hot spots” are not treated the higher temperatures accelerate insect activity until temperatures lethal to many species are reached, at which point the adults will move away to cooler parts of the bulk (Oxley and Howe, 1944). Lethal temperatures vary considerably and depend on factors such as species, stage of development, acclimation and relative humidity. Some storage species will die eventually at temperatures a little above 35°C, while at 55°C death will occur within a few minutes (Fields, 1992).

Dietary requirements. Much insect behaviour is directed towards the search for, selection and utilisation of suitable food materials that meet the dietary requirements of each particular species (Slansky and Rodriguez, 1987). Early studies on the dietary requirements of storage beetles showed that they are similar to those of other phytophagous insects except that they have adapted to develop and reproduce on comparatively dry foods, aided by their ability to utilise their own metabolic water which is a by-product of the breakdown of fats and carbohydrates (Fraenkel and Blewett, 1944). However, feeding on food with increased moisture content generally leads to faster developmental rates and increased body size (Baker and Loschiavo, 1987). The passive sorption of water vapour from ambient air has

also been shown to be an important source of body water in storage beetles such as the rice weevil, *Sitophilus oryzae* (L.) (Arlian, 1978; Arlian and Veselica, 1979). Most storage beetles, except those that develop entirely within grains, are also characterised by being able to develop and reproduce on a wide range of stored foodstuffs, both whole and processed (Sinha, 1969; LeCato and McCray, 1973; Loschiavo, 1976; Levinson and Levinson, 1978). When the internal grain feeder, *S. granarius*, was tested on five types of whole grain, developmental period from egg to adult were shortest on a diet of rice and longest on maize, while progeny production was highest on barley and lowest on oats (Schwartz and Burkholder, 1991). The proportions by weight of the main nutrient classes (amino acids, carbohydrates, fatty acids, minerals, vitamins and sterol) in synthetic diets that support good larval growth have been determined for *O. surinamensis* (Fraenkel and Blewett, 1943; Davis, 1973; Singh, 1977). A summary of data on the chemical and nutritional composition of cereal grains has been provided by Pomeranz (1978). In general, assimilation efficiencies for storage beetles are higher than those of leaf-feeding species while relative growth rates and growth efficiencies reflect the quality of food consumed (Slansky and Scriber, 1985). For example, maize contains lower levels of essential amino acids than other cereals (Shellenberger, 1971); *S. oryzae*, is more efficient in utilising wheat as a food and has a higher capacity for population increase than *S. granarius* (Birch, 1953, Baker, 1974; Evans, 1977). One reason for this appears to be the

higher amylase enzyme levels present in the midgut of *S. oryzae* compared to *S. granarius*. As well as its major role in the breakdown of starch, this enzyme may act as a detoxifying mechanism for the alpha-amylase inhibitors present in wheat (Baker, 1986). Relative growth rates, food utilisation efficiencies and energy budgets (relationship between energy utilisation and innate capacity for increase) on cereals have been calculated for *O. surinamensis*, *C. ferrugineus* and *S. granarius* (Baker and Mabie, 1973; Campbell et al., 1976; Campbell and Sinha, 1978; White and Sinha, 1981). Some attempts have been made to study the resistance of different varieties of wheat to attack by storage beetles, including *O. surinamensis* (Sinha, 1971). Differences in susceptibility have been attributed to chemical factors such as sterol and alpha-amylase inhibitor levels or to physical properties including thickness of the bran layer and hardness of the endosperm (Sarin and Sharma, 1979; Yetter *et al.*, 1979; Sudhakar and Pandey, 1982; Gatehouse *et al.*, 1986). However, seed coat puncture resistance does not appear to differ between wheat varieties (Sinha and Voisey, 1978). When given a choice, *S. granarius* were found to show a preference for laying eggs in varieties of wheat with large grains rather than in varieties with small ones, particularly when insect densities were low; as insect density increased, the number of eggs laid on each grain also increased, thereby reducing the prospects of larval survival (Ewer, 1945; Stejskal and Kucerova, 1996). The development and productivity of *O. surinamensis* on nine winter wheat varieties have been examined recently at

the Central Science Laboratory, York, UK (CSL) (Fleming, pers. comm.). Larval mortality was loosely associated with bran thickness and germ size. However, varietal differences were considered to be of limited practical significance as far as resistance to attack by *O. surinamensis* was concerned. The germ of wheat grains is often eaten by storage beetles, such as *O. surinamensis*, in preference to the endosperm (Surtees, 1964; Hurlock, 1967). This in part may be due to the germ area being the softest part of the grain (Sinha and Voisey, 1978). Cereal products with high germ content are also more attractive to grain beetles (Loschiavo, 1959).

Food volatiles. It is likely that most phytophagous insects, including grain beetles, use chemical cues to find sources of suitable food. Most plant-derived foods release hundreds of volatile chemicals, and many of those from grains have been identified as short-chain alcohols, aldehydes, fatty acids, ketones, esters, terpenes and heterocyclic compounds (Maga, 1978; Maarse, 1991; Seitz and Sauer, 1992). Food volatiles may act as attractants in their own right. For example, wheat germ contains at least 15% lipid, up to 60% of which is triglyceride (Pomeranz, 1978). Unsaturated triglycerides are particularly attractive and elicit aggregation responses in species such as *S. granarius* (Nawrot and Czaplicki, 1982). In laboratory tests using two-choice open arenas, the oat triglycerides and the fatty acids, mainly palmitic, oleic and linoleic acids, stimulated aggregation in *O. surinamensis*, probably by acting as arrestants and feeding stimulants (Freedman et al., 1982; Mikolajczak et

al., 1983); the most attractive volatiles isolated were (E)-2-nonenal and (E,E) -2,4-nonadienal (Mikolajczak et al., 1984). The attractancy of oat volatiles was increased at low insect densities and when beetles were more than 2 months old (Pierce et al., 1983). Adults of *O. surinamensis* were trapped successfully in laboratory assays using traps baited with a mixture of rolled oats extract, wheat germ oil and white mineral oil (Barak and Burkholder, 1985). Some fatty acids can induce positive gustatory responses and aggregation behaviour in storage beetles whilst others can act as repellents (Levinson and Levinson, 1978). Valeric acid had the lowest lower threshold for attraction at 0.1 mg for *O. surinamensis* (Pierce et al., 1990). For the same species, the most attractive volatile isolated from pods of the carob tree, *Ceratonia siliqua* L. (Leguminaceae), was hexanoic acid (Stubbs et al., 1985). The use of carob pod pieces and extracts has been shown to enhance trap catch for a range of storage beetles, including *O. surinamensis*, *S. granarius*, *S. zeamais*, *S. oryzae*, *C. ferrugineus* and *A. advena* (Pinniger et al., 1984; Cogan and Wakefield, 1987; Obeng-Ofori, 1993b; Wakefield, 1997; Wakefield, 1999). In laboratory tests with *O. surinamensis*, adult age but not sex affected the antennal and behavioural responses to carob extract, and attraction increased with age up to about 3 weeks post-emergence (White, 1989). Hexanoic acid isolated from rice and corn has also been reported to be the main attractant for the maize weevil, *S. zeamais* Mots., (Yamamoto et al., 1976). Other nutrients found in grain, such as maltose and amylopectin, also act as feeding

stimulants for storage beetles, including *S. oryzae*, while benzaldehyde from brewer's yeast is attractive to *Oryzaephilus* spp. (Loschiavo, 1965; Chippendale, 1972; Pierce *et al.*, 1981). In some beetles, feeding may also be dependent on ovipositional responses. For example, stored wheat contains higher levels of ovipositional and gustatory stimulants for *S. granarius* than does newly harvested wheat; pentane extracts of stored wheat induced longer arrestance than those of fresh grain, with females generally reacting more strongly than males (Kanaujia and Levinson, 1981; Levinson and Kanaujia, 1981). It may be possible to enhance lures by incorporating ovipositional stimulants that will be particularly attractive to females. Food volatiles can also enhance the activity of pheromones released by insects in contact with the food material. In laboratory studies, a combination of the three grain volatiles valeraldehyde, maltol and vanillin with the synthetic pheromone sitophinone (subsequently renamed sitophilure) was shown to be more attractive to *S. oryzae* than either the pheromone alone or the grain volatile mixture, while a combination of a soybean oil and wheat germ based food product with the pheromone 4,8-dimethyldecanal was more attractive to *T. castaneum* than either food alone or pheromone alone (Phillips *et al.*, 1993). Water vapour and carbon dioxide are also produced by stored grain during respiration, and the response of beetles to these gases relative to the other volatiles has not been established (Sinha *et al.*, 1986). However, Surtees (1965) found that in laboratory experiments *O. surinamensis* accumulated in pockets of

damp grain, providing sporulating moulds were not present. Also, in laboratory experiments with arenas containing wicks soaked in water, adults of this species were attracted to areas of high humidity (Stubbs and Griffin, 1983). It may be worth looking again at attraction to free water and areas of high humidity by some storage beetles; lures might be made more attractive by the addition of moisture, perhaps with the incorporation of mould inhibitors or humectants such as glycerol. The situation is further complicated by the gases and volatiles released by micro-organisms associated with the grain (Seitz and Sauer, 1992). For example, *C. ferrugineus* aggregated in large numbers and fed extensively on wheat kernels infected with the fungus *Nigrospora sphaerica* Mason and *Penicillium corymbiferum* Westling (Loschiavo and Sinha, 1966; Dolinski and Loschiavo, 1973). When triglycerides were extracted from *Nigrospora sphaerica* the monounsaturated triglyceride oleodipalmitin elicited the most aggregation activity in the flour beetle, *Tribolium confusum* du Val (Starratt and Loschiavo, 1971, 1972). Short-chain alcohols and ketones known to be produced by fungi were shown to be attractive to *O. surinamensis*, *C. ferrugineus* and *A. advena* in two-choice pitfall olfactometer tests; 3-Methylbutanol and 1-octen-3-ol were particularly attractive at the concentrations tested (Pierce *et al.*, 1991). Although attractants derived from food sources have not been used as widely as species specific pheromones for trapping storage beetles, food volatiles have great potential because some of them have been shown to attract adults and larvae of both sexes of the many different

species found in grain stores and to enhance the activity of pheromones. Whilst they are generally cheaper to produce than pheromones, the main drawback so far has been the lower levels of attractancy of food volatiles compared to species specific pheromones, particularly in the presence of a plentiful food supply. These and other aspects of the use of food attractants for storage beetles have been reviewed by Chambers (1990) and Pinniger (1990).

Multi-species pheromone components. The locomotory behaviour of beetle pests of stored food is associated mainly with finding suitable sexual partners, egg-laying sites and sources of food, together with defensive activities to protect them from adverse environmental conditions and natural enemies. This behaviour is modified by chemicals produced by the beetles themselves, such as oviposition deterrents, sex and aggregation pheromones. Over the last 30 years or so, there has been a steady increase in the efforts to use these semiochemicals to monitor and control stored food beetle pests. The published work on this subject has been reviewed in detail recently by Oehlschlager et al. (1988), Chambers (1990), Burkholder (1990), Levinson and Levinson (1995), Phillips (1994, 1997), Plarre (1998) and Plarre and Vanderwel (1999). Insect pheromones are usually species specific. In general terms, there are two main types of pheromone: sex pheromones usually produced by the female of short-lived species that attract males for mating, and aggregation pheromones usually produced by males of longer-lived species that attract adults of both sexes to a

suitable food source (Burkholder, 1990). Using species specific pheromones for pest detection and control purposes has the disadvantage that a different chemical must be deployed for each pest species present. Little is known about the interaction between pheromones from different species used in the same trap; a slight repellent effect caused by *T. castaneum* pheromone on the pitfall trap catch of the warehouse beetle *Trogoderma variable* (Ballion) has been reported by Dowdy and Mullen (1998), whereas Lindgren et al. (1985) showed that mixing together lures of the pheromones from *C. ferrugineus* and *T. castaneum* did not affect catches of either species. However, in the specialised habitat of the grain store, there are some pheromone components that attract more than one species and these provide a possible alternative to food attractants as a starting point for the development of effective multi-species lures. This is particularly true for males of the pyralid storage moths *Plodia interpunctella* (Hubner) and four *Ephestia* spp that are all attracted to a single chemical, (Z,E)-9,12-tetradecadien-1-yl acetate (Phelan and Baker, 1986). There are also some examples of interspecific cross-attraction amongst stored product beetles in the families Bostrichidae, Curculionidae, Dermestidae, Laemophloeidae, Silvanidae and Tenebrionidae. This cross-attraction may be due to one species being confused by the signals produced by another, usually closely related, species with a similar pheromone system but which fortuitously leads to the location of suitable habitats and food supplies. *A. advena*, *O. surinamensis*, *C. ferrugineus* and *S. granarius* are the only

beetle species commonly associated with stored grain in the northern hemisphere that have been shown to exhibit any cross-species attraction for pheromone components with other storage species. The pheromones of *Cryptolestes* spp other than *C. ferrugineus* have also been identified but so far no further examples of cross-species attraction in the cucujids and silvanids has been confirmed, even though the molecules concerned are closely related chemically (Oehlschlager *et al.*, 1988). Males of *S. granarius* produce an aggregation pheromone, (2S,3R)-3-hydroxy-2-methylpentanoate (Phillips *et al.*, 1989) and a second component not yet identified (Chambers *et al.*, 1996). So far, no definite cross-species attraction has been demonstrated for these compounds. The aggregation pheromone (5R,4S)-5-hydroxy-4-methyl-3-heptanone produced by both *S. oryzae* and *S. zeamais* is to some extent also attractive to *S. granarius* as demonstrated in trapping tests (Walgenbach *et al.*, 1983; Phillips *et al.*, 1985). However, electroantennogram studies failed to show any olfactory perception of this compound by *S. granarius* (Levinson *et al.*, 1990). One possible explanation for this could be that in *Sitophilus* species the antennae are not the main site for receptors. The addition of carob volatiles to the aggregation pheromone has been shown to be more effective than either carob or pheromone alone in attracting all three species of *Sitophilus* (Wakefield, 1997). Further studies to investigate the attractiveness of combinations of food volatiles and aggregation pheromones would appear to be worth considering. Amongst other beetle pests of stored

products, in the Bostrichidae only *Rhyzopertha dominica* (F.) regularly occurs on stored products in northern Europe where it can only survive mild winters in unheated premises (Solomon and Adamson, 1955; Bahr, 1978). Males produce an aggregation pheromone with two components, both of which are attractive to *Prostephanus truncatus* (Horn), even though males of this tropical species produce their own aggregation pheromone with two components, 1-methylethyl (E)-2-methyl-2-pentenoate and 1-methylethyl (E,E)-2,4-dimethyl-2,4-heptadienoate (Hodges *et al.*, 1983, 1984; Cork *et al.*, 1991). There have been suggestions recently that the *R. dominica* pheromone may be attractive to some *Dinoderus* spp which are bostrichid wood-borers and pests of dried root crops such as cassava, but this has yet to be confirmed (Borgemeister *et al.*, 1999). In the Dermestidae, cross-species attraction of males to female extracts occurs in the two carpet beetles, *Attagenus megatoma* (F.) and *Attagenus schaefferi* Beal (Barak and Burkholder, 1978). Similarly, males of the *Trogoderma* species *T. granarium* Everts, *T. inclusum* LeConte and *T. variabile* exhibit cross-species attraction to female extracts, all three species responding to the common pheromone component (Z)-14-methyl-8-hexadecen-1-ol (Levinson and Levinson, 1977). Although these beetles are considered minor pests in Britain and northern Europe, *T. granarium* can cause serious damage in warmer climates (Burges, 1962; Greenblatt *et al.*, 1977; Peacock, 1993). Males of three species of flour beetle in the Tenebrionidae produce the compound (4R, 8R)- 4,8-dimethyl-decanal which is

attractive to both sexes of all three species, namely *Tribolium castaneum*, *T. confusum* and *T. freemani* Hinton (Suzuki et al., 1987). These beetles are serious cosmopolitan pests but in northern Europe they are more usually associated with imported products or heated premises such as flour mills (Aitken, 1975; Bahr, 1978; Hill, 1978). Also, there may be examples of multi-species attraction in the Bruchidae, a family containing serious cosmopolitan pests of stored dried pulses. Cork et al. (1991) identified (2Z)-3-methyl-2-heptenoic acid as a pheromone component produced by female *Callosobruchus analis* (F.). However, another group of workers demonstrated that this same compound was one of a number of carboxylic acids produced by female *Callosobruchus maculatus* (F.) but they failed to find any evidence for cross-species attraction to *C. analis* (Phillips et al., 1996).

Interaction between insects and plant volatiles. Animal life cannot exist without green plants and wherever plants grow, insects are also found. About twenty-five years ago, volatile plant chemicals were suggested to be important in interactions with insects (Price *et al.*, 1980). Since then, the roles of odours in trophic level interactions have been extensively studied and it has been found that very specific interactions between plants and insects can be mediated by plant volatiles (Dicke, van Poecke and de Boer, 2003; Turlings and Wäckers, 2004). Plants display a multitude of different properties that characterize them, not only hundreds of different chemicals but also e.g. form, colour

and texture (Dethier, 1982; Raguso, 2004). Recognition of plants by insects depends not only on these characters but also on the detection and coding capabilities of the insect (Dethier, 1982). The process of finding and choosing a host plant for oviposition or feeding performed by herbivorous insects can be divided into different, but overlapping, stages: 1) orientation, 2) landing and 3) assessment (Renwick, 1989; Schoonhoven, Jermy and van Loon, 1998). Regarding larvae behaviour, in few studies was described their process of finding and choosing. Clearly most, if not all, plant- and host-seeking insects use multiple sensory modalities throughout this process (Prokopy, 1986). Olfactory and visual stimuli are most important in the first steps of host location, orientation and landing, while mechano-sensory and gustatory stimuli are considered to be most important during the final assessment (Prokopy and Owens, 1983; Schoonhoven, Jermy and van Loon, 1998). Visual and olfactory stimuli are probably also involved during assessment on the plant (Bernays and Chapman, 1994). Animal behaviour is dependent not only on stimuli perceived from the environment but also on internal factors. In insects, factors such as hunger state, age and mating status influence the behavioural decisions. The response to perceived stimuli can be described as a sum of positive and negative external stimuli and internal factors processed by the central nervous system of the insect (Dethier, 1982; Miller and Strickler, 1984). Most food sources release complex mixtures of volatiles (Knudsen, Tollsten and Bergström, 1993). Food scents alone or in combination with

visual cues can attract insect, guide the insect to the resource and elicit feeding behaviour (Blight *et al.*, 1997; Raguso, 2004). Many herbivorous insects are selective feeders, adapted to feed on only a limited number of plant families, and therefore need good abilities to discriminate between plant species. Furthermore, it can be important for insects to assess age, health and infestation status when searching for a suitable host plant for themselves or their offspring. For example, herbivorous insects specialised on Brassicaceae are attracted to isothiocyanates (Bartlett, 1996; Smart and Blight, 2000) and the onion fly is attracted to dipropylid sulphide (Harris, Keller and Miller, 1987). Most examples of attraction to plant volatiles have been found with monophagous or oligophagous insects, but examples of attraction to plant volatiles among generalist insects, for example among moths and beetles, have also been found (Bernays and Chapman, 1994). Attraction to induced plant volatiles has been found in some Coleoptera (Harari, Ben-Yakir and Rosen, 1994; Loughrin, Potter and Hamilton-Kemp, 1995; Bolter *et al.*, 1997). In some of these, the plant volatiles are probably used as aggregation cues.

Insect olfaction. The antennae are the primary olfactory organs, but odour receptors can also be found on the mouthparts of several insects (Keil, 1999). Insect antennae have in many cases evolved into sophisticated shapes, such as feather-like and clubshaped structures, to maximize the area carrying odour detecting organs (Keil, 1999). Olfactory receptor neurons (ORNs) are housed within small sensilla.

Apart from the ORNs, the olfactory sensilla consist of a number of auxillary (or enveloping) cells that have supportive functions and are involved in development of the sensilla during ontogeny. Depending on the cuticular structure, the sensilla are classified into different types (Hallberg and Hansson, 1999). The most common are (1) trichoid, long hair-like sensilla, (2) basiconic sensilla, also hairlike but normally shorter and thicker than the trichoid, (3) placoid sensilla, platelike and (4) coeloconic sensilla, which are short peg-like structures situated in a pit (Hallberg & Hansson, 1999). Odour molecules enter the sensilla through pores in the wall. The odour molecules are transported through the sensillum lymph by water-soluble odourant binding proteins (OBPs). The selectivity of insect odour detection is thought to depend partly on selectivity in these odour-OBP bindings, but mainly on specificity of receptor sites of the odour receptor neuron (Stengl *et al.*, 1999). The mechanisms involved in the transformation of chemical signals to electrical signals are only partly understood (Stengl *et al.*, 1999). Briefly, the odour-OBP complex interacts with receptor proteins in the dendritic membrane of the odour receptor neuron. These interactions give rise to a change in receptor potential and if the potential is above a threshold, action potentials are triggered in the neuron (Todd and Baker, 1999). After receptor activation the odour-OBP complex is deactivated and dissolved, followed by degradation of the odour molecule by enzymes in the lymph (Stengl *et al.*, 1999). The action potentials of different odour receptor neurons have different amplitudes

and waveforms, which often allow discrimination between recorded neurons within one sensillum (Todd and Baker, 1999). However, it is the frequency of action potentials rather than their size and shape that reflects the strength of the response and affects the behaviour. These action potentials are transmitted in the olfactory axons of the antennal nerves ending in the antennal lobe, where the olfactory signal is processed before being transferred to higher integrative centres of the brain (Todd and Baker, 1999).

Electroantennography. Electroantennography (EAG) is a bioassay widely used in experimental entomology for the detection of volatiles perceived by the antennal olfactory apparatus of insects. The method is based on the discovery by Schneider (1957), who recorded small voltage fluctuations between the tip and base of an insect antenna during stimulation with pheromones. Although the precise mechanism behind the EAG signal is not known, it is generally assumed that the measured voltage fluctuation is caused by electrical depolarisations of many olfactory neurons in the insects antenna. The amplitude of an EAG response increases with increasing stimulus concentrations until a saturation level is reached. The amplitude is further dependent on the nature of the stimulus, the insect species, its sex, and many less well defined factors. The EAG method can be used for many purposes like screening biologically active compounds, purification of extracts,

identification of active fractions, selection of active synthetic compounds, concentration measurements in the field, and as a detector in gas chromatography. The quality of EAG signals is dependent on many factors, which are not always well recognised, and the large variety of insects demands a flexible attitude and a sense for improvisation of the operator. Although the basic principles are always the same, EAG recording methods may differ in many details. The large variety of insects and the differences in antennal morphology make adaptations in the recording technique necessary and require an inventive attitude.

***Tenebrio molitor* L. (Coleoptera: Tenebrionidae)**

The yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is an important cosmopolitan stored-products pest, with infestations usually originating in poorly stored animal feed and stored grain (Sinha and Watters, 1985). Moreover, the yellow mealworm beetle is one of the most common species utilized as feed and food.

Biology. The life cycle of *T. molitor* is of variable length, from 280 to 630 days at 27-22°C. Larvae hatch after 10-12 days (at 18-20°C) and become mature after a variable number of instars, typically after 3-4 months (at ambient temperature) but the larva stage can last up to 18 months. *T. molitor* displays plasticity in the number of instars needed for its development (Esperk *et al.* 2007). Instars can vary from 8 to more than 20 (Cotton and St. George 1929)

depending on several factors including temperature (Ludwig 1956), humidity (Murray 1968, Urs and Hopkins 1973), photoperiod (Tyshchenko and Sheyk Ba 1986), oxygen concentration (Loudon 1988, Greenberg and Ar 1996), population density (Connat *et al.*, 1991), parental age (Ludwig 1956, Ludwig and Flore 1960), and food quality (Stellwaag-Kittler 1954). The number of instars in *T. molitor* increases in response to adverse conditions (Esperk *et al.*, 2007). Larval development time is reported to be affected by environmental factors in a similar way as the number of instars (Ludwig 1956, Murray 1968, Urs and Hopkins 1973, Tyshchenko and Sheyk Ba 1986, Loudon 1988). At high density, larvae have been observed to experience extra larval moults (Weaver and Macfarlane 1990; Connat *et al.*, 1991; Kotaki and Fujii 1995), increased mortality and cannibalism (Savvidou and Bell 1994). *T. molitor* are highly cannibalistic, a behavior that occurs especially following larval moults and during pupation (Tschinkel and Willson 1971, Weaver & McFarlane 1990 and Savvidou and Bell 1994). It has also been reported that nutrition has an effect on instar number and total development time (Stellwaag-Kittler 1954), but the effects of different diets on larval growth performances in *T. molitor* have not been statistically determined or specifically addressed by previous researches. Also, information on development time and stadia length are lacking. The mature larva is of a light yellow-brown colour, 20 to 32 mm long, and weighs 130 to 160 mg. Larvae firstly eat the germs of stored grains and can feed on a wide variety of plant products such as ground

grains, flour, tobacco and foodstuffs. Larvae are very voracious and highly resistant to low temperature; they can remain alive for 80 days at -5°C. The pupal stage lasts 7-9 days at 25°C and up to 20 days at lower temperatures. The adult lives for 2 to 3 months. They are black or dark brown, they are elongate, reaching 16 mm long. Females can lay up to 600 eggs during its lifetime. Male *T. molitor* beetles prefer mature to immature females. Infact, polygynandrous species, mating typically occurs after a period of maturation during which the oocytes grow (Ridley 1989). Thus, immature females tend to have underdeveloped oocytes, which may curtail their fecundity. Newly emerged female mealworm beetles do not reach sexual maturity until approximately 5 days posteclosion (Valentine 1931; Happ and Wheeler 1969). Although immature females are capable of mating, they seem to be less receptive than mature females (Drnevich *et al.*, 2001; Drnevich 2003).

Chemical ecology. The yellow mealworm beetle produces several different types of pheromones, including both primer pheromones that stimulate reproductive maturation (Happ *et al.*, 1970), and releaser pheromones that mediate aggregation (Weaver *et al.*, 1989), mating (reviewed in Plarre and Vanderwel, 1999; Bryning *et al.*, 2005), and epideictic (Weaver *et al.*, 1989; Weaver *et al.*, 1990) behavior. One of the female-produced sex pheromones has been identified as (4*R*)-(+)-4-methyl-1-nonanol (MNol) (Tanaka *et al.*, 1986 ; Tanaka *et al.*, 1989). There are several scientific articles regarding the role and regulation of the

female-produced sex pheromone in the yellow mealworm beetle (reviewed in Plarre and Vanderwel, 1999). In most of these studies pheromone production was quantified by bioassay with unambiguous interpretation of the results. Some studies have proposed even the existence of a male-produced volatile antiaphrodisiac that would act by deterring other mealworm males from attempting copulation with a recently mated female (Happ 1969). Furthermore, the proposed male-produced antiaphrodisiac has not yet been isolated or identified, and recent evidence argues against its existence (Seybold and Vanderwel 2003).

Rearing. Mealworms are omnivorous and can eat all kinds of plant material as well as animal products such as meat and feathers (Ramos-Elorduy *et al.*, 2002). They are typically fed on cereal bran or flour (wheat, oats, maize) supplemented with fresh fruits and vegetables (carrots, potatoes, lettuce) for moisture together with protein sources such as soybean flour, skimmed milk powder or yeast (Aguilar-Miranda *et al.*, 2002; Hardouin *et al.* 2003). Mealworms are a high quality feed. They contain large amounts of protein (45-60% DM) and fat (30-45% DM). Fresh larvae contain about 60% water. They are relatively poor in ash (less than 5% DM), and like other insects they have a low calcium content and a very low Ca:P ratio. The diet for their rearing, should be balanced to contain about 20% protein (DM basis) (Ramos-Elorduy *et al.*, 2002). It is preferable to provide them with a source of water for better productivity and to prevent cannibalism. Relative humidity

is linked positively with fertility and adult activity. It is necessary to monitor fresh feeds as they may turn mouldy (Hardouin *et al.*, 2003). Moreover *T. molitor* are easy to breed. For these reasons, they are produced industrially as feed for pets and zoo animals, including birds, reptiles, small mammals, batrachians and fish. They are usually fed live, but they are also sold canned, dried, or in powder form (Aguilar-Miranda *et al.*, 2002; Hardouin *et al.*, 2003; Veldkamp *et al.*, 2012). Using *T. molitor* as feed and food has also originated some safety concerns, especially related to microbiological and allergenic effects. Other aspects have also been considered for the optimization of rearing *T. molitor*, such as water supply, which has been found to have a significant importance on the growth rate (Hansen *et al.*, 2004), heat emission (Hansen *et al.*, 2004), egg harvesting (Ghaly and Alkoik, 2009) and the design of the specific rearing units. (Comparat *et al.*, 2016a, b). All of these studies demonstrate that rearing *T. molitor* should be considered as a whole system, and it is very difficult to optimize only one aspect (e.g. diet) without taking into consideration these other factors.

Nutritional needs. Early studies on diets for *T. molitor* were mainly focused on the design of the most suitable artificial medium and understanding the nutritional needs of this species; whereas more recent studies have focused on the utilisation of by-products and the overall lowering of the diets' costs. One of the most significant studies on the *T. molitor* nutritional needs was performed by Fraenkel in

1950. It was found that these beetles require 80-85% carbohydrates in their diet, the source being indifferently starch or glucose; the protein supply could be made by different sources as casein, lactalbumin, etc., whereas some others were found deleterious (zein, gliadin, etc.). Some important supplements were also identified, such as cholesterol at 1% and vitamin B. Those observations were then confirmed (Martin *et al.*, 1976) and additional discoveries, especially on the calcium intake (Hunt *et al.*, 2001; Klasing *et al.*, 2000) were added.

T. molitor as feed. Nutritional needs of monogastric species cannot exclude high quality proteins in sufficient amounts, with a high digestibility (Barrows *et al.*, 2008). Fishmeal and soymeal are the two types of proteins sources mostly used. The soymeal is the best source of proteins of plant origin; the fishmeal, on its hand, has a composition that suits more the nutritional needs of animal species. Nevertheless, the use of these two types of animal feed causes severe environmental problems (Sánchez-Muros *et al.*, 2014): the intensive cultivation of soy involves deforestation (Carvalho, 1999; Osava, 1999), excessive water consumption (Steinfeld *et al.*, 2006), the use of pesticides and fertilizers (Carvalho, 1999) and the development of new transgenic varieties (Garcia and Altieri, 2005). Fishmeal production is qualitatively and quantitatively variable, depending on the fishing practice (FAO, Animal Feed Resources Information System). Moreover, the deterioration of the marine environment and the fishing stripping have led

to a reduction of the production and the increase of the price. This situation has stressed the importance of alternative and renewable protein sources (Sánchez-Muros *et al.*, 2014). According to the International Feed Industry Federation (IFIF), the global feed production was of 720 million tons in 2010. Insects could integrate the conventional sources like soy, cereals and fishmeal. The species with the highest potential for a large scale production are the black soldier fly (*Hermetia illucens* L.) , the housefly (*Musca domestica*) and the mealworm (*T. molitor*). *T. molitor* dried larvae and meal have been tested as feed for different monogastric animals such as pigs, poultry and fishes. Dried mealworms comprised up to 6% in the diet of weaning pigs (Jin *et al.*, 2016). The supplementation of mealworms was found to linearly improve body weight, the average daily gain and the average daily feed intake. Also, as mealworms proportion was increased, nitrogen retention and digestibility of dry matter as well as crude protein were linearly increased, thus showing a clear beneficial effect of feeding weaning pigs on a diet composed up to 6% dried *T. molitor*. Several trials were performed on feeding poultry *T. molitor* meal. It was shown that *T. molitor* larvae (TML) meal improved the food conversion ratio compared to soybean meal and the albumin to globulin ratio, suggesting a higher immune response in broilers fed on TML meal (Bovera *et al.*, 2015). Another study (Klasing *et al.*, 2000) showed that *T. molitor* enriched in calcium contributes to the bone mineralisation of growing chicks, with the calcium bioavailability being measured at a

level of 76% in this study. Different species of fish have also been tested with the TML meal as main feed. Rainbow trout were shown to perform with a 30% weight gain and growth performance (Hubert, 2016); different results were observed for catfishes, however no precise meal preparation description was given (Roncarati *et al.*, 2015). *T. molitor* has been proposed as a source of protein for catfish (Ng *et al.* 2001) and broiler chicken production (Ramos-Elorduy *et al.* 2003).

T. molitor as food. For some people, insects are considered delicacies, for others (the majority), they are disgusting. Apart from the personal opinions, insects are assuming an important role in the human nutrition due to their accessibility, composition (they are rich of proteins, minerals, vitamins and chitin, now considered a fibre) and taste (Andersen *et al.*, 1995, López *et al.*, 1999). In the Western countries, insects are well known for the production of wax, silk and for their use as food colorants (scarlet red produced from Lacciferidae and *Margarodes polonicus* Cockerell) and pharmaceutical products like propolis, royal jelly and bees' poison (Schabel, 2010). Starting from the 1st January 2018, the Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, has entered into force. With Novel Food, it is intended any food that was not used for human consumption to a significant degree within the Union before the 15th May 1997. The new Regulation improves conditions so that food businesses can easily bring

new and innovative foods to the EU market, while maintaining a high level of food safety for European consumers. The regulation brought different features and improvement to the previous one, like the expansion of the categories of novel foods, the establishment of a Union list of authorised novel foods, the simplification of the authorisation procedure and the promotion of the scientific innovation. Insects are now considered in the new categories of novel foods. Numerous studies and programs have promoted the utilisation of edible insects for human nutrition; the short list of suitable insects usually includes *T. molitor* along with grasshoppers, crickets and few others. However, in order to introduce such an uncommon food source in Western countries, several regulations and public concerns should be well documented. Safety is one of the most important concerns, and toxicology, allergenic properties and microbial load have been extensively studied. The evaluation of toxicity of freeze dried TML was studied for different periods of time, 28 (Han *et al.*, 2014) and 90 (Han *et al.*, 2016) days, and evaluated on Sprague-Dawley rats. These studies have shown that intake of freeze-dried TML for 28 or 90 days had no adverse effects up to the maximum tested dose, i.e. 3,000 mg/kg/day intake. Therefore, TML were considered to be non-genotoxic and the no observed adverse effect level was determined to be 3,000 mg/kg/day. Regarding the allergenic aspects, the first study reported on the evaluation of allergies induced by ingestion and inhalation of *T. molitor* was performed on volunteers from the New York Entomological Society

(Freye *et al.*, 1996). It showed that allergic reaction was possible for patients already with other allergies to trees, dust, mites or cats. More recent studies, made with immunoglobulin E have confirmed the increased sensitivity of patients with known allergies to shellfishes or house dust mites (Verhoeckx *et al.*, 2013, 2014). The most important allergen in aqueous phase was identified to be arginine kinase, whereas tropomyosin was found in the solid part (Verhoeckx *et al.*, 2013). Moreover, it was shown that, even if thermal processing changes the solubility of *T. molitor* allergenic substances (Broekman *et al.*, 2015), it does not affect their allergenic potential (Broekman *et al.*, 2015, Van Broekhoven *et al.*, 2016). For some applications, it might be worthwhile to separate *T. molitor* proteins from oil, as oil becomes another product which requires proper valorisation. Studies focusing on the inner composition and possible utilisation of oil for food and feed purposes have found that it was mainly composed by unsaturated fatty acids, with a very interesting omega 3 to omega 6 ratio, moreover it exhibits a superior oxidative stability (Jeon *et al.*, 2016). All of these aspects makes this oil a valuable source of fatty acids for human and animal nutrition. Also, the potential of *T. molitor* as biodiesel source has been evaluated. It was found that this oil was of great value for the biodiesel industry, exhibiting several characteristics such as a cetane number (the cetane number or cetane rating is an indicator of the combustion speed of diesel fuel and compression needed for ignition) of 58, which is reportedly much better quality than, for example, the rapeseed biodiesel with the

cetane number of 45, and satisfying the requirements of being at least 51 (Zheng *et al.*, 2013), according to the EN 14214 norm that describes the requirements and test methods for FAME - the most common type of biodiesel.

Bioconversion. The process of bioconversion through *T. molitor* is a very attractive option, considering that it represents a potential valuable solution to two problems: food waste management on the one hand and, on the other, the rising global demand for food and feed. Mealworms have the ability to recycle waste and food byproducts of low quality into high-quality feed rich in energy, protein and fat in a relatively short time. More recent studies focused on the utilisation of by-products (by-products are goods and materials resulting from a manufacturing or extraction process, the primary aim of which is not the production of that item itself; the intent is not directly to discard the side stream but try to exploit or market on terms which are advantageous to it, in a subsequent process, without any further processing) such as brewer's grain, bread/cookie baking, potato processing, biodiesel production (Van Broekhoven *et al.*, 2015), pulp floor (Alves *et al.*, 2016) or even polystyrene (Yang *et al.*, 2015). They have shown these by-products to be feeding sources by highly adaptable *T. molitor*. The only substrate inducing significant change in growth of *T. molitor* was found to be T-2 toxin, known for its inhibitor capacity for the synthesis of proteins (Davis and Shieffer, 1982). The impact of different diets on the composition of *T. molitor* was also evaluated and the impact

on the protein content and quality has been found to be minimal. *T. molitor* was reported to possess a range of 45.1% on wet mass basis to 65% on dry mass basis (Alves *et al.*, 2016; Ghaly and Alkoik, 2009; Jones *et al.*, 1972; Martin *et al.*, 1976; Van Broekhoven *et al.*, 2015) of proteins; the diets have, however, significant influence on fat content, 18,9% (wet mass basis) to 50% (dry mass basis), and composition (Alves *et al.*, 2016; Ghaly and Alkoik, 2009; Jones *et al.*, 1972; Martin *et al.*, 1976; Van Broekhoven *et al.*, 2015). It remains very difficult to explain the whole metabolism of their transformation and to predict the fat content and composition of *T. molitor* based on diet substrates (Van Broekhoven *et al.*, 2015).

The circular economy view and the by-products used

With the growing understanding of the role of bio-economy within Europe and the need to apply circularity in the use of natural resources, recognizing that the waste can represent a new starting material for other industrial processes is now a must. In this sense, as suggested by the EU community, a circular economy should be applied improving economic benefits, contributing to innovation and growth, encouraging sustainability and competitiveness in the long term. According to the concept of circular economy, insects represent good candidates as food and feed since they can be cultured through environmental-friendly, cost-effective farming processes, on by-products/wastes (Henry *et al.*, 2015; Merida *et al.*, 2018). Moreover, it is estimated that more than one third of the global food supply is lost or

wasted between the farm and the fork, roughly 1.3 billion tons per year. There is a limited availability and heterogeneity of data, so it is quite difficult to precisely evaluate the extent of total food losses and waste. Food is wasted through the entire value chain, from initial agricultural production down to final household consumption. *Food losses* refer to a decrease in food quantity or quality in the early stages of the Food Supply Chain (FSC), reducing the amount of food suitable for the human consumption. *Food wastes*, on the other hand, often refer to later stages of the FSC, such as retail and households. While the firsts are thereby often connected to post-harvest activities with lacking systems or infrastructural capacities and they are not generally intentional, the latter are caused by human behaviour and they are, most of the time, intentional (Parfitt *et al.*, 2010). It is possible to work on technologies and scientific knowledge to try to limit the food losses, while for the food wastes is more a matter of behavioural education and information to the consumer. Food loss/waste levels increase with increasing regional average food consumption level, GDP per capita and urbanization level (Gustafsson *et al.*, 2013). In medium and high-income countries, like Europe and North America, food is to a greater extent wasted, meaning that is thrown away even if it is still suitable for human consumption. In low-income countries, like North Africa and South Asia, food is mainly lost during the early and middle stages of the food supply chain; much less food is wasted at the consumer level. The extent of this

wastefulness has several impacts: production of greenhouse gas emissions; soil degradation; waste of water resources; energy consumption. Only in Italy, fruits and vegetables thrown away at points of sale involve the consumption of over 73 million cubic meters of water: to understand, 36.5 billion two liter bottles. Wasting 1.3 billion tons of food causes huge economic losses and a lot of needless hunger, but there are climate environmental issues deeply connected to food waste, according to a report from the FAO. In *Food Wastage Footprint: Impacts on Natural Resources*, FAO analyses the impacts of global food wastage from an environmental perspective, looking specifically at its consequences for the climate, water and land use, and biodiversity. The carbon footprint of food produced and not eaten is estimated at 3.3 billion tons of greenhouse gases (GHG), making food wastage the third top GHG emitter after the U.S. and China. Globally, the blue water footprint (i.e. the consumption of surface and groundwater resources) of food wastage is about 250 km³, which is equivalent to the annual water discharge of the Volga river, or three times the volume of Lake Geneva. Produced but uneaten food occupies almost 1.4 billion hectares of land; representing nearly 30 percent of the world's agricultural land area. Fifty-four percent of the world's food wastage occurs "upstream" during production, post-harvest handling and storage. About 46% of it happens "downstream," at the processing, distribution and consumption stages. Beyond its environmental impacts, the direct economic consequences of the generation of food wastes to producers run about

\$750 billion annually. According to Galanakis, wastes and by-products from various sectors of the food industry can be divided into two main groups (plant and animal origin) and seven subcategories (cereals; roots and tubers; legumes and oil crops; fruits and vegetables; meat products; fish and seafood; dairy products). In particular for the cereals, nine main species are harvested all over the world (wheat, rye, barley, oat, rice, millet, maize, sorghum and triticale). Wheat is the dominant crop supply in medium- and high-income countries, and the consumer phase is the stage with largest losses, between 40-50% of total cereal food waste. During milling, pearling or production of flour, there is the removal of several part of the plant (straw, bran, germ) and the loss of valuable components (vitamins, phenolic compounds, fatty acids). The aim of FAO, and of the several other initiatives established recently, is to reduce the amount of losses and wastes of food along the value chain in relation to the prospects for feeding a population of nine billion people by 2050. A range of approaches exists for reducing them at all steps of the FSC. The possibility to use food wastes, residues and by-products as substrate for the recovery of valuable nutrients is becoming quite popular using in particular insects. Skins, pulp, seed, blood, are important sources of components that the consumer generally searches in a food product for their important characteristics, related in particular to health and general well-being. Traditionally, food by products and wastes were also used as natural fertilizers or animal feed: it all depends on their composition. While, in most of the cases, the

components present inside were considered positive to enrich the soil or provide the right nutrients to livestock, the presence of possible toxic compounds as well as deficiencies of certain compounds should be taken into account. In a circular economy view, two food by-product were used to carry out development studies and behavioural bioassays: wheatmeal and olive pomace. Both of them are available locally. Wheatmeal is a by-product, used mainly for animal feeding. To produce refined (white) wheat flour, grain is usually tempered (added with water) before milling, to optimize milling efficiency. This softens the starchy portion of the wheat kernel, which will be separated out in the milling process. The addition of moisture also stiffens the bran and ultimately reduces the energy input required to shatter the kernel, while at the same time avoiding the shattering of bran and germ particles to be separated out in this milling process by sieving or sifting. The moistened grain is first passed through the series of break rollers, then sieved to separate out the fine particles that make up white flour. The balances are intermediate particles of endosperm and coarse particles of bran and germ (wheatmeal). The middling then makes multiple passes through the reduction rolls, and is again sieved after each pass to maximize extraction of white flour from the endosperm, while removing coarser bran and germ particles. The olive pomace is a by-product of the olive oil extraction process. It remains after the olives crushing to obtain the Extra Virgin Olive Oil or the Virgin Olive Oil. It is composed by skins, pulp residues and kernel scraps. The pomace can be

recycled by the food industry to obtain a vegetable oil, due to the presence inside the paste of a consistent lipid fraction (it contains between 3% and 6% of its weight of oil). In addition, the exhausted pomace is a by-product largely used to produce renewable energy. In wide terms, the wastes of the olive oil production can be included in the combustible biomasses. The pomace can also be used to recover thermal energy to substitute the fossil fuels, reducing the CO₂ emissions. However, the profitability of the olive pomace can be limited by the high cost of transportation, the high content of water due to the centrifugation and the chosen extraction technology and the limited interest of the market; therefore, oil mills tend to consider the olive pomace a waste. For these reasons, the olive pomace is under the length of the EU, that is investing in the research to strength the use of the food by-products to reduce the amount of wastes.

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CHAPTER 1

Morphology of the maxillo-labial complex of the yellow mealworm beetle *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae)

Abstract

The yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is known to be an important stored products pest, even though its larvae are considered one of the most common feed and food edible insect. We investigated the maxillo-labial complex morphology of the last instar larvae, using Scanning Electron Microscopy technique. The maxillary palps are composed by three segments. On the second segment we observed two sensilla supposed to be mechanoreceptors, one dorsally and one externally located. On the third segment we found: a sensory complex composed by four different types of putative olfactory and gustative sensilla, located on the tip; up to four putative chemoreceptors and one putative mechanoreceptor located on the internal side; one digitiform sensilla, supposed to be hygroreceptor, located on the external side. The labial palps are composed by two segments. On the latero-ventral side of the second labial segment we found sensilla supposed to be gustative and on the tip we observed a sensory complex similar to the one described on the maxillary palp tip. The function of most of the sensilla was supposed by morphological similarities with sensilla in closely related insects. For this reason, further ultrastructure

investigations are necessary to better describe and understand the function of each sensillum and their correlation with *T. molitor* food preference behavior.

Keywords: mealworm, mouthparts, sensilla, morphology, *Tenebrio molitor*.

Introduction

Insect mouthparts are adapted for the ingestion of many types of foods and the most distinguishing characteristic of an insect's behavior lies in their functional mouthparts (Moon *et al.* 2008, 2014). Most of these structures are generally distributed on the labio-maxillary complex and exhibit a variety of forms and characteristics in relation to their various functions of contact chemoreception, mechanoreception and thermohygroreception (Benham and Ryan, 1978). In particular, the number and distribution of sensilla on the mouthparts play an important role in detecting feeding resources and evaluating their quality prior to ingestion (Shields, 1996; Zacharuk and Shields, 1991). Such a variety of sensilla provide the most important cues also to help them to locate a specific microhabitat and to participate in chemical communication (Hansson, 2002; Regnier and Law, 1968; Shorey, 1973). Previous studies of the mouthparts sensilla in Coleoptera order have mostly focused upon the external comparative morphology within a tribe or family with the aim of elucidating their feeding mechanisms and/or taxonomic relationships (Betz *et al.*,

2003). Well-studied examples include in Coleoptera: Carabidae (Di Giulio *et al.*, 2012; Merivee *et al.*, 2001; Merivee *et al.*, 2002), Chrysomelidae (Bartlet *et al.*, 1999; Zhang *et al.*, 2013), Curculionidae (Gao *et al.*, 2013; Liu *et al.*, 2010; Smith *et al.*, 1976; Yang *et al.*, 2009), Scolytidae (Hallberg, 1982; Peng *et al.*, 2012), and Cerambycidae (Crook *et al.*, 2003; Dai and Honda, 1990; Xiong *et al.*, 2014; Zhang *et al.*, 2013). The sensory structures of palps were previously studied in polyphagous beetle larvae such as *Oryzaephilus surinamensis* (L.) (Roppel *et al.*, 1972), *Entomoscelis americana* Brown (Mitchell *et al.*, 1979), *Speophyes lucidus* (Delar) (Corbière 1969; Corbière-Tichanè 1973) and *Ctenicera destructor* (Brown) (Zacharuk 1962, 1971, 1979; Zacharuk *et al.*, 1977). However, there are few studies of sensilla on mouthparts of Tenebrionidae, mainly concentrated on the distribution and abundance of sensilla on the larval mouthparts of *Tribolium confusum* du Val and *Tribolium castaneum* (Herbst) (Ryan and Behan, 1973; Behan and Ryan 1978). The yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is worldwide known because of its larvae, considered one of the most common feed and food edible insect (Van Huis *et al.*, 2013). It is an important laboratory test animal and at the same time, this beetle is also known to be a serious primary stored product pest (Sinha and Watters, 1985). Damages and subsequent economic losses, due to infestations of *T. molitor* are mainly due to their feeding activity. So far, although this species has been widely investigated, there is a lack of information about larvae mouthpart morphology.

This work shows preliminary investigations on the type, number and distribution of sensilla on the labio-maxillary complex of *T. molitor* larvae using Scanning electron microscopy.

Materials and methods

Insects. *Tenebrio molitor* larvae were obtained from cultures maintained on wheatmeal in a climatic chamber at the Entomology laboratory of Polytechnic University of Marche, Italy. Rearing conditions were set at 28 ± 0.5 °C, $60 \pm 10\%$ RH and a photoperiod of 0:24 h (L:D).

Scanning electron microscopy. Scanning electron microscopy (SEM) was performed on maxillary and labial palps of last instar larvae (n=20). Insects were anaesthetized by exposure to cold temperatures (-18°C) for 60 s, then they were dipped in 60% alcohol. The labio-maxillary complex were carefully excised with fine forceps under a stereomicroscope. Samples were dehydrated in a series of graded ethanol, from 60% to 99%, 15 min each. After dehydration, 99% ethanol was substituted with pure HMDS (Hexamethyldisilazane, Sigma®) and the specimens were allowed to dry under a hood, at room conditions; this step was repeated twice. They were then mounted on stubs with conductive doublesided black adhesive tape, coated, using a sputter coated (Balzers Union® SCD 040 unit Balzers, Vaduz, Liechtenstein), with gold, taking care to place them with different orientations in order to obtain a clear view of

the ventral, dorsal and lateral sides. The goldcoated samples were observed in a FE-SEM Zeiss® SUPRA 40 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and a Philips® XL 30 (Eindhoven, The Netherlands) operating at an accelerating voltage of 7–10 KV, WD 9–10 mm and analyzed by a SMART-SEM® software.

Statistical analysis. Measurements were taken with SMART-SEM® version 05.06 software (Carl Zeiss Microscopy Jena, Germany) on digitized images and processed as means \pm standard deviation. The shape, length, occurrence of grooves and socket joint were compared by SEM morphology.

Results

The head of *T. molitor* larva (Fig. 1.1) is prognathous and has a well-developed chewing mouthpart, that consists of a clearly visible labrum, forming a roof on the upper part of the mouth, a pairs of strongly sclerified mandibles with a triangular shape, and a big maxillo-labial complex ventrally located to close the down part of the mouth (Fig. 1.1). The maxillary-labial complex carries maxillary and labial palps (Fig. 1.1), inserted respectively on a large galea-lacinia complex and on a developed prementum. The maxillary palp is three-segmented and the labial palp is two-segmented (Fig. 1.1).

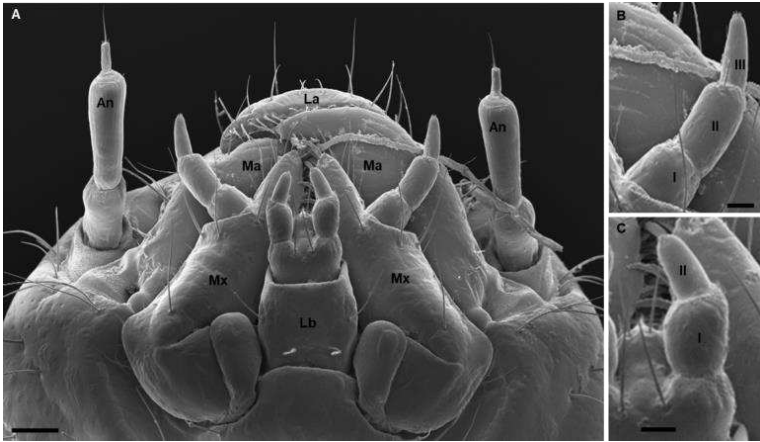


Figure 1.1 SEM images of chewing mouthpart of *T. molitor*. A) ventral view of chewing mouthpart; LA: labrum, MA: mandible, MX: maxilla, LB: labium, An: antennae; B) maxillary palp that consists of three segments (I, II, III); C) labial palp that consists of two segments (I, II).. Scale bars: a: 200 μ m; b,c: 100 μ m

Eight different types of sensilla are present on the apical article of both maxillary and labial palps: sensilla trichoidea type 1 and type 2 (Fig. 1.2), sensilla digitiformia, sensilla chaetica and sensilla basiconica type 1, type 2, type 3, type 4 (Fig. 1.2, 1.3). In particular, sensilla basiconica type 1, type 2, type 3 and type 4 form a sensory complex located on the tip of each maxillary palp (Fig. 1.3). While sensilla basiconica type 1, type 3 and type 4 form a sensory complex located on the tip of each labial palp. The number and the distribution of different type of sensilla basiconica vary between maxillary and labial palps.

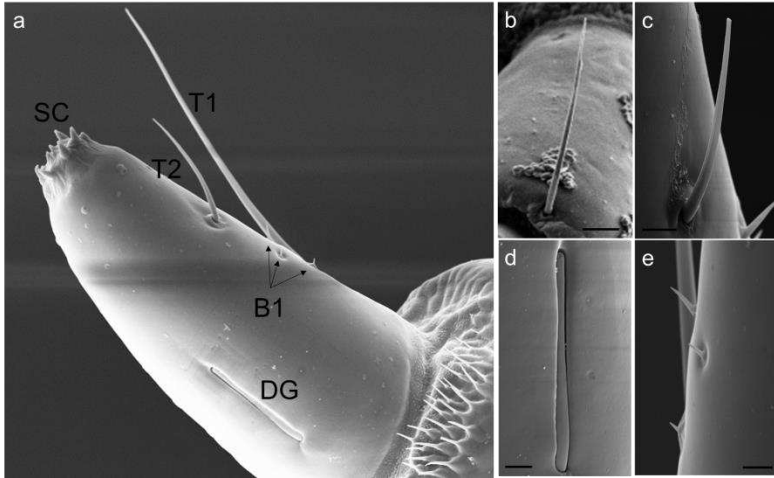


Figure 1.2 SEM images of maxillary palps of *T. molitor*. a) III segment of the maxillary palp; b) sensillum trichoideum type 1 (T1); c) sensillum trichoideum type 2 (T2); d) sensillum digitiformium (D); e) sensilla basiconica type 1 (B1). SC: sensory complex. Scale bars: a,b: 20 μ m; e: 5 μ m; c,d: 4 μ m.

Sensilla trichodea. Two types of sensilla trichoidea are present (Fig. 1.2):

Sensilla trichoidea type 1 are characterized by an elongated cuticular shaft $28.27 \pm 0.05 \mu\text{m}$ long ($n = 10$) that, from the base $1.24 \pm 0.01 \mu\text{m}$ width, decreases in diameter toward the apex, inserted in a specialized, flexible socket. On each maxillary palp, two sensilla trichoidea are ventrally located on the distal part of the second article.

Sensilla trichoidea type 2 are characterized by an elongated cuticular shaft $11.86 \pm 0.02 \mu\text{m}$ long ($n = 10$), strongly shorter than sensilla trichoidea type 2, that, from the base

0.55±0.02 µm width, decreases in diameter toward the apex, inserted in a specialized, flexible socket. On each maxillary palp, one sensilla trichoidea type 2 are located on the internal part of the third article, just above the sensilla chaetica.

Sensilla digitiformia. They appear as an elongate finger-like peg which lies in an individual groove, 39.53±0.16 µm long (n = 10) and 2.48±1.18 µm width (Fig. 1.2). On each maxillary palp, one sensillum digitiform is proximally located on the dorsal side.

Sensilla chaetica. They show a cuticular apparatus consisting of an outstanding hair shaft (Fig. 1.2), 2.58±0.24 µm long (n = 10) gradually tapering from the base (ca 0.81±0.07 µm in diameter) to a blunt tip with smooth walls (Fig), and inserted in a specialized, flexible socket (Fig. 1.2). On each maxillary palp, up to three sensilla chaetica are located on the apical article, proximally on the internal side, opposite to the digitiform sensillum.

Sensilla basiconica. Four types of sensilla basiconica are present (Fig. 1.3):

Sensilla basiconica type 1 have the shape of a upper cylinder, 1.10±0.17 µm long (n = 10) and 0.97±0.13 µm width, inserted on a bigger one 2.55±0.18 µm long (n = 10) and 2.55±0.18 µm width, rising on a rigid socket. On each maxillary and labial palps two sensilla basiconica type 1 are present in the apical sensory complex.

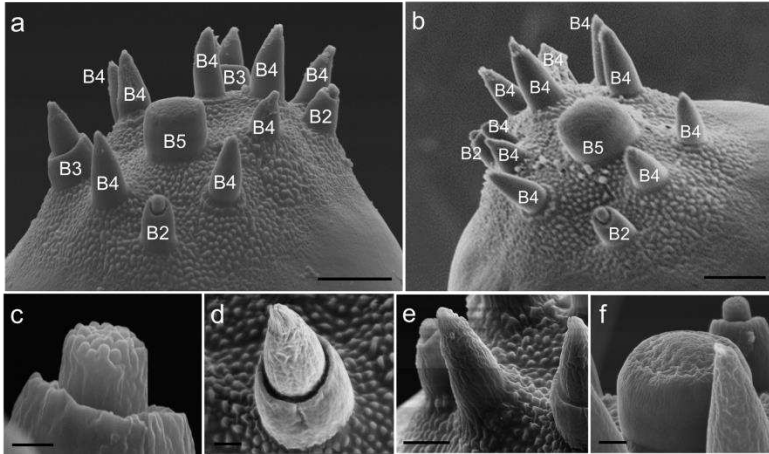


Figure 1.3 SEM images of sensory complex of *T. molitor*. a) sensory complex of maxillary palp; b) sensory complex of labial palp; c) sensillum basiconicum type 2 (B2); d) sensillum basiconicum type 3 (B3); e) sensillum basiconicum type 4 (B4); f) sensillum basiconicum type 5 (B5). Scale bars: a,b: 5 μ m; c: 2 μ m; d,f: 1 μ m; c: 400nm.

Sensilla basiconica type 2 appears as a cone shaped structure with apical finger-like cuticular projections, 4.34 ± 0.06 μ m long ($n = 10$) and 3.79 ± 0.08 μ m width, inserted on a cylinder 2.51 ± 0.17 μ m long ($n = 10$) and 3.79 ± 0.08 μ m width rising on a rigid socket. On each maxillary and labial palps two sensilla basiconica type 2 are present in the apical sensory complex.

Sensilla basiconica type 3 appear as a cone shaped structure with a rough surface, 5.58 ± 0.14 μ m long ($n = 10$) and 2.50 ± 0.03 μ m width. On maxillary and labial palps sensilla basiconica type 3 are present in the apical sensory complex

in 10 and 9 units respectively.

Sensilla basiconica type 4 appear as a globelike structure, $2.61 \pm 0.05 \mu\text{m}$ long ($n = 10$) and $4.70 \pm 0.03 \mu\text{m}$ width. On both maxillary and labial palps one unit is present in the center.

Discussion and conclusions

Together with the published data on the sensory organs of coleopterans larvae (totally, more than 125 species from 28 families) (Zacharuk, 1962; Scott and Zacharuk, 1971; Zaitseva and Elizarov, 1980; Bloom and Zacharuk, 1982; Bloom *et al.*, 1982; Zaitseva and Sinitsina, 1982; Bland, 1983; Speirs *et al.*, 1986; Chan *et al.*, 1988; Zacharuk and Shields, 1991; Chaika and Tomkovich, 1997; Dubinskas *et al.*, 1997; Tomkovich and Chaika, 2001; Giglio *et al.*, 2003; Sinitsina and Chaika, 2003; Giglio *et al.*, 2008; Farazmand and Chaika 2008; Giglio *et al.*, 2010; Eilers *et al.*, 2012; Giglio *et al.*, 2013; Xu *et al.*, 2017; ;) this work enables us to make some generalizations. The beetle larvae, inhabiting different biotopes and having a wide range of trophic relations, still display uniform topography of their sensory organs (Alekseev *et al.*, 2006). There is, in fact, a common apical group on both maxillary and labial palps in several coleopteran families (Thompson, 1992). In addition, literature describes one or several digitiform sensilla present on the lateral surface of maxillary (sometimes also labial) palps (Giglio *et al.*, 2003; Honomichl and Guse 1981). The mouthparts of *Tenebrio molitor* generally can be ascribed to those of other coleopteran species previously described

(Giglio et al., 2013; Farazmand and Chaika 2008). In fact, mouthpart of *T. molitor* displays both the presence of an apical group on the tips of palps and the presence of one digitiform sensillum on the lateral surface of maxillary palps. In general, the coleopteran order is characterized by a limited number of sensilla on the palps, on average from 4 to 10–12 (Giglio et al., 2013). In rare cases, for example in *Scarites terricola* and species of the genus *Carabus*, there are more than 20 sensilla, or the sensilla are absent at all (Giglio et al., 2003). The shape of the sensilla varies considerably, from small cones to typical basiconic sensilla, often with basal cuticular rings. In many species the sensilla are randomly positioned in the receptor field. The digitiform sensilla are either small and numerous, or large (present in the number of 7–8) and arranged at nearly the entire perimeter of both palps (Sinitsina and Chaika, 2003). The sensory organs of the maxillary and labial palps have very similar sets of sensilla. Most authors note that the number of sensilla is either the same on both palps, or different by 1–2 sensilla (Giglio et al., 2003; 2013). For example, there are 8 and 7 sensilla in *Hydaticus* sp. (Dytiscidae), 12 and 14 in *Anthrenus scrophulariae*, 10 and 9 in *Attagenus smirnovi* (Dermestidae) (Zaitseva and Elizarov, 1980), 16 and 11 in *Leptinotarsa decemlineata* (Chrysomelidae) (Mitchell and Schoonhoven, 1974), 12 and 9 in *Cassida* (Rosciszewska, 1981), and 10–13 and 8–10 in species of the family Curculionidae (Chaika and Tomkovich, 1997; Tomkovich and Chaika, 2001). In *T. molitor* it was found that sensilla number did not differ between maxillary and labial palps

but sensilla are present, with a different arrangement, only on the tip around the apex. In literature an increased number of sensilla in the palpal receptor groups was observed in species from the families Scarabaeidae, Elateridae and Cucujidae (Alekseev *et al.*, 2006). In particular, the sensilla are especially abundant in some predaceous forms from the families Elateridae and Coccinellidae. An increase in the number of sensilla in the receptor groups should obviously be considered a progressive trend, facilitating better perception of information from the environment (Alekseev *et al.*, 2006). Knowledge of the types and relative abundance of the sensory receptors of *T. molitor* larvae gives some insight into the possible role of the receptors in the heterospecific larval response. The function of *T. molitor* maxillo-labial sensilla described in this paper is supposed by morphological similarities with others closely related insects. Digitiform sensilla on mouthpart of *T. molitor* larvae were described by Honomichl and Guse (1981) as an aporous hairshaft basally inserted in a narrow canal of the cuticle and with its longer distal part positioned in a superficial groove. Corbière-Tichanè (1971), Zackaruk *et al.*, (1977), and Doane and Klinger (1978) have described digitiform sensilla on the distal segment of the maxillar and the labial palps in three species of Coleoptera. Honomichl (1980) has shown that these sensilla are widespread in Coleoptera. The presumed function of the digitiform sensilla is related to thermo-, hygro-, and CO₂- receptors (Honomichl and Guse 1981). Doane and Klinger (1978), Klinger (1966) and White *et al.*, (1974) describe the loci of

CO₂-reception in several larval Coleoptera and they find that the maxillar palps possess a greater number of CO₂-receptors than the labial palps. Such a distribution is corresponding to the arrangement of digitiform sensilla in adult and larval Coleoptera (Honomichl 1980). Digitiform sensillum is innervated by a single bipolar neuron, as it has previously been documented (Zacharuk *et al.*, 1977). The digitiform sensillum responds only to mechanostimulation including contact and vibratory stimuli (Zacharuk *et al.*, 1977). In literature the function of trichoidea sensilla is mainly described as mechanoreceptor (Schneider and Steinbrecht 1968), and both contact chemoreceptors and mechanoreceptors (Lewis 1970). The size and location in *T. molitor* mouthpart appendages of T1 and T2 suggest that the main function could be mechanoreceptors for T1 (Zacharuk, 1985; Giglio *et al.*, 2003; Farazmand and Chaika, 2008) and both mechanoreceptors and contact chemoreception for T2 (Mustaparta 1973; Giglio *et al.*, 2003; Farazmand and Chaika, 2008). Sensilla similar to T2 have been described on several species of curculionid beetles (Alm and Hall; 1986; Hatfield *et al.*, 1976; Isidoro and Solinas, 1992; Mustaparta, 1973). In electrophysiological experiments with *Hericum abietis*, Mustaparta (1975) found that this sensillar type was electrophysiologically responsive to odours. Chemoreceptors often observed on the palps of larvae and imaginal stages of insects, enable them to perceive several types of volatiles from both plants and feeding sources (Ishikawa and Hirao 1963; Ma, 1972; Nagnan-Le Meillour *et al.*, 2000; Tang *et al.*, 2014). The present study shows that

chemoreceptors on mouthpart appendages of *T. molitor* larvae are mainly located on the terminal disc of the distal segment of maxillary and labial palps. Such a sensillum arrangement is consistent to the localization of chemoreceptors at coleopteran larvae. The basic locations of contact chemoreceptors were on the sensory disc at the tip of maxillary and labial palps. The cuticle of these discs is less sclerotized, which allows sensilla to deeply move into distal segment when the palp contacts with a substrate. The sensilla chaetica are uniporous sensilla with a combined mechanosensory and gustatory function. In electrophysiological recordings they responded to host-plant chemicals, including glucosinolates (Isidoro *et al.*, 1998). Curculionid, scolytid and coccinellid beetles have been reported to bear antennal sensilla similar to the sensilla basiconica described here (Alm and Hall, 1986; Bland, 1981; Hateld *et al.*, 1976; Isidoro and Solinas, 1992; Jourdan *et al.*, 1995; Mustaparta, 1973; Smith *et al.*, 1976; Whitehead, 1981). The sensilla basiconica of *H. abietis* were responsive to odours in electrophysiological experiments (Mustaparta, 1975). Reduction of the cuticular parts of the sensilla is related to the fact that active movements of the palps in the process of ingesting hard food (tegument of the prey), would certainly damage the exposed sensilla. Moreover, the presence of an elastic cuticle on palp tips is a clue to confirm the above hypothesis. All these characteristics are found in *T. molitor* mouthpart. Mouthparts have evolved into a high diversity forms and functions (Jervis 1998) and research on their

morphology is needed to provide insight into feeding mechanisms (Ma *et al.*, 2013, Krenn 2007) and to provide characters for use in behavioural (Blaney and Chapman, 1969) and phylogenetic studies (Beutel 1993; Bologna and Pinto, 2001). Additional studies will be needed to determine the function of those sensilla through ultrastructural analysis. Moreover additional research is needed to further elucidate the correlation between morphology and behavioural studies.

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CHAPTER 2

Influence of different diets composed of organic by-products on growth and development of the Yellow Mealworm (Coleoptera: Tenebrionidae)

Abstract

Insects receive increasing attention as an alternative protein-rich food source for humans and animals. Producing edible insects on diets composed of organic by-products could increase sustainability. In addition, insect growth rate can be influenced by diet. *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is a cosmopolitan stored product pest and its larvae have the ability to recycle organic waste and food by-products of low-quality into high-quality feed and food rich in energy, protein and fat. In this study, *T. molitor* was grown on diets composed of organic by-products originating from mill and olive oil production; growth performances were monitored under four different diets: organic wheatmeal (WH), wheatmeal:olive-pomace, 3:1 (M1), wheatmeal:olive-pomace 1:1 (M2), wheatmeal:olive-pomace 1:3 (M3). For each diet tested, there were analyzed: i) the larval growth performances from eclosion to the last molt; ii) the number of instars; iii) the duration of larval stage; iv) the weight of last instar larvae and pupae; v) the overall survival rate. Diet affected mealworm development such that diets higher in wheatmeal appear favourable, with respect to shortening larval development time, reducing

mortality and increasing weight gain. The best larval growth performance was observed in WH diet. However, larvae of *T. molitor* can be grown successfully on diets composed up to 25% of organic olive pomace replacement. In conclusion, the results of this study highlight the importance of diets respect with larval growth performances in the set-up of a rearing systems for mass production.

Keywords: edible mealworm, yellow mealworm, larval development, survival, instars.

Introduction

The yellow mealworm beetle (YMB), *Tenebrio molitor* L. (Coleoptera, Tenebrionidae), is a cosmopolitan stored-products pest, with infestations usually originating in poorly stored animal feed and stored grain (Sinha & Watters, 1985). Damages and subsequent economic losses, due to infestations of *T. molitor* are mainly due to their feeding activity. *T. molitor* is worldwide known because of its larvae, considered one of the most common feed and food edible insect. YMB are omnivorous and can eat all kinds of plant material as well as animal products such as meat and feathers (Ramos-Elorduy *et al.*, 2002). *T. molitor* is typically fed on cereal bran or flour (wheat, oats, maize) supplemented with fresh fruits and vegetables (carrots, potatoes, lettuce) for moisture together with protein sources such as soybean flour, skimmed milk powder or yeast (Aguilar-Miranda *et al.*, 2002; Hardouin *et al.*, 2003). They

are produced industrially as feed for pets and zoo animals, including birds, reptiles, small mammals, batrachians and fish. They are usually fed live, but they are also sold canned, dried, or in powder form (Aguilar-Miranda *et al.*, 2002; Hardouin *et al.*, 2003; Veldkamp *et al.*, 2012). *T. molitor* has also been proposed as a source of protein for catfish (Ng *et al.*, 2001) and broiler chicken production (De Marco *et al.*, 2015; Ramos-Elorduy *et al.*, 2003). Additionally, *T. molitor* can be used as a host for in vivo mass production of entomopathogenic nematodes (Shapiro-Ilan *et al.*, 2002, 2008). YMB have the ability to recycle waste and food by-products of low quality into high-quality feed rich in energy, protein and fat in a relatively short time. The yellow mealworm is suitable for human consumption and its mass production is well-documented (Ghaly and Alkoaik, 2009, Van Huis, 2013). Mealworm species are considered suitable for introducing unaccustomed consumers to entomophagy since they feed on cereals directly used in food production. When introducing edible insects as a more sustainable alternative to conventional meat, it is advantageous to use diets from a local and more sustainable source than is currently the case. This can be achieved by producing the insects on diets composed of industrial by-products, for example from the food industry. Insect growth rate and body composition, and hence nutritional quality, can be altered by diet (Anderson, 2000; Davis and Sosulski, 1974). This offers opportunities to increase production and alter the nutritional composition of mealworms to better suit consumer needs. Literature is available on dietary effects on the growth and

chemical composition of *T. molitor* (Davis and Sosulski, 1974; Gao *et al.*, 2010; Morales-Ramos *et al.*, 2010; Ramos-Elorduy *et al.*, 2002) but it is still not well documented. The current state of the scientific research on YMB doesn't fully consider larval growth performances under different diets for the production of novel food. Moreover, information on development time and stadia length are lacking. The purpose of this study is to compare YMB larval growth performances under four different diets in order to optimize its rearing system using food by-products. Besides larval performances, pupal weight was compared among diets as it can be used as a measure for insect dietary quality (Chapman, 1998).

Materials and Methods

Insect source. The rearing stock of *T. molitor* was provided by a commercial pet shop PlanetFish&Co. [Ancona, Italy]. The mother colony was maintained at $28 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and 0:24 h (L:D) photoperiod on a supplement-free diet of organic wheatmeal. Water was provided twice a week through pieces of peeled organic carrots directly in the wheatmeal. Wheatmeal was added as needed. Pupae were separated from the diet by the use of a standard No. 6 sieve (3.35 mm openings) and allowed to complete development in plastic boxes (200 x 150 x 60 mm, L x W x H) lined with filter paper. Adult beetles were reared in plastic containers (400 x 300 x 60 mm L x W x H) on wheatmeal. These containers were sieved and replaced every 4 days to recover

the eggs that adult beetles had glued to the bottom of the pan. Eggs glued to the bottom were isolated using a brush and grouped according to the collection date in Petri dishes (14 cm diameter). Petri dishes were then monitored every 12 hours for hatching of first instars.

Insect diet preparation. Side streams were selected as ingredients for the experimental diets based on local availability and deemed suitability as feed for insects. The four diets used for mealworms rearing consisted of: wheatmeal (Molino del Conero, San Biagio, Osimo, Italy) and olive-pomace (Azienda Agricola “I tre filari”, Recanati, Italy) (Tab. 2.1) according to the following scheme: (WH) organic wheatmeal, (M1) 3:1 mix of organic wheatmeal and organic olive-pomace, (M2) 1:1 mix of organic wheatmeal and organic olive-pomace and (M3) 1:3 mix of organic wheatmeal and organic olive-pomace. Ingredients needed for the preparation of the diets, were collected from one batch. Olive pomace was stored in fridge at 1-2°C meanwhile wheatmeal was stored at room temperature. Diets were prepared by mixing fresh ingredients every week.

Table 2.1 Composition of the experimental diets made from organic by-products. WH: organic wheatmeal; M1: 3:1 mix of organic wheatmeal and organic olive pomace; M2: 1:1 mix of organic wheatmeal and organic olive pomace; M3: 1:3 mix of organic wheatmeal and organic olive pomace.

	WH (%)	M1 (%)	M2 (%)	M3 (%)
Wheatmeal	100	75	50	25
Olive pomace	-	25	50	75

Chemical analysis of diets. Analytical determinations of the diets were performed referring to official methodologies (AOAC, 2003; 2005; Atwater and Wood, 1896): moisture content, in an oven; protein, following Kjeldahl procedure using 6.25 as conversion coefficient; fat, using petroleum ether extraction in Soxhlet equipment; fibre, through acid and base solubilisation; NFE, by difference. The Atwater coefficients (4 kcal/g for proteins and 9 kcal/g for lipids) were used to estimate the energy value of each diet.

Larval growth and development experiments. First instar larvae (n=50) were collected using a brush and grouped according to the collection date in Petri dishes (14 cm diameter) (n=3), providing 10 g of the corresponding diet treatment and kept in an environmental chamber at $28 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH, and 0:24 h (L:D) photoperiod. Every week, head capsule width were recorded for each larva until pupation. Width of head capsule was recorded using a

stereomicroscope equipped with a photcamera wireless connected with a laptop. Photos were elaborated using the software [Leica LasEZ] measuring the width of head capsule in the larger part. Every time head capsule were measured, larvae were removed from the Petri dishes and introduced to new Petri dishes containing 10 g of new diet of the corresponding treatment with 2 g of peeled organic carrots; the old carrot pieces were removed and the old diet was discarded. Larval survival rate, the duration of larval stage and last instar larval weight and pupal weight were also recorded for each diet treatment.

Data analysis. Data consisting of larval survival and development time, last instar head capsule width, last instar weight and pupal weight, did not conform to the normal distribution and were analysed by a Kruskal-Wallis test at a significant level of 0.05 followed by Dunn's test for post hoc comparison applying Bonferroni correction. Linear regression analysis was used to determine if correlation existed between larval development time and head capsule width. Regression lines of head capsule width and developmental time from the different diet treatments were compared for differences in slope and intercept using the F statistic method for multiple lines comparisons and then compared among treatments by analysis of variance (ANOVA) of a significance level of 0.05 followed by Tukey method for comparing a family of four estimates. Correlation between larval survival and development time was analysed by Spearman's rank correlation coefficient.

Number of instars in each diet, were determined, analysing the frequency distribution of head capsule width data following Logan et al., 1998. All statistical procedures were done using “R” software, version 3.5.1.

Results

Chemical composition of the diets. Olive-pomace, used as feeding substrate mixed in different quantitative ratio with wheatmeal, showed the following analytical composition: 4.40% CP, 12.26% EE, Fibre 46.39%, NFE 32.64%, ash 4.30% on D.M. basis, and 259 kcal/100 g. WH, M1, M2, and M3 diets chemical composition are listed in Table 2.2. Protein content of the experimental diets ranged from 10.09% in M3 to 17.50% in WH diet treatment. Fat content of the experimental diets was between 4.08% in WH and 8.01% in M3 diet treatment. The chemical composition of the experimental diets (% DM) showed that the partial replacement of wheatmeal with olive-pomace influenced the feeding substrate composition with increase in moisture, fat and fibre content; a reduction in protein and Nitrogen Free Extract was also observed. Slight decrease in ash percentage and modest reduction in energy content were detected as the percentage of olive-pomace increased.

Table 2.2 Chemical composition (mean \pm std. dev.) of the experimental diets used for *T. molitor* rearing (% DM). WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace). DM: dry matter; EE: ether extract; NFE: nitrogen free extract.

	WH	M1	M2	M3
Moisture (%)	14.51 \pm 0.06	25.62 \pm 0.12	40.61 \pm 0.42	54.75 \pm 0.53
Protein (%)	17.50 \pm 0.06	16.13 \pm 0.09	14.42 \pm 0.24	10.09 \pm 0.07
Fat (%)	4.08 \pm 0.39	4.62 \pm 0.46	6.19 \pm 0.26	8.01 \pm 0.52
Fibre (%)	8.65 \pm 0.25	12.23 \pm 1.32	15.03 \pm 1.92	29.15 \pm 3.80
NFE (%)	64.08 \pm 0.16	61.51 \pm 1.69	58.90 \pm 2.36	47.39 \pm 4.19
Ash (%)	5.70 \pm 0.07	5.51 \pm 0.01	5.46 \pm 0.06	5.35 \pm 0.05
Energy (kcal/100g)	363 \pm 2.66	352 \pm 2.96	349 \pm 6.16	302 \pm 12.41

Number of larval instars. The frequency distribution of head capsule widths indicate distinct peaks, representing the number of instars (Fig. 2.1). The number of instars observed in all the replicates of WH and M1 were 13 (Fig. 2.1, A-B)

meanwhile the instar number in M2 and M3 diet treatments were 17 (Fig. 2.1, C-D) (Table 2.3).

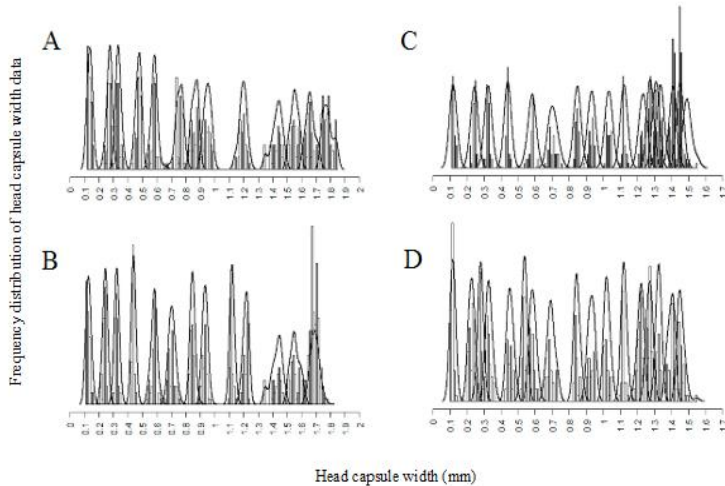


Figure 2.1 Head capsule width frequency distribution. Lines indicate distinct peaks that represent the individual instar distribution observed in the four diet treatments: organic wheatmeal (WH) (A); 3:1 mix of organic wheatmeal and organic olive pomace (M1) (B); 1:1 mix of organic wheatmeal and organic olive pomace (M2) (C) and 1:3 mix of organic wheatmeal and organic olive pomace (M3) (D).

Larval development time. Larval development was strongly affected by the diets (Tab. 2.3). Larvae grown on WH showed a significantly shorter larval stage ($P < 0.0001$) respect to all the other diet treatments.

Table 2.3 Larval development time (DT) and survival rate (SR) until the first pupae was observed, number of instar (NI), head capsule width (HW) of last instar stage, larval (LW) and pupal weight (PW). Values are given as mean \pm std. dev. except for the number of instars. Superscripts denote significant differences. WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace).

Diets	DT (days)	SR (%)	NI (n)	HW (mm)	LW (g)	PW (g)
WH	98 \pm 4.9 a	85 \pm 3.1 a	13	1.80 \pm 0.03 a	0,128 \pm 0.004 a	0.190 \pm 0.047 a
M1	105 \pm 4.9 b	78 \pm 2.0 b	13	1.71 \pm 0.03 b	0,131 \pm 0.004 a	0.192 \pm 0.042 a
M2	126 \pm 7.0 c	54 \pm 10.6 c	17	1.46 \pm 0.03 c	0,081 \pm 0.003 b	0.171 \pm 0.037 b
M3	133 \pm 7.0 c	58 \pm 4.0 c	17	1.45 \pm 0.03 c	0,070 \pm 0.003 b	0.153 \pm 0.030 c

Larval survival rate. Survival rate on WH was significantly higher ($P < 0.0001$) respect to all the other diet treatments (Tab. 2.3). Development time and survival rate was also correlated (Fig. 2.3). Most of the mortality occurred within the first 28-30 days after eclosion in all diets treatments.

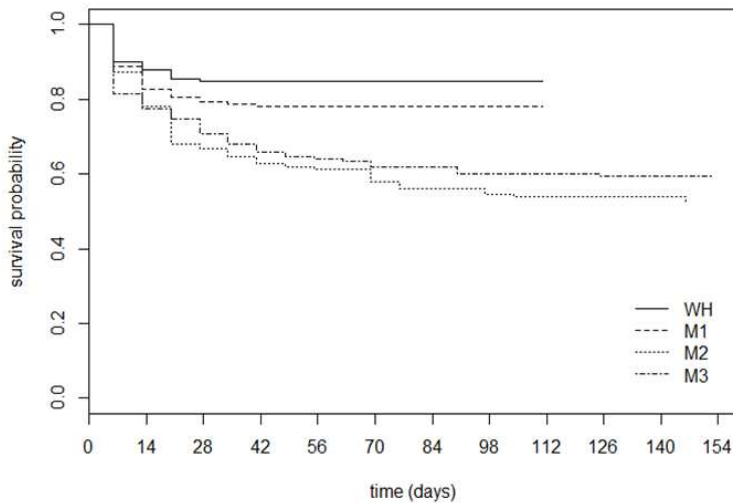


Figure 2.2. Average larval survival as percentage of the total number of larvae at week 0 (n=50) determined weekly until the first pupa was observed. WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace).

Head capsule width. Last instar head capsule width was significantly affected by the diet ($\chi^2 = 352.53$, $df = 3$, $P < 0.0001$) (Fig 2.3; Tab 2.3). Larvae reared on WH diet showed a significantly higher mean last instar head capsule width ($P < 0.0001$) respect to all other treatments. Larvae reared on M2 and M3 showed the lowest mean last instar head capsule width.

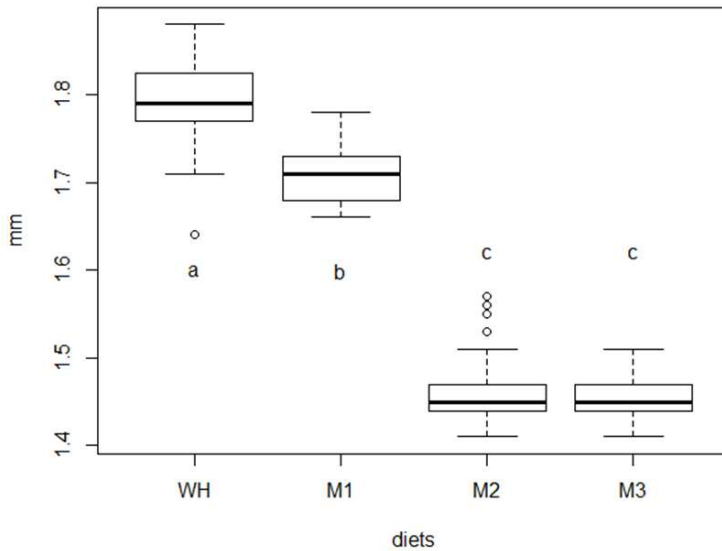


Figure 2.3 Last instar head capsule width according to the four diet treatments. Continuous line represents the mean and dashed line represents the median. Dots represent outlying points. Means with the same letter are not significantly different after Kruskal-Wallis test followed by Dunn's test for post hoc comparisons at significant level $P = 0.05$. WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace).

Last instar weight. Last instar larval weight was significantly higher in WH and M1 diet treatments ($\chi^2 = 113.96$; $df= 3$; $P < 0.0001$) than in all the other treatments (Fig 2.4; Tab 2.3). M1 diet treatment showed the highest mean larval weight followed by WH diet. M2 and M3 diet

treatments showed the worst performance for the last instar larval weights.

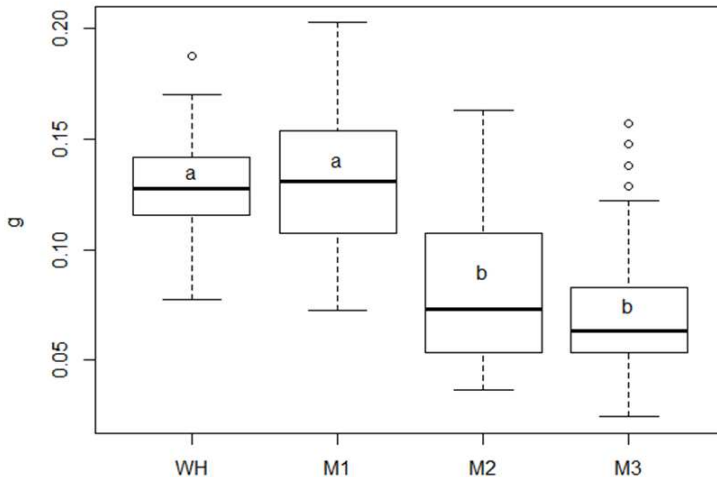


Figure 2.4 Mean last instar larval weight according to the four diets treatments. Continuous line represents the mean and dashed line represents the median. Dots represent outlying points Means with the same letter are not significantly different after Kruskal-Wallis test followed by Dunn's test for post hoc comparisons at significant level $p = 0.05$. WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace).

Pupal weight. Pupal weight was significantly different among diet treatments ($\chi^2 = 47.39$; $df = 3$; $P < 0.0001$). Pupal weight was significantly higher in diet WH and M1 than in all the other treatments, ($P < 0.0001$) (Fig 2.5; Tab 2.3).

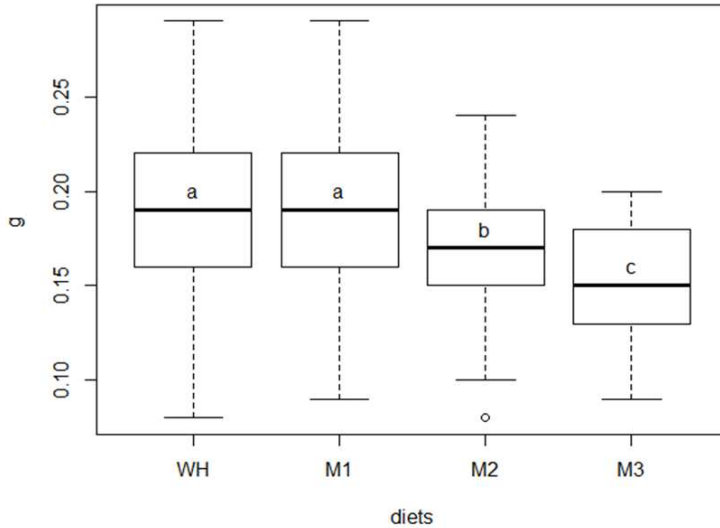


Figure 2.5 Mean pupal weight according to the four diets treatments. Continuous line represents the mean and dashed line represents the median. Dots represent outlying points. Means with the same letter are not significantly different after Kruskal-Wallis test, followed by Dunn's test for post hoc comparisons at significant level $p = 0.05$. WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace).

Larval growth performance. Larval growth performance was analysed by a linear regression analysis between development time and head capsule width ($F = 1471.1$; $df = 3$; $P < 0.0001$) (Fig 2.6). Larvae grown on WH and M1

showed a significantly better larval performances respect to all other treatments ($P < 0.0001$).

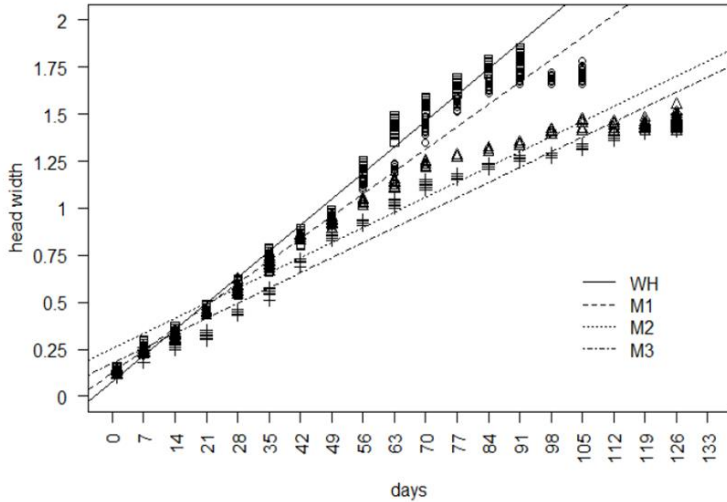


Figure 2.6 Linear regression analysis between mean head capsule width and development time. WH (organic wheatmeal); M1 (3:1 mix of organic wheatmeal and organic olive pomace); M2 (1:1 mix of organic wheatmeal and organic olive pomace); M3 (1:3 mix of organic wheatmeal and organic olive pomace).

Discussion

This study shows that diets can significantly affect the larval performances of the yellow mealworm. Larval mortality was very high on the diets with higher percentage of olive pomace. On the alternative, diets with higher percentage of wheatmeal showed higher survival and shorter development

time; this can be related to the higher percentage in the protein content of WH and M1 diets. Moreover in diets with higher content of olive pomace can be present phenolic compounds acting as antinutrient (Isikber *et al.*, 2009). This confirms the explanation that diet higher in olive pomace might not only lack nutrients, but might also contain compounds which are harder to digest or toxic to the mealworm. Induced detoxification of secondary plant metabolites commonly begins in earlier larval stadia in herbivorous insects (Glendinning, 2002; Yu and Hsu, 1993) and lower mortality is therefore expected in older larvae. In addition to retarded growth on diet M2 and M3, larvae were also subjected to higher number of molts respect to the other diets. Larvae grown on diets with higher inclusion of olive pomace showed also a smaller last instar head capsule width and lighter last instar larvae than those that consumed other diets. Possibly, protein quality of diet with higher percentage of olive pomace was lower for the yellow mealworm. This would cause the larvae to be more subjected to molts and more vulnerable to infection by pathogens, reducing their survival. Pupal weight can be used as a measure for insect dietary quality (Chapman, 1998). Pupal weight was lowest on diet with higher percentage of olive pomace; this is a further indication that this diet was of lower quality for development of the yellow mealworm. Regarding the number of instar, the determination of it, it is fundamental to both applied and basic research. Insect phenology and the resultant life-stage distribution is important in applied ecology because pest species are often

vulnerable to control methods for only restricted period in their life cycle. Determining instar distributions is required for life tables analysis, key factor analysis and other important ecological investigations. At the same time growth of insect larvae is discontinuous, with the most measurable change in size occurring following molts (Chapman 1982). Heavily sclerotized structures, such as head capsules, remain approximately the same size during a stadium, and can be used to differentiate larval instars (Daly 1985). The determination of the instar number can be done through the analysis of the frequency distribution of the head capsule width over time. The frequency distribution of widths indicate distinct peaks, representing instars. Diets with higher percentage of wheatmeal showed 13 instars meanwhile diets with high content of olive pomace showed 17 instars. Moreover head capsule width data showed that larvae grown on diets with higher inclusion of olive pomace were much smaller than the other diet treatments. These data could result from a variety of causes as the lack of nutrient causing a different number of molts that may result in size differences. Moreover larvae grow linearly in the diets treatment within the first 40 days post eclosion; after that period larvae started to differentiate their growth with a sharp increase in the growth rate in diets with higher percentage of wheatmeal. After 80 days post eclosion the growth rate seems to turn in a stable conditions. In conclusion, the mealworm can be grown successfully on diets composed of organic by-products, where diets high in wheatmeal appear favourable with respect to reduced larval

development time, reduced mortality and weight gain. Further studies are needed in order to understand better the nutrient composition of the larvae and to compose diets which support optimal growth and development time.

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CHAPTER 3

Behavioral responses to food volatiles in a four and two-choice arena bioassay by larvae of *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae)

Abstract

Behavioral responses of *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae to volatile organic compounds volatiles emitted by flour, wheatmeal, mixture of wheatmeal and pomace (50:50) was investigated using four and two-way choice. The larval behavior was analysed using a video tracking software. The results of this study showed that *T. molitor* larvae were significant attracted to volatiles emitted both by wheatmeal ($p = 0.023$) and mixture of wheatmeal and pomace ($p = 0.029$). This is the first study that evaluated the responses of *T. molitor* larvae to volatiles emitted by wheatmeal and pomace.

Keywords: yellow mealworm, behavioral response, arena, wheatmeal, olive pomace.

Introduction

Chemical senses are represented by the sense of taste and smell, depending on whether the stimulus is detected through contact or airborne (Ibba, 2010). The sense of smell is probably the oldest sensory modality in the animal kingdom

(Strausfeld and Hildebrand, 1999) and plays a central role in almost all tasks such as location of food enemies and mates. Insects are the most important group of terrestrial animals. They adapted very well to environmental changes, invading every niche except the benthic zone (Grimaldi and Engel, 2005). Insects are equipped with highly evolved sensory systems and olfaction is a key sense routing interspecific communication in insect communities. The role of infochemicals steering interactions among insects belonging to different trophic levels has been extensively investigated. Moreover research on the insect olfactory system has contributed to the control of insects that can be harmful to human health and agriculture. But also as it helps improving the positive functions that insects can have both for ecosystems as natural enemies and for humans as pollinators and honey producers (Karg and Suckling, 1999). Numerous studies are contributing in different ways to understanding behavioural responses elicited by olfactory stimuli (Visser, 1986; Suh *et al.*, 2004; Bruce *et al.*, 2005; Stockinger *et al.*, 2005; Billeter *et al.*, 2006; Carrasco *et al.*, 2015). Although many foodstuff insects are originally polyphagous, when foraging in anthropized habitats, they can rely on few different food resources and the association between pest species is common (Giunti *et al.*, 2018). Therefore, infochemicals are pivotal to detect suitable food sources and can improve pest searching efficiency, by shaping trophic interactions and competitive coexistence. Interspecific competition, either occurring as exploitation (i.e. without behavioural interactions) or interference (i.e. through

aggressive behaviours), is widespread among stored product insect pests and can lead to severe consequences for the involved species (Giunti *et al.*, 2018). Stored product protection is a key issue in the food production and processing contexts. Worldwide annual post-harvest losses attributable to stored product pests have been estimated at 10% of the total cereal grain yield, determining also severe weight losses (i.e. dry matter loss). In the last few decades, the intensive use of synthetic insecticides to control stored-product insect pests has caused many major concerns, such as the development of insect resistance, toxicity toward nontarget organisms, and adverse effects on the environment. Consequently, there is a need for new, effective, and environmentally friendly alternatives to reduce the use of synthetic pesticides in the pest control (Germinara *et al.*, 2008). Moreover the study can give an insight to the olive pomace disposal. The aim of this study was to investigate the behavioral responses of *T. molitor* larvae to volatile organic compounds volatiles emitted by flour, wheatmeal, mixture of wheatmeal and pomace (50:50) using four and two-way choice.

Materials and methods

Insects. *Tenebrio molitor* larvae were purchased from a commercial pet store (Planet Fish, Ancona – Italy). Isolated *T. molitor* eggs were placed on organic maize flour (neutral substrate) and maintained in a climatic cell at 28 ± 1 °C, 60

± 10% RH and 0:24 (L:D). *T. molitor* larvae of third and tenth instars were used in the bioassay in order to compare differences in their behavior between larval instars. Third instars larvae were easily distinguishable by their dark yellow coloration, which contrasted with the white coloration of first and second instars. Tenth instar larvae were selected according to the width of their cephalic capsule (see chapter 2).

Bioassays. A four and two choice bioassays were conducted to investigate the olfactory responses of *T. molitor* larvae to the volatiles emitted by: (A) organic flour, (B) organic wheatmeal, (C) organic mix of 50% pomace and 50% wheatmeal, (D) organic pomace. Organic flour and wheatmeal were purchased from a local mill factory [“Molino Agostini” - Italcere Soc. Coop. Agr, San Biagio di Osimo (AN), Italy]. Organic pomace was purchased from a local oil mill factory [Azienda Agricola “I tre filari” – Recanati – AN – Italy]. Organic flour and wheatmeal were stored at room temperature before use. Organic pomace was stored in a refrigerator room at 1.5°C and dried for 1 hour at 40°C before using in the bioassays. Each arena (16 cm Ø) (Petri dishes, Fisherbrand, Pittsburgh, PA) was covered at the bottom with filter paper (Whatman ® No. 1, Merck KGaA, Darmstadt, Germany) to facilitate insect movement (Pike *et al.*, 1994; Germinara *et al.*, 2002).

Four-way choice bioassays. Arena was divided into four equal sized wedge sections (Fig 3.1).

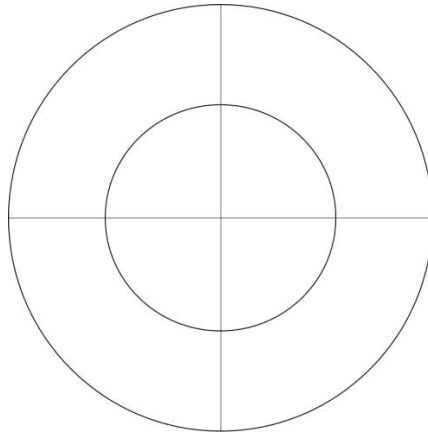


Figure 3.1 Representation of the division into sectors of the Petri dish.

4 g of each stimulus were randomly placed in the centre of each section (Baek *et al.*, 2015) at the beginning of each bioassay. Larvae were isolated from their feeding substrate and placed individually in small petri dishes (3 cm diameter x 1.2 cm high) at 28 ± 1 °C, $60 \pm 10\%$ Relative Humidity (RH), complete darkness. Each larva was starved for 12 h before being introduced into a Petri dish and allowed to acclimate for 30 minutes under an inverted Petri dish (3 cm diameter x 1.2 cm high) at the centre of the arena at 28 ± 1 °C, $60 \pm 10\%$ RH and under infrared lights (20 W red led placed 60 cm above workplace). A digital camera (Ikegami Tsushinki Co., Ltd – Japan) was suspended at 40 cm above the arena to record the larval behaviour. Insect that stopped on a stimulus for 8 seconds (s) was recorded as choice

(Baek *et al.*, 2015). For each larva the following times were recorded: (1) time to reach the stimulus (contact with the stimulus) (Cooperband & Allan 2009), (2) total time in which a larva resting/freezing (larva stayed immobile) in a sector (Zimba *et al.*, 2016; Obeysekara and Legrand 2014), (3) total time that the larva spend in motion in each section (Zimba *et al.*, 2016; Obeysekara and Legrand 2014), (4) time of larval contact with the stimulus less than eight seconds (Harvey *et al.*, 2008). For each behaviour was analysed: minimum and maximum time, average time, total duration, standard deviation, standard error, rate per minute/frequency, percentage, and latency. For these bioassays 132 larvae of third instar and 103 larvae of tenth instar were tested. Each larva was tested only once. The larvae that did not choose within 10 min were recorded as ‘no choice’.

Two-way choice bioassays. A Petri dish (16 cm Ø) (Fisherbrand, Pittsburgh, PA) was divided into two equal sized wedge sections. Petri dishes were covered with filter paper (Whatman ® No. 1, Merck KGaA, Darmstadt, Germany) to facilitate insect movements (Pike *et al.*, 1994; Germinara *et al.*, 2008). In the two sections 4 g of each stimulus were randomly placed (Baek *et al.*, 2015). Each food stimulus was placed in the centre of each section. Each larva was starved for 12 h before being introduced into a Petri dish and allowed to acclimate for 30 minutes under an inverted Petri dish (3 cm diameter x 1.2 cm high) at the centre of the arena. A digital video camcorder was

suspended approximately 40 cm above the Petri dish to record the searching behaviour and behavioural responses. Experiments were carried out at 28 ± 1 °C, $60 \pm 10\%$ RH and under infrared lights (20 W red led). Insect that stopped on a stimulus for 8 seconds (s) was recorded as choice (Baek *et al.*, 2015). For each larva also the time to reach the stimulus (contact with the stimulus) was recorded. For the bioassays 93 larvae of tenth instar were tested. Each larva was tested only once. The larvae that did not choose within 10 min were recorded as ‘no choice’.

Data analysis. Focal sampling and continuous recording techniques were used to score the behavior of the tested subjects for the 10 min recording session. Each testing session was analysed using The Observer XT analysis software [Noldus Information Technology, Wageningen, the Netherlands] for the behavioural responses. First choice was statistically compared using a chi-squared analysis (Zar, 1999). T-test was used for multiple comparisons between the means of the times of the different behavior. Data were filtered by removing outlier measurements (> 300 s). Third instar data were also filtered by removing the time of larval contact with the stimulus less than 8 seconds (s), due to low number of observations (taste). Shapiro-Wilk test was performed to assess normality of the data. Each behavior and related time spent in each arena section were analysed by two-ways ANOVA, followed by Kruskal-Wallis and Dunn’s *post hoc*. For statistical analysis Statistica 6.0 for Windows and R statistical environment were used.

Results

Third instar larvae first choice in four way bioassays. As concerns the third instar larvae that made a choice ($n=90$), 17 have chosen flour, 20 wheatmeal, 33 mixture and 20 pomace (Fig. 3.2).

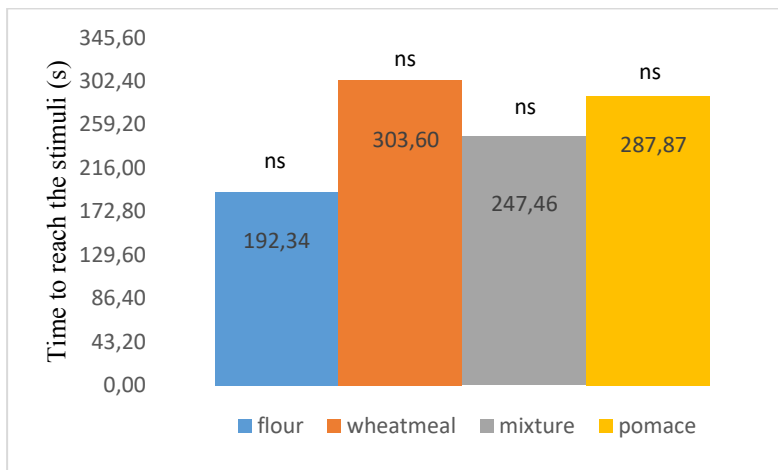


Figure 3.2 Behavioral responses of *Tenebrio molitor* third instar larvae that made a choice. Numbers indicate number of larvae that made a choice. (Chi-square analysis: ns, nonsignificant).

no significant differences ($p = 0.08$) was observed among the different stimuli (Figure 3.2). Difference was found when first choices were compared between mixture and flour ($p = 0.02$) (Tab. 3.1). No differences for the other comparisons have been showed (Tab. 3.1).

The average time for third instar larvae to reach the flour was 192.3 seconds; the average time to reach the wheatmeal

was 303.5 seconds; the average time to reach the mix was 247.4 seconds; the average time to reach the olive pomace was 288.3 seconds. No differences were showed among the times to reach the different stimuli (Fig. 3.3).



No differences among resident times (both immobile and moving) spent in the different sectors were observed (Fig. 3.4).

Table 3.1 Multiple comparisons between the number of third instar larvae that made a choice. df: degree of freedom; p: level of significance (* = $p < 0.05$). (Chi-square analysis).

Stimulus	Observed	Expected	df	p
Flour	17	18.5	1	0.622
Wheatmeal	20	18.5	1	
Flour	17	25	1	0.023*
Mix	33	25	1	
Flour	17	18,5	1	0.622
Pomace	20	18,5	1	
Wheatmeal	20	26,5	1	0.074
Mix	33	26,5	1	
Wheatmeal	20	20	1	1
Pomace	20	20	1	
Mix	33	26.5	1	0.074
Pomace	20	26.5	1	

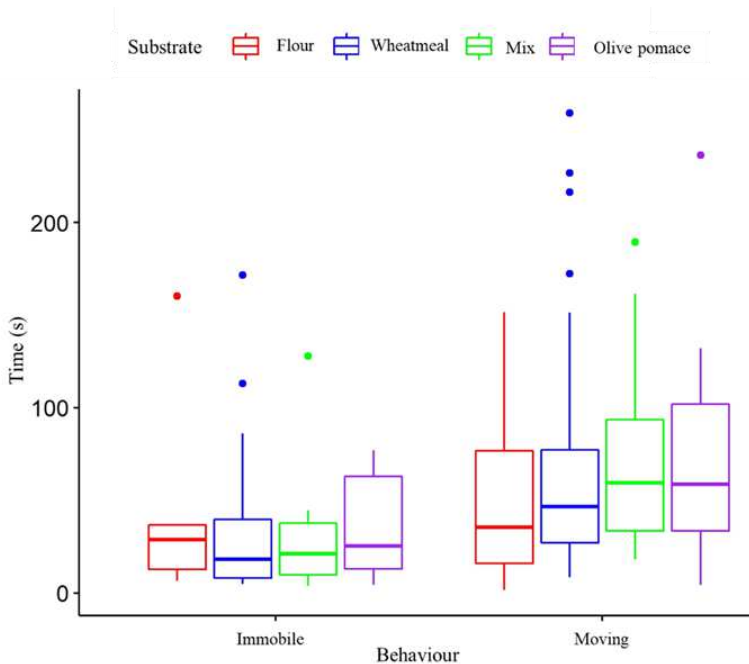


Figure 3.4 Resident time spent in the four sectors by the third instar larvae. Immobile: larva stayed immobile; moving: larva spend in motion. (Two-ways ANOVA, followed by Kruskal-Wallis and Dunn's post hoc).

Tenth instar larvae first choice in four way bioassays. As concerns the tenth instar larvae that made a choice ($n=90$), 15 have chosen flour, 28 wheatmeal, 31 mixture and 16 pomace (Fig. 3.5) significant differences ($df = 3$; $p = 0,03$) were observed in the choice of the tenth instar larvae.

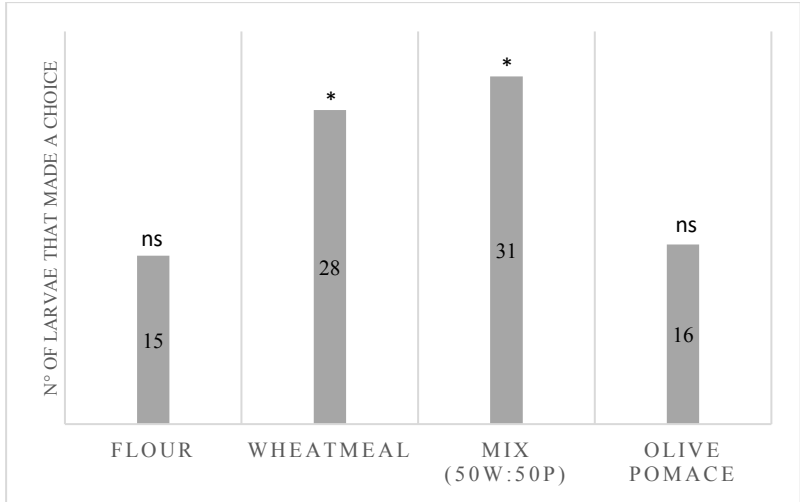


Figure 3.5 Behavioral responses of *Tenebrio molitor* tenth instar larvae that made a choice. Numbers indicate number of larvae that made a choice. (Chi-square analysis: ns, not significant).

A significant result was identified between flour (15) and wheatmeal (28) ($p=0.04$), flour and mix (31) ($p=0.02$) and mix and pomace (16) ($p=0.03$). All the other comparisons were not statistically significant. The results are showed in Table 3.2.

The average times for tenth instar larvae to reach stimuli were: 97.8 s for flour, 96.4 s for wheatmeal, 116.5 s for mix and 124.1 s for olive pomace.

No differences were showed among the times to reach the different stimuli (Fig. 3.6)

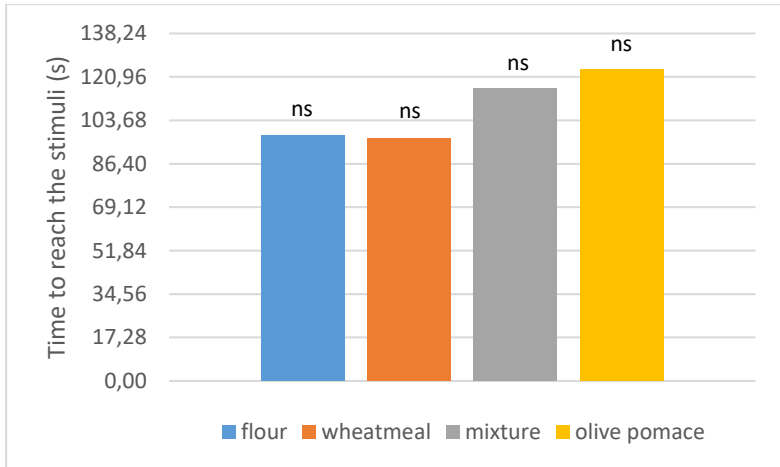


Figure 3.6 Multiple comparison among means of time to reach the stimuli. (Chi-square analysis: ns, not significant)

No differences among resident times (immobile, moving and taste) spent in the different sectors were observed (Fig. 3.7).

Table 3.2 Multiple comparisons between the number of tenth instar larvae that made a choice. df: degree of freedom; p: level of significance ($p < 0.05^*$). (Chi-square analysis).

Stimulus	Observed	Expected	df	P
Flour	15	21.5	1	0.047*
Wheatmeal	28	21.5	1	
Flour	15	23	1	0.018*
Mix	31	23	1	
Flour	15	15.5	1	0.857
Pomace	16	15.5	1	
Wheatmeal	28	29.5	1	0.696
Mix	31	29.5	1	
Wheatmeal	28	22	1	0.070
Pomace	16	22	1	
Mix	31	23.5	1	0.029*
Pomace	16	23.5	1	

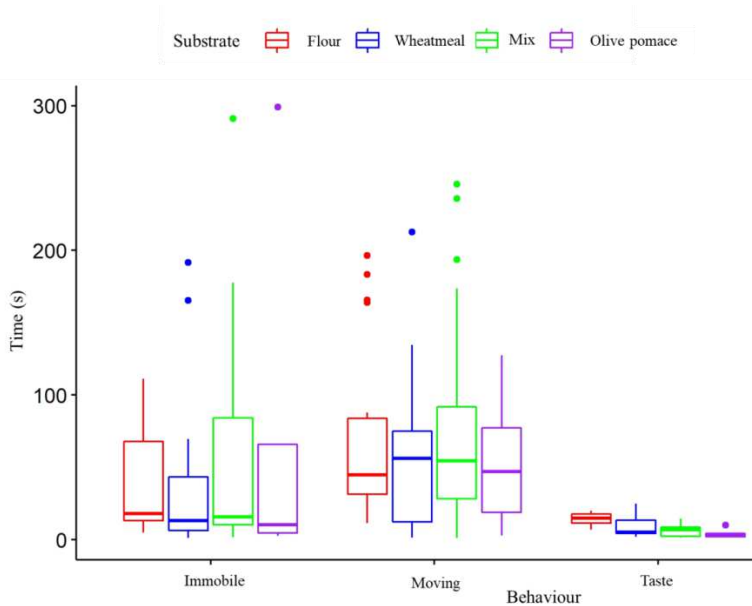


Figure 3.7 Resident time spent in the four sectors by the tenth instar larvae. Immobile: larva stayed immobile; moving: larva spend in motion. Taste: larva stayed for less then 8 s on the stimuli (Two-ways ANOVA, followed by Kruskal-Wallis and Dunn’s post hoc).

Comparison of the time to reach the stimuli between larval instar in four way bioassays. The time spent by third and tenth larval instar for the first choice differs significantly (Fig 3.8) in table values are showed (Tab 3.3).

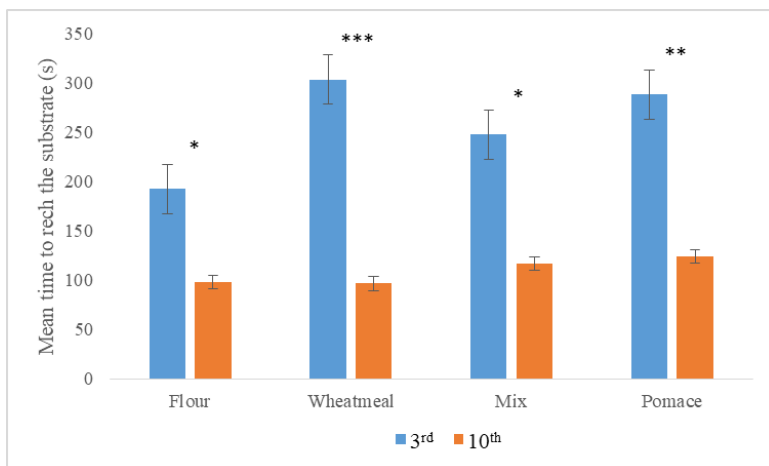


Figure 3.8 Mean (\pm s.e.m.) time to reach the stimuli of *T. molitor* in both instars. Asterisks indicate values that differed significantly between the instars among the stimuli ($p < 0.05^*$).

Table 3.3 Multiple comparisons between the means of the time to reach the stimuli between instars. df: degree of freedom; t: t value; p level of significance.

Stimulus	df	t	P
Flour	30	2.2	0.03*
Wheatmeal	46	4.8	0.0001***
Mix	62	3.3	0.001*
Olive pomace	34	3.9	0.0005**

Tenth instar larvae choice in two way bioassays. As concerns the larvae that made a choice in a two way bioassay there was significant difference among the six combinations of stimuli. *T. molitor* larvae showed significant preference for organic wheatmeal versus organic flour ($\chi^2 = 0.0105$, $df= 3$, $P < 0.05$) respectively with 22 and 8 first food choices.

Table 3.4 Multiple comparisons between the number of tenth instar larvae that made a choice. df : degree of freedom; p : level of significance ($p < 0.05^*$). (Chi-square analysis).

Stimulus	Observed	Expected	df	p
Flour	8	15	1	0.010*
Wheatmeal	22	15	1	
Flour	19	15	1	0.144
Mix	11	15	1	
Flour	20	15	1	0.067
Pomace	10	15	1	
Wheatmeal	18	15	1	0.273
Mix	12	15	1	
Wheatmeal	22	15	1	0.010*
Pomace	8	15	1	
Mix	18	15	1	0.273
Pomace	12	15	1	

Moreover larvae showed significant preference for organic wheatmeal versus organic olive pomace ($\chi^2 = 0.0105$, $df = 3$, $P < 0.05$) respectively with 22 and 8 first food choices. In the other combinations no significant differences were found (Tab. 3.4).

Discussion and conclusions

The olfactory system detects and process chemical volatiles from the environment. One of the main questions in olfaction is how these neural olfactory components translate into appropriate behavioural responses e.g. attraction to different odorants that are vital for finding food sources, mates, oviposition sites etc. In our study the volatiles emitted by wheatmeal were clearly attractive for *T. molitor* larvae. This result was expected as *T. molitor* is a stored product pest and wheatmeal represent one of the infested product. Moreover volatiles emitted by flour were not significant attractive for this pest. Volatiles can give information on the quality of the feeding substrate and insects assess the quality of a feeding source through VOCs. An expected result was that mixture represented the most chosen stimulus. Olive pomace does not cover the volatiles coming from wheatmeal and it does not have any repellent effect. Germinara et al., (2002, 2008) for other stored product pests, such as *S. granarius*, showed that volatiles emitted by wheat and their derivatives significantly attracted these insects. Moreover, Germinara et al. (2015) demonstrated that certain substances, which are repellent at given amount, become attractive when provided at increased quantities or if they

are mixed with other components. In conclusion, this study demonstrated that the mixture and the wheatmeal were always the most chosen stimuli for both instars showing a significant attractiveness. However, further research on VOCs emitted by olive pomace has needed to better understand their behaviour.

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CHAPTER 4

Behavioral responses to food volatiles in a two-choice pitfall bioassay by adults of *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae).

Abstract

In the present study, the behavioral responses of adults of the yellow mealworm beetle *Tenebrio molitor* to Volatile Organic Compounds (VOCs) emitted by wheatmeal were investigated in a two-choice pitfall olfactometer. Five doses, ranging from 1 g to 200 g of wheatmeal were tested. The data showed that adults of the yellow mealworm beetle have the ability to respond behaviorally to cereal volatiles emitted by the wheatmeal and that responses may change as a function of concentration of these volatiles. All doses elicited positive responses to beetles. Females showed higher Response Index (RI) to higher doses of wheatmeal respect to males which were attracted at the same level to the different doses. In the pitfall bioassays increasing doses of organic wheatmeal attracted adults of this pest. Moreover a preliminary electroantennography tests on 16 different VOCs extracted from wheatmeal on this adult beetle is presented. An understanding of how the beetle respond to such volatiles could be useful for the development of effective integrated pest management strategies

Keywords: yellow mealworm, Coleoptera, Tenebrionidae, behavioral bioassay, wheatmealvolatiles, pitfall olfactometer, electrophysiology.

Introduction

The yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is a serious pest of stored cereals worldwide. Infestations not only cause significant losses due to the consumption of stored products; they also result in elevated temperature and moisture conditions that lead to an accelerated growth of molds, including toxigenic species (Sauer *et al.*, 1984; Magan *et al.*, 2003). Recent legislation that limits the use of fumigants and broad-spectrum contact insecticides, with increasing consumer demand for safe food, makes the control of such storage pests difficult. Furthermore, as larvae develop in the substrates, any effective control strategy requires the early and accurate monitoring of adult beetles (Germinara *et al.*, 2002, 2008). The use of semiochemicals has stimulated interest, as they may be used to improve current monitoring as well as direct means of controlling stored-product insect pests (Phillips 1997; Cox 2004, Germinara *et al.*, 2002, 2008). There are very few studies on *T. molitor* behavior to volatile emitted by cereals for its pest management. In literature it was demonstrated that phytophagous insects use volatiles from plant materials to locate suitable substrates (Dickens 1984; Visser 1986), and it has been shown that extracts from carob pods and peanuts (Collins *et al.*, 2004,

2007; Wakefield *et al.*, 2005) as well as specific components attract (Collins *et al.*, 2007) as well as specific components attract granary weevil adults (Collins *et al.* 2007) Moreover in literature, granary weevil adults have been more investigated; studies on the granary weevil adults showed that they orient to volatile blends emitted by grains of several cereal species (Levinson and Kanaujia 1981; Rietdorf and Steidle 2002), and the presence of phagostimulatory compounds are considered crucial in the infestation process by this pest (Kanaujia and Levinson 1981). To determine the release of semiochemicals that can route insect orientation, and to identify Volatile Organic Compounds (VOCs) acting as attractants, the emissions of wheatmeal were sampled by Head-Space Solid Phase Micro-Extraction (HS-SPME) and analysed through Gas-chromatography/Mass spectrometry (GC/MS) techniques. Electrophysiological investigation were carried out on antennae of *T. molitor* adults. In particular electroantennography (EAG) is a bioassay widely used in experimental entomology for the detection of volatiles perceived by the antennal olfactory apparatus of insects (Roelofs, 1984; Schneider 1957). To better understand the behavior of stored product pest electrophysiology investigations are carried out to better analysis their responses to Volatiles Organic Compounds (VOCs) emitted by different sources. Although the precise mechanism behind the EAG signal is not known, it is generally assumed that the measured voltage fluctuation is caused by electrical depolarisations of many olfactory neurons in the insects

antenna. The amplitude of an EAG response increases with increasing stimulus concentrations until a saturation level is reached. The amplitude is further dependent on the nature of the stimulus, the insect species and many less well defined factors (Schneider *et al.*, 1967). The EAG method can be used for many purposes like screening biologically active compounds, purification of extracts, identification of active fractions, selection of active synthetic compounds, concentration measurements in the field, and as a detector in gas chromatography. The quality of EAG signals is dependent on many factors, which are not always well recognised, and the large variety of insects demands a flexible attitude and a sense for improvisation of the operator. Although the basic principles are always the same, EAG recording methods may differ in many details. The large variety of insects and the differences in antennal morphology make adaptations in the recording technique necessary and require an inventive attitude. In literature, male and female of the granary weevil respond to various extracts from stored winter wheat (Levinson and Kanaujia 1982) and to lipid fractions that consist mostly of triacylglycerols and fatty acids (Nawrot *et al.*, 1995). Moreover, Chambers *et al.* (1996) showed that a wheat extract mixture was electroantennographically active but showed no specific peaks of activity in using the combined gas chromatography–electroannographic detection approach to the granary weevil. In previous studies, Germinara *et al.*, 2002 observed that both male and female *S. granarius* antennae responded to a wide range of synthetic compounds

found in cereal volatiles. Despite this behavioral evidence and the identification of many volatiles emitted by grains of several cereal species (Maga 1978; Zhou *et al.*, 1999; Sides *et al.*, 2001), little attention has been given to the response of *T. molitor* adults to compounds from wheatmeal. In this study, we examined the behavioral response of *T. molitor* adults to different doses of volatiles emitted by wheatmeal useful for the yellow mealworm pest management. Information are available about the morphology of the antennal sensory apparatus of this pest. Antennae bear sensory sensilla; these structures play an important role in the recognition of various stimuli for finding suitable habitats and food and for locating mates. Several reports have characterized the antennal sensilla of *T. molitor* both of larvae and adults, including their morphology through microscopy techniques (Harbach *et al.*, 1987; Bloom *et al.*, 1982; Bloom *et al.*, 1989). These previous morphological studies will help us to carry out electrofisiological investigations, in order to define new control methods for this stored product pest. The olfactory sensitivity of *T. molitor* adults to VOCs extracted from wheatmeal was investigated in more detail by behavioural and electrophysiological studies, and the responses elicited by single compounds were recorded.

Materials and methods

Pitfall olfactometer tests

Insect source. *T. molitor* were reared on wheatmeal for several generations in continuous dark at $28\pm 1^{\circ}\text{C}$ and $60\pm 5\%$ relative humidity (r.h.). New emerged adult beetles of 3-4 days old, divided by sex, were used for the experiments.

Pitfall bioassays. The behavioral response of *T. molitor* adults to different doses of wheatmeal was measured by using a two-choice pitfall bioassay, similar to those described by Phillips *et al.* (1993), Pike *et al.* (1994) and Germinara *et al.* (2008). The test arena was a steel container (32 cm diameter \times 7 cm high) with two diametrically opposed holes (3 cm diameter) located 3 cm from the side wall. Test stimuli were placed in glass flasks (500 ml) positioned under each hole, and the inside surfaces of their necks were coated with mineral oil to prevent captured insects from returning to the arena. The floor of the arena was covered with filter paper (Whatman No. 1) to provide a uniform surface and to facilitate insect movements (Pike *et al.*, 1994; Germinara *et al.*, 2008). Twenty insects, divided by sex, deprived of food for at least 4 h, were placed under an inverted Petri dish (5 cm diameter \times 1.2 cm high) at the center of the arena and allowed 30 min to acclimate. They were then released and tested for 3 h in the dark at $28 \pm 1^{\circ}\text{C}$ and $60 \pm 5\%$ r.h. Experiments were carried out between

10:00 A.M. and 4:00 P.M. During the assay, the arena was covered with a steel lid to prevent insects from escaping. Insects were given a choice between a specific dose of wheatmeal (1, 10, 50, 100 and 200 g) and air, used as a control. There were five replicates/sex of each assay, and insects were only used once.

Data analysis. In each trial, a response index (RI) was calculated by using $RI = [(T - C) / Tot] \times 100$, where T is the number responding to the treatment, C is the number responding to the control, and Tot is the total number of insects released (Phillips *et al.*, 1993). Positive values of RI indicate attraction to the treatment, while negative ones indicate repellence. The significance of the mean RI in each treatment of the two-choice pitfall bioassay was evaluated by the Student's t test for paired comparisons (Phillips *et al.*, 1993). Data were submitted to linear regression analysis to evaluate the effect of the dose on the insects' response. The most significant positive or negative mean values of RI were first analyzed by an analysis of variance and subsequently ranked by using the least significant difference (LSD) multiple range test ($P=0.05$).

Odour Stimuli

Chemical analysis. Different sample of wheatmeal (each 2 g) were placed in glass bottles (250 mL) for 2 h to confine the odor source and then, headspace volatiles were collected using a solid-phase microextraction (SPME) fiber. A 2 cm

manual injector and a $\sim 50/30\ \mu\text{m}$ DVB/CAR/PDMS StableFlex fiber head was inserted into the mouth of the bottle. Volatiles were extracted for 40 min at $80\ ^\circ\text{C}$, then the fiber head was quickly removed and inserted into the injection port of the gas chromatograph (GC) ($250\ ^\circ\text{C}$, run in splitless sampling mode) (Germinara *et al.*, 2002; 2008). Thermal desorption was carried out for 5 min. The collected volatiles were analyzed by GC-MS (HP6890/5975C, Agilent Technologies). The chromatographic column was a ZB-5MSI 5% phenyl-95% dimethylpolysiloxane elastic quartz capillary vessel column ($30\ \text{m} \times 0.25\ \text{mm} \times 0.25\ \mu\text{m}$); the gas chromatograph was operated at an initial temperature of $40\ ^\circ\text{C}$ for 2 min, then increased at $5\ ^\circ\text{C}/\text{min}$ to $255\ ^\circ\text{C}$, which was maintained for 2 min. The temperatures of the vaporizing chamber, interface, and quadrupole rod were $250\ ^\circ\text{C}$, $280\ ^\circ\text{C}$, and $150\ ^\circ\text{C}$, respectively. To identify compounds, we compared the mass spectra of compounds with those in databases and with mass spectra data of an home made dataset of compounds previously analysed. The carrier gas was high-purity helium (99.999%) and the column head pressure was 7.62 psi to ensure that the flow rate of carrier gas was 1.0 mL/min. Additional parameters were as follows: delay time of solvent, 1.0 min; ion source, EI; temperature, $230\ ^\circ\text{C}$; ionization potential, 70 eV; emission current, $34.6\ \mu\text{A}$; voltage of the multiplier, 1671 V; and scanning from 29 to 500 atomic mass units (Germinara *et al.*, 2008).

Electrophysiology investigations

Insect source. Insect used in this study were derived from a laboratory colony at the Wageningen University (The Netherlands). The rearing of *T. molitor* were maintained in a climatic chamber at 28 °C, 60%RH with a 24L:0D photoperiod. Pupae were isolated until emergence. Three-four days post emergence, adults of *T. molitor* were isolated 12h before the electrophysiology test and placed in Petri Dishes (3.5 cm diameter) in a dark environment at 28°C and 60% RH in starving conditions.

Electroantennography. Test compounds were 16 VOCs selected to represent two different chemical classes (alcohols and aldehydes) extracted from wheatmeal (Table 4.2). The chemicals used for the EAG assay were obtained from Sigma-Aldrich Co. The recording electrode was bowl shaped and the last half segment of the antenna inserted into the recording electrode, and the indifferent electrode was inserted into the head. In the experiments, 20 µL of each test solution was absorbed onto a filter paper strip (4× 40 mm) and placed into a Pasteur pipette (with a diameter of 10 mm and a length of 15 cm) to serve as an odor cartridge after the solvent (hexadecane) had completely evaporated from the paper. Controls consisting of a filter paper strip treated with 20 µL of hexadecane alone were prepared. A Pasteur pipette with a test compound was inserted into a small hole in the wall of a steel tube (with a diameter of 15 mm diameter and a length of 15 cm), which was connected to a

stimulus air controller (model CS-05b, Syntech, the Netherlands) to deliver a constant flow of humidified air at a rate of 40 mL/min. The open end of the steel tube was positioned 10 mm from the antenna. During the stimulation, air at a rate of 20 mL/min was applied through the pipette into the main airflow for 0.2 s. An interval of at least 1 min between puffs was used to ensure complete antenna recovery. Serial dilutions of each compound from the lowest to the highest concentration were included 0,1%, 1% and 10% (Delorme and Payne 1990; Den Otter et al. 1996). An air control, an hexadecane-only control and a standard solution (cis-3-hexenylacetate at 10% in hexadecane) were tested before and after each test compounds (Zhang *et al.*, 2010). Each compound at each concentration was tested reaching a total of 8 complete random series. In total, 51 stimuli (48 odorants, plus a solvent control, air control and standard control), consisting of compounds extracted from wheatmeal were used. The EAG responses will be corrected for solvent and other background effects by subtracting the mean response to the solvent-only controls before and after exposure to each sample from the response to the test compound. To compensate for the decline in the sensitivity of the antennae during the experiment and individual differences in the test insects, the EAG data were standardized by calculating the EAGs as percentages relative to the response to the standard solution, which allowed direct comparisons of the responses obtained from different preparations.

Data analysis. Mann–Whitney tests were performed using SPSS (1999) to determine the significance of differences in the relative EAG responses between the sexes. Chi square tests were performed using SPSS (1999) to assess the differences in the responses to the different compounds measured through the olfactometer bioassays. In the dose-response curves, the activation threshold will be considered to be the lowest dose at which the lower limit of the standard error of the mean response was greater than the upper limit of the standard error for the lowest dilution tested; the saturation level will be taken as the lowest dose at which the mean response was equal to or less than the previous dose. EAG responses to each dose of the VOCs tested were compared using Student's t test (with significant differences corresponding to $P \sim 0.05$).

Results

Pitfall bioassay. This study showed that adults beetle from both sex are attracted to increase doses of volatiles emitted by wheatmeal. In particular with an increase in the dose of wheatmeal there is an increase in the response index both for males and females. Males showed higher mean RI than females ranging from values of RI from 49 in the lower dose to 77 higher dose (Fig 4.2). RI in females range from 46 in 1g dose to 72 in 200g dose of wheatmeal (Fig 4.1). There was no significant difference between sexes ($df= 1$; $F = 3.85$; $p = 0.085$). However, among doses, it was found a

significant difference for the higher dose in females ($df = 24$; $F = 13.07$; $p = 0.00145$) (Tab 4.1).

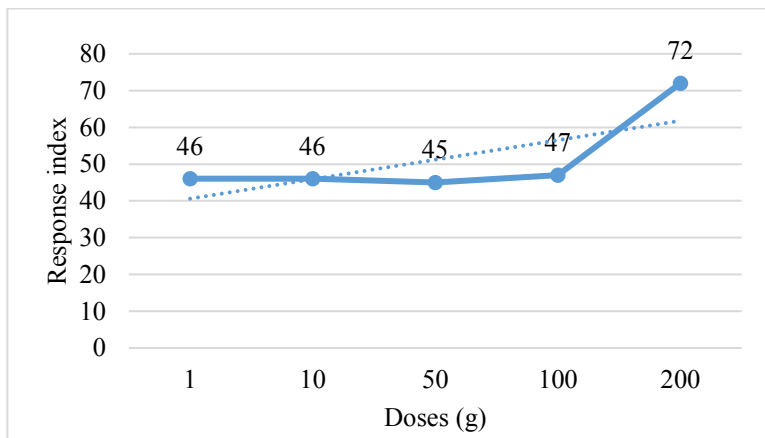


Figure 4.1 Response Index curve in females.

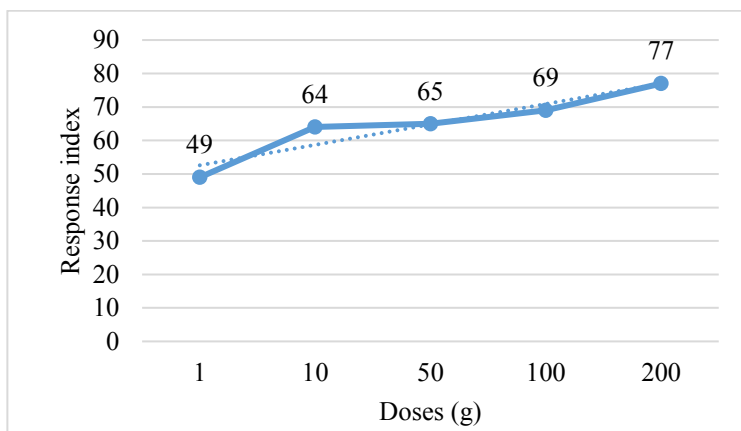


Figure 4.2 Response Index curve in males.

Table 4.1. Response of adult to increasing doses of wheatmeal volatiles in two-choice pitfall bioassays, determination coefficient (R^2) of log (dose)–response linear regression analysis and its significance (p)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (significant response to experimental stimulus; paired-sample t test); ^a $N = 5$

Sex	Response Index (Mean \pm SE) ^a					R^2	p
	1g	10g	50g	100g	200g		
Males	49 \pm	64 \pm	65 \pm	69 \pm	77 \pm	0.14	0.035
	18.50	27.92	18.37	9.61	7.58		
Females	46 \pm	46 \pm	45 \pm	47 \pm	72 \pm	0.33	0.001
	10.83	10.83	20.92	9.74	5.7 ***		

Chemical analysis. The collected volatiles were analyzed by GC-MS (HP6890/5975C, Agilent Technologies). In table 4.2 are listed the 11 most abundant compounds identified in the headspace of the wheatmeal and then tested on antennae of adults.

Electroantennography responses. EAG tests showed that the antennal olfactory systems of *T. molitor* are able to perceive several VOCs emitted by wheatmeal (Fig 4.3). In particular the largest EAG amplitudes were recorded for 1-heptanol, 1-hexanol, (E)-2-hexenal (E)-2-heptenal, hexanal, (E,E)-2,4-heptadienal (Tab. 4.3). The responses to these compounds were not significantly different from each other ($P > 0.05$, Tukey test). Further statistical analysis are needed to better understand their responses.

Table 4.2 Chemical purity of VOCs extracted by GC-MS from wheatmeal tested in electroantennography tests.

Retention time	Peack number	Compound name	Area tot %
7.241	1	Hexanal	35.231
7.994	2	Hexanol	10.556
9.233	3	Heptanal	1.108
9.988	4	Hexanoic acid	2.214
11.397	5	(2E,4E)-2,4-Nonadienal	1.369
12.566	6	E-2-octenal	2.901
13.205	7	Heptanal	23.219
14.401	8	E-2-nonenal	2.087
16.162	9	Octanal	4.145
16.322	10	Nonanal	1.854
17.851	11	Tridecane	11.125

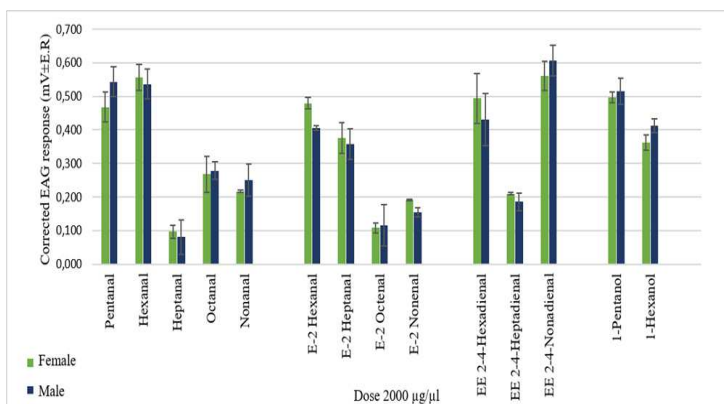


Figure 4.3 EAG response profile of adult of *T. molitor* to a range of VOCs emitted by the wheatmeal.

Table 4.3 EAG responses of *T. molitor* adults to a range of volatile organic compounds (VOCs).

Class compound	Chemical purity (%)	Absolute EAG response in mV (Mean \pm SE)	
		Females	Males
<i>Aliphatic aldehydes</i>			
Pentanal	95.0	0.468 \pm 0.04	0.544 \pm 0.05
Hexanal	98.0	0.556 \pm 0.04	0.536 \pm 0.04
Heptanal	95.0	0.097 \pm 0.02	0.081 \pm 0.05
Octanal	99.0	0.268 \pm 0.05	0.278 \pm 0.03
Nonanal	95.0	0.217 \pm 0.00	0.251 \pm 0.05
E-2 Hexanal	99.0	0.479 \pm 0.02	0.405 \pm 0.01
E-2 Heptanal	97.0	0.376 \pm 0.05	0.358 \pm 0.05
E-2 Octenal	94.0	0.108 \pm 0.01	0.116 \pm 0.06
E-2 Nonenal	97.0	0.191 \pm 0.00	0.154 \pm 0.01
EE 2-4-Hexadienal	95.0	0.494 \pm 0.07	0.431 \pm 0.08
EE 2-4-Heptadienal	88.0	0.210 \pm 0.00	0.186 \pm 0.03
EE 2-4-Nonadienal	85.0	0.561 \pm 0.04	0.607 \pm 0.05
<i>Aliphatic alcohols</i>			
1-Pentanol	99.0	0.496 \pm 0.02	0.515 \pm 0.04
1-Hexanol	98.0	0.363 \pm 0.02	0.412 \pm 0.02

Discussion

Our results show that many wheatmeal volatiles, known to be detected by antennal sensilla of other stored product pests (Germinara *et al.*, 2008), also elicit a behavioral response at all doses in *T. molitor* adults. For the first time, males and

females were analysed separately and compared. Females were attracted by higher doses of wheatmeal, meanwhile males did not show any significant correlation among doses. It can be assumed that the females choice depends to the necessity to find a suitable oviposition site. In insect such as beetles, which feed as adults on the same source for their offspring, no clear discrimination can be made between orientation to feeding or oviposition sites. Wheatmeal can be a source of nutrition needed for egg maturation and at the same time provide an oviposition site. On the other hand attraction or anemotaxis to volatiles by female insects might not be directly related to oviposition but rather reflect orientation to food sources. The dose of 200g of wheatmeal was significantly attractive to *T. molitor* adults for both males and females. The quantity of substrate is clearly influencing the choice, probably because a higher amount of volatiles compounds should be associated to a higher quality of the feeding substrate for larvae development. The yellow mealworm beetle produces several different types of pheromones, including those which mediate the aggregation (Weaver *et al.*, 1989). In fact, males of *T. molitor* were also attracted by lower doses of wheatmeal and this can be related to the production of those aggregation pheromones. Pheromone produced by males of *T. molitor* stimulates locomotor activity in the female and also stimulates a group of females to assemble (aggregate) in the vicinity of the male (August, 1970). The results reported here are of significance from a management perspective. Specific blends of synthetic attractants may be used to monitor

and/or trap the adult beetles. These could be deployed alone or in combination with the aggregation pheromone of *T. molitor* as synergistic or additive effects have been reported for several species (Landolt and Phillips 1997). Consequently, a clear understanding of the biological activity of different wheatmeal volatiles, individually and in combination, will be essential for developing semiochemical for the management strategies. Furthermore, as noted above, considerable attention will not only have to be given to the selection of the actual compounds but also to the specific concentration to be used. Furthermore, one must also consider the impact that individual compounds or blends might have on other species that utilize the same resources to ensure that compounds to repel one pest is not an attractant for another. Such differential responses to the same semiochemicals that originate from a heterogeneous food substrate undoubtedly play an important role in niche partitioning within the stored-cereal ecosystem (Phillips *et al.*, 1993). However, a failure to understand these interactions when deploying lures to control one species may lead to an increase in the density of other pests. Regarding the preliminary results from the electroantennogram it can be supposed that *T. molitor* behavior is related to specific doses of single compounds that can act as attractant to adults of this pest. An electroantennogram is the summation of receptor potentials evoked by an olfactory stimulus from various sensilla on the antenna that are tuned to the chemical tested. Compounds that are EAG active to an insect species are frequently of

ecological significance and, therefore, may act as insect behavior-modifying compounds. However further analysis using electrophysiology techniques are needed to clarify their behavior for an efficient control of this pest. The EAG experiments indicated that the antennae of *T. molitor* are able to detect a broad range of volatiles emitted by the wheatmeal. Olfactory responses of antennae of *T. molitor* adults were compared in only a few studies with contrasting conclusions. However some of the compounds which were described for *S. granarius* (Germinara *et al.*, 2008) were in a good extent the same compounds perceived by *T. molitor*. In conclusion, as in other generalist stored product pest, the olfactory receptor system of of *T. molitor* is adapted to perceive a wide variety of cereal volatiles. Elucidation of the behavioural activity of the EAG-active compounds reported here will contribute to set up *T. molitor*-based control strategies. For example, attractant chemicals could be used to improve the monitoring and control methods of this pest.

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FUTURE RESEARCH

Insects can be foreseen as the future perspective to feed the 9 billion people in 2050 as well as possible way to try to reduce the amount of food wastes generated by the industry. Most of the by-product that nowadays are produced are reach of nutritional compounds, suitable as feeding substrates for insects, as *Tenebrio molitor*. The knowledge of the larval performances over time, as well as its behaviour response to different sources of volatiles it's fundamental to better understand its ecology and to guarantee the best growth and the best nutritional composition for an optimization of its rearing. Moreover the inclusion of a right amount of a waste in the diet of the insect can help us to dispose the waste faster and in an efficient way in an optic of a circular economy. Further deeper studies on the VOCs emitted by the different stimuli are needed. Knowing the right composition and VOCs, of olive pomace in particular, will allow us to understand its impact on the insect both in development and behavioural aspects. Moreover further behavioural bioassays and electrophysiology tests, using in particular wheatmeal and olive pomace volatiles, are needed to better understand their impact on the behaviour and on the sensory structure of this pest, in order to implement new control and management methods.

In conclusion, further researches are needed to provide more information to explore: (1) the potential of by-products as feeding substrates to improve its rearing (2) the inclusion of

waste in diets for their disposal (3) volatiles emitted by olive pomace and wheatmeal in order to use them in behavioural and electrophysiology tests to better understand *Tenebrio molitor* biology.

Scientific research on the potential of nature is the only way to preserve our future, to improve our quality of life, where the humankind will be able to satisfy its growing needs without affecting the planet.