Quality and safety aspects of mealworms as human food

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Abstract

Insects are consumed in the tropics in many parts of the world. However, they do not form a part of the diet in Western countries. Nowadays, strong arguments exist to expand the consumption of edible insects to the Western world. The world population is growing and with a growing welfare, the demand for animal protein is increasing. Production of conventional livestock is associated with detrimental effects on the environment, such as global warming, land degradation and loss of biodiversity. The nutritional value of edible insects is comparable to that of conventional meat, and insect production has several benefits over the production of conventional livestock. Insects emit less greenhouse gases per unit of growth, need less land area to be produced, and can be grown on organic by-products. In addition, insects do not invest energy in keeping a constant body temperature and can therefore invest more energy in growth. This means they require relatively less feed to gain biomass, compared to conventional livestock.

This thesis focuses on three mealworm species: the Yellow mealworm (*Tenebrio molitor* L.), the Giant mealworm (*Zophobas morio* Fab.) and the Lesser mealworm (*Alphitobius diaperinus* Panzer). Mealworms are already mass-produced as feed for pets such as reptiles and birds. In addition, they are promising candidates for human consumption. The aim of this thesis is to 1) explore the possibility to produce mealworms more sustainably on certain organic by-products and 2) to investigate potential food safety risks associated with edible mealworms. The thesis starts with a brief introduction on the concept of insects for human consumption, followed by an overview of the nutritional value of the three mealworm species. Possible food safety risks of edible insects, with an emphasis on mealworms, are discussed. In Chapter 2, the three mealworm species were produced on diets composed of organic by-products, differing in protein and starch content. Large differences in growth and development of mealworms were observed on the different diets. Furthermore, diet affected feed conversion efficiency and fatty acid profile of the insects. The results show that diet can be used to alter mealworm growth speed and harvest weight, and, to a certain extent, their fatty acid profile. In Chapter 3, risk of contamination of *T. molitor* with a common mycotoxin, deoxynivalenol, was investigated. The mycotoxin, present in the larval diet in naturally realistic concentrations, did not affect growth and survival of *T. molitor*. After harvest, the mycotoxin was not detected in the larval bodies, and appeared to be in part excreted through larval faeces. Chapters 4 and 5 explored the potential of allergic cross-reactivity of mealworm protein. In Chapter 4, the allergic potential of *T. molitor* was assessed *in vitro* using sera from individuals allergic to House dust mites and crustaceans. Results showed that sera of these allergic individuals did cross-react with protein from *T. molitor*, which means the possibility of an allergic reaction upon consumption of this mealworm species is realistic. Chapter 5 expands on the study of

Chapter 4 by assessing the allergic potential of all three mealworm species *in vitro* using sera from individuals allergic to either House dust mites or crustaceans. In addition, influence of the food processing methods boiling, frying and lyophilisation on allergenicity are determined, as well the effect of *in vitro* digestion. Results show that protein of all three mealworm species did cross-react with sera from the allergic individuals. Heat processing and *in vitro* digestion did reduce, but not eliminate this reaction. The final chapter of this thesis discusses the obtained results in a broader context and identifies topics of further research contributing to the introduction of edible insects. Furthermore, different aspects that may create barriers to the introduction of edible insects are discussed, such as consumer acceptance and legislation.

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Chapter 1

General introduction

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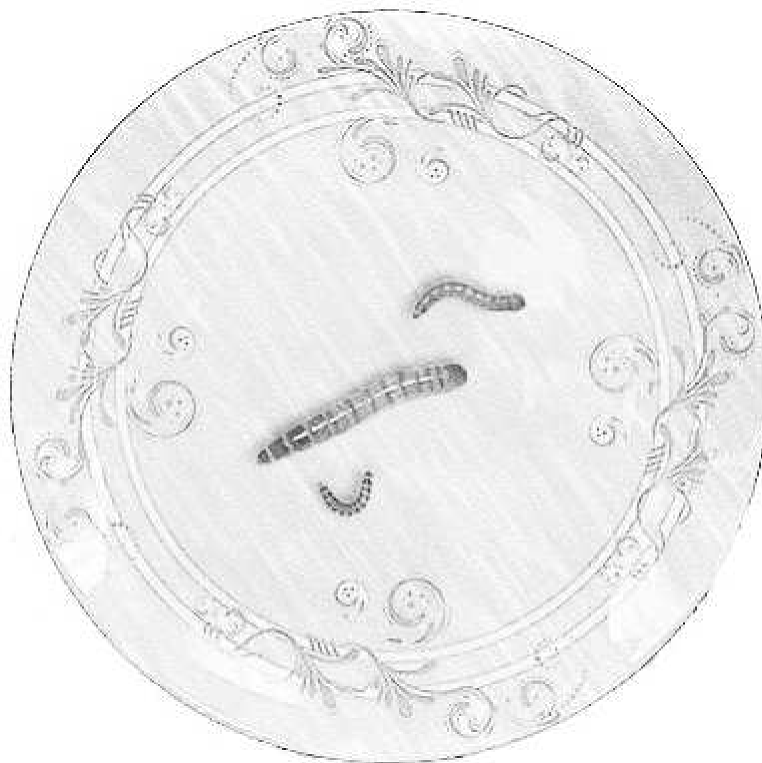

Insects as food

The consumption of insects as food by human beings, also called entomophagy, is as old as mankind. In his 1951 "Insects as human food, a chapter on the ecology of man", Bodenheimer, (1951) extensively reviewed records of insects being consumed throughout human history. For example, several insect species suitable for human consumption are listed in the Bible (Leviticus) such as crickets and grasshoppers. The ancient Greeks and Romans were familiar with eating cicadas and mentioned locusts being consumed by different tribes (Bequaert, 1921; Bodenheimer, 1951).

Today, entomophagy is still practised in tropical and subtropical countries in Africa (Ramos-Elorduy, 2009; Van Huis, 2003), Asia (Meyer-Rochow and Changkija, 1997; Yen, 2015; Yhoung-Aree et al., 1997) and Latin America (Cerda et al., 2001; Costa-Neto, 2015; Ramos-Elorduy, 2009), and by aboriginal people of Australia (Meyer-Rochow and Changkija, 1997; Yen, 2009b, 2015). Worldwide, over 2000 species of insects are consumed by humans (Jongema, 2014). Insects are most often collected from the wild, although (semi-)cultivation systems are used for the production of some species (Van Itterbeeck and Van Huis, 2012), such as the rearing of Domesticated silkworm pupae (*Bombyx mori* L.) in Asia (DeFoliart, 1995) which are a by-product of silk production and the harvesting of eggs from aquatic water bugs (Hemiptera: Corixidae) for human consumption in Mexico (Ramos-Elorduy, 2006). In the West, insects are no longer a part of the human diet and the practice of entomophagy is often considered primitive and disgusting (DeFoliart, 1999; Yen, 2009a).

In the 19th century, both Riley (1877) and Holt (1885) advocated the consumption of insects by humans in the Western world. Both authors were aware of the practice of entomophagy in other cultures. When observing problems caused by pest insects consuming food crops, they suggested the insects as a nutritious and palatable alternative food source which would aid in averting starvation, especially among the rural population. Both authors were also aware of the Western bias against insects as food. In the early 20th century, Bequaert (1921) acknowledged the widespread practice of entomophagy among indigenous people in tropical countries, but in contrast to Holt, he did not advocate the consumption of insects in the West, because of the bias.

More recently, awareness of entomophagy was created by among others DeFoliart (1992, 1995, 1999), Ramos-Elorduy (1990, 1997), Van Huis (2003, 2005) and Meyer-Rochow (1997). Due to the negative Western view on entomophagy, there is a risk the practice will go out of favour amongst indigenous people, while an alternative protein source to replace insects in their diet is often not available (DeFoliart, 1999; Ramos-Elorduy, 1997). In addition to being an important protein source in tropical countries, strong arguments exist to expand the practice of entomophagy to the Western world. The world population is expected to exceed 9 billion in 2050 and the demand for animal derived

protein is expected to increase with 75% (Alexandratos and Bruinsma, 2012). Production of conventional livestock is associated with several detrimental environmental effects, including global warming, land degradation, and loss of biodiversity (Steinfeld et al., 2006). Insects emit less greenhouse gases per unit of growth (Oonincx et al., 2010) and need less land area to be produced compared with conventional livestock (Oonincx and De Boer, 2012). Furthermore, as insects are poikilotherm (cold blooded) they do not invest energy in keeping a constant body temperature, and can therefore invest more energy in growth (Nakagaki and DeFoliart, 1991), requiring relatively less feed to gain biomass. This makes insects more efficient production animals than conventional livestock. Nutritional value of insects varies depending on species and developmental stage, though many species are rich in protein and fat, essential amino acids and fatty acids as well as vitamins and minerals (Bukkens, 1997; Rumpold and Schlüter, 2013).

In the Western world, edible insects need to be produced in closed farming systems to allow for year-round production. Promising candidates to commence the introduction of edible insects are species that are already being produced as pet food and for use in laboratories, such as crickets, locusts and mealworms. Nutritional value as well as food safety risks need to be addressed when introducing insect species on the Western market for human consumption (Belluco et al., 2013; Rumpold and Schlüter, 2013).

Mealworms

Mealworms are the larval stage of Darkling beetles (Coleoptera: Tenebrionidae; Figure 1). The insects are commercially produced in the Western world as pet food for reptiles and birds and as fish bait. In addition, they are promising species for human consumption. Recently, several companies in The Netherlands have set up separate production lines to produce mealworms for human consumption. This thesis focusses on three species of edible mealworms: the Yellow mealworm (*Tenebrio molitor* L.), the Giant mealworm (*Zophobas atratus* Fab.) and the Lesser mealworm (*Alphitobius diaperinus* Panzer), all three of which are produced in The Netherlands. Presently, only *T. molitor* and *A. diaperinus* are produced for human consumption; however, *Z. atratus* is edible (Ramos-Elorduy, 2009).

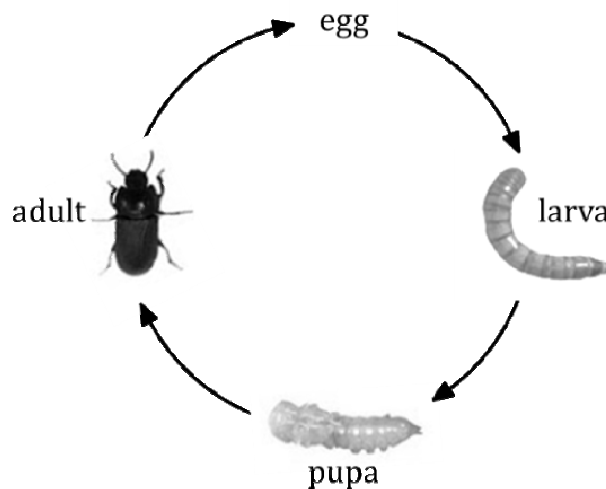

Figure 1: Life cycle of mealworm species (here *Tenebrio molitor*).

Tenebrio molitor grows to a body length of about 2.5 cm and is a common stored product pest feeding on grain and cereal products. *Zophobas atratus* is originally from the American continent and is often found in bat guano and other organic litter in nature (Tschinkel, 1984). This species resembles *T. molitor* in shape and colour, but grows to a larger size of about 3.8 to 5.7 cm (Quennedey et al., 1995). *Alphitobius diaperinus* is a tropical beetle. It frequently inhabits poultry houses in colder climates, where it feeds on poultry fodder, manure and dead poultry (Despins et al., 1994; Dunford and Kaufman, 2006). The species grows to about 0.75 cm. Development time of these species depends on different factors including temperature (Rueda and Axtell, 1996; Wilson and Miner, 1969), moisture (Murray, 1968; Urs and Hopkins, 1973a, b), diet quality (Fraenkel et al., 1950; Hosen et al., 2004; Morales-Ramos et al., 2010; Ramos-Elorduy et al., 2002), larval density (Barnes and Siva-Jothy, 2000; Quennedey et al., 1995; Weaver and McFarlane, 1990) and strain differences (Urs and Hopkins, 1973a, b). In addition, mealworms show plasticity in number of larval instars, which appears to be influenced by the same parameters as overall development time.

With respect to nutritional research on mealworm species, most research has focused on *T. molitor* as a feed insect for insectivorous animals (Barker et al., 1998; Finke, 2002; Pennino et al., 1991; Ramos-Elorduy et al., 2002) and, to a lesser extent, for human consumption (Ghaly and Alkoaik, 2009). Several studies compared the nutritional value of *T. molitor* with *Z. atratus* (Barker et al., 1998; Finke, 2002; Pennino et al., 1991). Research on the nutritional value of *A. diaperinus* is scarce (Despins and Axtell, 1995). Table 1 summarises the approximate nutrient composition as found in the abovementioned literature. Moisture content of *T. molitor* and *Z. atratus* is comparable. Of the three species, *A. diaperinus* has the highest protein content and *Z. atratus* has the highest fat content. Carbohydrate content was calculated by subtracting the other nutritional components from

total dry weight. A higher carbohydrate content was found for *T. molitor* than for *Z. atratus*. Chitin, a complex carbohydrate and main component of the insect exoskeleton, was included in fibre. A wide range in fibre content was reported for *T. molitor*, with the value from Ramos-Elorduy et al. (5.0; 2002) being lower than the values found by Barker et al. (14.5; 1998), Finke (21.6; 2002) and Pennino et al. (18.9; 1991). Differences in values can occur due to differences in insect strains, diets provided to the insects, and analytical methods used to determine nutritional components.

Table 1: Approximate nutrient composition (minimum – maximum or average values reported as % DM unless stated otherwise) of *Tenebrio molitor*, *Zophobas atratus* and *Alphitobius diaperinus*.

Component	*Tenebrio molitor*			*Zophobas atratus*			*Alphitobius diaperinus*
Moisture (% of fresh weight)	58.1	–	61.9	55.6	–	57.9	-
Crude protein	45.0	–	68.9	40.6	–	46.8	67.9
Crude fat	23.0	–	37.7	40.8	–	44.9	20.7
Carbohydrates			7.1			2.6	-
Fibre	5.0	–	21.6	13.0	–	13.6	7.3
Ash	2.4	–	4.3	2.4	–	8.6	5.0

-: No information available.
Sources: moisture content for *T. molitor* and *Z. atratus* (Barker et al., 1998; Finke, 2002; Pennino et al., 1991), *T. molitor* only (Ghaly and Alkoaik, 2009); crude protein, crude fat and ash content for *T. molitor* and *Z. atratus* (Barker et al., 1998; Finke, 2002; Pennino et al., 1991), *T. molitor* only (Ghaly and Alkoaik, 2009; Ramos-Elorduy et al., 2002), *A. diaperinus* (Despins and Axtell, 1995); carbohydates for *T. molitor* and *Z. atratus* (Finke, 2002), *T. molitor* only (Ramos-Elorduy et al., 2002); fibre for *T. molitor* and *Z. atratus* (Barker et al., 1998; Finke, 2002; Pennino et al., 1991), *T. molitor* only (Ramos-Elorduy et al., 2002) and *A. diaperinus* (Despins and Axtell, 1995).

Amino acid content of the different mealworm species was analysed by Finke (2002) for *T. molitor* and *Z. atratus* and by Despins and Axtell (1995) for *A. diaperinus* (Table 2). Values were comparable between species for most amino acids, except *A. diaperinus* appeared higher in methionine, tryptophan and histidine than the other two species. *Tenebrio molitor* was lower in glutamic acid than the other two species, but higher in taurine than *Z. atratus*. Taurine was not determined for *A. diaperinus*.

Fatty acid composition was only determined by Finke (2002) for *T. molitor* and *Z. atratus* (Table 3). The two mealworm species were predominantly high in palmitic, oleic and linoleic acid. *Zophobas atratus* was almost twice as high in palmitic and stearic acid as *T. molitor*, while *T. molitor* was higher in palmitoleic and linoleic acid.

Amino acid composition of all three mealworm species and fatty acid composition of *T. molitor* and *A. diaperinus* was more recently also determined by Yi et al. (2013) and Tzompa-Sosa et al. (2014) respectively. Because these studies and those reported in this thesis were part of the same research project, they will be discussed more elaborately in relevant experimental chapters and in the general discussion.

Table 2: Average amino acid content (g/kg DM unless stated otherwise) of *Tenebrio molitor*, *Zophobas atratus* and *Alphitobius diaperinus*.

Amino acid	***Tenebrio molitor***	***Zophobas atratus***	***Alphitobius diaperinus***
Essential			
Isoleucine	24.7	22.1	25.6
Leucine	52.2	45.4	40.3
Lysine	26.8	24.5	39.0
Methionine	6.3	5.0	18.7
Phenylalanine	17.3	16.2	27.9
Threonine	25.2	18.5	23.4
Tryptophan	3.9	4.3	10.9
Valine	28.9	24.5	32.9
Semi-essential			
Arginine	25.5	22.8	33.5
Histidine	15.5	14.3	26.0
Methionine + cysteine	10.5	8.6	23.9
Tyrosine	36.0	32.5	47.6
Non-essential			
Alanine	40.4	34.0	39.3
Aspartic acid	40.0	37.5	48.3
Cysteine	4.2	3.6	5.2
Glycine	27.3	22.6	28.9
Glutamic acid	71.7	53.7	70.3
Proline	34.1	25.6	35.6
Serine	25.2	21.9	25.6
Taurine (mg/kg)	210	ND	-

- : No information available.
ND: Not detected.
Sources: Finke (*T. molitor* and *Z. atratus*; 2002) and Despins and Axtell (*A. diaperinus*; 1995)

Table 3: Fatty acid content (g/kg DM) of *Tenebrio molitor* and *Zophobas atratus*.

Fatty acid			*Tenebrio molitor*	*Zophobas atratus*
Common name	**Lipid number**	**ω-n**		
Unsaturated				
Lauric acid	12:0		ND	ND
Myristic acid	14:0		7.6	4.0
Pentadecanoic acid	15:0		ND	1.0
Palmitic acid	16:0		60.1	125.4
Heptadecanoic acid	17:0		ND	1.7
Stearic acid	18:0		10.2	30.0
Arachidic acid	20:0		0.8	1.0
Monounsaturated				
Palmitoleic acid	16:1	ω-7	9.2	1.7
Heptadecenoic acid	17:1	ω-7	0.8	0.6
Oleic acid	18:1	ω-9	141.5	156.8
Polyunsaturated				
Linoleic acid	18:2	ω-6	91.3	78.1
Linolenic acid	18:3	ω-9	3.7	2.6
All others			0.5	0.5

ND: Not detected.
Source: Finke (2002).

Insect growth and development as well as nutritional composition can be altered by diet to certain extents (Anderson, 2000; Davis and Sosulski, 1974; Finke, 2002). This offers possibilities for adjusting mealworm production rate as well as nutritional composition to suit consumer's needs. Chapman (1998) states that *T. molitor* needs a dietary carbohydrate content of at least 40% in order to develop, and optimal growth is reached on a diet containing 70% carbohydrates. Additionally, larvae grow and develop faster when a source of moisture is available (Urs and Hopkins, 1973a, b). Larvae reared in the presence of moisture are heavier and this difference in weight is not due to a higher water content, but due to a higher fat content as well as a higher fat-free dry weight. However, there was no difference in larval fatty acid content compared with larvae grown on diets of low moisture content (Urs and Hopkins, 1973b). According to Davis and Sosulski (1974), *T. molitor* larvae gain twice as much weight when fed ground wheat containing 10% brewer's yeast (*Saccharomyces cerevisiae*) than when fed ground wheat only. Brewer's yeast is a source of protein (Aghdamshariar et al., 2006; Rumsey et al., 1991) and vitamins of the B complex (Copping and Honora Roscoe, 1937; Fraenkel et al., 1950). Davis and Sosulski (1974) determined *T. molitor* growth when fed an artificial diet containing 10% protein of either soybean, sunflower, safflower, turnip rape, rape or flax. Larvae fed diets containing protein from turnip rape or sunflower performed better than larvae fed diets containing flax protein. Rape- and safflower protein scored intermediately. Differences in amino acid composition could be an explanation for the differences in growth of the larvae on the different diets.

Ramos-Elorduy et al. (2002) reared larvae of *T. molitor* on different diets consisting of yeast, *T. molitor* excreta and different proportions of organic wastes. Larvae grew better on diets with a higher protein content derived from organic wastes and excreta than on diets with a higher protein content derived from yeast. Diets with relatively more protein derived from organic wastes and excreta contained almost as much protein as the wheat bran with yeast control diet, and larvae grown on these diets contained subsequently more protein than those fed the control diet. Hosen et al. (2004) tested the suitability of wheat, barley, corn and rice flour as diet for rearing *A. diaperinus* larvae, using whole meal flour supplemented with yeast as control diet. Larvae performed best on barley and wheat flour, and gained the least weight on rice flour. Subsequent differences in insect body composition were not determined. At present, no data on effects of diet on development and body composition of *Z. atratus* appear to be available.

Food safety risks

Food safety is of special importance when dealing with new food sources. In the context of edible insects, there are four ways through which food safety risks can arise, i.e. 1) the insect itself could be toxic; 2) the insect could have acquired toxic substances or human pathogens from its environment during its life cycle; 3) the insect could become spoiled after harvest; and 4) consumers could experience an allergic reaction to the insect. Research directly focussing on safety of mealworms as food for human consumption is scarce. However, literature is available on mealworms in relation to human pathogenic microbes, toxic substances and allergic reactions.

Toxicity of mealworms

Tenebrionid beetles possess defensive secretions that contain 1,4-benzoquinones (Attygalle et al., 1991; Hill and Tschinkel, 1985; Tschinkel, 1969; Tseng et al., 1971). These benzoquinones are toxic and possibly carcinogenic as was shown in animal experiments reviewed by Lis et al. (2011). The International Agency for Research on Cancer (IARC, 1999) has classified 1,4-benzoquinones as a Group 3 substance, meaning they are not classifiable as to its carcinogenicity to humans (IARC, 1999). Benzoquinones are not known to be present in the larval stages of Tenebrionidae, which are used for human consumption. Han et al. (2014) evaluated genotoxicity of powder made from freeze-dried *T. molitor* larvae. They did not observe any adverse effects both *in vitro* and *in vivo* after 28 days oral administration in rats.

Acquisition of harmful microbes and toxic substances

Mealworms can potentially acquire toxic substances, as well as microbes pathogenic to humans, from their environment, in particular through their diet. In nature, *A. diaperinus* is frequently found in poultry houses where it feeds on poultry feed as well as dead poultry and poultry waste which can be contaminated with pathogens (Despins et al., 1994; McAllister et al., 1994). Despins et al. (1994) found that *A. diaperinus* larvae exposed to turkey faeces containing enterovirus and rotavirus spread the pathogens to turkeys that fed on the larvae, even when the larvae were surface-sterilised. Beetles fed faeces infected with turkey coronavirus remained infective for 1 hour after exposure (Watson et al., 2000). Templeton et al. (2006) and Strother et al. (2005) discovered *A. diaperinus* to be short time carriers of *Campylobacter jejuni*. The bacteria were found on the outside of the body surface of both larvae and adults for up to 12 hours after exposure. After ingestion of a solution containing *C. jejuni*, the larvae carried live bacteria for up to 72 hours and shed the bacteria in their faeces for 12 hours, while the adults carried live bacteria for up to 15 hours and shed them in their faeces for 6 hours. After these time periods, no live bacteria could be detected (Strother et al., 2005). Furthermore, *A. diaperinus* can carry detectable live *Salmonella typhimurium* for up to 28 days after ingestion (McAllister et al., 1994). Adults shed the bacteria in their faeces for 28 days while larvae shed them for 13 days, including after moulting. No explanation was given for the difference in shedding time between adult and larval *A. diaperinus*. According to Davies & Wray (1995), beetles were not found to carry *S. enteritidis* after ingestion. However, Crippen et al. (2009) discovered that beetles were capable of acquiring *S. enteritidis* and could harbour them in their digestive tract. *Alphitobius diaperinus* was found to carry *Escherichia coli* for 10 to 12 days (McAllister et al., 1996). Detectable live bacteria were shed in the faeces of the larvae and adults for 6 and 10 days, respectively. Larvae stopped shedding bacteria after they moulted.

Similar to *A. diaperinus*, *Z. atratus* feeds on organic litter in nature which might harbour pathogens. However, research on *Z. atratus* harbouring or spreading human pathogens is unavailable. Research on *T. molitor* in relation to pathogenic microbes has focussed more on the effect of exposure to fungi and their toxic secondary metabolites (mycotoxins) rather than bacteria. *Tenebrio molitor* larvae showed reduced growth, but no increased mortality on diets containing fumonisin B_1 (Abado-Becognee et al., 1998) or at least 16 ppm of T-2 toxin (Davis and Schiefer, 1982). Davis et al. (1975) described varying effects of several fungal strains on growth of *T. molitor* larvae, where some strains impaired growth while others improved growth depending on the cereal type the fungus had been growing on. The authors did not give an explanation for this phenomenon, but a possible explanation could be that the fungi produced certain growth-promoting nutrients on some cereals they could not produce on others. In a study of Chuku et al. (2007), both *T. molitor* and *A. diaperinus* were found to actively feed on fungi such as *Penicillium italicum*, *Rhizopus stolonifer* and *Aspergillus niger* on coconut. No deleterious effects on larvae were observed after feeding

for 48 h. In the above-mentioned studies, it was not investigated whether fungal spores or mycotoxins stayed behind in the larval body.

Non-organic toxic substances can also be acquired by insects from the environment. For example, insects can bioaccumulate ingested metals which might not cause mortality, but could have a toxic effect on insectivores (Ballan-Dufrançais, 2002). Vijver et al. (2003) showed that larvae of *T. molitor* accumulate cadmium and lead in their body when feeding on organic matter in soils harbouring these metals. Exposed larvae increased significantly in fresh weight, but very little in dry weight and after 14 days of exposure, mortality was 45 percent. However, Lindqvist and Block (1995) discovered that larvae lost small amounts of cadmium after each moult and larger amounts after metamorphosis. Selenium is an essential trace element; however, in dietary excess concentrations it has a toxic effect. Hogan and Razniak (1991) exposed adult *T. molitor* to different concentrations of sodium selenite, which is an abundant form of selenium found in nature. The compound had a negative effect on beetle survival and most selenium was found to be accumulated in the Malpighian tubules, followed by the digestive tract and the reproductive tissue.

Mealworms, when produced in closed farming systems, will not feed on animal waste or organic litter as found in nature. Hence, there is a reduced chance of exposure to certain pathogenic microbes or toxic compounds. However, knowing the insect's capacity to feed on or harbour harmful compounds is relevant because contamination – in particular with mycotoxins – can also occur in grain, which is commonly used as feed for mealworms. In addition, insect diets composed of organic by-products could contain contaminants.

Risk of spoilage

Literature is available on spoilage of edible insects after harvest. This topic is especially relevant in tropical countries where the insects are traditionally sun-dried and not refrigerated. For example, several species of fungi such as *Fusarium* spp., *Penicillium* spp. and *Aspergillus niger* were found on larvae of the Rhinoceros beetle *Oryctes monoceros* when stored at room temperature (Banjo et al., 2006). On deteriorating Mopane worm (*Imbrasia belina*), several bacteria were found such as *Artrobacter* sp., *Bacillus* spp. and *Pseudomonas* sp., as well as several fungi such as *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. (Simpanya et al., 2000). Mpuchane et al. (2000) also screened for microbes associated with deteriorating *I. belina* and found predominantly *Bacillus* spp. and *Aspergillus* sp.. *Aspergillus flavus* and *A. parasiticus* are known producers of aflatoxin. In some *I. belina* samples, aflatoxin concentrations as high as 50 μg/kg were found, which is more than twice the maximum acceptable concentration in most countries (Mpuchane et al., 1996). Contamination of edible insects mostly occurs due to bad hygiene practices during drying, storage and preparation of the insects (Mpuchane et al., 2000). Only one study looked into microbial contamination of mealworms after harvest. Klunder et al. (2012) found Enterobacteriaceae in *T. molitor*, which were largely eliminated by blanching

followed by roasting of the insects. Fermentation inactivated Enterobacteriaceae but not spore forming bacteria, although the latter were unable to germinate.

Risk of allergenicity

Allergic reactions following the consumption of edible insects have been described, though literature is scarce. Cases of food allergy have been described upon consumption of Mopane worm (Okezie et al., 2010), Domesticated silkworm pupae (Ji et al., 2008), Teak caterpillar (*Hyblaea puera*; Lukiwati, 2008) and food containing carmine dye produced from cochineal (*Dactylopius coccus*; Acero et al., 1998). Symptoms ranged from skin rash and mild swelling to anaphylactic shock. With respect to mealworm species, occupational allergy has been described for laboratory workers and handlers of live fish bait who come in frequent contact with these insects (Schroeckenstein et al., 1990; Schroeckenstein et al., 1988; Senti et al., 2000; Siracusa et al., 2003). This concerned mostly allergic reactions upon inhalation or skin contact and symptoms included skin rash, itching and inflammation of the eyes and nose. Only one study described a case of food allergy following consumption of *T. molitor* (Freye et al., 1996), where the patient, known to suffer from occupational allergy to mealworms, experienced anaphylaxis.

Legislation concerning introduction of edible insects

The European Union has strict regulations for introducing new food and food ingredients on the market, as was reviewed by Belluco et al. (2013). According to the Regulation (EC) 258/1997 of the European Parliament and of the Council, novel foods are classified as "foods and food ingredients which have not hitherto (May 15, 1997) been used for human consumption to a significant degree". When a food is classified as a novel food, a safety risk assessment has to be performed prior to introducing the food to the European market. Although insects are sold as food already in some European countries, they are not yet a common food item. It is not clearly described in EC regulation 258/1997 what qualifies as "consumption to a significant degree" and at present, it is not yet decided whether edible insects will qualify as a novel food. Nonetheless, insects sold for human consumption should be free of contaminants and need to be correctly labelled for allergen information, in order to protect the consumer. For this reason, when introducing mealworms as an alternative protein source for human consumption, research on their allergic potential, as well as possible contamination risks, should be expanded.

Objectives of this thesis

This thesis was performed as part of the project "Sustainable production of insect proteins for human consumption" (SUPRO2). The aim of the SUPRO2 project was to investigate the possibility of producing insects as a sustainable source of animal protein for human consumption. One of my aims in this project was to produce edible mealworms of high nutritional quality on diets composed of organic by-products, as this would contribute to a more sustainable production process. Diet composition affects insect feed conversion efficiency, where an increased efficiency also contributes to a more sustainable production process.

A second aim of my project was to investigate potential food safety risks associated with edible mealworms. Risks could originate from the presence of toxic or microbial hazards in the insects, as well as from potential allergenicity. For this thesis I focussed on two food safety risks. First, when insects are produced on organic by-products, a food safety risk could occur if these by-products are contaminated. Mycotoxins are not eliminated by food processing methods and pose a substantial risk in by-products originating from the food industry. In this thesis I aim to investigate whether mycotoxins in their diet are sequestered by mealworms, thereby forming a threat to the consumer. Second, I focus on a potential food safety risk originating from the insect itself, by assessing the allergic potential of mealworms. Allergic reactions could occur when the consumer has been directly sensitised to insect protein, or indirectly through cross-reactivity between proteins originating from different phylogenetically related species. In this thesis, I focus on allergic potential through possible cross-reactivity.

Thesis outline

Chapter 2 investigates the potential of producing three edible mealworm species *T. molitor*, *Z. atratus* and *A. diaperinus* on diets composed of organic by-products from the food and bio-ethanol industry. Larval growth, development and feed conversion efficiency were monitored, and larval protein content and fat composition were assessed.

Chapter 3 focusses on mycotoxins as a possible contamination risk. *Tenebrio molitor* larvae were exposed to wheat flour naturally contaminated with a mixture of mycotoxins, predominantly deoxynivalenol (DON) as well as to clean wheat flour spiked with a high concentration of DON. The possibility of DON sequestration and excretion was assessed by analysing DON concentration in larval bodies as well as in larval faeces.

Chapter 4 explores another food safety risk associated with the consumption of edible insects. The allergic potential of *T. molitor* protein was assessed *in vitro* using sera from

patients allergic to both House dust mites and crustaceans to indicate possible allergic cross-reactivity.

Chapter 5 expands the previous study by determining the allergic potential of *T. molitor* as well as *Z. atratus* and *Z. morio*. Allergic cross-reactivity was determined *in vitro* for protein from mealworms with sera from patients allergic to either House dust mites or crustaceans. In addition, the influence of food processing methods as well as *in vitro* digestion on the allergenicity was determined.

Chapter 6 provides a general discussion and conclusions based on the research presented in this thesis. The findings are put in a wider perspective and priorities for future research on production of edible insects as well as on food safety concerns are discussed.

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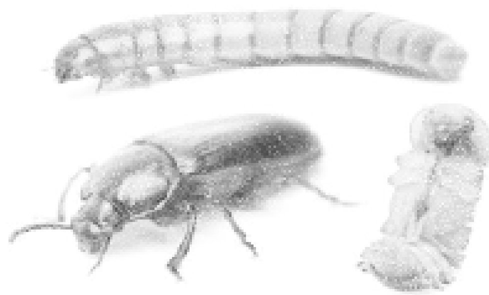
Chapter 2

Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products



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Abstract

Insects receive increasing attention as an alternative protein-rich food source for humans. Producing edible insects on diets composed of organic by-products could increase sustainability. In addition, insect growth rate and body composition, and hence nutritional quality, can be altered by diet.
Three edible mealworm species *Tenebrio molitor* L., *Zophobas atratus* Fab. and *Alphitobius diaperinus* Panzer were grown on diets composed of organic by-products originating from beer brewing, bread/cookie baking, potato processing and bioethanol production. Experimental diets differed with respect to protein and starch content. Larval growth and survival was monitored. Moreover, effects of dietary composition on feed conversion efficiency and mealworm crude protein and fatty acid profile were assessed. Diet affected mealworm development and feed conversion efficiency such that diets high in yeast-derived protein appear favourable, compared to diets used by commercial breeders, with respect to shortening larval development time, reducing mortality and increasing weight gain. Diet also affected the chemical composition of mealworms. Larval protein content was stable on diets that differed 2-3 fold in protein content, whereas dietary fat did have an effect on larval fat content and fatty acid profile. However, larval fatty acid profile did not necessarily follow the same trend as dietary fatty acid composition. Diets that allowed for fast larval growth and low mortality in this study led to a comparable or less favourable n6/n3 fatty acid ratio compared to control diets used by commercial breeders. In conclusion, the mealworm species used in this study can be grown successfully on diets composed of organic by-products. Diet composition did not influence larval protein content, but did alter larval fat composition to a certain extent.

Keywords: edible mealworms, larval development, survival, feed conversion efficiency, crude protein, fatty acids

Introduction

Insects are consumed in most tropical countries, whereas in the Western world they currently do not form a significant part of the human diet. Due to a growing world population and increasing welfare, there is a rising demand for animal-derived protein, and the consumption of insects (entomophagy) receives increasing attention as an alternative protein-rich food source (Van Huis, 2013; Van Huis et al., 2013).

Production of conventional livestock is associated with detrimental environmental effects such as global warming, land degradation, air and water pollution, and loss of biodiversity (Mekonnen and Hoekstra, 2010; Steinfeld et al., 2006). Insects, being poikilotherms, do not use metabolic energy to maintain a constant body temperature as homeotherms do and can therefore invest more energy in growth, resulting in a higher feed conversion efficiency (Nakagaki and DeFoliart, 1991). Furthermore, compared to conventional livestock, insects require less land (Oonincx and De Boer, 2012), are expected to use less water (Van Huis, 2013) and emit less greenhouse gases (Oonincx et al., 2010), making them a more sustainable source of animal protein.

In the Western world, insects are produced in closed farming systems rather than harvested from nature. For example, three species of edible larvae of the beetle family Tenebrionidae, better known as mealworms, are currently commercially produced: the Yellow mealworm (*Tenebrio molitor* L.), the Giant mealworm (*Zophobas atratus* Fab.) and the Lesser mealworm (*Alphitobius diaperinus* Panzer). These insects are commonly produced on mixed grain diets. Recently, separate production lines have been set up in The Netherlands to facilitate the production of *T. molitor* and *A. diaperinus* for human consumption. *Zophobas atratus* is currently not yet produced for human consumption; however, larvae of this species are suitable for human consumption. Mealworm 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 is very scarce for *A. diaperinus* (Hosen et al., 2004) and seems unavailable for *Z. atratus*. Furthermore, it is thus far unknown how diet composition

influences feed conversion efficiency of these insects. In this study, growth performance, feed conversion efficiency and nutritional composition of the three mealworm species on diets composed of organic by-products were determined.

Materials and methods

Insects

Newly hatched larvae of *T. molitor*, *Z. atratus* and *A. diaperinus* were obtained from the insect rearing company Kreca (Ermelo, The Netherlands). During the experiment, insects were maintained in a climate chamber (28°C, 65% RH, 12 h photoperiod).

Diet preparation

Organic by-products were selected as ingredients for the experimental diets based on local availability and deemed suitability as feed for insects and included: spent grains and beer yeast (*Saccharomyces cerevisiae* Meyen *ex* Hansen; Anheuser-Busch, Dommelen, The Netherlands), bread remains (Bakkersland BV, Hedel, The Netherlands), cookie remains (Banketbakkerij Van Strien, Oud-Beijerland, The Netherlands), potato steam peelings (Hedimix BV, Boxmeer, The Netherlands) and maize distillers' dried grains with solubles (DDGS; Groan BV, Giessen, The Netherlands). The ingredients were lyophilised, ground and then mixed to compose four diets either high in both protein and starch (HPHS), high in protein and low in starch (HPLS), low in protein and high in starch (LPHS) and low in both protein and starch (LPLS) (Table 1). Because high starch diets based on cookie remains caused high larval mortality, they were replaced with high starch diets based on potato steam peelings (see Results and Discussion). Diets obtained from commercial insect rearing companies (referred to as A and B) were used as control diets. Company A uses the same diet for *T. molitor* and *Z. atratus*, but does not produce *A. diaperinus*. Hence, that same diet (control diet A) was used for this species in this experiment. Company B also uses the same diet for *T. molitor* and *Z. atratus* (control diet B-Tm/Za), but a different diet for *A. diaperinus* (control diet B-Ad). Diets were stored at -20°C until use.

Table 1: Composition of experimental diets made from organic by-products, and approximate composition of experimental diets and control diets.

Ingredient (%)	HPHS	HPHS[b]	HPLS	LPHS	LPHS[b]	LPLS	Control A	Control B-Tm/Za	Control B-Ad
Maize DDGS	10	10	20	-	-	-			
Beer yeast	40	40	40	5	5	10			
Bread remains	10	10	10	10	10	50			
Spent grains	-	-	30	-	-	40			
Potato steam peelings	40	-	-	85	-	-			
Cookie remains	-	40	-	-	85	-			
Approximate composition (%)[a]									
Crude protein	24.1	26.4	32.5	10.7	10.7	20.0	18.8	15.5	16.0
Crude fat	4.0	7.1	7.0	1.8	8.4	6.2	6.0	4.0	4.4
Starch	28.4	26.9	7.4	49.8	46.7	19.4	43.6	23.0	~

~: No information available.

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

[a] Values calculated based on available values for organic by-products (www.duyniebeuker.nl, www.groan.nl).

[b] Discontinued.

Larval growth and development experiment

Fifty newly hatched larvae were transferred to a plastic container (17.5 x 9.3 x 6.3 cm) with aeration slits in the sides. Each container contained 4 g of diet and 1 g of carrot. Per diet and species, five replicate containers were used. Larvae were allowed to feed *ad libitum* and diet was refreshed when needed, based on visual observation of remaining diet and accumulated faeces. To provide moisture, 2 g of fresh carrot was added twice a week. Old carrot pieces were removed.

Larvae were allowed to feed undisturbed for four weeks. After four weeks, larval weight and survival were monitored weekly as a group until 50% of the surviving larvae had pupated. Because *Z. atratus* larvae failed to pupate under crowded condition, individual larvae were moved to containers containing 1 g of diet and 0.25 g of carrot once 50% of the larvae reached or exceeded a body length of 5 cm. Pupae were collected, weighed and kept separate until adult eclosion after which adult weight was determined.

Feed conversion efficiency experiment

Diet LPLS was excluded from further experiments because larvae failed to consume large portions of it (personal observation). Control diet A was also excluded from further experiments, because of relatively low survival and because this diet was not used by commercial companies to produce *A. diaperinus*.

For each diet, batches of newly hatched larvae were allowed to feed *ad libitum* prior to the experiment. The experimental period was chosen because mortality among newly hatched larvae was higher than in later larval stages and growth rates can vary considerably

between individual larvae. The larval age during which the experiment was conducted was based on the results from the growth and development experiment and differed per species, but was equal in duration for each diet: from day 45 to day 60 for *T. molitor*; from day 70 to day 112 for *Z. atratus*; and from day 25 to day 40 for *A. diaperinus*. Per replicate, 50 larvae for *T. molitor*, 30 larvae for *Z. atratus* and 70 larvae for *A. diaperinus* were weighed as a group at the start of the experimental period. They were subsequently placed in a plastic container (17.5 x 9.3 x 6.3 cm) on 5 g diet for *T. molitor*, 7 g diet for *Z. atratus* and 3 g diet for *A. diaperinus*. Per diet and species, five replicate containers were set up. Throughout the experiment, carrot (2 g for *T. molitor*, 3 g for *Z. atratus* and 1 g for *A. diaperinus*) was replaced twice a week. Non-consumed carrot was removed and dried at 100°C until constant weight, which was then compared to the dry weight of a carrot piece of the same original fresh weight cut from the same carrot as the pieces used in the experiment. Before the diet was completely consumed (determined based on visual observation of diet and faeces), larvae were transferred to a container with fresh diet and carrot. The residue, consisting of a mixture of leftover diet and faeces, was removed and stored at -20 °C. After termination of the experiment, larvae were starved for 24 h and were then killed by freezing at -20°C and stored at this temperature until further analysis.

In order to determine diet consumption, for each diet-species combination, a separate batch of larvae was allowed to consume the diet and carrot entirely. Larvae were then removed and pure faeces were stored at -20°C. Uric acid analysis (see respective section) was performed on pure faeces and on residues to quantify diet consumption in the feed conversion experiment. Thereafter, diets, pure faeces and residues were dried at 100°C to a constant weight. Uric acid concentration in pure faeces and diets was corrected for dry weight percentage. Feed conversion efficiency was expressed on a dry matter base as the Efficiency of Conversion of Ingested food (ECI; Waldbauer, 1968), calculated as:

$$ECI = \frac{weight\ gained}{weight\ of\ ingested\ food} \times 100\%$$

and expressed on a fresh matter base as the Feed Conversion Ratio (FCR), calculated as:

$$FCR = \frac{weight\ of\ ingested\ food}{weight\ gained}$$

Uric acid analysis

Ten milligram of either residue or pure faeces sample was extracted in 50 mL of 0.5% borax solution for 2 h, after which the uric acid concentration was determined by spectrophotometry at OD 450 nm according to Van Handel (1975). Uric acid content of the residues was compared to uric acid content of pure faeces to determine the amount of faeces in the residues, calculated as:

$$\text{weight of pure faeces in the residue} = \frac{\text{amount of uric acid in the residue}}{\text{amount of uric acid in the pure faeces}}$$

Analysis of nutrient composition

After termination of the feed conversion experiment, insects were harvested and pooled for each species and diet. Insect samples were then lyophilised at -50°C and 1.5 mbar. Total lipid content was determined as described by Folch et al. (1957) and fatty acid composition was determined according to Metcalfe et al. (1966). Nitrogen content was determined according to Novozamsky et al. (1984). Crude protein content was calculated by multiplying nitrogen content by 6.25.

Statistical analysis

Data of pupal and adult weight were distributed normally and analysed by One way Analysis of Variance (ANOVA) at a significance level of 0.05, followed by a Šidák correction for multiple comparisons. Data of larval survival and development time, percentage of eclosed adults, as well as uric acid concentration of faeces, ECI, FCR and consumed carrot/food ratio did not conform to the normal distribution and were analysed by a Kruskal-Wallis test at a significance level of 0.05, followed by Mann-Whitney U tests with applying Šidák correction. For larval survival and development time and percentage of eclosed adults, the level of significance was corrected to $1-(1-0.05)^{1/5}= 0.010$ for post-hoc analysis. For uric acid concentration of faeces, ECI and FCR, the level of significance was corrected to $1-(1-0.05)^{1/3} = 0.017$ for post-hoc analysis. Correlation between larval survival and development time was analysed by Spearman's rank correlation coefficient. All statistical analyses were performed using IBM SPSS statistics v. 20.

Results

Diet nutrient composition

Diets were prepared to differ in protein and starch content (Table 1). Calculated approximate protein content of high protein diets ranged from 24.1% to 32.5% and was 10.7% for both LPHS diets. Approximate starch content was 26.9% and 28.4% for the HPHS diets, 7.4% for diet HPLS and 46.7% and 49.8% for the LPHS diets. Diet LPLS was only slightly lower in both protein and starch (20% and 19.4% respectively) than the HPHS diets. Approximate fat content of the experimental diets was between 6.2% and 8.4% for all experimental diets except for the potato-based high protein diets (4.0% for HPHS and 1.8% for LPHS).

Protein and fat content was determined for diets used for the feed conversion experiment. Protein content of high protein diets was between 33% and 39% and was 17-

18% in control diets (Table 2). The control diets and HPHS had similar DM contents, whereas diets HPLS and LPHS had a DM content of *ca.* 95%. High protein diets contained 5-6% fat whereas diet LPHS and control diets contained 5% or less. Analysis of fatty acid composition showed that linoleic acid was prevalent in all diets, in particular in control diet B-Tm/Za (Table 3). Other predominant fatty acids were palmitic and oleic acid. Oleic acid content in control diet B-Ad and the experimental diets exceeded 20% of total fatty acids but was *ca.* 13% for control diet B-Tm/Za. The ratio between ω-6 and ω-3 polyunsaturated fatty acids (n6/n3 ratio) ranged from 10:1 to 18:1 for the control diets and high protein diets, and was 5:1 for diet LPHS.

Table 2: Dry matter (DM) percentage, crude protein and crude fat content of different diets.

Diet	DM[a] (% of whole)	Crude protein[b] (% DM)	Crude fat[a] (% DM)
Control B-Tm/Za	89.0	17.1	3.0
Control B-Ad	87.4	17.8	5.0
HPHS	86.5	32.7	5.5
HPLS	95.1	39.1	5.8
LPHS	95.7	11.9	2.3
carrot[c]	11.7	7.9	2.1

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch).
[a] Values based on single analysis.
[b] Values based on analysis in duplo.
[c] Values for carrot are based on USDA SR-12 nutrient data for carrot.

Larval survival

The original, and discontinued high starch diets contained cookie remains as starch source. For all species reared on diets containing cookie remains, survival was 0% on diet LPHS and < 40% on diet HPHS (results not shown). Therefore, the experiment was repeated using new high starch diets with potato steam peelings as starch source.

For all three mealworm species, diets affected survival (Table 4, Figures 1, 2, 3; $p = 0.034$ for *T. molitor*, $p < 0.001$ for *Z. atratus* and *A. diaperinus*) and strongly affected development time ($p < 0.001$). For *T. molitor*, survival on diets HPLS and LPHS was higher (> 80%) than on control diet A (71%; $p = 0.008$). For *Z. atratus*, survival was higher on experimental high protein diets (≥ 84%) than on control diets (≤ 78%; $p = 0.008$). Survival on diet LPHS was very low compared to other diets (27%; $p = 0.008$). For *A. diaperinus*, survival was lower on control diet A (ca. 80%) than on experimental diets (> 90%, $p = 0.008$).

Table 3: Fatty acid profile of control diets and experimental diets. Values are in g/100 g of total fatty acids. Fatty acids not detected in any of the diets were excluded. Values based on single analysis.

Fatty acid			**Diet**					
Common name	Lipid number	ω-n	control B-Tm/Za	control B-Ad	HPHS	HPLS	LPHS	carrot[a]
Saturated								
Lauric acid	C 12:0		-	-	0.09	-	0.41	-
Myristic acid	C 14:0		-	-	0.41	0.28	1.21	-
Pentadecanoic acid	C 15:0		-	-	0.16	-	-	-
Palmitic acid	C 16:0		17.04	13.50	13.16	14.90	14.04	14.93
Margaric acid	C 17:0		-	-	0.32	0.20	1.33	-
Stearic acid	C 18:0		0.76	3.13	2.35	2.09	2.90	0.87
Arachidic acid	C 20:0		-	-	0.27	0.33	0.57	-
Behenic acid	C 22:0		0.17	-	0.18	-	0.60	-
Lignoceric acid	C 24:0		-	-	0.24	0.24	0.54	-
Monounsaturated								
Myristoleic acid	C 14:1	ω-5	-	-	0.08	-	-	-
Palmitoleic acid	C 16:1	ω-7	0.17	-	3.28	1.79	3.31	0.87
Oleic acid	C 18:1	ω-9	12.58	23.68	26.20	24.48	22.88	5.13
Vaccenic acid	C 18:1	ω-7	1.06	1.19	0.67	0.65	1.45	-
Gadoleic acid	C 20:1	ω-12	0.83	-	-	-	-	-
Gondoic acid	C 20:1	ω-9	-	-	0.26	0.39	0.42	-
Nervonic acid	C 24:1	ω-9	0.22	-	0.36	0.24	-	-
Polyunsaturated								
Hexadecadienoic acid	C 16:2	ω-4	-	-	0.27	-	1.31	-
Hexadecatrienoic acid	C 16:3	ω-4	-	-	0.48	0.26	0.72	-
Linoleic acid	C 18:2	ω-6	60.66	53.87	47.28	51.03	32.02	49.00
α-Linolenic acid	C 18:3	ω-3	5.90	4.63	2.67	2.86	4.32	0.87
Stearidonic acid	C 18:4	ω-3	-	-	0.23	-	2.34	-
Eicosadienoic acid	C 20:2	ω-6	-	-	0.08	-	0.68	-
Eicosatetraenoic acid	C 20:4	ω-3	-	-	-	-	0.42	-
n6/n3 ratio			10:1	12:1	16:1	18:1	5:1	56:1

- : not detected.
Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch).
[a] Values for carrot are based on USDA SR-12 nutrient data for carrot.

Development time

For *T. molitor* and *Z. atratus*, development time until 50% pupation was similar on both control diets but lower on high protein experimental diets (p = 0.008; Table 4). For *A. diaperinus*, development time on control diet B-Ad was longer than on diet HPLS and shorter than on diet LPLS (p = 0.008). Development time on control diet A was longer than on control diet B and experimental diets HPHS, HPLS and LPLS (p = 0.008). For all three species, development time on diet LPHS was much longer than on the other diets (p = 0.008). Furthermore, larvae grown on diet LPHS were lighter in colour than larvae grown

on the other diets, while faeces were darker. Both diets LPHS and HPHS containing potato steam peelings were light in colour as were control diets A and B-Ad, and diet LPLS. Development time and survival were only correlated for *Z. atratus* ($\rho = -0.759$, $p < 0.001$).

Table 4: Average development time and survival at 50% pupation of three mealworm species grown on different diets. Values are given as mean ± SD. Superscripts denote significant differences; n = 5.

Diet	Development time (days)	Survival (%)
Tenebrio molitor		
Control A	117^{a} ± 1.5	71^{a} ± 12.7
Control B-Tm/Za	123^{a} ± 2.4	86^{ab} ± 9.6
HPHS	79^{b} ± 3.2	88^{ab} ± 5.2
HPLS	95^{c} ± 3.6	92^{b} ± 2.6
LPHS	168^{d} ± 11.5	88^{b} ± 0.9
LPLS	95^{c} ± 7.1	84^{b} ± 10.5
Zophobas atratus		
Control A	139^{a} ± 8.5	68^{a} ± 3.8
Control B-Tm/Za	140^{a} ± 11.4	78^{ab} ± 11.4
HPHS	117^{b} ± 2.9	96^{c} ± 2.6
HPLS	103^{c} ± 1.7	91^{cd} ± 2.3
LPHS	225^{d} ± 6.1	27^{e} ± 16.9
LPLS	152^{a} ± 7.9	84^{bd} ± 6.2
Alphitobius diaperinus		
Control A	66^{a} ± 0.9	79^{a} ± 6.6
Control B-Ad	42^{b} ± 1.7	82^{ab} ± 15.4
HPHS	44^{bc} ± 2.2	95^{b} ± 4.1
HPLS	38^{d} ± 1.5	94^{b} ± 2.6
LPHS	106^{e} ± 8.1	91^{b} ± 5.2
LPLS	48^{c} ± 2.6	97^{b} ± 1.1

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

Table 4 (continued): Pupal weight, adult weight and percentage of successfully eclosed adults of three mealworm species grown on different diets. Values are given as mean ± SD. Superscripts denote significant differences; n = 5.

Diet	Pupal weight (g)		Adult weight (g)		Adults (% of pupae)	
Tenebrio molitor						
Control A	0.149^{ab}	± 0.022	0.133^{ab}	± 0.020	94^{a}	± 0.071
Control B-Tm/Za	0.144^{ab}	± 0.023	0.127^{a}	± 0.019	95^{a}	± 0.048
HPHS	0.146^{ab}	± 0.021	0.126^{a}	± 0.018	90^{a}	± 0.114
HPLS	0.161^{b}	± 0.023	0.140^{b}	± 0.020	97^{a}	± 0.054
LPHS	0.117^{c}	± 0.017	0.100^{c}	± 0.015	93^{a}	± 0.068
LPLS	0.145^{b}	± 0.021	0.127^{a}	± 0.020	98^{a}	± 0.028
Zophobas atratus						
Control A	0.603^{a}	± 0.063	0.499^{a}	± 0.049	86^{a}	± 0.074
Control B-Tm/Za	0.584^{a}	± 0.065	0.485^{a}	± 0.056	85^{a}	± 0.147
HPHS	0.664^{b}	± 0.059	0.551^{b}	± 0.047	94^{a}	± 0.092
HPLS	0.722^{c}	± 0.062	0.604^{c}	± 0.054	98^{a}	± 0.032
LPHS	0.482^{d}	± 0.114	0.391^{d}	± 0.074	80^{a}	± 0.326
LPLS	0.651^{b}	± 0.038	0.538^{b}	± 0.037	95^{a}	± 0.075
Alphitobius diaperinus						
Control A	0.021^{a}	± 0.003	0.018^{a}	± 0.003	89^{ab}	± 0.078
Control B-Ad	0.023^{a}	± 0.004	0.019^{a}	± 0.003	74^{ab}	± 0.112
HPHS	0.022^{a}	± 0.003	0.019^{a}	± 0.003	90^{ab}	± 0.076
HPLS	0.021^{a}	± 0.003	0.020^{a}	± 0.013	77^{a}	± 0.078
LPHS	0.018^{b}	± 0.003	0.015^{b}	± 0.003	91^{b}	± 0.031
LPLS	0.023^{a}	± 0.003	0.019^{a}	± 0.003	86^{ab}	± 0.092

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

Pupal and adult weight

Number of days from pupation until adult eclosion was not influenced by diet and was 7 days for *T. molitor*, 12 days for *Z. atratus* and 5 days for *A. diaperinus*.

For *T. molitor*, pupal weight was higher on diet HPLS (Table 4), and lower on diet LPHS compared to the control diets ($p \leq 0.001$). Adult weight was lower on diet LPHS than on control diet A ($p < 0.01$) and higher on diets HPLS and LPLS than on control diet B-Tm/Za ($p < 0.001$). For *Z. atratus*, both pupal weight and adult weight differed between the control diets and the experimental diets, where weight was higher on both high protein diets and diet LPLS, and lower on diet LPHS ($p < 0.001$). For *A. diaperinus*, pupal weight was lower on diet LPHS than on control diets ($p < 0.001$), as was adult weight ($p = 0.004$ for control diet A and $p < 0.001$ for control diet B-Ad). Rather than turning black in colour, *A. diaperinus* adults remained dark brown when larvae had consumed diet LPHS. Percentage of adults eclosing intact (viz. normal elytra and wings) was over 90% for *T. molitor* and over 80% for *Z. atratus*. No difference was observed between diets. For *A. diaperinus*,

successful eclosion was lower for diet HPLS (77%) than for diet LPHS (91%, p = 0.008). No differences were observed when comparing other diets.

Feed conversion efficiency

Uric acid concentration in pure mealworm faeces differed depending on species and diet consumed (Table 5). For all three species, more uric acid was present in faeces when larvae fed on diet HPLS than on LPHS or control diet. For *A. diaperinus*, faeces produced on diet LPHS contained less uric acid compared to both high protein diets.

For all three mealworm species, diet had an effect on the ECI (Table 6). For *T. molitor*, ECI was lowest on diet LPHS (p = 0.008) but did not differ between the other diets. For *Z. atratus*, ECI was highest on both high protein diets (p = 0.008). ECI on diet LPHS was approximately half of that for the other experimental diets (*ca.* 15 vs. 30%, p = 0.016). For *A. diaperinus*, ECI was highest on diet HPHS (p = 0.016) and very low on diet LPHS (6.36%, p = 0.008). A high ECI corresponded to a low FCR. However, for *T. molitor*, FCR on diet HPLS was lower than on the control diet and diet HPHS (p = 0.008 and p = 0.016 respectively), whereas ECI values were similar. For *A. diaperinus*, FCR on diet HPLS did not differ from the control diet and diet HPHS.

Differences were observed in the amount of carrot consumed per gram of diet (DM base, Table 6). Larvae of *T. molitor* consumed more carrot per gram of diet on diet LPHS than on control diet (p = 0.008). For *Z. atratus*, carrot consumption was lowest on diet HPHS and highest on diet LPHS compared to the other diets (p = 0.008 and p = 0.016 respectively). Large differences were observed for *A. diaperinus*, where carrot consumption per gram of diet on control diet B-Ad and diet LPHS was approximately twice as high as on the high protein diets.

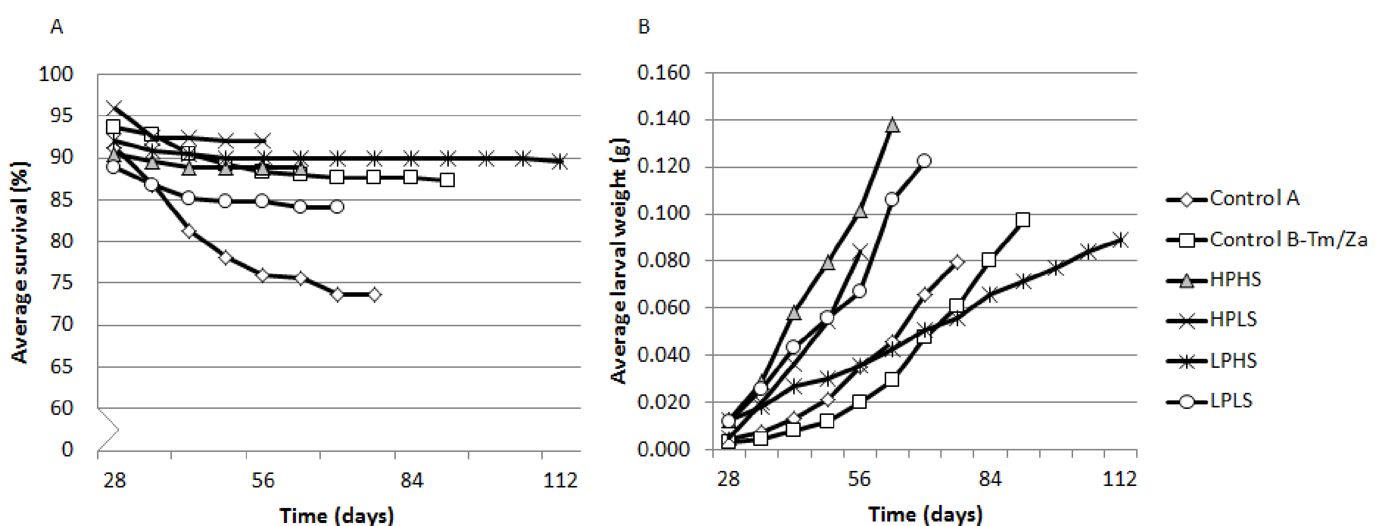

Figure 1: Average larval survival (A) as percentage of the total number of larvae at week 0 (n = 50) and average larval weight (B) of *Tenebrio molitor*, determined weekly until the first pupa was observed. HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

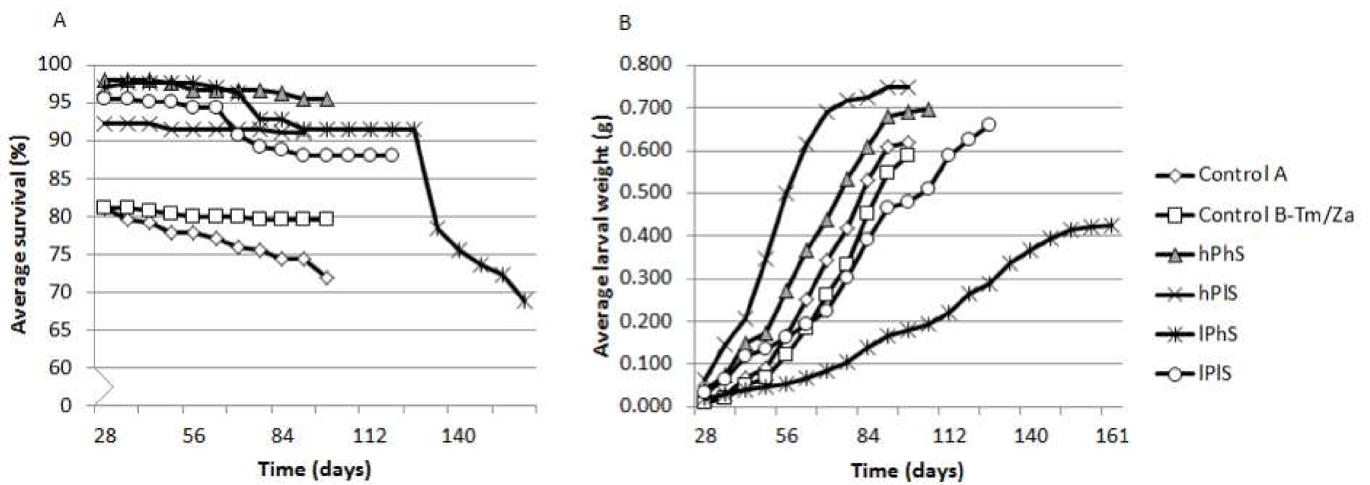

Figure 2: Average larval survival (A) as percentage of the total number of larvae at week 0 (n = 50) and average larval weight (B) of *Zophobas atratus*, determined weekly until the first pupa was observed. HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

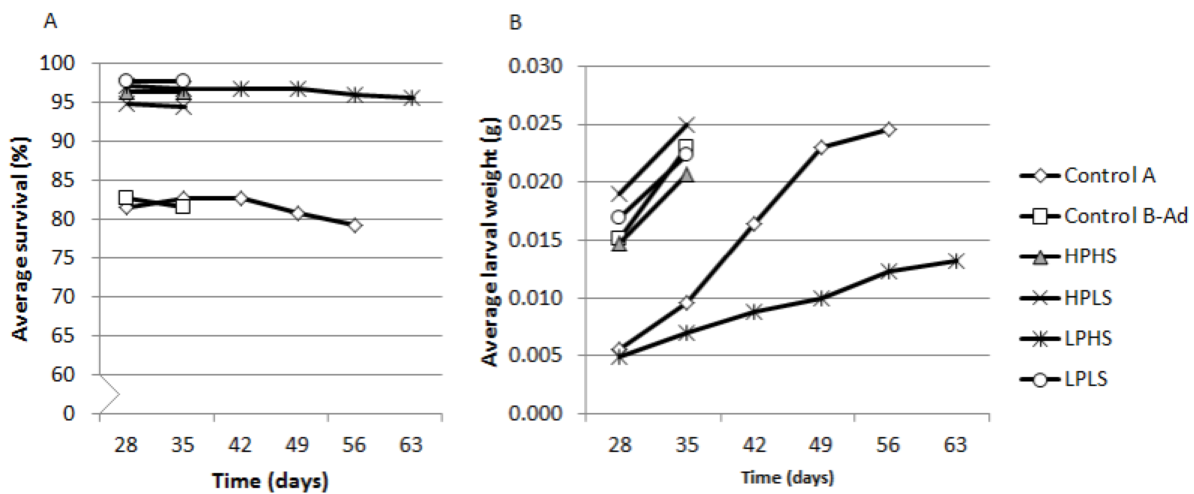

Figure 3: Average larval survival (A) as percentage of the total number of larvae at week 0 (n = 50) and average larval weight (B) of *Alphitobius diaperinus*, determined weekly until the first pupa was observed. HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

Table 5: Uric acid concentration in pure faeces (mg/mg DM) produced by three mealworm species grown on different diets. Values are given as mean ± SD. Superscripts denote significant differences; n = 4.

Diet	*Tenebrio molitor*		*Zophobas atratus*		*Alphitobius diaperinus*	
Control B-Tm/Zm	0.042^{ac}	± 0.002	0.043^{ac}	± 0.003	-	
Control B-Ad	-		-		0.108^{ab}	± 0.004
HPHS	0.151^{abc}	± 0.008	0.073^{abc}	± 0.001	0.186^{b}	± 0.012
HPLS	0.188^{b}	± 0.012	0.182^{b}	± 0.002	0.202^{b}	± 0.017
LPHS	0.041^{c}	± 0.002	0.049^{c}	± 0.004	0.050^{a}	± 0.001

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch); LPLS (low protein, low starch).

Table 6: Feed conversion efficiency (ECI) and carrot consumed per gram diet on dry matter (DM) basis and feed conversion ratio (FCR) on fresh weight (FW) basis. Values are given as mean ± SD. Superscripts denote significant differences; n = 5.

Diet	ECI (DM) (%)		FCR (FW)		Carrot consumed per g diet (g DM)	
Tenebrio molitor						
Control B-Tm/Za	18.96[a]	± 0.70	3.44[a]	± 0.24	0.159[ab]	± 0.011
HPHS	28.93[a]	± 3.56	3.04[a]	± 0.21	0.211[ac]	± 0.036
HPLS	28.47[a]	± 0.75	2.62[b]	± 0.10	0.155[b]	± 0.007
LPHS	16.76[b]	± 0.77	6.05[c]	± 0.44	0.248[c]	± 0.013
Zophobas atratus						
Control B-Tm/Za	23.78[a]	± 0.90	3.64[a]	± 0.17	0.186[a]	± 0.008
HPHS	28.93[b]	± 1.41	3.11[b]	± 0.14	0.162[b]	± 0.002
HPLS	33.33[c]	± 2.37	2.73[c]	± 0.12	0.177[a]	± 0.011
LPHS	15.76[d]	± 1.45	5.63[d]	± 0.64	0.226[c]	± 0.017
Alphitobius diaperinus						
Control B-Ad	23.03[a]	± 6.93	8.11[a]	± 2.69	0.508[a]	± 0.149
HPHS	34.37[b]	± 6.09	3.01[b]	± 0.34	0.257[b]	± 0.047
HPLS	25.41[a]	± 2.21	3.24[ab]	± 0.48	0.208[b]	± 0.037
LPHS	6.36[c]	± 1.73	24.60[c]	± 6.86	0.424[a]	± 0.082

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch).

Nutritional composition

Dry matter content of the three mealworm species was *ca.* 30% (Table 7). Crude protein content was *ca.* 47% for *T. molitor*, *ca.* 40% for *Z. atratus* and *ca.* 64% for *A. diaperinus*. Fat content was *ca*. 25% for *T. molitor*, *ca.* 38% for *Z. atratus* and *ca.* 19% for *A. diaperinus*.

The predominant fatty acids in all three species were palmitic acid, oleic acid and linoleic acid, together comprising 72 - 91% of total fatty acids (Table 8). Fatty acid data of *A. diaperinus* larvae on diet HPHS were excluded because of insufficient quality of the sample, creating a high background in gas chromatography. Palmitic acid content was *ca.* 16% for *T. molitor*, whereas for *Z. atratus* this was *ca.* 25% on diet HPLS to 33% on diet LPHS. For *A. diaperinus*, palmitic acid concentrations showed a wider range (*ca.* 16% on diet HPLS to 25% on control diet B-Ad). Oleic acid content was *ca.* 40% on diet HPLS to 58% on diet LPHS for *T. molitor*, *ca.* 31% on diet HPLS to 45% on diet LPHS for *Z. atratus* and *ca.* 20% on diet HPLS to 44% on diet LPHS for *A. diaperinus*. Linoleic acid content showed a wide range for all three species and was *ca.* 15% on diet LPHS to 31% on diet HPLS for *T. molitor*, *ca.* 10% on diet LPHS to 29% on diet HPLS for *Z. atratus* and *ca.* 17% on diet LPHS to 36% on diet HPLS for *A. diaperinus*. Other fatty acids comprised ≤ 3.19% of total fatty acids, with the exception of stearic acid in *Z. atratus* and *A. diaperinus* (*ca*. 7-8% and 9-11%, respectively).

The ratio between ω-6 and ω-3 polyunsaturated fatty acids (n6/n3 ratio) was *ca.* 19:1 for larvae on control diets, 19-25:1 on diet HPLS and *ca.* 30:1 for larvae on high starch diets.

For *T. molitor* on diet LPHS, no ω-3 fatty acids were present in values above the detection limit and an n6/n3 ratio could therefore not be calculated.

Table 7: Dry matter (DM) percentage, crude protein and crude fat content of three mealworm species grown on different diets.

Diet	DM[a] (% of fresh weight)	Crude protein[b] (% DM)	Crude fat[a] (% DM)
Tenebrio molitor			
Control B-Tm/Za	27.3	45.1	25.0
HPHS	33.4	48.6	26.3
HPLS	29.4	47.5	27.6
LPHS	33.3	46.9	18.9
Zophobas atratus			
Control B-Tm/Za	33.3	41.5	36.2
HPHS	36.7	41.1	43.5
HPLS	35.7	42.5	40.0
LPHS	30.8	34.2	32.8
Alphitobius diaperinus			
Control B-Ad	33.3	61.7	24.3
HPHS	31.8	64.3	21.8
HPLS	30.0	65.0	18.1
LPHS	33.3	-	13.4

- : Values not available due to insufficient sample.
[a] Values based on single analysis.
[b] Values based on analysis in duplo.
Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch).

Table 8: Fatty acid profile of *Tenebrio molitor* and *Zophobas atratus* grown on different diets. Values are in g/100 g of total fatty acids. Fatty acids not detected in any of the diets were excluded. Values based on single analysis.

Fatty acid			***Tenebrio molitor***				***Zophobas atratus***			
Common name	Lipid number	ω-n	control B-Tm/Za	HPHS	HPLS	LPHS	control B-Tm/Za	HPHS	HPLS	LPHS
Saturated										
Caprylic acid	C 8:0		-	-	-	-	0.15	0.13	0.12	0.15
Capric acid	C 10:0		-	-	-	-	-	-	-	-
Lauric acid	C 12:0		-	0.38	-	-	-	-	-	-
Myristic acid	C 14:0		2.32	3.19	2.20	2.79	0.78	0.76	0.70	0.75
Pentadecanoic acid	C 15:0		-	0.19	-	-	0.16	0.24	0.31	0.13
Palmitic acid	C 16:0		16.19	16.96	16.13	16.67	31.09	28.90	25.04	33.00
Margaric acid	C 17:0		-	0.34	0.49	-	0.29	0.84	1.10	0.22
Stearic acid	C 18:0		2.97	2.72	2.64	-	7.73	7.06	6.77	7.14
Arachidic acid	C 20:0		-	0.16	-	-	0.18	0.18	0.17	0.13
Behenic acid	C 22:0		-	-	-	-	-	-	-	-
Monounsaturated										
Palmitoleic acid	C 16:1	ω-7	1.56	2.88	2.67	1.56	0.77	1.88	1.85	1.85
Oleic acid	C 18:1	ω-9	46.41	48.68	39.78	57.63	33.17	39.56	30.72	44.72
Vaccenic acid	C 18:1	ω-7	0.39	0.26	0.40	0.20	0.36	0.25	0.36	0.34
Gondoic acid	C 20:1	ω-9	-	-	-	-	0.13	-	0.12	-
Polyunsaturated										
Hexadecatrienoic acid	C 16:3	ω-4	-	0.37	-	-	-	-	-	-
Linoleic acid	C 18:2	ω-6	27.83	20.99	31.25	15.45	22.54	17.84	29.31	9.86
γ-Linolenic acid	C 18:3	ω-6	-	-	-	-	-	-	-	-
α-Linolenic acid	C 18:3	ω-3	1.48	0.67	1.29	-	1.25	0.62	1.16	0.29
Eicosadienoic acid	C 20:2	ω-6	-	0.10	0.34	-	-	-	-	-
Dihomo-γ-linolenic acid	C 20:3	ω-6	-	-	-	-	-	-	-	-
Eicosatetraenoic acid	C 20:4	ω-3	-	-	-	-	-	-	-	-
Eicosapentaenoic acid	C 20:5	ω-3	-	-	0.21	-	-	-	-	-
Docosadienoic acid	C 22:2	ω-6	-	-	0.24	-	-	-	-	-
Docosatrienoic acid	C 22:3	ω-3	-	-	-	-	-	-	-	-
N6/n3 ratio			19:1	32:1	21:1	-	18:1	29:1	25:1	34:1

- : Not detected.

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch).

[a] Due to insufficient quality of *Alphitobius diaperinus* material grown on diet HPHS, data were excluded.

Table 8 (continued): Fatty acid profile of *Alphitobius diaperinus* grown on different diets. Values are in g/100 g of total fatty acids. Fatty acids not detected in any of the diets were excluded. Values based on single analysis.

Fatty acid			***Alphitobius diaperinus***[a]		
Common name	Lipid number	ω-n	control B-Ad	HPLS	LPHS
Saturated					
Caprylic acid	C 8:0		-	0.28	-
Capric acid	C 10:0		-	0.24	-
Lauric acid	C 12:0		-	-	-
Myristic acid	C 14:0		0.57	0.57	0.82
Pentadecanoic acid	C 15:0		-	0.22	0.21
Palmitic acid	C 16:0		24.89	15.73	22.30
Margaric acid	C 17:0		-	1.14	0.53
Stearic acid	C 18:0		9.50	8.95	10.44
Arachidic acid	C 20:0		0.49	0.34	0.55
Behenic acid	C 22:0		-	0.16	-
Monounsaturated					
Palmitoleic acid	C 16:1	ω-7	-	0.51	0.67
Oleic acid	C 18:1	ω-9	37.25	20.55	44.25
Vaccenic acid	C 18:1	ω-7	-	0.24	-
Gondoic acid	C 20:1	ω-9	-	-	-
Polyunsaturated					
Hexadecatrienoic acid	C 16:3	ω-4	-	0.44	0.28
Linoleic acid	C 18:2	ω-6	22.65	36.41	16.84
γ-Linolenic acid	C 18:3	ω-6	-	-	0.41
α-Linolenic acid	C 18:3	ω-3	0.70	0.65	0.38
Eicosadienoic acid	C 20:2	ω-6	-	0.18	-
Dihomo-γ-linolenic acid	C 20:3	ω-6	-	0.23	0.18
Eicosatetraenoic acid	C 20:4	ω-3	-	0.31	-
Eicosapentaenoic acid	C 20:5	ω-3	-	-	-
Docosadienoic acid	C 22:2	ω-6	-	1.55	0.18
Docosatrienoic acid	C 22:3	ω-3	0.50	1.03	0.19
N6/n3 ratio			19:1	19:1	31:1

- : Not detected.

Diet abbreviations: HPHS (high protein, high starch); HPLS (high protein, low starch); LPHS (low protein, high starch).

[a] Due to insufficient quality of *Alphitobius diaperinus* material grown on diet HPHS, data were excluded.

Discussion

This study shows that when the three edible mealworm species were produced on diets composed of organic by-products, diet affected larval performance and feed conversion efficiency. Furthermore, larval chemical composition differed between species and dietary treatments.

Larval mortality was very high on the original high starch diets containing cookie remains. These diets smelled strongly of spices such as cinnamon and clove, of which the vapours can be toxic to insects (George et al., 2010; Işıkber et al., 2009). On the alternative high starch diets, containing potato steam peelings as starch source, larval mortality was similar to that observed on the other diets.

Larvae showed higher survival and shorter development time on diets higher in protein and lower survival and longer development time on diet LPHS, compared to control diet used by commercial mealworm producers. Several studies reported increased growth rate of mealworms on high protein diets, in particular when derived from yeast (Davis and Sosulski, 1974; Morales-Ramos et al., 2010). High protein diets in the present study contained 40% beer yeast. In addition to protein (Aghdamshariar et al., 2006; Rumsey et al., 1991), yeast supplies B vitamins (Copping and Honora Roscoe, 1937; Fraenkel et al., 1950) and works as a feeding stimulant for *T. molitor*, as does wheat flour (Murray, 1960). Similar literature data are lacking for *Z. atratus* and *A. diaperinus*. Suboptimal performance of larvae can be due to low feeding stimulation, rather than a low nutritional value or the presence of deterring components. For this reason, each experimental diet in the present study contained at least 10% bread remains and 5% yeast. Similar to protein source, starch source could influence larval performance, rather than the absolute amount of starch. Hosen (2004) observed differences in performance of *A. diaperinus* when grown on different types of cereal flour, despite minor differences in carbohydrate levels. Potato starch is more resistant to digestion by Tenebrionidae than starch from wheat or maize (Applebaum, 1966; Mereiles et al., 2009). Furthermore, potato glycoalkaloids, which persist after processing (Po and Sinha, 2010) can have a toxic effect on insects that do not consume potato in nature (Nenaah, 2011; Ventrella et al., 2014). Long-time exposure to a high content of potato steam peelings could in part explain the high mortality of *Z. atratus* on diet LPHS, which predominantly occurred after 20 weeks. 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. However, chronic toxicity resulting in among others reduced growth and lower feed conversion efficiency has been described (Chaubey, 2008; Wheeler and Isman, 2001). In addition to retarded growth on diet LPHS, larvae of all three species and beetles of *A. diaperinus* were lighter in colour than those that consumed other diets. Lee et al. (2008) observed that larvae of *Spodoptera littoralis* (Boisduval) had less strongly melanised cuticles when feeding on diets lower in protein quality. Stronger melanisation is associated

with increased direct immune function (Barnes and Siva-Jothy, 2000; Lee et al., 2008). Possibly, protein quality of diet LPHS was lower for mealworms, hence causing decreased melanisation of the cuticle. This would cause the larvae to be more vulnerable to infection by pathogens, reducing their survival.

Pupal weight can be used as a measure for insect dietary quality (Chapman, 1998). For all three mealworm species, pupal weight, and concomitantly adult weight, was lowest on diet LPHS. This is a further indication that this diet was of lower quality for development of the three mealworm species. However, diet LPHS did not have a detrimental effect on successful eclosion of adults.

Carrot consumption was significantly higher on diet LPHS than on the other diets for all three species. Possibly, larvae consumed more carrot to compensate for nutrients lacking in the diet.

Feed conversion efficiency was higher on high protein diets and lower on diet LPHS. This confirms the explanation that diet LPHS might not only lack nutrients, but might also contain compounds which are harder to digest or toxic to mealworms. Feed conversion ratio (FCR) on the high protein diets was *ca.* 3 and was higher than the value reported by Oonincx and De Boer for *T. molitor* (2.2; Oonincx and De Boer, 2012), although carrot consumption was not taken into account in this study, and thus values found in the present study are expected to be higher. No FCR values have been published for *Z. atratus* and *A. diaperinus*. The extremely high FCRs found for *A. diaperinus* on diet LPHS and control diet B are in part due to the large amounts of carrot consumed. Other ways of providing water could dramatically decrease the FCR, provided that carrot was mostly consumed as a source of moisture and not as a source of other nutrients. However, results from the present study suggest that carrot is likely consumed as a source of nutrients to compensate for poor diet quality. When comparing the FCRs of mealworm species on high protein diets (*ca.* 3) with FCRs for conventional livestock, mealworms are comparable to poultry (2.0) and pigs (3.6), and compare favourably to beef (7.8; Wilkinson, 2011). However, the edible portion of mealworms (100%) is greater than that of poultry and pigs (*ca.* 50%; De Vries and De Boer, 2010), making them more efficient production animals.

Larval crude protein content (*ca*. 47% for *T. molitor*, 40% for *Z. atratus* and 64% for *A. diaperinus*) fell within the ranges reported in literature (Barker et al., 1998; Despins and Axtell, 1995; Finke, 2002; Ghaly and Alkoaik, 2009; Pennino et al., 1991; Ramos-Elorduy et al., 2002; Yi et al., 2013). Despite the 2 - 3-fold differences in dietary crude protein contents, larval protein content was similar among the dietary treatment groups, except for the 18% lower value for *Z. atratus* on diet LPHS. Similarly, Ramos-Elorduy et al. (2002) and Gao et al. (2010) observed small differences in *T. molitor* protein content when grown on different diets. This suggests that the mealworms are well able to regulate body protein content. Insects regulate dietary intake of nutrients in order to obtain their nutrient target (Behmer, 2009; Raubenheimer and Simpson, 1999). Being restricted to one diet complicates intake regulation, although Behmer (2009) lists several modes of post-ingestive

regulation. For example, locusts (*Locusta migratoria* L.) excrete excess ingested protein in the form of uric acid and ammonium. In the present study, larvae feeding on diet HPLS excreted more uric acid through faeces than larvae feeding on control diet or diet LPHS, indicating mealworms species might use a similar strategy to cope with excess dietary protein.

Larval fat content as well as fatty acid profile were more strongly influenced by diet. Fat content in this study (*ca.* 19-28% for *T. molitor*, 33-44% for *Z. atratus* and 13-24% for *A. diaperinus*) fell within the ranges reported in literature (*ca.* 23-40% for T. molitor, 40-45% for Z. atratus and 21-24% for A. diaperinus; Barker et al., 1998; Despins and Axtell, 1995; Finke, 2002; Ghaly and Alkoaik, 2009; Pennino et al., 1991; Ramos-Elorduy et al., 2002; Tzompa-Sosa et al., 2014; Yi et al., 2013). However, larvae grown on diet LPHS had a lower fat content, as did the diet itself. In addition, larvae use fat reserves for energy when diet nutritional quality is low (Arrese and Soulages, 2010). A low nutritional quality of diet LPHS could have contributed to the lower fat content of larvae on this diet. Raubenheimer and Simpson (2003) observed a rather stable lipid content in the desert locust *Schistocerca gregaria* (Forsskål) on increasingly protein deficient diets. A similar result was not observed in the present study. Insects can synthesise lipids out of different dietary components such as carbohydrates. Possibly, the more difficult to digest potato starch present in diet LPHS interfered with biosynthesis of lipids out of carbohydrates.

With respect to fatty acid composition, larvae of all three species were predominantly high in palmitic acid, oleic acid and linoleic acid. This corresponds to results from previous studies (Finke, 2002; Howard and Stanley-Samuelson, 1996; Tzompa-Sosa et al., 2014), although values for the three fatty acids reported by Finke (2002) were relatively low compared to those reported in the present study. Diet composition was, however, not specified by Finke (2002). The present results show a rather wide range of individual fatty acid contents depending on the diet, in particular for oleic acid and linoleic acid. Though larval fatty acid composition was altered by diet, it did not necessarily follow the same trend as dietary fatty acid composition in this study, indicating physiological regulation of larval fatty acid composition. Control diet B-Tm/Za contained the highest amount of palmitic acid and linoleic acid, but larvae fed on these diets did not contain the highest amount of these fatty acids. Larval oleic acid content was highest on diet LPHS, whereas this diet contained the lowest amount of oleic acid. In contrast, linoleic acid content was lowest in diet LPHS, and also in the larvae fed on this diet. These results show that larval fatty acid composition can be altered by diet, but to what extent differs between individual fatty acids and mealworm species. Insects are known to synthesise certain fatty acids *de novo*, such as palmitic, oleic and stearic acid (Beenakkers et al., 1985; Canavoso et al., 2001; Stanley-Samuelson et al., 1988). This could be an explanation for larval levels of palmitic and oleic acid not following a similar pattern to dietary levels. In contrast, linoleic acid is more likely to be an essential fatty acids which needs to be obtained from a dietary source. According to Stanley-Samuelson et al. (1988), increasing levels of dietary

polyunsaturated fatty acids generally leads to a higher proportion of insect tissue polyunsaturated fatty acids and a lower proportion of monounsaturated fatty acids. This also appeared to be the case in the present study, where higher proportions of dietary polyunsaturated C18 fatty acids led to relatively lower proportion of larval C18 monounsaturated fatty acids (results not shown).

On all diets in this study, the three mealworm species had a high n6/n3 ratio, ranging from *ca.* 18:1 on control diet to >30:1 on diet LPHS. This was unexpected as diet LPHS had a lower n6/n3 ratio (5:1) than the other diets (*ca.* 10-12:1). However, the n6/n3 ratio of carrot exceeded 50:1. Possibly, high carrot consumption contributed to the high n6/n3 ratio of mealworms fed on diet LPHS. The n3/n6 ratios found in this study were comparable to the ratios found by Tzompa-Sosa et al. (*ca.* 25:1 for *T. molitor* and *ca.* 20:1 for *A. diaperinus*; 2014). An n6/n3 ratio of ≤ 5:1 is considered optimal for the human diet (Kouba and Mourot, 2011). When producing mealworm species for human consumption, it would improve dietary quality to lower the n6/n3 ratio through dietary fatty acid composition. Controlling fatty acid composition is, however, difficult to achieve when using organic by-products. In conventional meat, the ratio ranges from 10:1 to 15:1 (Kouba and Mourot, 2011), but can be lowered through diet. The extent to which the n6/n3 ratio can be lowered for the mealworm species used in this study remains a topic of investigation.

In conclusion, the mealworm species used in this study can be grown successfully on diets composed of organic by-products. Diet affects mealworm growth, development and feed conversion efficiency, where diets high in yeast-derived protein appear favourable with respect to reduced larval development time, reduced mortality and increased weight gain. However, studies spanning several insect generations should be performed to determine the effect of diet composition on adult fecundity. Dietary protein content had a minor effect on mealworm protein content, whereas larval fat content and fatty acid composition varied over a wider range. Diets that allowed for fast larval growth and low mortality in this study led to a less favourable n6/n3 ratio than control diets. Further studies are needed in order to compose diets which support optimal growth and development rate, while simultaneously facilitating a more optimal nutritional composition for human consumption.

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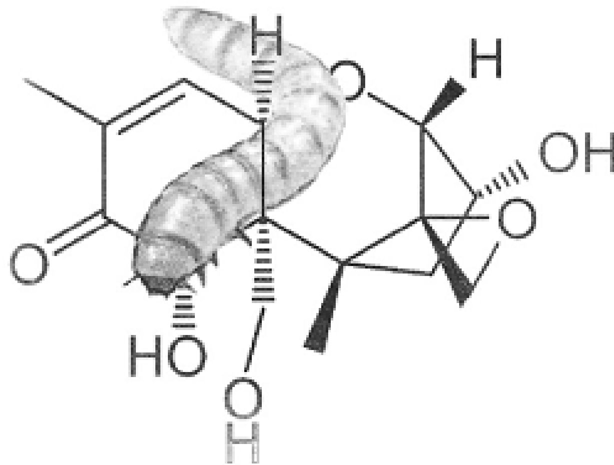
Chapter 3

Effect of mycotoxin contaminated diet on edible Yellow mealworm (*Tenebrio molitor* L.) and possible contamination risk



Van Broekhoven, S., Mota Gutierrez, J., De Rijk, T.C., De Nijs, W.C.M. and Van Loon, J.J.A. (2015) Effect of mycotoxin contaminated diet on edible Yellow mealworm (*Tenebrio molitor* L.) and possible contamination risk. *To be submitted*.

Abstract

The world population is growing, leading to an increased demand for animal protein. Several environmental problems are associated with conventional meat production, such as greenhouse gas emissions. Insects could be an alternative and a more sustainable source of animal protein. Yellow mealworms (*Tenebrio molitor* L.) are being produced in The Netherlands for human consumption. The larvae can be grown on diets made from organic by-products from the food and bio-ethanol industry, which would contribute to the circular economy. However, organic by-products can be contaminated with mycotoxins. Previous research showed that mycotoxin contamination of mealworm diet had little to no effect on mealworm growth and development. Hence, contamination of edible mealworms could go unnoticed, posing a possible threat to the consumer. Thus far, little is known about possible retention, excretion or detoxification of mycotoxins by edible insects.

Tenebrio molitor larvae were grown on wheat flour naturally contaminated with mycotoxins (predominantly deoxynivalenol (DON)), wheat flour spiked with 8 mg/kg pure DON, and uncontaminated wheat flour. Larval survival and weight gain on the three diets were compared. Presence of mycotoxins in larvae and larval faeces was analysed using LC-MS/MS.

Presence of dietary DON had no effect on *T. molitor* growth and survival. No DON or DON derivatives were detected in unmetabolised form in *T. molitor* after harvest. Excretion of DON in larval faeces was observed. These are promising results with respect to food safety. However, metabolism of DON in *T. molitor*, as well as possible toxicity of metabolites, remains to be investigated.

Keywords: deoxynivalenol, edible mealworm, contamination, survival, excretion

Introduction

A growing world population and increasing welfare are leading to an increased demand for animal protein (Van Huis, 2013; Van Huis et al., 2013). Several environmental disadvantages are associated with conventional meat production, such as greenhouse gas emission, land and water use (Mekonnen and Hoekstra, 2010; Steinfeld et al., 2006). The production of mini-livestock, such as edible insects, could provide an alternative protein source. Edible insects provide protein of similar quality to conventional livestock (Rumpold and Schlüter 2013), but compare favourably to conventional meat in terms of greenhouse gas production and land use (Oonincx and De Boer, 2012; Oonincx et al., 2010). Insects are commonly consumed in the tropics; however, efforts are ongoing to introduce insects into the Western diet. In Western countries, rather than collected from nature, edible insects are produced in closed farming systems. Promising candidate species for human consumption include species that are already commercially produced as pet food for fish, birds and reptiles, such as cricket and mealworm species. Several commercial rearing companies in The Netherlands have started to produce these insects for human consumption.

In addition to nutritional information and environmental benefits, food safety is important to consider when introducing new food products on the market (Van der Spiegel et al., 2013). Food safety risks of edible insects were reviewed by Van der Spiegel et al. (2013), Rumpold and Schlüter (2013) and Belluco et al. (2013). Most studies deal with food safety risks associated with edible insects in the tropics, such as contamination with bacteria and fungi. Such contamination can occur either during the insect's lifecycle when it feeds on contaminated substrate, or after harvest by contamination during processing or storage by spoilage under unrefrigerated conditions. In the West, commercially produced edible insects will not acquire harmful compounds or pathogens from nature and risk of post-harvest spoilage is decreased. However, a risk of acquiring contaminants during the insect's life cycle is still possible when produced on contaminated feed. Especially mycotoxins may pose a risk.

Mycotoxin contamination of grains has been on the rise in recent years, among others due to climatic changes (McCormick et al., 2011). According to Gurnari (2015), mycotoxins are one of the most important challenges in the food industry today. In addition, mycotoxins are prevalent in grain used as animal feed, with deoxynivalenol and ochratoxin A being most often detected in feed samples from Europe (Rodrigues and Naehrer, 2012).

Yellow mealworms (*Tenebrio molitor* L.) are commonly produced on wheat bran. Alternatively, these insects could be produced more sustainably and efficiently on diets derived from organic by-products (Ramos-Elorduy et al., 2002; Van Broekhoven et al., 2015). Wheat bran, as well as organic by-products used as insect feed, could be contaminated with mycotoxins. Mycotoxins could have a deleterious effect directly on the insects or, indirectly, on the consumer of the insect. Elaborate research has been performed to determine the risk of mycotoxin contamination of feed for conventional livestock (EFSA,

2004, 2011, 2013; Pestka, 2007). However, research on the risk of mycotoxin contamination of edible insects is limited. Mycotoxigenic fungi such as *Aspergillus*, *Penicillium* and *Fusarium* species were isolated from samples of edible caterpillars *Imbrasia belina* (Simpanya et al., 2000) and *Bunaea alcinoa* (Braide et al., 2011) in Africa. Presence of mycotoxins was, however, not detected. Abadon-Becognee et al. (1998) investigated the effect of fumonisin B_1 on the growth and metabolism of Yellow mealworm. The presence of fumonisin B_1 at a concentration of 450 µg/g diet reduced larval growth and metabolism, but did not increase mortality. About 40% of the ingested fumonisin B_1 was excreted through faeces. Davis and Schiefer (1982) reported reduced larval growth of Yellow mealworm feeding on diets containing up to 64 µg/g of the trichothecene T-2 toxin, but no increased mortality. A study by Guo et al. (2014) showed that *T. molitor* larvae readily fed on grain infested with several different *Fusarium* species. Several mycotoxins produced by these *Fusarium* species were detected in the larval bodies after the feeding experiment, including zearalenone, fumonisin B_1 and several enniatins.

The fact that *T. molitor* can consume mycotoxin-contaminated feed without any visible adverse effects on larval growth and mortality suggests mycotoxin contamination may go unnoticed by the insect producer. This could pose a health risk to the consumer of edible insects. In this study, the effect of feed contaminated with deoxinivalenol (DON) on *T. molitor* larvae was determined. DON, also known as vomitoxin, is a mycotoxin in the group B of trichothecenes. It is produced by *Fusarium graminearum*, *F. pseudograminearum* and *F. culmorum* (Glenn, 2007). DON is a polar organic molecule and contains a carbonyl and epoxy group (Figure 1) which have been associated with its toxicity (Nagy et al., 2005). Two acetylated forms of DON co-occur with DON: 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), both of which have lower toxicity than DON. Furthermore, glucosidic plant metabolites can co-occur in contaminated crops (Pestka, 2010). Acute exposure to DON can lead to gastrointestinal symptoms such as vomiting in both humans and animals (EFSA, 2013). Long term dietary exposure can lead to decreased weight gain, decreased feed intake and altered nutritional efficiency. However, there is no evidence for mutagenic of carcinogenic effects. The Tolerable Daily Intake (TDI) for DON as established by the Scientific Committee on Food is 1µg/kg body weight per day (EFSA, 2013).

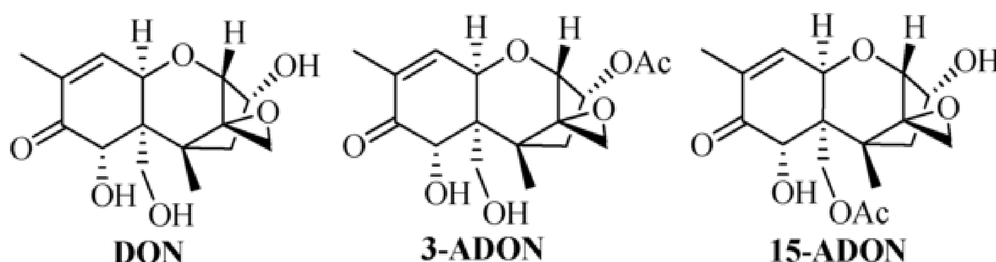


Figure 1: Chemical structure of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). Source: Fruhmann et al. (2014).

In this study, *Tenebrio molitor* larvae were grown on wheat flour naturally contaminated with DON and DON-derivatives; wheat flour spiked with pure DON; and clean wheat flour.

The effect of dietary DON on larval growth performance and survival was monitored. Furthermore, in order to determine whether DON and DON-derivatives are sequestered or excreted by the larvae, harvested larvae and larval faeces were analysed for the presence of DON and DON derivatives.

Materials and methods

Materials

Five weeks old Yellow mealworm larvae were obtained from commercial insect producing company Van de Ven (Deurne, The Netherlands). Naturally contaminated wholegrain wheat flour (*Triticum aestivum* L.) was obtained from RIKILT (Wageningen, The Netherlands). Clean wholegrain wheat flour was obtained from windmill De Vlijt (Wageningen, The Netherlands). Pure DON was obtained from Romer Labs® (Tulln, Austria).

Diet preparation

Clean wheat flour served as control diet. Experimental diets consisted of naturally contaminated wheat flour containing, among others, 4900 µg/kg DON (Table 1); and clean wheat flour spiked with 8000 µg/kg pure DON. In order to prepare the spiked diet, 750 g clean wheat flour was mixed with 1500 mL water using an ultra-turrax. During mixing, 30 mL 200 µg/mL DON in acetonitrile was added, after which the slurry was mixed by head over head rotation for 1 h. The slurry was then lyophilised for seven days until stabile pressure. The lyophilised material was ground using a food processor. Three samples were taken for analysis of homogeneity using LC-MS/MS.

Table 1: Mycotoxins present in naturally contaminated wheat flour obtained from RIKILT.

Mycotoxin	Concentration (µg/kg)
Deoxynivalenol (DON)	4900
15-ADON	86
DON-3G	300
Beauvericin	13
Enniatin A1	45
Enniatin B	490
Enniatin B1	170
Nivalenol	270
Zearalenone	73
Ergotamine	5
Ergotaminine	16

Experimental setup

Five weeks old larvae with an average weight of 58 mg were randomly assigned to either of the three diets. Each diet treatment comprised six replicates. Each replicate consisted of a plastic container (17.5 x 9.3 x 6.3 cm) with aeration slits in the sides. Each container contained 50 larvae on 2 g of diet and 1 g of carrot. Carrot was replaced twice a week. Once larvae had consumed all diet (based on visual estimation of diet and faeces), faeces were removed and stored at -20°C until analysis, and fresh diet was added.

Larvae were allowed to grow for two weeks, after which they were at harvest weight (> 100 mg). Larvae were cleaned of faeces and counted, after which larvae and faeces were weighed separately. For each replicate, half of surviving larvae were killed immediately by freezing at -20°C, and half were allowed to fast for 24 h in empty containers. Larvae were subsequently killed by freezing at -20°C. Larvae and faeces were oven dried at 50°C until constant weight. All material was then weighed and ground to powder in a grinder (Tomado TM-1287).

LC-MS/MS analysis

Presence of DON and derivatives (DON-3-glucoside, 3-acetyl-DON and 15-acetyl-DON) in larvae and faeces was determined by LC-MS/MS analysis at RIKILT using a AB SCIEX QTRAP® 5500 System according to the method described by Van Asselt et al. (2012) with a limit of detection (LOD) of 100 µg/kg. For each treatment, two experimental replicates were pooled to obtain three biological replicates in total.

Calculations and Statistical analysis

Excreted DON as percentage of ingested DON was calculated as:

$$excreted\ DON = \frac{amount\ of\ faeces\ produced\ \times amount\ of\ DON\ in\ faeces}{amount\ of\ diet\ provided\ \times amount\ of\ DON\ in\ diet} \times 100\%$$

Where, based on visual evaluation, it was assumed that larvae had consumed all diet provided.

Data of larval survival and weight gain did not conform to the normal distribution and were analysed by a Kruskal-Wallis test at a significance level of 0.05, followed by Mann-Whitney U tests with applying Šidák correction, where the level of significance was corrected to $1-(1-0.05)^{1/2}= 0.025$. Where DON and DON derivative concentrations were below detectable levels, statistics could not be applied. All statistical analyses were performed using IBM SPSS statistics v. 20.

Results

Larval growth and survival

Tenebrio molitor larvae were observed to readily consume all three diets, which were similar in appearance (personal observation). Larval survival was high on both clean Control diet (98.33%) and the two DON-contaminated diets (98.33% for Naturally contaminated and 99.33% for DON-spiked, respectively; Figure 2). No significant difference in survival was observed between the different diet treatments (p = 0.230).

Average weight increase was 49.80 mg for larvae on Control diet, 63.26 mg on Naturally contaminated diet and 48.55 mg on DON-spiked diet, with medians of (Figure 3). No significant difference was observed between diet treatments (p = 0.091).

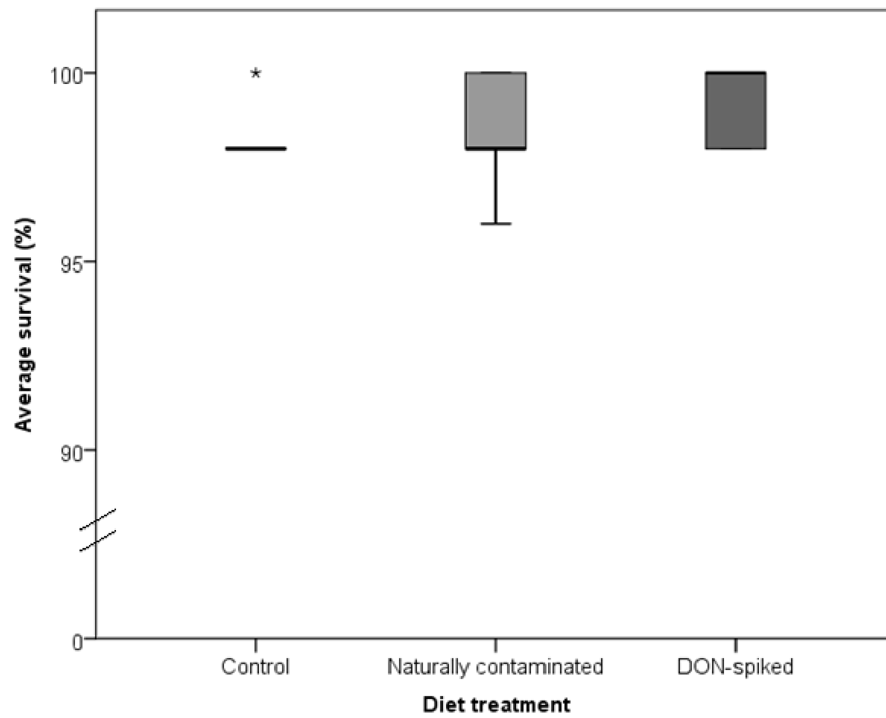


Figure 2: Average larval survival of Yellow mealworm (*Tenebrio molitor*) as percentage of the total number of larvae at the start of the experiment (n = 50) after feeding on either clean control diet, diet naturally contaminated with deoxynivalenol (DON) and diet spiked with 8000 µg/kg DON. A star represents an outlier.

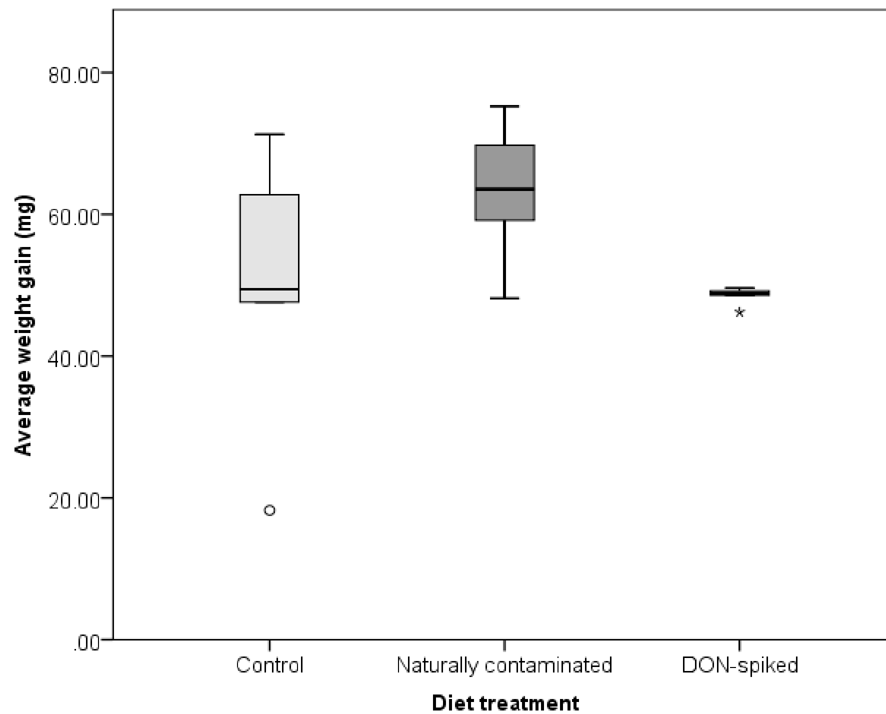


Figure 3: Average larval weight gain of Yellow mealworm (*Tenebrio molitor*) at the end of the experiment after feeding on either clean control diet, diet naturally contaminated with deoxynivalenol (DON) and diet spiked with 8000 µg/kg DON. A star represents an outlier; a circle represents an extreme value.

Analysis of mycotoxins

Larval and faecal samples were analysed for presence of DON and DON derivatives. No DON derivatives were detected in any sample (data not shown). DON was not present in detectable levels in both directly harvested larvae and larvae that fasted for 24 h. However, faeces of larvae on DON-contaminated diets contained DON in levels that far exceeded the limit of detection (1140 µg/kg for Naturally contaminated and 4980 µg/kg for DON-spiked, respectively).

The percentage of excreted DON in faeces from larvae on DON-spiked diet was higher (*ca.* 41%) than in faeces from larvae on Naturally contaminated diet (*ca.* 14%; Table 3).

Table 2: Concentration of deoxynivalenol (DON) in Yellow mealworm (*Tenebrio molitor*) larvae and faeces after feeding on clean or DON-contaminated diet, analysed by LC-MS/MS. LOD was 100 µg/kg. Values are given as mean ± SD; n = 3.

Diet	**DON (µg/kg)**
Larvae, directly harvested	
Control	ND
Naturally contaminated	ND
DON-spiked	ND
Larvae, fasted	
Control	ND
Naturally contaminated	ND
DON-spiked	ND
Faeces	
Control	ND
Naturally contaminated	1140 ± 141.8
DON-spiked	4980 ± 209.3

ND: Not detected.
DON derivatives were excluded because these were not detected in any of the samples.

Table 3: Amount and percentage of dietary deoxynivalenol (DON) ingested and excreted by *Tenebrio molitor* larvae combined in pooled replications. Values are given as mean ± SD; n = 3.

	Diet			
	Naturally contaminated		**DON-spiked**	
DON ingested (µg)	99.666	± 5.669	144.177	± 0.046
DON excreted (µg)	13.953	± 1.664	58.572	± 1.464
% excreted	14.027	± 1.767	40.625	± 1.018

Discussion

This study shows that feeding on high concentrations of DON (up to 8,000 µg/kg) has no direct detrimental effects on *T. molitor* survival and weight gain. DON was partly excreted through larval faeces and no detectable levels of this mycotoxin remained in the larval body in unmetabolised form.

Larvae of *T. molitor* were allowed to feed on clean wheat flour, naturally contaminated wheat flour containing several mycotoxins including 4900 µg/kg, and wheat flour spiked with 8,000 µg/kg DON. The larvae readily consumed all diets (personal observation). Presence of DON did not increase larval mortality. Similarly, Abado-Becognee et al. (1998) and Davis and Schiefer (1982) and Van Broekhoven et al. (2013) did not observe increased mortality when *T. molitor* larvae consumed diet contaminated with fumonisin B_1 and T2-toxin. Guo et al. (2014) observed that *T. molitor* larvae feeding on wheat kernels infested with *Fusarium proliferatum* or *F. poae* showed similar survival rates as larvae feeding on uncontaminated wheat. In contrast, the presence of *F. culmorum*, *F. avenaceum* or *Beauveria bassiana* did increase mortality. Compared to the *Fusarium* species that did not increase larval mortality, *F. culmorum* contained high levels of zearalenone (>210,000 µg/kg) and deoxynivalenol (10,240 µg/kg), while *F. avenaceum* contained high levels of enniatins (> 90,000 µg/kg). *Beauveria bassiana* did not contain mycotoxins in high quantities, but the fungus can multiply within the insect body and possibly kill the larvae by different mechanisms. The highest concentration of DON in the present study was lower than the level found for *F. culmorum* infested wheat in the study of Guo et al. (2014). Larvae in both studies were allowed to feed on contaminated diet for a comparable amount of time (14 vs 15 days). Possibly, 8,000 µg/kg is still too low for *T. molitor* to affect mortality. Alternatively, the mortality observed by Guo et al. (2014) was due to zearalenone rather than DON, or a combined effect of both mycotoxins. Zearalenone was also present in the naturally contaminated wheat flour used in the present study, but in a much lower concentration (73 µg/kg; Table 1). Similarly, the concentration of enniatins (up to 490 µg/kg; Table 1) was much lower in the present study than in the study by Guo et al. (2014). An even higher concentration of DON (25, 000 µg/kg) was administered by Dowd et al (1989) to the moth species Corn earworm (*Helicoverpa zea*) and Fall armyworm (*Spodoptera frugiperda;* both Lepidoptera: Noctuidae). Reductions in weight were observed, but no significant differences in mortality. With lower concentrations, little negative effect was observed.

The presence of DON did not lead to decreased weight gain for *T. molitor. Tenebrio molitor* has been found to consume fungus-infested material (Chuku et al., 2007; Davis et al., 1975; Guo et al., 2014). Not only can larvae prefer fungus-infested diet over uncontaminated diet (Guo et al., 2014), the presence of fungus can even promote larval growth, depending on the fungal species and the substrate the fungus is growing on (Davis et al., 1975; Guo et al., 2014). The presence of fungus in diet might provide the larvae with

nutritional factors contributing to growth. This might compensate for the effect of mycotoxins and other anti-nutritional factors produced by the same fungus, which would cause a problem for the insect producer and a possible health risk for the consumer.

After the feeding period, *T. molitor* larvae were either directly harvested, or were allowed to fast for 24 h to clear the digestive tract. No DON or DON derivatives were detected in larval bodies of both groups. Possibly, the mycotoxins were not sequestered in the larval bodies in unmetabolised form in quantities exceeding the detection limit. Similarly, Guo et al. (2014) did not detect DON in larvae that had been feeding on *Fusarium* infected wheat containing a high level of DON. Mycotoxins that were detected were present in low concentrations compared to the levels found in the infected wheat, with the exception of Enniatin A (30 µg/kg in the diet vs 10 µg/kg in the larvae).

The present study shows that *ca.* 14% of DON ingested through naturally contaminated wheat flour was excreted through faeces. For DON-spiked wheat flour, this was *ca.* 41%. For both diets, the remaining fraction of DON could not be detected. This suggests that it was metabolised by *T. molitor*. Metabolites could be either sequestered or excreted through faeces. It is not known why a smaller proportion of DON was excreted after feeding on naturally contaminated wheat flour, compared to DON-spiked wheat flour. Possibly, the presence of other fungal metabolites in the naturally contaminated flour interfered with excretion of DON in *T. molitor* larvae.

Exposure and metabolism of DON is well documented for conventional livestock. Sensitivity to DON is related to the extent to which the animal is capable of metabolising DON to compounds with decreased or lost toxicity (Pestka, 2007). The major metabolite detected in the urine and faeces of animals exposed to DON is de-epoxy DON (DOM-1), produced by microbes present in the intestine (Dänicke et al., 2004; Pestka, 2007). DOM-1 was, however, not detected in human faeces (Sundstøl Eriksen and Pettersson, 2003) and also does not contribute much to DON detoxification in pigs, the most sensitive species (Dänicke et al., 2004). Another mode of DON detoxification is the conjugation to DON-glucuronides in the liver (Pestka, 2007). No information is available on detoxification of DON or other mycotoxins by *T. molitor*. However, detoxification has been studied to some extent in different insect species. Dowd (Dowd, 1990) found p-nitroanisole (PNA) O-demethylation and CDNB glutathione conjugation activity in response to > 25 000 µg/kg DON for *H. zea*. In contrast, PNA O-demethylation activity was found for *S. frugiperda*, whereas CDNB glutathione conjugation activity was inhibited. Presence of O-demethylated or glutathione-conjugated DON metabolites was not investigated. Niu et al. (2008) found that the P450 monooxygenase CYP321A1 contributed to the detoxification of aflatoxin B1 in *H. zea* by transforming it to aflatoxin P1, an O-demethylated product of aflatoxin B1. P450 monooxygenases (P450s) are known to contribute to detoxification of xenobiotics in all organisms including insects (Niu et al., 2008). In contrast, P450s can also increase toxicity. In *H. zea*, the P450 CYP2A6 contributes to the toxicity of aflatoxin B1 (Zeng et

al., 2006). Another invertebrate species, the earthworm *Lumbricus terrestris*, interacts with soil microorganisms to degrade DON (Oldenburg et al., 2008).

Enzymes involved in detoxification of xenobiotics, such as P450s and glutathione-S-transferases, are present in *T. molitor* and related species (Kostaropoulos et al., 1996; Richards et al., 2008; Silva et al., 2015). These enzymes are possibly involved in detoxification of mycotoxins. Which enzymes are responsible for metabolism of DON in *T. molitor* and which metabolites are produced remains a topic of investigation.

In conclusion, *T. molitor* larvae did not experience significant detrimental effects in response to dietary DON concentrations up to 8000 µg/kg. DON was partly excreted through faeces in unmetabolised form. These are promising results with respect to food safety. However, the remaining portion is likely metabolised into different compounds. Further studies should focus on the metabolic pathway in response to DON exposure in *T. molitor*, as well as the possible toxicity of the resulting DON metabolites.

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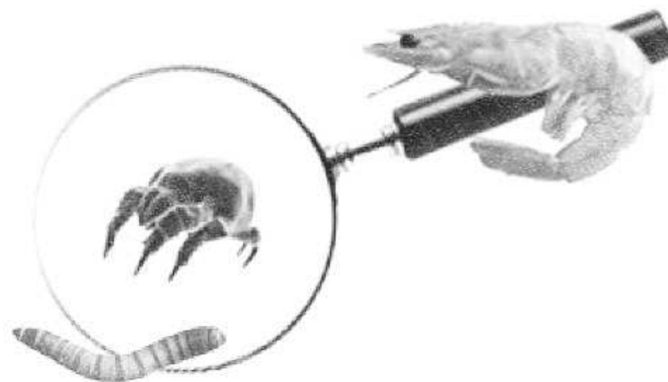
Chapter 4

House dust mite (Der p 10) and crustacean allergic patients may react to food containing Yellow mealworm proteins



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Abstract:

Due to the imminent growth of the world population, shortage of protein sources for human consumption will arise in the near future. Alternative and sustainable protein sources (e.g. insects) are being explored for the production of food and feed. In this project, the safety of Yellow mealworms (*Tenebrio molitor* L.) for human consumption was tested using approaches as advised by the European Food Safety Authority for allergenicity risk assessment.

Different *T. molitor* protein fractions were prepared, characterized, and tested for cross-reactivity using sera from patients with an inhalation or food allergy to biologically related species (house dust mite (HDM) and crustaceans) by immunoblotting and basophil activation. Furthermore, the stability was investigated using an *in vitro* pepsin digestion test.

IgE from HDM- and crustacean allergic patients cross-reacted with *T. molitor* proteins. This cross-reactivity was functional, as shown by the induction of basophil activation. The major cross-reactive proteins were identified as tropomyosin and arginine kinase, which are well known allergens in arthropods. These proteins were moderately stable in the pepsin stability test.

Based on these cross-reactivity studies, there is a realistic possibility that HDM- and crustacean allergic patients may react to food containing *T. molitor* proteins.

Key words: food allergy, risk assessment, Yellow mealworm, cross-reactivity, crustaceans, House dust mite

Introduction

Due to the imminent growth of the world population and increasingly more demanding consumers (Tilman et al., 2011), shortage of protein sources for human consumption is to be expected in the near future (Van Huis, 2013). Because available energy supplies, clean water and land are declining, new initiatives are initiated to find more sustainable protein sources than conventional meat for the food and feed industry, including the production of mini-livestock such as edible insects (Van Huis, 2013; Van Huis et al., 2013).

The introduction of new food sources is regulated by the Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997, concerning novel foods and novel food ingredients. All food and food products that were not commonly used for consumption before 1997 must be labelled as novel foods (http://eur-lex.europa.eu/). For new food products, it is important to assess food safety in terms of microbial, nutritional, toxicological, and allergenic risks.

Food allergy is a major health concern in the Western society. The prevalence of food allergy is around 3-4 % in the general population (Sicherer, 2011). Symptoms of food allergy range from oral allergy to anaphylactic shock. In addition to direct sensitization to proteins in foods, food allergy can also result from IgE cross-reactivity between proteins in other food products or inhalant allergens from other species. Cross-reactivity is well-known for various related proteins (e.g. vicilins, PR-10 proteins, tropomyosins; Radauer et al., 2008).

New proteins and genetically modified foods are currently assessed for their allergenic potential using an allergenicity assessment strategy (decision tree) advised by the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) (FAO/WHO, 2001) or the weight of evidence approach described by the European Food Safety Authority (EFSA, 2010). Both approaches focus on the source of the gene, the similarity of the amino acids sequence of the new protein with that of known allergens, cross-reactivity with human sera from food allergic patients, and the stability of the protein tested in a static *in vitro* digestion model with pepsin.

In this study, the allergenicity of Yellow mealworm (*Tenebrio molitor* L.) protein was assessed. The mealworm is the larval stage of the Yellow mealworm beetle and is commercially produced as feed for animals such as fish, reptiles and birds. It could additionally be considered an alternative protein source for humans (Van Huis, 2013). To our knowledge, little is known about the allergenic potency of *T. molitor* proteins. A number of publications report occupational allergy in sensitised individuals, with symptoms such as asthma, rhinoconjunctivitis, and contact urticaria. These reactions were mostly observed in people frequently working with *T. molitor* (Bernstein et al., 1983; Schroeckenstein et al., 1990). Only one publication reported anaphylaxis following the ingestion of *T. molitor* by an individual with a known inhalant allergy to this insect (Freye

et al., 1996). A few cases have been described of allergic reactions upon the consumption of other insect species, including Mopane worms (*Imbrasia belina*; Okezie et al., 2010) and Domestic silkworm pupae (*Bombyx mori*; Liu et al., 2009).

Because *T. molitor* may be introduced as food for human consumption, the safety with respect to the potential development of food allergy needs to be assessed. In this study, the weight of evidence approach was used to assess the allergenic potency of *T. molitor* proteins.

Materials and Methods

Materials

All reagents were obtained from Sigma (St-Louis, USA), unless stated otherwise. *T. molitor* in final larval instars was obtained from insect producing company Kreca (Ermelo, the Netherlands).

Patient sera

Sera were collected at the University Medical Centre Utrecht (Utrecht, the Netherlands) from well characterized allergic patients that were positive for specific IgE measured by ImmunCAP ISAC (Immuno Solid-Phase Allergen Chip). In this study, sera were used from seven patients allergic to crustaceans and House dust mite (HDM, *Dermatophagoides pteronyssinus* Trouessart) Der p 10. Negative control sera were used from patients (in total 15 patients) allergic to grass pollen, peanuts, fish or eggs and/or milk. These patients were not allergic to crustaceans or HDM Der p 10. This study was approved by the ethical committee of the University Medical Centre Utrecht.

Preparation of *Tenebrio molitor* extracts

Larvae were killed by freezing at -20 °C. Ten grams of frozen larvae were disrupted in 70 mL ice cold extraction buffer (20 mM Tris buffer pH 7.6, 1 mM phenylthiocarbamide and Halt Protease Inhibitor Cocktail (Pierce Protein Biology Products) using an ultra-turrax. After centrifugation (10 min 16000*g* at 4°C), the supernatant was split into two fractions. One fraction was frozen immediately (SRN1) while the other was dialysed overnight (SRN2) against cold 20 mM Tris buffer pH 7.6 using a 3500 Da dialysis membrane. Both fractions were stored at -20°C.

The water insoluble residue was washed twice with extraction buffer followed by overnight extraction at 4°C in 70 mL 6 M urea in extraction buffer (SRN3). The sample was centrifuged (10 min 16000*g* at 4°C) and the supernatant was stored at -20°C. The protein concentration was measured using the Bradford method (Bio-rad).

Identification of proteins in *Tenebrio molitor* extract

The three individual extracts (380 µg protein) were digested overnight with trypsin (protein: trypsin 380 : 7.5) after reduction and alkylation according to the standard digestion protocol (see "Identification of cross-reactive proteins"). Digestion was confirmed by Coomassie-stained SDS-PAGE. Prior to LC-MS analysis, samples were dried using a vacuum concentrator (MAXI Dry Plus, Heto-Holten, Denmark) and reconstituted in 0.1 % formic acid (FA) in water. Ten µL of extract (20 µg protein) was injected on an Atlantis® dc18 column (1.0 x 150 mm, 3 µm, Waters) using gradient elution with a constant flow of 50 µL min^{-1}. The gradient started with 95 % A (0.1 % *v/v* FA in water) for 5 minutes followed by a linear increase to 45 % B (0.1 % *v/v* FA in MeCN) which was achieved in 25 minutes. This was followed by a linear increase to 95 % B in 5 minutes and was kept at this gradient for another 5 minutes. The LC-MS system consisted of a Thermo Orbitrap mass spectrometer (Breda, The Netherlands) coupled to a Thermo Accela auto sampler and pump. The electrospray interface was used in positive-ion mode and an ion-spray voltage of 4.5 kV was applied. Capillary temperature was set to 350°C. The orbitrap was operated in data-dependent mode, selecting the top 5 ions for MS/MS scans at 35 % collision energy units. See for protein identification "Identification of cross-reactive proteins".

Determination of allergic potential

The allergic potential of proteins identified in *T. molitor* extracts of which the sequence is known, as well as that of *T. molitor* proteins with a known sequence present in UniProt database, was predicted using Allermatch™ (www.allermatch.org). Comparison in Allermatch™ was based on UniProt as well as the WHO-IUIS database. An 80 amino acid sliding window alignment was performed with a 35 % cut-off percentage (Fiers et al., 2004).

SDS-PAGE and Immunoblotting

All three individual *T. molitor* protein extracts (3 µg) or digests thereof were diluted with sample buffer (50 mM Tris pH 6.8, 2 % SDS, 10 % glycerol, 2 % β-mercaptoethanol) and analysed using 15 % acrylamide/Tris-HCl gels (Criterion, Biorad, Germany). Control samples (extracts of shrimp/lobster, peanut, grass pollen, herring/cod, egg from ALK, Denmark) and a Protein marker (Bio-Rad) were run on each gel. Proteins were visualised using Coomassie gel staining (Expedion, UK) or used for immunoblotting. For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 4 % (w/v) Protifar (Nutricia, The Netherlands) in PBS/0.1 % Tween 20 for 60 min after which they were incubated for 1 hour with diluted patient sera (dilution depended on IgE titers and ranged from 1:25 to 1:200) at room temperature. Bound IgE was detected with 1:30000 diluted peroxidase-conjugated goat anti-human IgE (KPL, USA). Visualisation was performed using a chemiluminescent peroxidase substrate kit and blots were scanned using a Chemidoc XRS+ image scanner

with Imagelab software (Bio-Rad). Bands of interest were excised from gel for identification of cross-reacting proteins.

Immunoprecipitation

For immunoprecipitation, Dynabeads M-280 Tosylactivated (10 mg, Invitrogen) were used according to the manufacturer's protocol. Beads were coated with 0.2 mg Goat anti-Hu IgE (Invitrogen). Conjugated beads were incubated for 1 hour at 37˚C with 1 mL of a mixture of patient sera (bead 1) from patients 2, 5 and 6 (2:3:3) or 1 mL serum from patient 1 (bead 2). Conjugated beads were cross-linked with 5 mM BS^3 (Pierce) according to the manufacturer's protocol to ensure reusability of the beads. The beads were washed three times with 0.1% Tween 20 in PBS pH 7.4, followed by overnight incubation at 37˚C with either 100 µL of a mixture of SRN1 + SRN2 with bead 1 or 100 µL SRN 3 (500 µg protein) with bead 2. Proteins were eluted with 2 times 100 µL 0.1 M glycine and the pH of the solution was neutralized using 20 µL of 1 M tris-HCL pH 8.5. Incubation with Yellow mealworm extract was repeated twice and all elutates were pooled, freeze dried and stored at -80˚C until further analysis.

Identification of cross-reactive proteins

Gel bands for mass spectrometric analysis were processed according to (Shevchenko et al., 2006). Gel pieces were washed with 100 mM NH_4HCO_3 and HPLC-grade acetonitrile (1:1, v/v) (buffer A). Proteins were in-gel reduced by 10 mM DTT in buffer A, and subsequently alkylated with 55 mM iodoacetamide in buffer A. Proteins were digested overnight at 37°C in digestion buffer (40 mM NH_4HCO_3, 10% acetonitrile) containing 12.5 ng/µl proteomics-grade trypsin. Peptides were extracted with 100 µl 2:1 (v/v) ACN : 5 % FA. Extracts were dried in a vacuum centrifuge and reconstituted in 10 µl of mobile phase A (2.8.3).

Samples from Immunoprecipitation were reconstituted in 250 µL HPLC-grade water, reduced with 10 mM DTT (1 h, 37° C), alkylated with 24 mM iodoacetamide (1 h, 37° C) and digested with 600 ng proteomics-grade trypsin after quenching with 2 mM DTT (20 min, 37 ^{o} C). Peptides were purified by strong-cation exchange stage tips and subsequently injected for mass spectrometric analysis.

Chromatography was performed on an Easy LC 1000 nanoscale liquid chromatography (nanoLC) system (Thermo Fisher Scientific, Denmark). Four µL peptide mixture was loaded at 500 nl/min directly onto a pulled silica capillary (75 µm i.d.), in-house packed to a length of 10 cm with 3 µm C_{18} silica particles (Dr. Maisch, Germany). For gel band analysis, gradient elution was achieved at 350 nl/min flow rate, and ramped from 100 % A (0.1 % FA in 2 % acetonitrile), to 30 % B (0.1 % FA, 80 % acetonitrile) in 15 min, then from 30 % B to 100 % B in 5 min. MS detection was performed on the Q-Exactive (Thermo Fisher Scientific, Germany) operating in positive ion mode, with nanoelectrospray (nESI) potential at 1800 V. Data-dependent acquisition was performed using a top-5 method. Mass window for precursor I on isolation was 2.0 m/z, whereas normalized

collision energy was 30. For in-solution digests, the following parameters were changed with respect to the gel spot method: (i) nanoLC gradient was ramped from 100 % A to 60 % A in 100 min. Data were processed by Proteome Discoverer 1.3 (Thermo Fisher Scientific, Germany), using Sequest as search engine and the Swiss Prot database accessed on February 2013 as sequence database. The following search parameters were used: MS tolerance 7 ppm; MS/MS tolerance 0.02 Da; fixed modifications carbamidomethyl cysteine; enzyme trypsin; max. missed cleavages 2; taxonomy *Metazoa* (for gel bands) *or Insecta* (for immunoprecipitated extracts). Protein hits based on two successful peptide identifications (Xcorr> 2.0 for doubly charged peptides, >2.5 for triply charged peptides, and >3.0 for peptides having a charge state >3) were considered valid.

<u>Indirect basophil activation test (BAT)</u>
BAT was performed as described by (Koppelman et al., 2004) with minor adaptations. Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation with Ficoll (Amersham, Sweden). Two mL of lactic acid was used to strip basophils of IgE. Basophils were reloaded with specific IgE in incubation buffer with 15 % patient serum. Cells (1×10^6) were incubated with equal volumes of i) RPMI (with 2 ng mL^{-1} IL-3 (R&D systems, USA) and 1 % HSA) as negative control; ii) a 10-fold serial dilution of allergen (from 100 µg mL^{-1} to 1 ng mL^{-1}); or iii) goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, USA) as a positive control (3 µg mL^{-1}, 1 µg mL^{-1} and 0.1 µg mL^{-1}). Cells were subsequently incubated in the dark (30 min, 37°C). Activation was stopped with 25 µL ice cold PBS plus 20 mM EDTA after which the cells were incubated in the dark (30 min, 4°C) with fluorescent labelled antibodies against CD63, CD123 and CD203c (Biolegend, USA). CD63, CD123 and CD203c expression was analysed by flow cytometry using a FACSCanto II (BD Biosciences, USA), and FACSDiva software. Basophils were identified on forward-side scatter histograms as $CD203c^{+}CD123^{+}$ and activated basophils as $CD203c^{+}CD63^{+}$. The cut-off percentage for positive basophil activation was determined as two times the percentage of activated basophils observed for RPMI + IL3 only.

<u>Simulated Gastric Fluid digestion</u>
Protein extracts (5 mg) were suspended in 10 mL water containing 23 mM citric acid and 0.38 mM Na_2HCO_3. The pH of the solution was set at 2.5. After incubation (5 min, 37°C) T=0 sample was collected (150 µL) and 900 units of porcine pepsin (1 : 0.07 protein : pepsin) was added. Digest samples were collected at 15 and 30 seconds, and at 1, 5, 10, 30, and 60 min and analysed using immunoblotting with sera from patient 1.

Results

Tenebrio molitor is taxonomically related to crustaceans and House dust mite (HDM)

Taxonomically, insects belong to the subphylum Hexapoda, which is one of the four subphyla of the phylum Arthropoda (Figure 1). Within the arthropods, several pan-allergens are known, including tropomyosin (Reese et al., 1999), arginine kinase (Binder et al., 2001) and glutathione S-transferase (Galindo et al., 2001). For these allergens, cross-reactivity has been observed not only between species within the same subphylum, but also between species from different arthropod subphyla, for example between crustacean species (e.g. shrimp, crab), chelicerates (e.g. mites) and several insects species (Binder et al., 2001; Galindo et al., 2001; Liu et al., 2009; Santos et al., 1999).

These findings suggest that people with a crustacean- or HDM allergy might experience allergic reactions upon consumption of *T. molitor*.

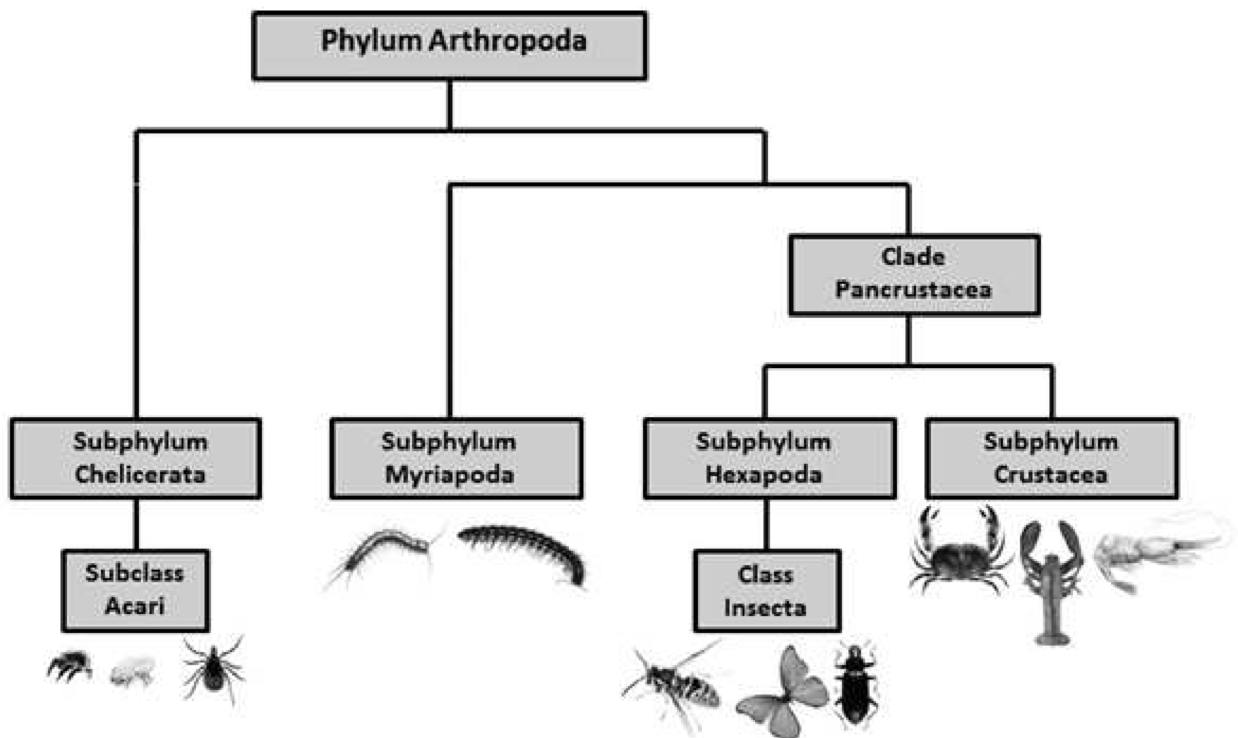

Figure 1: Simplified representation of the phylogeny of the phylum Arthropoda, based on Regier et al. (2010), Rota-Stabelli et al. (2011).

Table 1: Proteins identified in *Tenebrio molitor* protein extracts.

Protein	Accession	Score	Queries matched	Mass (kDa)	Sequence coverage (%)
SRN1					
Cationic trypsin	**P00760**	**554**	**16**	**26.5**	**45**
Calcium-transporting ATPase sarcoplasmic/ER type	Q292Q0	511	15	109.9	**11**
Arginine kinase	**P48610**	**438**	**11**	**40.1**	**17**
Actin	P49871	366	15	42.1	28
POTE ankyrin domain family member F	A5A3E0	248	9	123.0	6
Tubulin α-1 chain	**P06603**	**219**	**6**	**50.6**	**12**
Catalase	P00432	212	3	60.1	3
Alpha-amylase	**P56634**	**196**	**7**	**51.7**	**16**
Alpha-actinin	P18091	161	6	107.6	6
Muscle-specific protein 20	P14318	135	2	20.3	7
Glyceraldehyde-3-phosphate dehydrogenase	Q28259	127	4	36.1	8
Ovalbumin-like	**P01012**	**127**	**2**	**43.2**	8
Tubulin β chain	P41386	119	5	38.6	14
SRN2					
Cationic trypsin	**P00760**	**496**	**12**	**26.5**	**34**
Alpha-amylase	**P56634**	**370**	**10**	**51.7**	**20**
Arginine kinase	**P48610**	**344**	**9**	**40.1**	**14**
Calcium-transporting ATPase sarcoplasmic/ER type	Q292Q0	316	8	109.9	7
Actin	P49871	313	13	42.1	23
ATP synthase subunit β	Q05825	286	6	54.1	8
14-3-3 protein zeta	Q1HR36	280	4	28.3	21
POTE ankyrin domain family member E	Q6S8J3	225	8	122.9	7
Catalase	P00432	224	3	60.1	3
Alpha-actinin	P18091	153	5	107.6	4
SRN3					
Myosin heavy chain	P05661	2115	71	225.4	20
Actin	P83969	517	27	42.1	33
Cationic trypsin	**P00760**	**348**	**11**	**26.5**	20
Larval cuticle protein F1	Q9TXD9	335	7	14.9	51
Larval cuticle protein A1A	P80681	237	8	17.7	33
Late histone H2A	P16886	201	2	13.4	18
Tropomyosin-1	**Q1HPU0**	**200**	**5**	**32.6**	**16**
Pupal cuticle protein G1A	P80685	192	4	20.8	
Ovalbumin-like	**P01012**	**191**	**3**	**43.2**	**8**
Tropomyosin-2	**Q1HPQ0**	**154**	**10**	**32.8**	**23**
Myosin-2	P12845	134	3	223.0	1

Proteins identified by LC-MS/MS with a score of 100 and higher found in the water soluble protein fraction (SRN1), water soluble dialyzed protein fraction (SRN2) and in the urea soluble protein fraction (SRN3). Identification was based on homology with metazoan proteins in Swiss Prot database. Putative allergens are listed in bold.

Table 2: Sensitisation pattern of the different patients tested in this study.

Patient	Other[a] arthropods tropomyosin	HDM tropomyosin	HDM[b]	Fish parvalbumin	Milk[c]	Egg[d]	Pollen[e]	Peanut[f]	PR-10[g] food
Shrimp/lobster/HDM allergy									
patient 1	3	3	3	3	1	0	3	0	3
patient 2	3	3	3	0	0	0	3	0	1
patient 3	2	2	0	1	0	0	3	3	1
patient 4	3	3	3	0	2	2	3	3	3
patient 5	3	3	3	0	2	2	3	0	3
patient 6	2	2	0	0	2	3	3	3	2
patient 7	2	2	3	2	1	1	3	3	3
Grass pollen + food allergy									
patient 8	0	0	0	1	0	0	3	0	1
patient 9	0	0	0	1	0	0	3	0	3
patient 10	0	0	0	0	0	0	3	0	0
Grass pollen without food allergy									
patient 11	0	0	0	1	0	0	3	1	0
patient 12	0	0	0	1	0	0	3	0	0
patient 13	0	0	0	0	0	0	3	2	0
Peanut allergy									
patient 14	0	0	1	0	0	0	0	2	0
patient 15	0	0	0	0	0	0	0	2	0
patient 16	0	0	1	0	0	0	1	3	0
Fish allergy									
patient 18	0	0	3	2	0	0	2	0	1
patient 19	0	0	3	3	0	0	3	0	3
patient 20	0	0	0	2	0	0	0	0	0
Egg/milk allergy									
patient 21	0	0	0	0	1	2	3	0	3
patient 22	0	0	3	0	2	2	3	0	2
patient 23	0	0	0	0	2	0	0	0	0

IgE antibody levels correspond to ISAC Standardized Units (ISU) as follows: 0 (undetectable or very low, < 0.3 ISU); 1 (low, ≥ 0.3 - < 1 ISU); 2 (moderate to high, ≥ 1 - < 15 ISU); 3 (very high. ≥ 15 ISU).
a) Pen a 1, Pen i 1, Pen m 1,Bla g 7, Ani s 3
b) Der p 1, Der p 2, Der f 1, Der f 2, Eur m 2
c) β-lactoglobulin, casein, lactoferrin
d) ovomucoid, ovalbumin
e) Cyn d 1, Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 11, Phl p 12, Bet v 1, Aln g 1, Cor a 1.0101
f) Ara h 1, Ara h 2, Ara h 3
g) Ara h 8, Cor a 1.0401, Act d 8, Api g 1, Dau c 1, Gly m 4, Mal d 1, Pru p 1

Tenebrio molitor extracts contain various putative allergens

Highly abundant proteins in the *T. molitor* extracts SRN1+2 (water soluble) and SRN3 (urea soluble) were identified using LC-MS/MS. Only a few proteins were identified as

specific for *T. molitor* (α-amylase and larval- and pupal cuticle proteins), due to the absence of a good database for *T. molitor* proteins. Therefore, most proteins were identified based on homology with other metazoan species.

Proteins identified in both water soluble extracts were highly similar (Table 1). Identified proteins which are also known allergens in other species are cationic trypsin (e.g. mites), arginine kinase (mites, crustaceans, insects), ovalbumin-like protein (chicken eggs), α-tubulin (mites) and α-amylase (mites, insects).

For the urea soluble extract these were cationic trypsin, ovalbumin-like protein and tropomyosin (mites, crustaceans, insects). Based on the above-mentioned results as well as the taxonomic relationship (Fig. 1), sera from crustacean and/or HDM allergic patients were selected for cross-reactivity studies (Table 2). In addition, sera from food allergic patients not allergic to crustaceans or HDM Der p 10 were also tested.

Table 3: Potentially allergenic *Tenebrio molitor* proteins according to Allermatch™. Only *T. molitor* proteins with a known sequence were analysed.

Protein	**Sequence identity in Allermatch™**					
	Allergen	**80 AA sliding window analysis**		**Full sequence alignment**		
		# hits >35% identity	**% hits >35% identity**	**%**	**overlap (AA)**	**E**
Alpha-amylase (P56634)	Eur m 4	392	100.00	50.20	494	$1.0e^{-68}$
	Der p 4	392	100.00	49.70	493	$2.7e^{-68}$
Putative trypsin-like proteinase (A1XG56)	Blo t 3	179	100.00	47.47	257	$4.2e^{-46}$
	Eur m 3	162	90.50	46.36	220	$1.9e^{-39}$
	Der p 3	150	83.80	46.36	220	$2.6e^{-39}$
	Der f 3	120	67.04	44.09	220	$1.4e^{-38}$
	Tyr p 3	117	65.36	40.15	264	$7.2e^{-35}$
	Der p 9	109	60.89	40.44	225	$4.0e^{-29}$
	Der f 6	99	55.31	36.68	229	$2.8e^{-22}$
Putative serine proteinase truncated (A1XG64)	Blo t 3	21	80.77	39.39	99	$1.0e^{-09}$
	Der f 6	18	69.23	42.65	68	$2.8e^{-08}$
	Der p 9	13	50.00	42.03	69	$1.3e^{-07}$
Cockroach allergen-like protein (Q7YZB8)	Bla g 1	340	65.89	35.92	412	$1.5e\text{-}^{42}$
	Per a 1	259	50.15	35.59	413	$4.0e^{-40}$

Expect value (E) indicates the number of hits one can expect to see by chance when searching a database of a particular size. Eur m (*Euroglyphus maynei*); Der p (*Dermatophagoides pteronyssinus*); Blo t (*Blomia tropicalis*); Der f (*Dermatophagoides farinae*); Tyr p (*Tyrophagus putrescentiae*); Bla g (*Blatella germanica*); Per a (*Periplaneta americana*).

Allergic potential of *T. molitor* proteins

The allergenic potential of identified *T. molitor* proteins with a known sequence, as well we all *T. molitor* proteins in the UniProt database was predicted using Allermatch™. A protein can be considered potentially allergenic when it shows more than 35 % identity with a

known allergen within a window of 80 amino acids or more. For example, 392 hits > 35 % between *T. molitor* α-amylase and Eur m 4 allergen indicates that for *T. molitor* α-amylase, Allermatch^{TM} found 392 times > 35 % sequence identity within 80 amino acid windows with Eur m 4. Potentially allergenic proteins based on above-mentioned criteria are listed in Table 3.

Proteins in *T. molitor* extract cross-react with IgE from HDM and crustacean allergic patients

On immunoblot, six out of seven sera from HDM Der p 10- and crustacean allergic patients showed IgE binding to all *T. molitor* extracts (Table 4, Fig 2). IgE binding was observed for protein bands with MW between 25 and 40 kDa in both the water soluble fractions and the urea soluble fraction. Serum of patient 3 showed no IgE binding to any of the extracts tested, which could be explained by lower IgE titers in the serum. Fifteen sera from patients with an allergy to codfish, egg, milk, peanut or grass pollen and no allergy to HDM Der p 10 and crustaceans were also tested. These sera showed IgE binding to their respective controls, but not to proteins in *T. molitor* extracts. A representative blot is shown in Figure 2.

To determine whether cross reactivity observed in the immunoblots was functional, five sera from crustacean/HDM Der p 10 allergic patients showing the strongest IgE binding to *T. molitor* extracts were included in the indirect basophil activation test (BAT). Positive basophil activation in response to *T. molitor* extracts and shrimp extract was observed for all tested sera (Table 4). Sera from a grass pollen-, peanut- and a fish allergic patient were used as negative controls. Figure 2 shows three representative BAT results of one crustacean/HDM Der p 10 allergic patient (patient 1) who reacted strongly to urea soluble extract and one crustacean/HDM Der p 10 allergic patient who reacted more strongly to water soluble extract (patient 5). Patient 16 is a peanut allergic patient who did not react to any of the *T. molitor* extracts, but did react to peanut extract. These results are representative for the other sera tested.

Table 4: Results of immunoblots (Blot) using crustacean/HDM Der p 10-allergic patient sera and *Tenebrio molitor* protein extracts.

Patients	Blot SRN1	Blot SRN2	Blot SRN3	Blot tropomyosin	Blot shrimp/lobster
Patient 1	+	+	+	+	+
Patient 2	+	+	+	+	+
Patient 3	-	-	-	-	-
Patient 4	+	+	+	+	+
Patient 5	+	+	+	+	+
Patient 6	+	+	±	±	+
Patient 7	±	±	+	+	+

Response was identified as positive response (+), mild (-/+) or no response (-) to Yellow mealworm extracts (water soluble; SRN1, water soluble dialysed; SRN2, urea soluble; SRN3), shrimp/lobster extract and tropomyosin.

Table 4 (continued): Results of indirect basophil activation tests (BAT) using crustacean/HDM Der p 10-allergic patient sera and *Tenebrio molitor* protein extracts.

Patients	BAT SRN1	BAT SRN2	BAT SRN3	BAT tropomyosin	BAT shrimp/lobster
Patient 1	+	+	+	+	+
Patient 2	+	+	+	+	+
Patient 3	NT	NT	NT	NT	NT
Patient 4	+	+	+	+	+
Patient 5	+	+	±	±	+
Patient 6	+	+	+	±	±
Patient 7	NT	NT	NT	NT	NT

Response was identified as positive response (+), mild (-/+) or no response (-) to Yellow mealworm extracts (water soluble; SRN1, water soluble dialysed; SRN2, urea soluble; SRN3), shrimp/lobster extract and tropomyosin. NT = not tested.

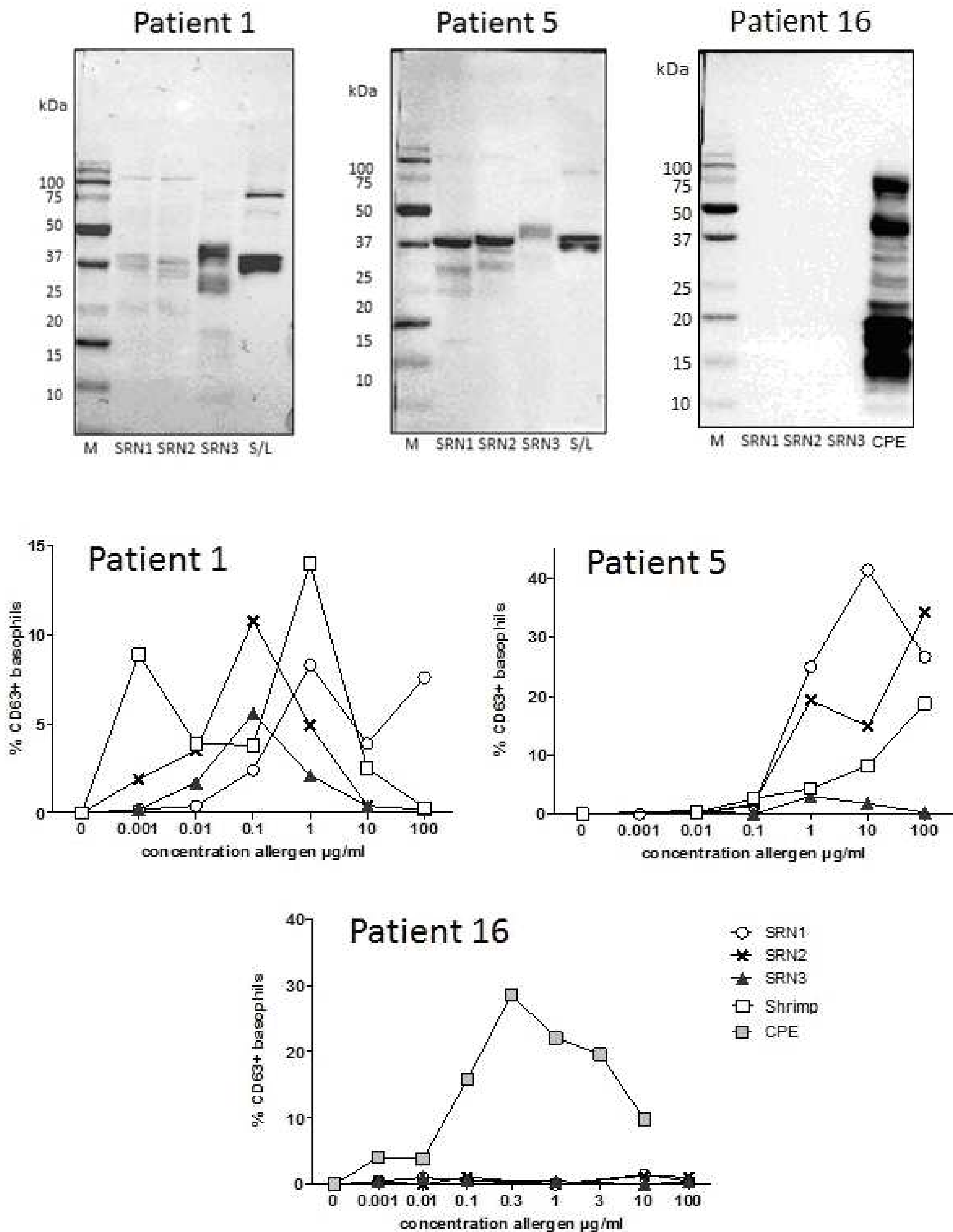

Figure 2: Cross-reactivity shown by immunoblot (above) and indirect basophil activation test (below) of *Tenebrio molitor* extracts with sera from crustacean/HDM Der p 10-allergic patients (patient 1 and 5) and a peanut-allergic patient, not allergic to crustaceans or HDM Der p 10 (patient 16). These results are representative for other patient sera tested (n=7 crustacean/HDM Der p 10-allergic and n=15 allergic to either codfish, egg, milk, peanut or grass pollen) SRN1 = water soluble protein; SRN2 = water soluble, dialysed protein; SRN3 = urea soluble protein; S/L = shrimp/lobster extract; CPE = crude peanut extract.

<u>Arginine kinase and tropomyosin were identified as major cross-reactive allergens in *T. molitor*</u>

Cross-reactive proteins were identified using LC-MS/MS in two ways: 1) excision of bands from SDS-PAGE gels (Fig. 3, Table 5) and 2) immunoprecipitation with IgE from crustacean/HDM Der p 10-allergic patients (Table 6). In most cases, more than one protein was identified in the samples.

In both water soluble extracts (SRN1 and 2), arginine kinase was identified. In addition, actin was identified in the undialysed fraction (SRN1) while fructose-biphosphate aldolase was identified in the dialysed fraction (SRN2). In the urea soluble protein fraction (SRN3), actin and tropomyosin were identified.

Immunoprecipitation of *T. molitor* protein extracts was performed to identify more cross-reactive proteins including those that could not be identified by immunoblotting. More putative allergens were found by immunoprecipitation. The top five based on identification score and number of identified peptides is listed in Table 6, in addition to identified proteins known to be allergenic in other species. For the water soluble fractions, arginine kinase was identified with the highest score, corresponding with the results of protein band identification. Actin was also identified using both techniques. Fructose-biphosphate aldolase was not identified within the top five. For the urea soluble extract, both actin and tropomyosin were identified using both techniques. In addition, a troponin-T -like protein, α- and β-tubulin and a light chain myosin-like protein were identified.

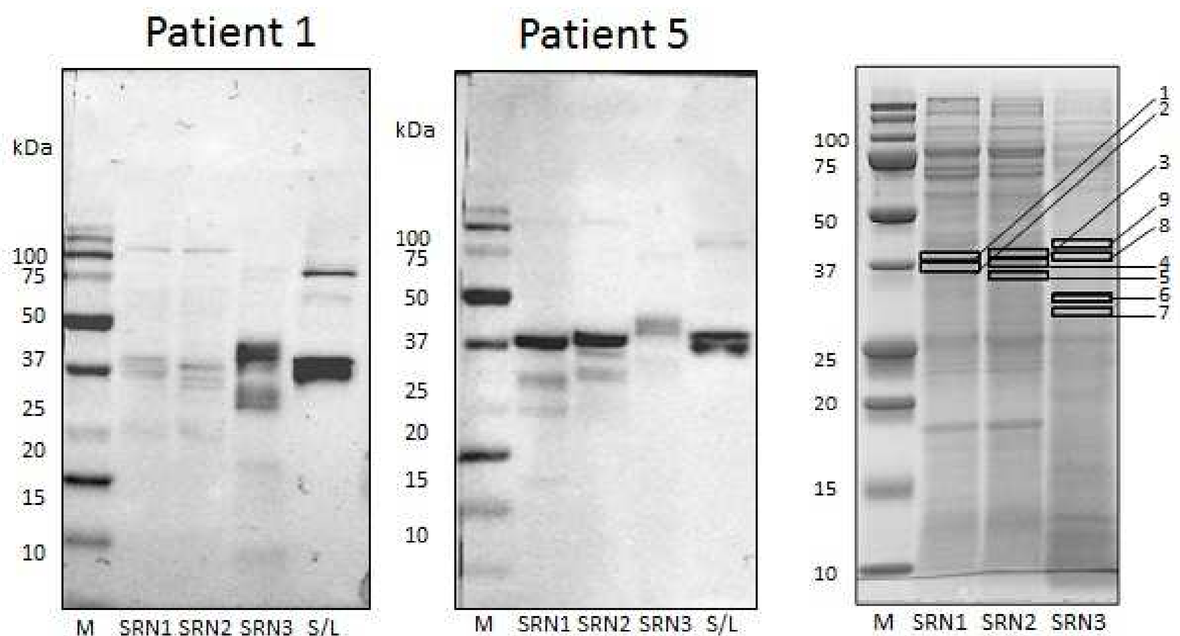

Figure 3: *Tenebrio molitor* protein bands showing cross-reactivity in immunoblot (left) excised from Coomassie-stained gel (right). SRN1 = water soluble protein; SRN2 = water soluble, dialyzed protein; SRN3 = urea soluble protein; S/L = shrimp/lobster extract. Numbers of bands correspond with Table 4.

Table 5: *Tenebrio molitor* proteins identified in excised bands using LC-MS/MS and metazoan database.

		LC-MS/MS analysis			Theoretical	
Band	**Protein**	**Accession**	**Score**	**Mass (kDa)**	**No. of peptides (unique/matched)**	**Sequence coverage (%)**
SRN1						
1	Arginine kinase	Q9U9J4	244	40.6	2/8	22
	Actin, muscle	P49871	149	42.1	0/6	19
2	Actin	O16808	101	42.1	1/4	19
	Arginine kinase	Q9U9J4	100	40.6	2/4	9
SRN2						
3	Arginine kinase	Q9U9J4	242	40.6	2/8	22
	Fructose-biphosphate aldolase	P07764	56	39.3	1/1	2
4	Fructose-biphosphate aldolase	P07764	55	39.3	1/1	2
	Arginine kinase	Q9U9J4	46	40.6	1/1	2
5	Arginine kinase	P48610	112	40.1	1/4	16
SRN3						
6	Tropomyosin	Q9NG56	54	32.9	0/2	8
7	Tropomyosin	O96764	39	32.6	0/1	4
8	Actin, indirect flight muscle	P83969	195	42.1	0/6	19
	Tropomyosin-1 isoforms 9A/A/B	P06754	89	39.4	0/4	10
9	Actin, muscle	P49871	120	42.1	0/6	19
	Tropomyosin-1 isoforms 9A/A/B	P06754	89	39.4	0/4	10

SRN1 = water soluble fraction, SRN2 = water soluble dialysed fraction, SRN3 = urea soluble fraction.

Table 6: Top five proteins based on score and proteins known to be allergenic (bold) identified in immuno-precipitation exacts of *Tenebrio molitor*.

	LC-MS/MS analysis			Theoretical	
Protein	**Accession**	**Score**	**Mass (kDa)**	**No. of peptides (unique/matched)**	**Sequence coverage (%)**
Bead 1: SRN1 + SRN 2 with serum patients 2, 5 and 6					
Arginine kinase	**D9YT56**	**38.49**	**39.6**	**2/5**	**16.06**
Myosin heavy-chain like	D6WVJ3	35.23	262.1	15/15	7.13
Titin-like	D6WIF5	15.52	2048.6	7/7	0.44
Actin-87E	B0WEY5	13.53	41.8	4/4	13.83
Limpet-like	D6WJL5	13.40	46.5	5/5	13.55
Beta-tubulin	**Q1PC35**	**10.31**	**26.8**	**3/3**	**13.19**
Alpha-amylase	**P56634**	**6.67**	**51.2**	**3/3**	**6.79**
Tropomyosin-like	**D6X4X2**	**6.26**	**75.2**	**2/2**	**3.37**
Paramyosin	**I4DIM8**	**5.48**	**102.3**	**3/3**	**3.77**
Alpha-tubulin (fragment)	**I6T3A6**	**4.83**	**35.0**	**2/2**	**6.07**
Cockroach allergen-like protein	**Q7YZB8**	**4.59**	**65.4**	**2/2**	**6.72**
Glutathione S-transferase-like	**D6WH21**	**2.00**	**23.6**	**2/2**	**9.31**
Bead 2: SRN3 with serum patient 1					
Myosin heavy chain-like	D6WVJ3	975.94	262.1	28/85	29.56
Titin-like	D6WIF5	286.63	2048.6	53/62	4.45
Actin, muscle	E2B152	242.60	41.7	3/14	39.10
Troponin T-like	D6W953	154.25	45.7	15/15	23.30
Tropomyosin-like	**D6X4X3**	**117.99**	**32.3**	**7/14**	**35.56**
Paramyosin-like	**J3JWD1**	**75.20**	**101.9**	**5/9**	**10.34**
Arginine kinase (fragment)	**D5L6P4**	**26.54**	**27.0**	**4/6**	**31.51**
Heat shock protein 70	**D2Y0Z5**	**21.94**	**71.0**	**4/6**	**12.02**
Chitinase	**Q8MP05**	**20.78**	**321.2**	**8/8**	**3.56**
Troponin C-like	**D6WZP8**	**16.72**	**17.5**	**3/6**	**20.39**
Beta-tubulin-like	**H9JHY3**	**14.38**	**45.5**	**5/5**	**13.73**
Heat shock protein 90-like	**K7IS89**	**12.05**	**82.0**	**3/4**	**16.34**
Alpha-tubulin (fragment)	**I6TYI6**	**10.71**	**41.4**	**4/4**	**5.17**
Alpha-amylase	**P56634**	**6.96**	**51.2**	**3/3**	**14.59**
Myosin light chain-like	**D6WZU7**	**5.56**	**31.3**	**2/2**	**6.79**

Identification was based on homology with known insect sequences. High scores indicate high probability of protein/ peptide identification. SRN1 = water soluble fraction, SRN2 = water soluble dialysed fraction, SRN3 = urea soluble fraction.

Cross-reactive proteins were moderately stable

Proteins in the water soluble fractions were partly digested after 15 seconds, after which they stayed relatively stable until 10 minutes. After 30 minutes, proteins were further digested. Digestion was, however, not completed after 60 minutes and the fragments could still bind IgE. In the urea soluble fraction, protein bands of approximately 32 kDa were

completely digested after 10 minutes, while a protein of approximately 40 kDa followed the same kinetics as the proteins in the water soluble fractions (Fig. 4).

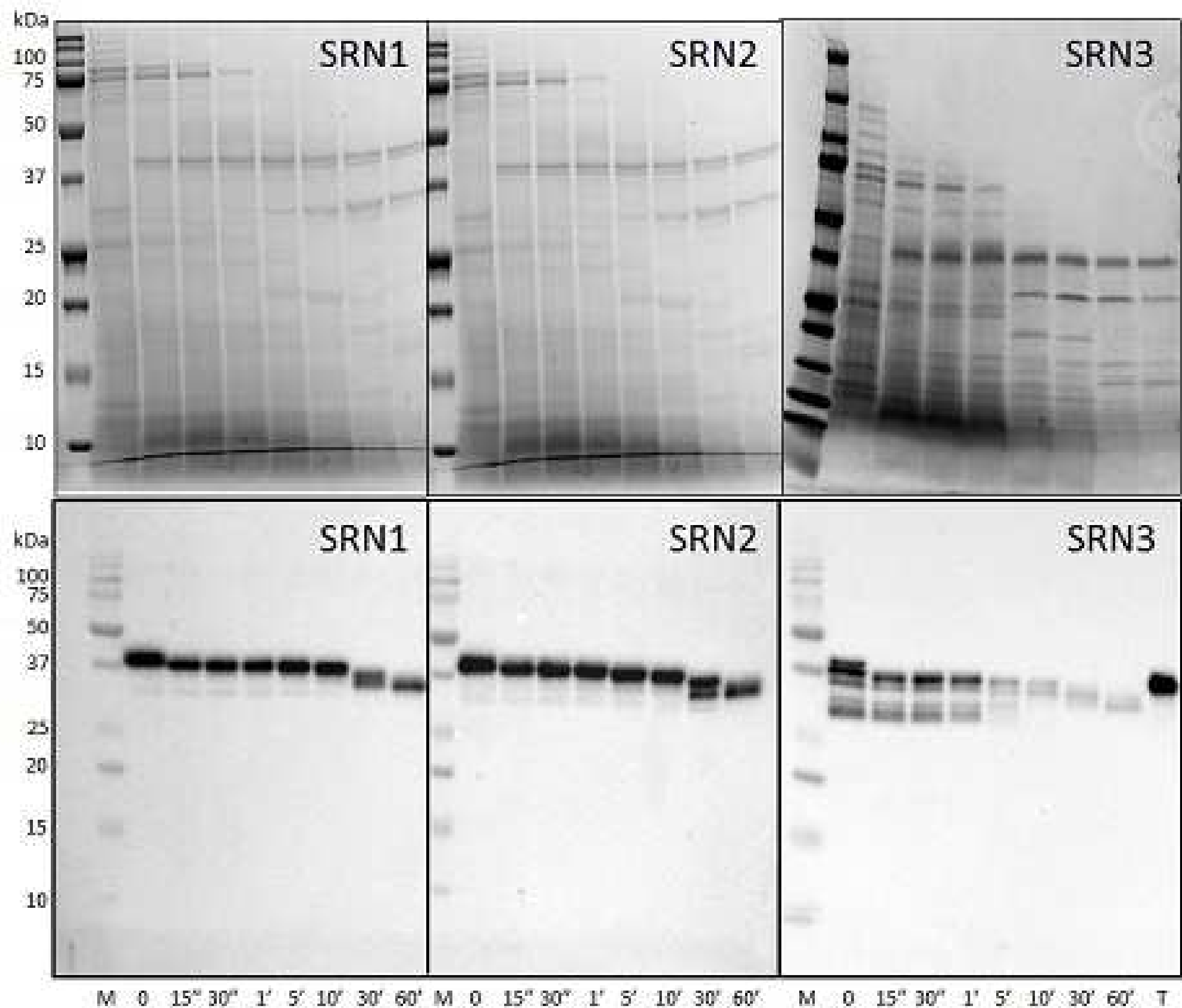

Figure 4: Digestion kinetics of *Tenebrio molitor* extracts SRN1 (water soluble fraction), SRN2 (water soluble, dialyzed fraction) and SRN3 (urea soluble fraction) in a static pepsin digestion model shown on Coomassie-stained gel (above) and immunoblot (below) with serum from a representative crustacean/HDM Der p 10 allergic patient (1).

Discussion

In this study, the weight of evidence approach as described by the European Food Safety Authority (EFSA, 2010), was used to assess the allergenic potency of *T. molitor* proteins. To this end, proteins were identified in *T. molitor* extract. The allergic potential of *T. molitor* proteins with a known sequence was determined. Cross reactivity of *T. molitor* proteins with IgE from crustacean and House dust mite (HDM) Der p 10 allergic patients

was assessed *in vitro* and the stability of the cross-reactive proteins was determined in a static pepsin digestion test.

Proteins were identified based on homology with other metazoan species, because only few sequences from *T. molitor* proteins are available in the protein database. The water soluble fractions contained putative allergens cationic trypsin, arginine kinase, α-tubulin, α-amylase and an ovalbumin-like protein. The urea soluble fraction contained putative allergens cationic trypsin, tropomyosin and an ovalbumin-like protein. Arginine kinase is an enzyme most often present in invertebrates and allergic cross-reactivity has been observed between different crustaceans (*e.g.* Black tiger shrimp pen m 2, *Penaeus monodon*; Whiteleg shrimp Lit v 2, *Litopenaeus vannamei*; Mud crab Scy pa 2, *Scylla paramamosain*), HDM (Der p 20) and insect species such as Indian meal moth (Plo i 1, *Plodia interpunctella*), Domesticated silkworm (Bomb m 1, *Bombyx mori*) and two cockroach species (Bla g 9, *Blatella germanica*; Per a 9, *Periplaneta americana*; Binder et al., 2001; Liu et al., 2009; Yu et al., 2013). Trypsin-like enzymes were described as HDM allergens Der f 3 (*Dermatophagoides farinae*) and Der p 3 (Ando et al., 1993; Steward et al., 1992). Alpha-tubulin has been described as an allergen in storage mites *Tyrophagus petruscentiae* and *Lepidoglyphus destructor* (Jeong et al., 2005; Saarne et al., 2003) while the enzyme α-amylase is known as HDM allergens Blo t 4 (*Blomia tropicalis*), Der p 4 (*D. pteronyssinus*), and Eur m 4 (*Euroglyphus maynei*; Thomas et al., 2010; Yan Chua et al., 2007). Tropomyosins are highly conserved key regulatory proteins involved in the contraction of muscle and non-muscle cells (Behrmann et al., 2012). Cross-reactivity has been observed between tropomyosins from crustaceans, mites and several insect species (Santos et al., 1999). While being a major crustacean allergen (Gámez et al., 2011), tropomyosin is a minor allergen in HDM. The dominant HDM allergens, Der p 1 and Der p 2, are recognized by over 90% of HDM allergic patients (Bronnert et al., 2012; Weghofer et al., 2005) while only 5 – 15% of patients recognize Der p 10 (Asturias et al., 1998; Resch et al., 2011). However, combined HDM and crustacean allergic patients express higher levels of IgE to Der p 10 than to the major HDM allergens (Bronnert et al., 2012). This was also the case for most patients in our database with an allergy to crustacean tropomyosin.

All *T. molitor* proteins with a known sequence in UniProt database were analyzed for allergic potential in Allermatch™. Proteins indicated to be potentially allergenic were α-amylase, a putative trypsin-like proteinase and a putative serine proteinase, which show sequence identity to several known mite allergens. Additionally, cockroach allergen-like protein shows sequence identity to cockroach allergens and was indicated to be potentially allergenic. Several known pan-allergens which were identified in *T. molitor* extracts, such as tropomyosin and arginine kinase, could not be analyzed in Allermatch™ because at this moment no sequence information is available for these proteins from *T. molitor*.

Choice of patient sera was based on arthropod phylogeny and proteins identified in *T. molitor* extracts. All *T. molitor* extracts showed cross-reactivity with sera from crustacean/HDM Der p 10 allergic patients on immunoblot. Cross-reactivity of *T. molitor*

proteins with IgE from crustacean/HDM Der p 10-allergic patients was proven functional in indirect BAT. Degranulation was between 10-40 %, depending on the sera tested. The positive control (shrimp extract) showed degranulation results comparable to *T. molitor* extracts. Low degranulation in response to *T. molitor* extracts still exceeded background degranulation (two times the percentage of activated basophils observed for RPMI + IL3 only, results not shown). The observed cross-reactivity was expected, because both fractions contain proteins (e.g. tropomyosin and arginine kinase), which are important cross-reacting arthropod pan-allergens (Binder et al., 2001; Reese et al., 1999). Sequence identities between these allergens in arthropods were at least 65 % (Barletta et al., 2005; Santos et al., 1999).

LC-MS/MS analysis identified the major cross-reactive *T. molitor* proteins as arginine kinase in the water soluble fraction and tropomyosin in the urea soluble fraction. In addition, actin was revealed as cross-reactive protein. Actin is not known as an allergen in arthropods. Sequence analysis of identified actins in Allermatch™ did not show sequence similarity to known allergens (results not shown). However, actin binds strongly to tropomyosin, forming a complex which is essential in muscle contraction (Behrmann et al., 2012). It is possible that identification of actin was caused by immunoprecipitation of the tropomyosin-actin complex. In addition, actin co-elutes with tropomyosin on SDS-PAGE gel. Insect actins might be putative allergens, but this remains to be proven. Several other allergens were identified, such as troponin T, tubulins and light chain myosin. Tubulins and light chain myosin are known arthropod allergens (Ayuso et al., 2008). It is unknown whether troponin T could act as an allergen.

Cross-reactive Yellow mealworm proteins were moderately stable in the static pepsin digestion model compared to Ara h 1, which was completely degraded within 15 seconds and Ara h 2, which was not completely digested even after 60 minutes using the same experimental conditions (data not shown).

To our knowledge, this is the first study to determine cross-reactivity between *T. molitor* proteins and IgE from crustacean/HDM Der p 10 allergic patients. Because *T. molitor* is not commonly eaten yet, sensitization to *T. molitor* protein through the oral route is unlikely for the tested patients. Unfortunately, sera from patients allergic to *T. molitor* protein are not readily available. It is therefore not yet possible to determine the risk of sensitization to mealworm proteins. Sensitization is always a risk with novel proteins, and cross-reactivity can occur without direct sensitization. This is illustrated by Fernandes et al. (2003), who described IgE reactivity to shrimp tropomyosin in orthodox Jews who were allergic to household allergens such as HDM and cockroach, but had no previous exposure to shellfish.

The results we presented in this paper indicate that crustacean/HDM Der p 10 allergic patients may experience an allergic reaction when consuming products containing Yellow mealworm protein. However, to confirm this, it is imperative to challenge patients with

Yellow mealworm extract by means of a double-blind placebo-controlled food challenge, which is the gold standard in allergy diagnosis.

In conclusion, there is a realistic possibility that HDM- and crustacean allergic patients may react to food containing Yellow mealworm proteins.

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Chapter 5

Influence of processing and *in vitro* digestion on the allergic cross-reactivity of three mealworm species

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Abstract

Edible insects are currently evaluated as an alternative and more sustainable protein source for humans. The introduction of new food sources can lead to development of novel allergies. Because in the Western world, insects are unlikely to be consumed raw, it is important to know how processing and *in vitro* digestion might influence their allergenicity. Three edible mealworm species (*Tenebrio molitor* L., *Zophobas atratus* Fab. and *Alphitobius diaperinus* Panzer) subjected to processing and *in vitro* digestion were analysed for their abilities to IgE cross-react. Immunoblot and MALDI-MS/MS analyses revealed that IgE from crustaceans or House Dust Mite (HDM) allergic patients showed cross-reactivity to mealworm tropomyosin or α-amylase, hexamerin 1B precursor and muscle myosin respectively. Heat processing as well as *in vitro* digestion did diminish, but not eliminate HDM or tropomyosin IgE cross-reactivity.
Results show that individuals allergic to HDM or crustaceans might be at risk when consuming mealworms, even after heat processing.

Keywords: food allergy, mealworms, food processing, *in vitro* digestion, IgE cross-reactivity, crustaceans, House dust mite

Introduction

Edible insects are currently under evaluation as a potential protein source that would provide more sustainable alternative dietary proteins for humans (Rumpold and Schlüter, 2012; Van Huis, 2013). Insects are already consumed in many parts of the world and initiatives are ongoing to introduce them into the diet in the Western world (Van Huis, 2013). In The Netherlands, mealworms (i.e. the larval stages of beetles in the family Tenebrionidae) are commercially produced as pet food and more recently, for human consumption.

Because the introduction of new food sources can lead to development of new allergies, it is important to determine the allergenic risk of newly introduced food sources. Research focusing on allergenicity of insect protein as dietary component is limited. Case studies reported clinical allergic reactions following the consumption of among others Domesticated silk worm pupae (Bombyx mori; Ji et al., 2008) and Mopani worms (*Imbrasia belina*`; \ Okezie, 2010}. Most studies on mealworm allergy report occupational allergy, most frequently experienced by cereal workers as well as people handling mealworms as pet food or fish bait (Bernstein et al., 1983; Schroeckenstein et al., 1990; Schroeckenstein et al., 1988; Siracusa et al., 2003). Only one study reported an allergic reaction in response to the consumption of *Tenebrio molitor* and *Zophobas atratus* (Freye et al., 1996).

In addition to primary sensitisation, allergies can also develop through cross-sensitisation to proteins related to the allergen (secondary sensitisation; Radauer et al., 2008). Within arthropods, several pan-allergens are known such as tropomyosin (Reese et al., 1999) and arginine kinase (Binder et al., 2001). Allergic cross-reactivity between proteins from different species of arthropods has been described in several studies (Barletta et al., 2005; Liu et al., 2009; Santos et al., 1999). Verhoeckx et al. (2014) reported *in vitro* cross-reactivity of protein extracted from raw *T. molitor* with IgE from patients with a combined crustacean and House dust mite allergy, proving that individuals with an allergy to other arthropod species might be at risk of an allergic response when consuming food containing *T. molitor* protein.

In the Western world, insects are unlikely to be consumed in raw form, and it is unknown how processing of mealworm species might influence protein integrity and thus allergenicity. Resistance to denaturation and digestion are important characteristics of many food allergens (Bannon, 2004). Several studies have shown that some allergenic proteins are stable to heat processing or digestion, while others are readily inactivated (Besler et al., 2001; Kamath et al., 2013; Yu et al., 2013). In contrast, heat processing might even cause other proteins to become more allergenic (Besler et al., 2001).

In this study, IgE cross-reactivity of protein, isolated from three edible mealworm species Yellow mealworm (Tenebrio molitor L), Giant mealworm (Zophobas atratus Fab.) and Lesser mealworm (Alphitobius diaperinus Panzer) was assessed with sera from patients

allergic to either crustaceans or House dust mite (HDM). Additionally, the effect of processing and in vitro digestion of whole mealworms on IgE-reactivity was determined.

Materials and methods

Materials

Reagents were obtained from Sigma Aldrich (St. Louis, USA) unless stated otherwise. *Tenebrio molitor*, *Z. atratus* and *A. diaperinus* in their final larval instars were obtained from the insect production company Kreca (Ermelo, The Netherlands).

Patient sera

Sera from crustacean allergic patients without any clinical history of mite allergy were provided by Erasmus Medical Centre (Rotterdam, The Netherlands). Rijnstate Hospital (Arnhem, The Netherlands) and Queen Beatrix Hospital (Winterswijk, The Netherlands) provided the sera of HDM allergic patients. Likewise, the HDM allergic patients had no clinical history of crustacean allergy. Sera from non-atopic volunteers were provided by co-workers from Wageningen University and Research Centre (Wageningen, The Netherlands). Dot-blot was performed using individual sera from patients experiencing clinical allergic symptoms to HDM (n = 11) or crustaceans (n = 8) as well as from donors with no clinical symptoms of allergy (n = 15). SDS-PAGE Immunoblot was performed using pooled sera from patients allergic to either HDM (n = 7) or crustaceans (n = 6) (Table 1). Serum from non-atopic volunteers was used as a pooled control (n = 6).

Processing of larvae

Larvae of the above-mentioned mealworm species were killed by freezing at -20°C and subsequently randomly divided over four batches. Three batches were each subjected to a different processing method: boiling, frying or lyophilising. The fourth batch was left unprocessed (raw). Larvae were either boiled for 5 min in tap water (40 g larvae in 500 mL water); fried for 5 min at 180°C in vegetable frying oil (containing sunflower oil, palm oil and rapeseed oil, ratio not specified) bought at a local supermarket (120 g larvae in 2 L oil); or lyophilised at -50°C and 1.5 mbar. Raw and processed larvae were stored at -20°C.

Preparation of protein extracts

Soluble and insoluble protein was extracted as previously described by Verhoeckx *et al.* (2014) with minor adjustments. In short, frozen larvae were mixed with ice cold extraction buffer (20 mM Tris buffer pH 7.6, 1 mM phenylthiocarbamide and 10 µl mL^{-1} Halt Protease Inhibitor Cocktail (Piercenet, Rockford, USA)) 1:7 and homogenised using an Ultra-Turrax T25 (IKA®, Staufen, Germany). Subsequently, the suspension was centrifuged (30 min, 4000 rpm, 4°C) and the supernatant was stored at -80°C. To extract water-insoluble protein,

the residue was washed twice with extraction buffer followed by overnight incubation at 4°C in 6 M urea in extraction buffer. The supernatant, obtained by centrifugation (30 min, 4000 rpm, 4°C) was stored at -80°C. Shrimp protein was extracted from Whiteleg shrimp (*Litopenaeus vannamei* Boone), purchased at a local supermarket, using the same method. Protein concentration in all extracts was measured using the Bradford assay (Thermo Scientific, Waltham, USA). For further analysis, water soluble and urea soluble protein was mixed in a 1:1 protein concentration ratio. Lyophilised HDM (*Dermatophagoides pteronyssinus* Trouessart) extract was obtained from Citeq Biologics (Groningen, The Netherlands) and was diluted according to the manufacturer's guidelines.

In vitro digestion

Larvae were digested *in vitro* as described by Vreeburg *et al*. (2012) with additions. In short, 15 g raw or boiled larvae or 6 g lyophilised or fried larvae (assuming 40% dry weight) were homogenised using an Ultra-Turrax T25 in 30 mL salt solution (140 mM NaCl, 5 mM KCl). Samples were kept dark by wrapping in aluminium foil. Twenty g slurry was adjusted to pH 2 with 1 M HCL, after which 0.667 mL of 40 mg mL^{-1} porcine pepsin in 0.1 M HCl was added. Samples were incubated at 37°C for 1h under continuous agitation, after which the pH was adjusted to 5.8 with 1 M $NaHCO_3$. One mL of 4 mg mL^{-1} porcine pancreatin with 1 mg mL^{-1} porcine lipase and 5.9 units mL^{-1} of bovine α-chymotrypsin in 0.1 M $NaHCO_3$ was added. Then, 0.5 mL bile salt (94.6 mg mL^{-1} sodium taurocholate and 83 mg mL^{-1} sodium glycodeoxycholate in 0.1 M $NaHCO_3$) was added and the pH was adjusted to 6.5 with 1 M $NaHCO_3$. The headspace was flushed with N_2, after which the samples were incubated at 37°C for 2h under continuous agitation. Subsequently, the pH was adjusted to 7.5 and the samples were centrifuged (30 min, 4000 rpm, 4°C). The supernatant was collected in 50 mL tubes of which the headspace was flushed with N_2 before storing at -80°C.

Dot-blot

To screen for suitable patient sera to use for immunoblotting, 1 µL droplets of 500 ng μL^{-1} protein in TBS were spotted on Whatman Protran BA 83 nitrocellulose membrane. Membranes were dried for 1h at 37°C and were subsequently blocked with 1% BSA in TBS for 1h at RT. Membranes were washed five times by hand rotation in TBS and incubated overnight with five times diluted patient serum in TBS. Membranes incubated with TBS only served as antibody control. Membranes were then washed five times with TBS and incubated with 1:1000 diluted polyclonal rabbit anti-IgE antibody for 1 h (Dako, Glostrup, Denmark). After another washing step, membranes were incubated with 1:20,000 diluted AP-conjugated polyclonal goat anti-rabbit antibody. Membranes were again subjected to a washing step after which spots were visualised by incubating for 10 minutes in SigmaFast™ BCIP®/NBT. After washing in MQ, membranes were dried for 1h at 37°C. Colour intensity of the spots (integrated intensity of all pixels in a spot, expressed as

arbitrary units) was determined using BIO-RAD Universal Hood II Gel Imager and Imagelab 4.1 software. Results were analysed by subtracting the spot intensity of the antibody control from the spot intensity observed for membranes incubated with serum.

SDS- PAGE and Immunoblot

Five µg protein in TBS buffer was separated by SDS-PAGE on NuPage® 10% BIS/TRIS gel according to the manufacturer's instructions (Invitrogen, Carlsbad, USA) under denaturing conditions. For Immunoblot, proteins were transferred from gel to 0.45 µm nitrocellulose membrane (LKB, Bromma, Sweden). After transfer, the membrane was washed in TBS 0.1% Tween-20 (TBST) for 10 min, after which the membrane was blocked with 2% BSA for 1 h at 4°C. Next, the membrane was washed two times with TBST for 10 min and subsequently incubated overnight with five times diluted pooled patient serum (Table 1). The membrane was then washed five times with TBST for 5 min, after which it was incubated with 1:2000 diluted polyclonal rabbit anti-IgE antibody (Dako, Glostrup, Denmark) for 1 h. After five more washing steps, the membrane was incubated with 1:2000 diluted polyclonal goat anti-rabbit HRP-conjugated antibody (Dako, Glostrup, Denmark) in TBS for 1 h. The membrane was again washed five times and was subsequently exposed to Pierce® ECL Immunoblotting Substrate (Invitrogen, Carlsbad, USA) for 1 min. IgE binding was visualised using BIO-RAD Universal Hood II Gel Imager and ImageLab 4.1 software.

Identification of cross-reacting proteins

Bands of interest were excised from SDS-PAGE gel and sent to Alphalyse (Odense, Denmark) for protein identification by MALDI-MS/MS.

Determination of allergic potential

The allergic potential of cross-reacting proteins identified in mealworm extracts was verified using Allermatch™ (http://www.allermatch.org). Comparison in Allermatch™ was based on UniProt as well as the WHO–IUIS database. An 80 amino acid sliding window alignment was performed with a 35% cut-off percentage (Fiers, 2004).

Results

Screening for sera by dot-blot

Sera responding to mealworm protein were selected by dot-blot (Table 1, Figure 1). Though serum IgE of most patients bound to one or more mealworm protein fractions on dot-blot, individual sensitisation differences could be observed. Serum IgE of about half of the HDM allergic patients showed at least a medium response (spot intensity 10,000 - 15,000 arb. units) in binding to protein of raw as well as processed mealworm species, with the

exception of lyophilised *A. diaperinus*. IgE of other HDM allergic patients, such as #5 and #6, bound to raw or lyophilised fractions, but not to heat processed fractions. In case of crustacean allergic patients, only serum IgE of patient #12 bound to boiled *T. molitor* protein. IgE from most patients bound, however, to boiled fractions of the other two mealworm species. Similar to HDM allergic patients, serum of most crustacean allergic patients reacted hardly or not to lyophilised *A. diaperinus*.

Sera showing the strongest binding to mealworm protein were selected for immunoblot. Control sera responding to mealworm species, *L. vannamei* or *D. pteronyssinus* protein were excluded (results not shown). The antibody control indicated that one or both of the secondary or conjugated tertiary antibodies bound to protein of heat processed samples. This was taken into account when selecting suitable sera based on spot colour intensity.

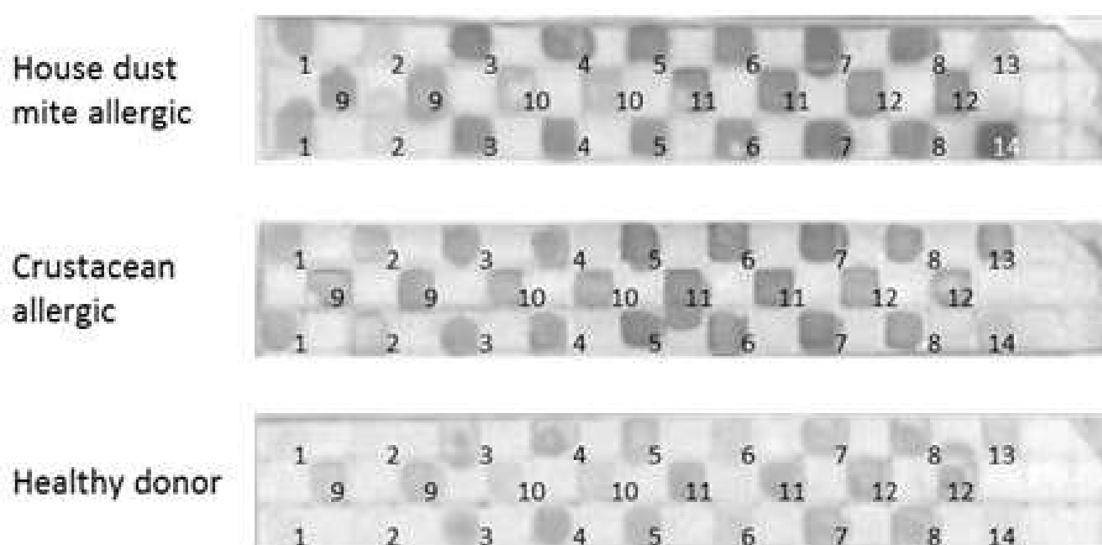


Figure 1: Dot-blots showing serum IgE binding of a representative House dust mite allergic patient (Table 1, #2), a crustacean allergic patient (Table 1, #17) and a healthy donor to raw (1), lyophilised (2), boiled (3) and fried (4) Yellow mealworm (*Tenebrio molitor*) protein; raw (5), lyophilised (6), boiled (7) and fried (8) Giant mealworm (*Zophobas atratus*) protein; raw (9), lyophilised (10), boiled (11) and fried (12) Lesser mealworm (*Alphitobius diaperinus*) protein; House dust mite (*Dermatophagoides farinae*) extract (13) and Whiteleg shrimp (*Litopenaeus vannamei*) protein (14).

Table 1: Response of individual HDM allergic and crustacean allergic patients to extracts from *Dermatophagoides pteronyssinus*, *Litopenaeus vannamei* and mealworm protein on dot-blot.

	Patient number HDM allergic patient sera											Patient number crustacean allergic patient sera							
Extract	**1**	**2**	**3**	**4**	**5**	**6**	**7**	8	9	*10*	11	**12**	**13**	**14**	**15**	**16**	**17**	18	19
Dermatophagoides pteronyssinus	+	+	±	+	-	-	-	+	+	*+*	+	-	±	-	-	-	-	-	-
Litopenaeus vannamei	±	+	±	±	-	-	±	-	-	*±*	-	±	+	+	-	+	+	±	-
Tenebrio molitor																			
Raw	+	+	±	±	±	±	-	±	±	*+*	-	±	±	±	-	±	+	-	-
Lyophilised	±	+	+	±	±	±	-	±	-	*+*	-	±	±	+	-	±	+	±	-
Boiled	±	±	±	+	-	-	±	-	-	*-*	-	±	-	-	-	-	-	-	-
Fried	±	±	±	+	-	-	+	-	-	*±*	-	-	-	-	±	±	±	-	-
Zophobas atratus																			
Raw	+	+	±	+	+	±	±	±	±	*+*	-	±	±	±	±	±	+	±	-
Lyophilised	+	+	-	+	±	-	±	-	-	*±*	-	-	-	-	-	±	+	-	-
Boiled	+	+	±	+	-	-	±	-	±	*±*	-	+	±	-	±	+	+	-	-
Fried	+	±	±	+	-	-	+	-	±	*+*	±	±	±	±	±	±	±	-	-
Alphitobius diaperinus																			
Raw	+	+	+	+	-	-	±	±	±	*±*	-	+	±	±	-	+	+	-	-
Lyophilised	-	-	-	-	±	+	-	-	-	*-*	-	-	-	-	-	-	±	±	-
Boiled	+	+	±	±	-	-	±	±	±	*±*	-	+	±	±	±	+	+	-	-
Fried	±	±	-	±	-	-	±	-	-	*±*	-	-	±	-	±	±	±	-	-

Patients listed in bold were selected for Immunoblot serum pools. Patient 10 listed in italics showed a very dark background on dot-blot and was for this reason excluded.
+ = high response (spot intensity > 15,000 arb. units); ± = medium response (spot intensity 10,000 - 15,000 arb. units); - = low or no response (spot intensity < 10,000 arb. units). Yellow mealworm (*T. molitor*), Giant mealworm (*Z. atratus*), Lesser mealworm (*A. diaperinus*).

Heat processing and *in vitro* digestion alters cross-reactivity of mealworm proteins

Immunoblot was performed using three sera pools as well as with secondary/tertiary antibodies only. The latter, as well as the control sera pool (obtained from non-allergic patients), confirmed direct unspecific binding of antibodies to heat processed mealworm protein (Figure 2). Specific binding was observed for IgE from the HDM allergic patient sera pool to proteins with MW between 25 kDa and 70 kDa (Figure 3A). IgE cross-reactivity appeared to vary between the different mealworm species on immunoblot. Some cross-reacting proteins appeared to be still reactive in heat processed samples, such as a *ca.* 35 kDa protein in *T. molitor* and *Z. atratus*. Cross-reactivity on immunoblot was not increased by heat processing but was in some cases diminished, as was the case for a *ca.* 25 kDa protein in fried samples. Most cross-reactive proteins appeared to be degraded by *in vitro* digestion. However, a protein with MW of *ca.* 25 kDa in raw and lyophilised *Z. atratus* remained strongly reactive.

In case of IgE from the crustacean allergic patient sera pool, specific binding was observed in all fractions for a protein with a MW of *ca.* 35 kDa, and in addition to this, a

protein of *ca.* 45 kDa in fractions from heat processed samples (Figure 3B). The 45 kDa protein appeared to be degraded after *in vitro* digestion. The 35 kDa protein remained strongly reactive in raw, lyophilised and boiled samples. In addition, cross-reactive proteins of *ca*. 25 kDa and 16 kDa appeared upon boiling of samples.

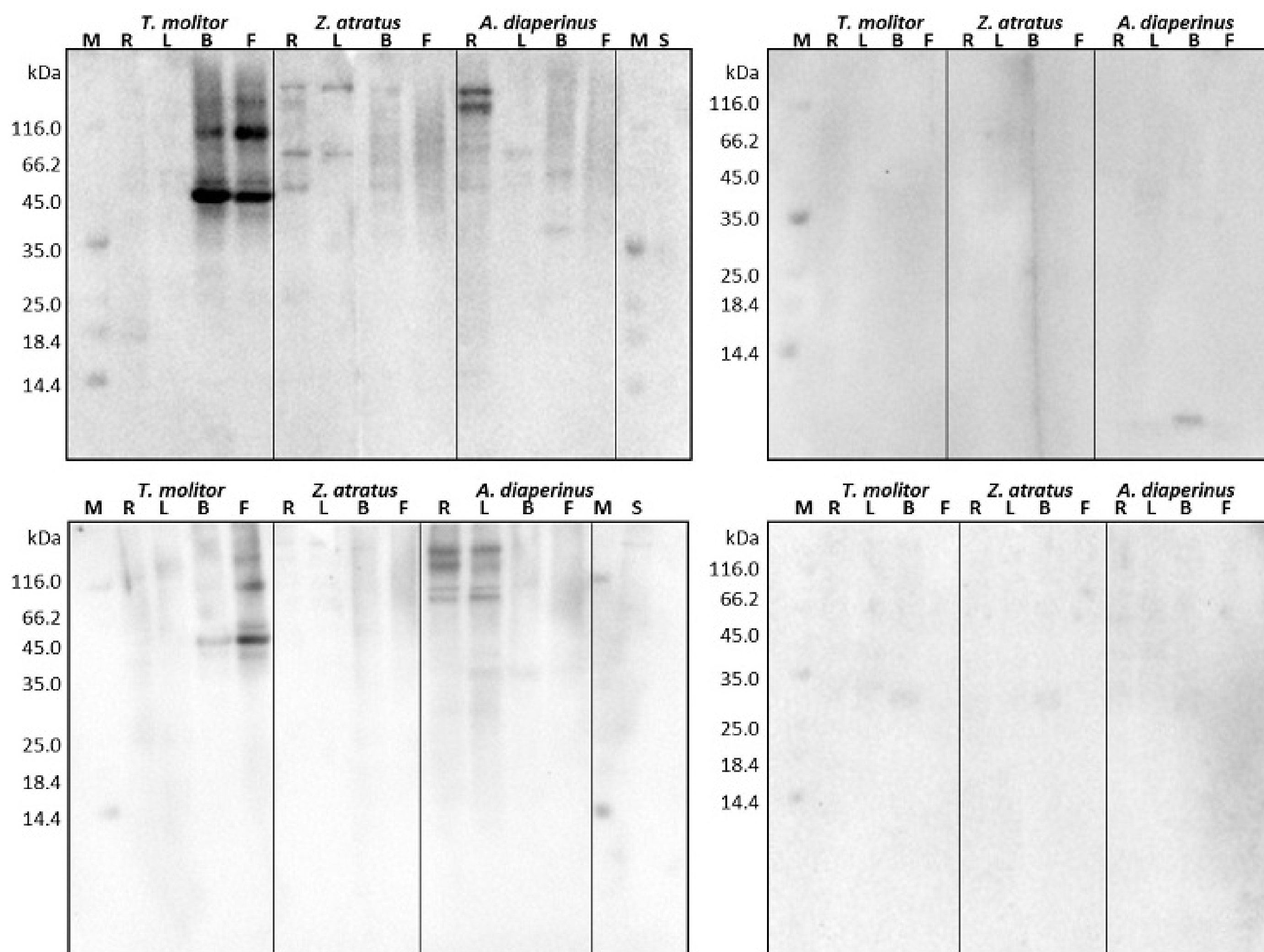


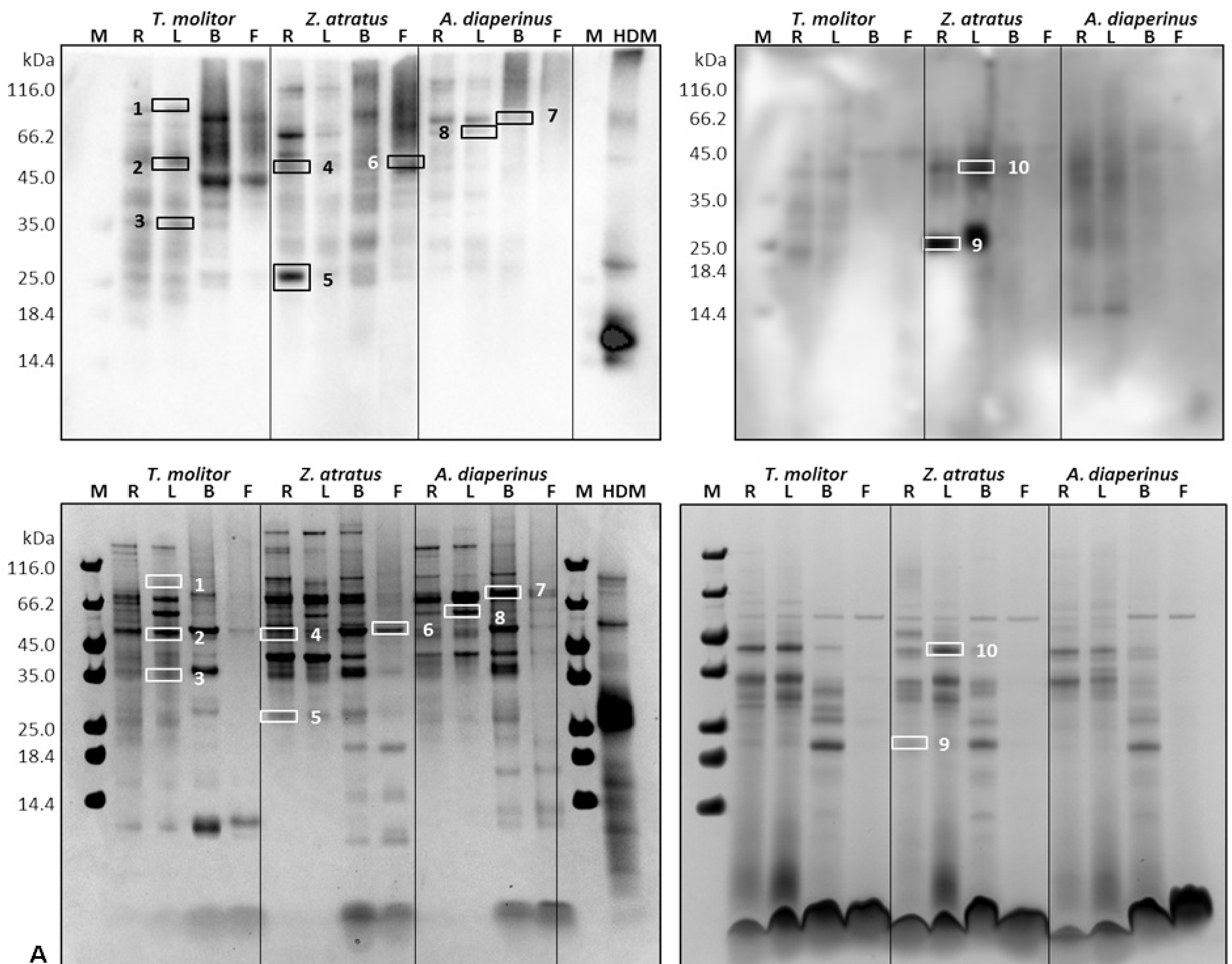
Figure 2: Immunoblot of non-digested (left panels) and *in vitro* digested (right panels) protein of three mealworm species treated with control sera (upper panels) and antibodies only (lower panels), showing non-specific binding. M = marker, R = raw, L = lyophilised, B = boiled, F = fried, S = Whiteleg shrimp (*Litopenaeus vannamei*) extract.

<u>Identification of cross-reacting proteins</u>

Several cross-reacting protein bands on immunoblot were excised from the corresponding Coomassie-stained SDS-PAGE gels (Figure 3) and were sent for identification by MALDI-MS/MS. Protein identification results of the excised bands are listed in Table 2. Proteins identified to cross-react with IgE from HDM allergic patients were a predicted long form paramyosin (*ca.* 100 kDa, band #1), α-amylase (*ca.* 52 kDa, band #2), actin (*ca.* 42 kDa, bands #3 and #4), a predicted larval cuticle protein (*ca.* 27 kDa, band #6), hexamerin 1B precursor (*ca.* 94 kDa, band #8) and muscle myosin (*ca.* 135 kDa, bands #9 and #10). No

significant protein hit was found in the database search for excised bands #5 (raw *Z. atratus* protein *ca.* 25 kDa) and #7 (boiled *A. diaperinus* protein *ca.* 67 kDa).

Proteins identified to cross-react with IgE from crustacean allergic patients were actin (*ca. 35* kDa, band #1) and tropomyosin (*ca.* 30 kDa, bands #2, #5 and #6). However, no significant protein hit was found in the database search for excised bands #3 (boiled *Z. atratus* protein *ca.* 45 kDa), #4 (boiled *A. diaperinus* protein *ca.* 116 kDa) and #7 (*in vitro* digested boiled *T. molitor* protein *ca.* 16 kDa).



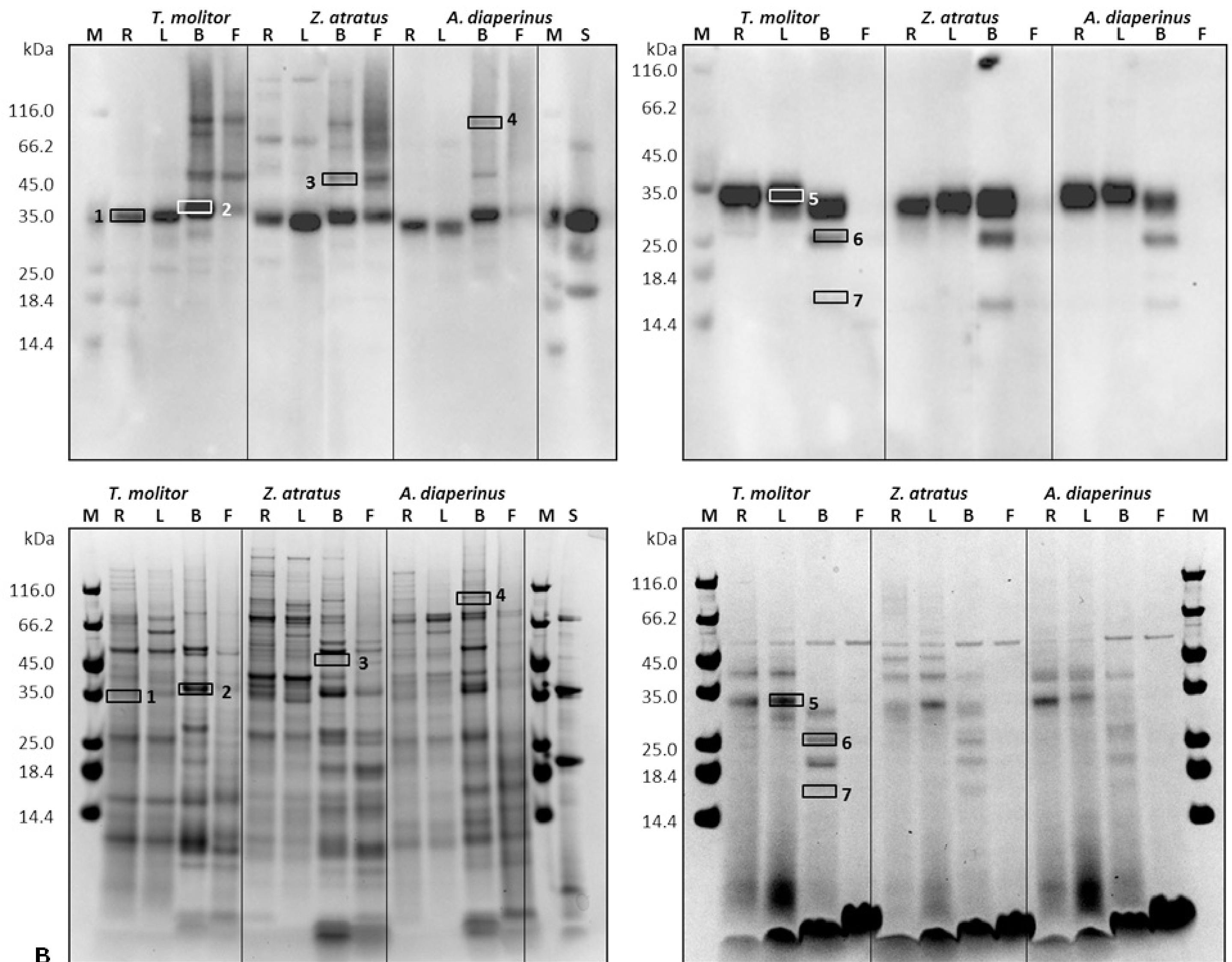


Figure 3: Non-digested protein (left panels) and *in vitro* digested protein (right panels) of three mealworm species showing cross-reactivity with sera from House dust mite allergic patients (A) or crustacean allergic patients (B) on Immunoblot (upper panels). Bands of interest were excised from Coomassie-stained SDS-PAGE gel (lower panels). Yellow mealworm (*T. molitor*), Giant mealworm (*Z. atratus*), Lesser mealworm (*A. diaperinus*); M = marker, R = raw, L = lyophilised, B = boiled, F = fried, HDM = House dust mite (*Dermatophagoides pteronyssinus*) extract, S = White shrimp (*Litopenaeus vannamei*) extract. Numbers of bands correspond with table 2.

Table 2: Proteins cross-reacting with HDM allergic or crustacean allergic serum pool identified in excised bands using MALDI-MS/MS and NCBI database. Band numbers correspond with figure 2A (HDM allergic) and 2B (crustacean allergic).

Band #	Protein	Accession	Calculated MW (kDa)	Score	Sequence coverage (%)
Cross-reacting with HDM allergic serum pool					
1	Predicted: paramyosin long form	XP_970719.1	101.90	128	12
2	Chain A, Structure of *Tenebrio molitor* larval α-amylase in complex with Ragi bifunctional inhibitor	1TMQ_A	51.70	555	39
3	Actin	P90689	41.97	228	33
4	Actin-2	Q9Y707	41.98	81	21
5	-	-	-	-	-
6	Predicted: similar to Larval cuticle protein A1A (TM-A1A) (TM-LCP A1A)	XP_975673	26.71	66	9
7	-	-	-	-	-
8	Hexamerin 1B precursor	XP_966959	93.78	362	17
9	Muscle myosin heavy chain	CAA37309	135.63	269	12
10	Muscle myosin heavy chain	CAA37309	135.63	410	14
Cross-reacting with crustacean allergic serum pool					
1	Actin beta/gamma 1	GAA36800	35.71	235	43
2	Tropomyosin 1, isoform A	NP_524360	29.34	152	38
3	-	-	-	-	-
4	-	-	-	-	-
5	Tropomyosin	P31816	32.45	200	40
6	Tropomyosin, partial	AGJ71763	30.79	139	31
7	-	-	-	-	-

Hyphens indicate no significant protein hit was found in the database search.

Allergic potential of cross-reacting proteins

Of the proteins identified as cross-reacting with sera from HDM allergic patients on Immunoblot, the predicted long form paramyosin (band #1) and muscle myosin (bands #9 and #10) showed significant identity to group 11 allergens of several HDM species and the parasitic nematode *Anisakis simplex* (Rudolphi) allergen Ani s 2 (Table 3). Alpha amylase (band #2) showed identity to HDM allergens Der p 4 (*Dermatophagoides pteronyssinus*) and Eur m 4 (*Euroglyphus maynei* Cooreman 1950). Hexamerin 1B precursor (band #8) showed identity to American cockroach (*Periplaneta americana* L.) allergen Per a 3. Actin and the predicted larval cuticle protein A1A did not show identity to any known allergens. Of the proteins identified as cross-reacting with sera from crustacean allergic patients, the tropomyosins showed significant identity to allergenic tropomyosins from, among others, several crustacean, mite and insect species. Due to the large number of known allergens found to show sequence identity to tropomyosins, only a limited number of results are represented in Table 3. Sequence identity of tropomyosin 1 isoform A (band #2) to known

allergens was lower (*ca.* 60%) than for the other two tropomyosins identified (*ca.* 80-90%). Beta/gamma-actin (band #1) did not show identity to known allergens.

Several peptides identified by MALDI-MS/MS partly matched known crustacean tropomyosin epitopes (Table 4, Figure 4). Two out of eight epitopes were matched by peptides found to resemble tropomyosin 1, isoform A (band #2). For tropomyosin (band #5 and #6), five out of eight and four out of eight epitopes were matched, respectively. Save for tropomyosin epitopes, insufficient information on epitope sequences is available in literature to compare with peptides found for the other putative allergenic mealworm proteins in this study.

Table 3: Sequence identity of allergenic proteins identified (Table 2) with known allergens according to Allermatch™.

Band #	**Protein**	**Sequence identity in Allermatch™**					
		Allergen	**88 AA sliding window analysis**		**Full sequence alignment**		
			# hits >35% identity	**% hits >35% identity**	**%**	**Overlap (AA)**	***E*[1]**
Cross-reacting with HDM allergic serum pool							
1	Predicted: paramyosin long form (XP_970719.1)	Der p 11	798	100.00	65.02	872	$1.4e^{-201}$
		Blo t 11	798	100.00	64.11	872	$1.2e^{-198}$
		Der f 11	685	85.84	64.31	692	$9.8e^{-159}$
		Ani s 2	787	98.62	49.08	870	$4.9e^{-148}$
2	Chain A, structure of *Tenebrio molitor* larval α-amylase In complex with Ragi bifunctional inhibitor (1TMQ_A)	Der p 4	392	100.00	50.20	494	$1.1e^{-68}$
		Eur m 4	392	100.00	49.70	493	$2.9e^{-68}$
3	Actin (P90689)	-	-	-	-	-	-
4	Actin-2 (Q9Y707)	-	-	-	-	-	-
6	Predicted: Similar to larval cuticle protein A1A (TM-A1A) (TM-LCP A1A) (XP_975673)	-	-	-	-	-	-
8	Hexamerin 1B precursor (NP_001164358)	Per a 3.0201	441	63.91	39.88	652	$3.1e^{-43}$
		Per a 3.0101	446	64.64	39.34	671	$2.1e^{-43}$
9 and 10	Muscle myosin heavy chain (CAA37309)	Blo t 11	637	58.12	41.41	821	$3.0e^{-86}$
		Der p 11	593	54.11	41.34	820	$1.2e^{-86}$
		Ani s 2	560	51.09	39.71	831	$1.7e^{-86}$

[1]Expected value (*E*) indicates the number of hits one can expect to observe by chance when searching a database of a particular size.
[2]Because the large number of matches found for this protein, only the top five matches found are listed here.
Hyphens indicate no sequence identity with known allergens was found in Allermatch™.
Der p (*Dermatophagoides pteronyssinus*); Blo t (*Blomia tropicalis*); Der f (*Dermatophagoides farinae*); Ani s (*Anisakis simplex*); Eur m (*Euroglyphus maynei*); Per a (*Periplaneta americana*); Cha f (*Chironomus kiiensis*); Pen m (*Penaeus monodon*); Pan b (*Pandalus borealis*); Lit v (*Litopenaeus vannamei*); Hom a (*Homarus americanus*).

Table 3 (continued): Sequence identity of allergenic proteins identified (Table 2) with known allergens according to Allermatch™.

Band #	Protein	Sequence identity in Allermatch™					
		Allergen	88 AA sliding window analysis		Full sequence alignment		
			# hits >35% identity	% hits >35% identity	%	Overlap (AA)	*E*[1]
Cross-reacting with crustacean allergic serum pool							
1	Actin beta/gamma 1 (GAA36800)	-	-	-	-	-	-
2	Tropomyosin 1, isoform A[2] (NP_524360)	Cha f 1	173	100.00	65.74	236	$1.4e^{-47}$
		Pen m 1	173	100.00	61.25	240	$8.1e^{-49}$
		Pan b 1	173	100.00	61.44	236	$6.1e^{-49}$
		Lit v 1	173	100.00	61.25	240	$8.1e^{-49}$
		Hom a 1 b	173	100.00	61.44	236	$6.1e^{-49}$
5	Tropomyosin[2] (P31816)	Chi k 10	204	100.00	91.17	283	$2.1e^{-83}$
		Per a 7 b	204	100.00	90.43	282	$4.0e^{-83}$
		Pen m 1	204	100.00	81.85	281	$1.0e^{-74}$
		Pan b 1	204	100.00	81.85	281	$1.6e^{-74}$
		Lit v 1	204	100.00	81.85	281	$1.0e^{-74}$
6	Tropomyosin, partial[2] (AGJ71763)	Chi k 10	188	100.00	91.39	267	$1.8e^{-75}$
		Per a 7 b	188	100.00	93.63	267	$1.8e^{-78}$
		Per a 7 a	188	100.00	92.88	267	$1.4e^{-77}$
		Pen m 1	188	100.00	85.02	267	$1.5e^{-70}$
		Pan b 1	188	100.00	85.39	267	$6.5e^{-71}$

[1]Expected value (*E*) indicates the number of hits one can expect to observe by chance when searching a database of a particular size.
[2]Because the large number of matches found for this protein, only the top five matches found are listed here.
Hyphens indicate no sequence identity with known allergens was found in Allermatch™.
Der p (*Dermatophagoides pteronyssinus*); Blo t (*Blomia tropicalis*); Der f (*Dermatophagoides farinae*); Ani s (*Anisakis simplex*); Eur m (*Euroglyphus maynei*); Per a (*Periplaneta americana*); Cha f (*Chironomus kiiensis*); Pen m (*Penaeus monodon*); Pan b (*Pandalus borealis*); Lit v (*Litopenaeus vannamei*); Hom a (*Homarus americanus*).

Table 4: Sequence identity of peptides in tropomyosins identified (Table 2) with known tropomyosin epitopes as described by Ayuso et al. (2002). Shaded areas represent epitope cores as described by Reese et al. (2005).

Epitope number	Sequence (AA)	Peptides identified	Band #	Tropomyosin identified	Accession
1	VHNLQKRMQQLEN	-		-	-
2	ALNRRIQLLEEDLER	RIQLLEEDLER... RIQLLEEDLER... RIQLLEEDLER...	2 5 6	Tropomyosin 1, isoform A Tropomyosin Tropomyosin, partial	NP_524360 P31816 AGJ71763
3a	RSLSDEERMDALEN	SLADEERMDALEN... RSLADEERMDALEN...	5 6	Tropomyosin Tropomyosin, partial	P31816
3b	ERMDALENQLKEARF	...ERMDALENQLK ...ERMDALENQLK	5 6	Tropomyosin Tropomyosin, partial	P31816 AGJ71763
4	ESKIVELEEEL	IVELEEEL... IVELEEEL... IVELEEEL...	2 5 6	Tropomyosin 1, isoform A Tropomyosin Tropomyosin, partial	NP_524360 P31816 AGJ71763
5a	LQKEVDRLEDEL	LEDEL...	5	Tropomyosin	P31816
5b	KYKSITDE	-		-	
5c	ELDQTFSEL	-		-	

Hyphens indicate no peptides were identified similar to the epitope. Only those parts of peptides matching epitopes are shown. Dots indicate peptides extended in this direction.

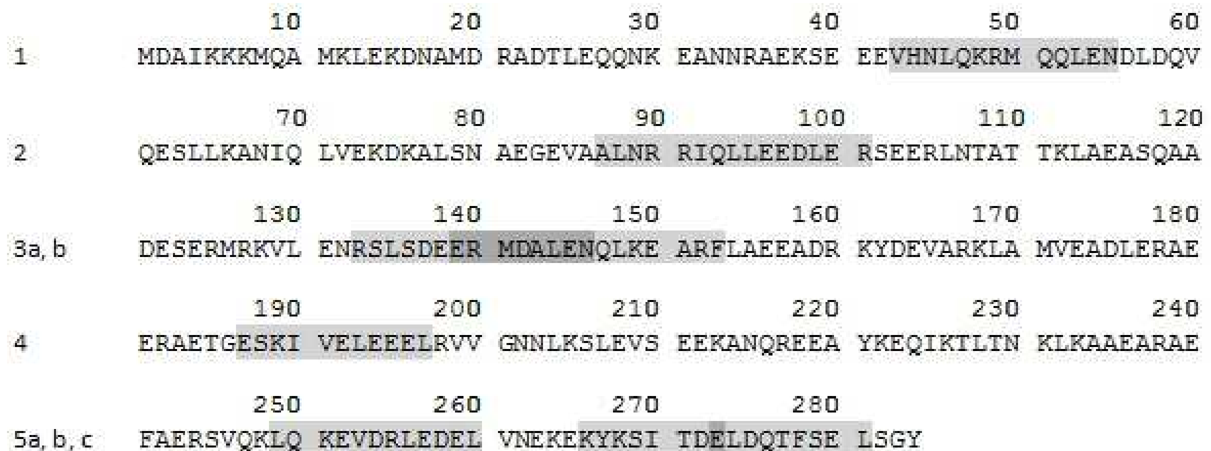


Figure 4: Full sequence of Whiteleg shrimp (*Litopenaeus vannamei*) allergenic tropomyosin Lit v 1, showing individual epitopes shaded in grey. Overlapping epitopes are marked dark grey.

Discussion

In this study, the effect of processing and *in vitro* digestion on the allergenicity of three edible mealworm species was determined. A study by Verhoeckx et al. (2014) demonstrated cross-reactivity between protein of unprocessed *T. molitor* and IgE from patients with a crustacean/House dust mite Der p 10 allergy *in vitro*. The present study shows that IgE from patients allergic to House dust mite (HDM) or crustaceans is also able to bind to proteins from *Z. atratus* and *A. diaperinus in vitro*. Furthermore, depending on

the type of protein, this cross-reactivity was altered by processing and *in vitro* digestion of mealworms.

Suitable sera for this study were pre-selected by dot-blot. Serum IgE of some non-atopic donors showed unexpected cross-reactivity to protein from *D. pteronyssinus*, *L. vannamei* or the mealworm species (results not shown). The dot-blot screening method detects single as well as multiple binding of serum IgE to protein. However, a clinical allergic reaction will only occur when two or more IgE molecules are cross-linked by an allergen. Hence, sensitisation to HDM or crustaceans (resulting in specific IgE production) might occur without clinical relevance. Only those sera not showing a reaction to abovementioned proteins were selected as control sera for further immunoblot studies. Serum IgE of three HDM allergic patients showed low response to *D. pteronyssinus* extract, even though these patients were described as exhibiting clinical allergic response to HDM. *Dermatophagoides pteronyssinus* extract does, however, not contain all known allergens of this species, as it was produced from whole bodies, while some allergens originate from mite faeces. Two crustacean allergic patients showed low serum IgE response to *L. vannamei* extract. These patients are possibly allergic to a less conserved crustacean allergen which might not be present, or might have a very different structure in *L. vannamei*. Sera of patients #6, #7 and #15 were nevertheless included in the respective pools for immunoblot studies because of a high response observed to one or more mealworm protein fractions. Serum of patient number #10 was excluded as it created a dark background on dot-blot, which can interfere with immunoblot visibility. Serum IgE of most allergic patients bound to one or more protein fractions for all three mealworm species, though differences in degree of sensitisation could be observed. Few patients responded to lyophilised *A. diaperinus*, which was also visible on immunoblot for HDM allergic patients. A strong response was observed on immunoblot for boiled *T. molitor* to crustacean allergic patient serum IgE. Compared with dot-blot results, the observed response likely represents prominent binding of patient #12 serum IgE, because IgE of other patients in this serum pool showed no response to boiled *T. molitor* on dot-blot. An alternative explanation might be that, on dot-blot, the high colour intensity caused by non-specific binding of one or more antibodies to boiled *T. molitor* protein partly masked the response of serum IgE of most patients to this protein fraction.

Protein of raw as well as processed mealworm species used in this study showed cross-reactivity with IgE from selected patient sera pools on immunoblot, while not showing cross-reactivity with IgE from the non-atopic serum pool. For the crustacean allergic serum pool, cross-reactivity appeared to be predominantly caused by tropomyosin, occurring in all tested species, of which the epitopes are stable to lyophilisation, boiling and *in vitro* digestion. Several different proteins cross-reacted with IgE from HDM allergic patients. Most of these proteins and their epitopes appeared to be degraded by *in vitro* digestion. Not all cross-reacting proteins could be identified by MALDI-MS/MS. This is possibly because not many proteins of *Z. atratus* and *A. diaperinus* have been presently described in protein databases. Proteins identified to cross-react with IgE from HDM allergic patients were

predicted long form paramyosin, α-amylase, actin, a predicted larval cuticle protein, hexamerin 1B precursor and heavy chain muscle myosin. Predicted long form paramyosin showed approximately 65% sequence identity to HDM allergens Der p 11 (*D. pteronyssinus*), Der f 11 (*D. farninae* Hughes) and Blo t 11 (*Blomia tropicalis* Bronwijk, Cock and Oshima) and 50% identity to *A. simplex* allergen Ani s 2. These allergens are all paramyosins (Pérez-Pérez et al., 2000; Tsai et al., 2000; Tsai et al., 2005), which are invertebrate muscle proteins (Hooper and Thuma, 2005). Alpha-amylase is a known allergen in HDM and other mite species (Mills et al., 1999; Thomas et al., 2010; Yan Chua et al., 2007). *Tenebrio molitor* α-amylase identified as cross-reactive protein showed approximately 50% sequence identity with HDM α-amylases Der p 4 and Eur m 4 (*Euroglyphus maynei*). Alpha-amylases have a rather high degree of sequence similarity between mites, insects and mammals (*ca.* 50%; Mills et al., 1999); hence the observed sequence identity does not have to result in allergic cross-reactivity. However, Allermatch^{TM} results indicated that 100% of 80 amino acid windows showed over 35% similarity of *T. molitor* α-amylase to HDM α-amylases. This increases the chance of potential binding sites for IgE antibodies and hence the chance of clinical allergic cross-reactivity.
Hexamerins are storage proteins present in the insect haemolymph, as are arylphorins (Burmester, 1999). Cockroach arylphorins have previously been described as allergens (American cockroach Per a 3, *Periplaneta americana*; Mindykowski et al., 2010). Hexamerin 1B precursor showed approximately 40% sequence identity to Per a 3. Similar to HDM, cockroaches are common sources of indoor allergens (Arshad, 2010). Possibly, one or more patients of which serum was used in this study were sensitised to cockroach in addition to HDM. Although Mindykowski et al. (2010) described Per a 3 as heat stable, no IgE cross-linking to a hexamerin protein was observed in the heat processed fractions in the present study. Thus far, heavy chain muscle myosin, actin and larval cuticle proteins are not known to be allergens. Long chain myosin does, however, show sequence identity to HDM paramyosins. Muscle protein was possibly partially broken down by *in vitro* digestion, thereby exposing or creating epitopes to serum IgE. Alternatively, long chain muscle myosin and actin are part of a myosin complex (Behrmann et al., 2012) and might have co-eluted with an allergenic muscle protein on SDS-PAGE, although it is surprising that this strong response on immunoblot was only observed for the *Z. atratus*. Heavy chain muscle myosin, actin and larval cuticle proteins could be novel allergens in mealworm species, but this remains to be proven.

The major protein identified to cross-react with IgE from crustacean allergic patients was tropomyosin. This was expected because tropomyosin is a well-known pan-allergen in arthropods (Reese et al., 1999). Within crustaceans, tropomyosins show over 90% homology (Reese et al., 1999). Furthermore, cross-reactivity of crustacean tropomyosins has been demonstrated with tropomyosins from insect species such as cockroach (*ca.* 80%; Asturias et al., 1999; Santos et al., 1999) and silverfish (*ca.* 65%; Barletta et al., 2005). Tropomyosins identified in mealworm showed *ca.* 60-90% identity to tropomyosins from

other insect species and crustaceans. For all three tropomyosins, Allermatch^{TM} results indicated 100% of 80 amino acid windows showed over 35% similarity to known allergenic tropomyosins. Of the eight tropomyosin epitopes described by Ayuso et al. (2002) and Reese et al. (2005), up to five epitopes were partly matched by peptides identified by MALDI-MS/MS in cross-reactive mealworm protein. These included peptides identified in boiled and *in vitro* digested protein fractions. This confirms that tropomyosin allergenicity appears to be stable to boiling (Besler et al., 2001; Kamath et al., 2014) and *in vitro* digestion (Guo et al., 2009). Mostly similar epitopes were matched by peptides in tropomyosin identified from protein bands #5 and #6, with the exception of epitope 5a, which was only found in protein band #5. This could be explained by tropomyosin being further degraded by digestion in band #6, which corresponds with the lower molecular weight. Fewer peptides matched epitopes for the tropomyosin identified in protein band #2. Possibly, this tropomyosin is a different isoform in mealworm species than the tropomyosin identified in the boiled, digested fraction. This would also explain the lower sequence identity observed in Allermatch^{TM} analysis, compared to the tropomyosin identified in protein bands #5 and #6.

In the present study, tropomyosin allergenicity appeared to be decreased in fried samples and disappeared in fried, *in vitro* digested samples. Fewer proteins were visible on SDS-PAGE for fried samples. Possibly, some protein was lost in the frying oil. Protein was also observed to leak into the boiling water during boiling of mealworms (personal observation) and as tropomyosin was still observed to be cross-reactive in these fractions, an alternative explanation could be that tropomyosin is less stable to digestion when exposed to high temperatures used for frying (180°C).

Tropomyosin was also observed to be a major allergen in the study by Verhoeckx et al. (2014), where the protein was identified in patients allergic to HDM. Serum IgE of HDM allergic patients in the present study did not cross-react with tropomyosin. Bronnert et al. (2012) stated that patients with a combined HDM/crustacean allergy are more likely to be sensitised to HDM tropomyosin (Der p 10) while patients allergic to HDM only tend to be sensitised to other HDM allergens. HDM allergic patients in this study were not allergic to crustaceans.

To our knowledge, this is the first study to determine the putative allergic cross-reactivity of serum IgE from patients allergic to crustaceans or HDM to proteins from *Z. atratus* and *A. diaperinus in vitro*, and the first study to examine the influence of food processing on the allergenicity of edible mealworm species. Results show that patients allergic to HDM or crustaceans might experience an allergic reaction when consuming mealworm species. Heat processing might decrease, but does not eliminate this risk.

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Chapter 6

General discussion

Sarah van Broekhoven

Insects as food - the present situation

Since the renewed interest in insects as food and feed during the 1990s and the early 2000s, research on edible insects has dramatically increased (Van Huis, 2013; Van Huis et al., 2013). Research expanded from ethno-entomological studies of insect consumption as an indigenous practice in many tropical countries to a possible solution to the world food problem. A growing world population and the increasing demand for meat result in a need for alternative, more sustainable protein sources, because production of conventional livestock is associated with environmental problems such as global warming, land degradation, and loss of biodiversity. Edible insects could provide an alternative source of animal protein. A first international conference entitled "Insects to feed the world", organised by the Food and Agriculture Organization of the United Nations (FAO) and Wageningen University and Research Centre in 2014 attracted about 450 attendants from 45 different countries (Van Huis and Vantomme, 2014; Vantomme et al., 2014). Benefits of edible insects over traditional livestock reviewed by among others Van Huis (2013) and Van Huis et al. (2013) include a good nutritional value, lower greenhouse gas emission and more efficient use of land and water, but also less risk of zoonosis and better feed conversion efficiency. Hence, arguments for introduction of entomophagy in Western countries are largely based on health as well as environmental reasons. Data on nutrient composition of different insect species, food-, feed- and industrial application as well as possible food safety risks become increasingly available. However, there are still many knowledge gaps that need to be filled. This thesis, as part of the project "Sustainable production of insect proteins for human consumption" (SUPRO2) aimed to contribute to the growing research field of insects as food. With a focus on three mealworm species suitable for human consumption, this thesis explored the possibility of producing the insects more sustainably on diets composed of organic by-products, and its effect on feed conversion efficiency and nutrient composition. Although not the objective of this study, the results may contribute to a better understanding of using these insects as a means to sustainably produce protein as feed for pets, farm animals and fish. Furthermore, with food safety being a factor of major importance when introducing new food items to the Western market, this thesis investigated two potential food safety risks: contamination with mycotoxins and allergenicity.

Sustainable production and feed conversion efficiency

One of the recurring arguments in favour of introducing edible insects into the Western world is that they can be produced more sustainably than conventional livestock. An important factor contributing to this sustainability is that due to being poikilothermic, insects do not need to invest metabolic energy in maintaining a constant body temperature.

This allows them to invest more energy from feed into growth, and hence insects are expected to have a higher feed conversion efficiency than conventional animals. For example, for House crickets (*Acheta domesticus*), the feed conversion ratio (FCR; kg of feed needed to produce one kg of biomass) was found to be 1.7 (Collavo et al., 2005). Considering that the edible portion of *A. domesticus* is greater than that of conventional livestock, *A. domesticus* was reported to be twice as efficient as chickens, four times more efficient than pigs, and ten times more efficient than cows at converting feed to biomass (Van Huis, 2013). Insect diet has a major effect on feed conversion efficiency. Optimisation of diets can thus improve sustainability of insect production. Sustainability can be further improved when diets are composed of locally produced organic by-products, for example originating from the food industry, although such diets could compromise insect development and feed conversion efficiency. This was shown in a study by Lundy and Parrella on House crickets (2015). The insects survived well on human food waste that had been subjected to an aerobic digestion process. However, development time took longer, harvest weight was lower and FCR was increased from 1.47 on control diet to 1.91 on food waste. Nadeau (2014) calculated that if the use of organic by-products as insect feed would result in only half of the production yield compared to using an optimal diet, then 15,586 ha of mealworm production facilities could produce all energy needed to erase the food deficit in the world. Assuming the organic by-products used would not require extra land for production, this 15,586 ha represents only 0.0003% of the agricultural land in the world (Nadeau et al., 2014). Of course it has to be kept in mind that production of mealworms, like production of conventional production animals, has an economic footprint.

In Chapter 2 (Van Broekhoven et al., 2015b), *T. molitor*, *Z. atratus* and *A. diaperinus* were produced on diets composed of organic by-products originating from the Dutch food and bio-ethanol industry. Diet composition had a major effect on larval performance. Diets with a high yeast-derived protein content resulted in faster development, a lower larval mortality and higher pupal weight, compared to control diets used by insect breeding companies. This offers interesting opportunities for breeders to improve mealworm production. Feed conversion efficiency was also higher on diets with a higher protein content, which could contribute to a more sustainable production process. The lowest feed conversion efficiency found (*ca.* 3) was comparable to the lowest value found for *T. molitor* by Oonincx et al. (3.8; Oonincx et al., submitted 2015). In addition to dietary differences between the two studies, experimental setup has likely influenced the feed conversion ratio. In Chapter 2, feed conversion efficiency was only determined for part of the larval stage, while it was determined for the entire larval stage by Oonincx et al. (Oonincx et al., submitted 2015). The feed conversion ratio of *ca.* 3 is comparable to the value for pigs (3.6) but somewhat higher than the value for poultry (2.0; Wilkinson, 2011). More research is needed to further optimise diets in order to decrease mealworm feed conversion ratio. Rather than observing larval performance on different diets with varying nutrient contents, as was done in Chapter 2 and by Oonincx et al. (submitted 2015), a suitable approach

would be to first determine the macronutrient requirements of the insects. Rho and Lee (2014) studied the dietary protein and carbohydrate requirements for adult *T. molitor* and observed the beetles self-selected these macronutrients in a protein:carbohydrate ratio of 1:1. The study was not performed for the larval stage, but it is expected that the protein:carbohydrate ratio would be higher than for adults to facilitate fast tissue growth. This would correspond with results from Chapter 2 where diets high in protein resulted in faster growth. However, the protein content of these diets might have been in excess of larval requirement. Larvae on high protein content excreted faeces higher in uric acid (Chapter 2) and did not appear to consume their own exuviae, contrary to larvae on diets lower in protein (personal observation). According to Mira (2000), cockroaches (*Periplaneta americana*) exhibit higher exuviae feeding on diets lower in protein. Feeding excess protein is not desirable because protein rich organic by-products are often more costly. It would therefore be advantageous to first experimentally determine the macronutrient intake target of edible insects, and then design diets that match this target as closely as possible. It has to be kept in mind, however, that insect performance is not solely determined by absolute amounts of macronutrients. With respect to carbohydrates, it might make a difference whether the carbohydrate content is primarily derived from sugars or starch. Furthermore, starch source would have an impact because not all starch sources are equally digestible (Hosen et al., 2004; Mereiles et al., 2009). Similarly, amino acid profile largely determines protein nutritional quality. In addition, organic by-products contain micronutrients and might contain secondary plant compounds with an anti-nutritive effect. This was observed for diets containing cookie remains in Chapter 2, and by Dastranj et al. (2013) who found extracts of field bean and different wheat cultivars inhibited *T. molitor* digestive enzyme activity. Another factor that might complicate the use of organic by-products to compose insect diets is the possibility of batch or seasonal variability, which would impact nutritional composition of the by-product. Similarly to conventional livestock, constant quality of organic by-products used for edible insect production would have to be ensured.

In addition to studying larval performance on different diets, studies spanning several insect generations should be performed to determine the effect of diet composition on adult fecundity. In Chapter 2, successful adult eclosion was observed for all experimental diets, but this does not give an indication for adult fecundity. Possible detrimental effects of diet on insect development might only become manifest in subsequent insect generations. Therefore, diets optimal for fast larval development could be different from diets optimal for adult fecundity. In such case, it would be an option for insect breeders to supply insects kept for reproduction with a different diet than the insects produced to be sold as food.

Nutritional quality

Edible insect nutrient composition has been extensively reviewed by Bukkens (1997) and more recently by Rumpold and Schlüter (2013a), Belluco at al. (2013) and Finke and Oonincx (2014). There is considerable variation in nutrient composition not only between species, but also between life stages. Insects are predominantly high in protein and fat. In general, larval stages contain more protein and fat than adults. In this thesis, protein content and fatty acid profile of three mealworm species was determined after larvae had been feeding on different diets composed of organic by-products (Chapter 2: Van Broekhoven et al., 2015b). Protein content was relatively stable regardless of diet, which corresponds with the findings of Ramos-Elorduy et al. (2002) and Gao et al. (2010) where *T. molitor* larvae were produced on different diets composed of organic and non-organic waste materials, respectively. Amino acid composition, which provides important information on protein quality, was not determined in Chapter 2. Larvae in the study of Ramos-Elorduy et al. (2002) differed in amino acid profile depending on diet, with levels of isoleucine, leucine, tryptophan and methionine showing the most variation. However, amino acid profile was not determined for the different diets. Yi et al. (2013) determined the amino acid profile of different insect species including the mealworm species studied in this thesis, when obtained from a commercial insect rearing company. The total of essential amino acids was comparable to soybean, but lower than for casein. However, quantities were sufficient for humans according to FAO/WHO/UNO recommendations for adults. Yi et al. (2013) furthermore analysed functional properties of insect protein, which is important when this protein is to be used in the food industry. Water soluble fractions were used to study foaming and gelation capacity. Out of the three mealworm species, only *Z. atratus* protein formed stable foams, but protein from all three species was able to form a gel. These are promising first results for future food industry application. Other researchers have also studied the possibilities of insect-derived compounds for application in the food industry. For example, Ulanova and Kravchenko (2014) used House fly (*Musca domestica*) protein to develop a milk substitute. The product was similar to whole milk in terms of colour, odour and viscosity. The protein content was similar to cow's milk with respect to protein content and had a good amino acid score compared to FAO recommendations. Mariod and Fadul (2014) used gelatine extracted from melon bug (*Coridius viduatus*) and sorghum bug (*Adonoscelis versicoloratus versicoloratus*) for ice cream preparation. However, ice cream made with insect gelatine scored lower for taste and texture in a sensory test than that made with commercial gelatine. Digestive proteinases from *T. molitor* are active against wheat gluten and could potentially be used medicinally to treat celiac disease (Elpidina and Goptar, 2007; Mika et al., 2014)

In Chapter 2, mealworm fat content and fatty acid profile varied depending on the diet consumed, though larvae on all diets were predominantly high in palmitic acid, oleic acid and linoleic acid. This corresponds to previous studies such as those by Finke (2002) and

Tzompa-Sosa et al. (2014). Polyunsaturated fatty acid (PUFA) content ranged between 20 and 30% of total fat. PUFAs are important for human health (Kouba and Mourot, 2011). Wang et al. (2014) investigated the effect of *T. molitor* PUFA on obesity and found the PUFA to reduce body weight and cholesterol in a study using obese mice. In addition to total PUFA content, the ratio between ω-6 and ω-3 PUFAs (n6/n3 ratio) is of importance for human health, with an optimal ratio being 5:1 or lower (Kouba and Mourot, 2011). Mealworm n6/n3 ratio exceeded 18:1 (Chapter 2: Van Broekhoven et al., 2015; Tzompa-Sosa et al., 2014), which is much higher than the optimal ratio and the FAO recommendation of 10:1 (FAO, 2010; Tzompa-Sosa et al., 2014). Fatty acid profile can be altered by diet and thus, possibilities exist to lower mealworm n6/n3 ratio in order to obtain a healthier product. To lower the insect n6/n3 ratio, Tzompa-Sosa et al. (2014) proposed an insect diet low in linoleic acid, an ω-6 fatty acid. However, in Chapter 2, diets low in linoleic acid compared to control diet did not produce mealworms lower in n6/n3 ratio. A higher amount of α-linolenic acid, contributing to a higher ω-3 concentration in the diet, might have a stronger effect on mealworm n6/n3 ratio. This should, however, be subject to further study. An important aspect to consider is that altering edible insect fatty acid profile will likely influence the sensory characteristics as well as the shelf life of the insects. In meat from conventional livestock (Díaz et al., 2011; Wood et al., 2008; Wood et al., 2004), as well as in fish (Waagbø et al., 1993), fatty acid profile influences flavour and texture. A high ω-3 content resulted in a more rancid flavour especially after longer storage time, caused by oxidation of fatty acids. Anti-oxidants such as vitamin E can prevent quality deterioration. When altering insect diet to obtain a more favourable n6/n3 fatty acid ratio, it might thus be important to also provide an higher amount of vitamin E in the diet.

In addition to diet, extraction methods can influence insect fatty acid profile as was shown by Tzompa-Sosa et al. (2014). The Folch method, which was also used in Chapter 2, showed a total fat extraction similar to Soxhlet extraction, but higher than aqueous extraction. Furthermore, extraction by the Folch method showed higher amounts of total polyunsaturated fatty acids (PUFAs) than the other methods, but a lower ω-3 PUFA concentration than aqueous extraction. The Folch method is widely used on laboratory scale, but Soxhlet and aqueous extraction have more industrial relevance. It is therefore important to realise that insect fatty acid profile might differ when fat is extracted on industrial scale than when determined on laboratory scale. When extracted insect fat is used in the food industry or for other applications, fatty acid profile should be determined and optimal extraction methods might depend on the application. However, this does not play a role when whole insects are used whether in recognisable form or ground to powder.

Nowak et al. (2014) compiled nutritional data of *T. molitor* to confirm claims on its nutritional value. According to the WHO and FAO limits for the labels 'source of' and 'high in', *Tenebrio molitor* larvae can be considered a source of calcium and zinc, and high in protein and magnesium. A limitation of the studies determining the nutrient composition of *T. molitor* and other mealworms is that raw insects were analysed. Humans in Western

societies do no consume mealworms raw, and food processing methods can influence nutrient composition. For example, Kinyuru et al. (2009) studied the effect of the traditional processing methods toasting and sun-drying on the vitamin content of an edible winged termite (*Macrotermes subhylanus*) and an edible katydid species (*Ruspolia differens*) and found a general decrease in vitamin content for both species, which was especially evident after toasting. It is at present unknown how processing might influence nutrient composition of mealworm species.

In addition to nutrient composition, digestibility plays an important role in determining nutritional value of edible insects for human consumption. Data on digestibility of mealworm species for humans is at present limited. Yi et al. (unpublised) determined the digestibility of *T. molitor in vitro* using a human gastric-duodenal system. Protein fractions containing low molecular weight hemolymph proteins were more rapidly digested than fractions containing muscle proteins. Bosch et al. (2014) determined the digestibility of several insect species, including the three mealworm species discussed in this thesis, for pet animals using a canine *in vitro* digestion system. The organic matter digestibility was higher for mealworms than for the reference substrates poultry meat meal, fish meal and soybean meal. In general, chitin is considered fibre in studies determining insect nutrient composition. Insect chitin is tightly cross-linked with proteins (Cauchie, 2002), which might make these proteins inaccessible for humans. However, chitinolytic enzymes have been found in the human stomach (Paoletti et al., 2007) and colon, where they are produced by chitinolytic bacteria (Dušková et al., 2011). Further research still has to be performed to determine whether the amounts of these enzymes are sufficient to digest insect chitin. Furthermore, processing of insects can influence their digestibility, as was shown by Kinyuru et al. (2009), where toasting as well as sun-drying resulted in a decreased *in vitro* protein digestibility for *R. differens*, but not for *M. subhylanus*.

Food safety

Compared to the number of studies on nutritional properties, research on the food safety aspects of edible insects is still limited, although it has increased in recent years (Belluco et al., 2015; Rumpold and Schlüter, 2013a, b). In Western societies, food security is of less concern and food safety has a large impact on consumer trust, among others due to longer and more complex food processing chains. Furthermore, when new food products or ingredients are introduced on the market, their food safety needs to be assessed in terms of microbial, toxicological and allergenic risks. This thesis explored two food safety aspects: risk of mycotoxin contamination and risk of allergic cross-reactivity.

Contamination risk

Contamination of edible insects with pathogens or toxic compounds can occur during the insect's life cycle as well as after harvest of the insect. Research on the presence of toxic compounds and pathogens, present in either freshly harvested or processed and stored edible insects, has recently been reviewed (Belluco et al., 2013; Van der Spiegel et al., 2013; Van Huis, 2013). However, most studies concern insects harvested from nature in tropical and sub-tropical countries. When insects are collected from nature, contamination can occur in the form of pesticides, heavy metals and soil-borne pathogens (Van der Spiegel et al., 2013; Van Huis, 2013). Post-harvest spoilage is a relevant risk in countries where edible insects are traditionally sun-dried and not refrigerated. Even though the above-mentioned concerns do not apply in Western countries where edible insects are produced in closed farming systems and food products can be refrigerated, the risk of contamination should not be underestimated. Studies showed that *A. diaperinus* is capable of harbouring human pathogens for a time span of several hours in case of *Campylobacter jejuni* (Strother et al., 2005; Templeton et al., 2006) to several days for *Salmonella typhimurium* (McAllister et al., 1994). *Tenebrio molitor* was found to consume fungi without any visible adverse effect (Chuku et al., 2007; Davis et al., 1975). Contamination of edible insects can occur during the production process when contaminated organic by-products are used as substrate, or when hygienic practices are applied insufficiently. The Hazard Analysis and Critical Control Points (HACCP) system is a widely used approach aiming to ensure food safety by preventing physical, chemical and biological contamination during the food production process (Gurnari, 2015; Van Huis et al., 2013). The HACCP system should be adopted by commercial edible insect producers and companies developing insect-based food products in order to assure a food product free of contamination. This will also increase consumer confidence in edible insect products (Van Huis et al., 2013). Gurnari (2015) stresses the importance of the HACCP system, and also indicates the problem of microbial toxins such as mycotoxins as one of the most important challenges in the food industry today. A worldwide survey by Rodrigues and Naehrer (2012) shows that mycotoxins are also prevalent in grain used as animal feed. One or more mycotoxins were present in 65% to 81% of samples tested, depending on the sensitivity of the detection method. The most prevalent mycotoxins found in samples from Europe were deoxynivalenol and ochratoxin A. Several studies examined the effect of mycotoxins on *T. molitor* (Abado-Becognee et al., 1998; Davis and Schiefer, 1982; Davis et al., 1975). Because the objectives of these studies were not to assess the safety of *T. molitor* as food, the presence of mycotoxins in the larval bodies was not determined. However, larval growth and mortality were not affected up to relatively high concentrations of mycotoxins. This fact, together with mycotoxins still being of concern in the food and feed industry today, emphasises the importance of studying the risk of mycotoxin contamination in commercially produced edible insects. In this thesis, *T. molitor* was fed wheat flour containing deoxynivalenol (Chapter 3) and presence of this mycotoxin in the larval bodies,

as well as excretion through faeces, were assessed. Recently, Guo et al. (2014), in a study on food choice, weight gain and mortality of *T. molitor* larvae on wheat colonised with several different *Fusarium* species, determined the presence of mycotoxins in the larvae, but not in the faeces. Similar to Chapter 3, levels of deoxinivalenol found in the larvae were below the detection limit in the study by Guo et al. (2014). Several other mycotoxins, including zearalenone, fumonisin B1 and several enniatins, were detected in concentrations <2 µg/g in larval samples, but 13 µg/g enantianin was detected in larvae that had been feeding on wheat containing >90 µg/g enniatin B. Chapter 3 shows that up to about 40% of ingested deoxynivalenol was excreted in the faeces. The remaining portion was likely metabolised to compounds not determined in Chapter 3. More research is needed to determine the fate of mycotoxins in mealworms, especially because it is unknown whether possible metabolites could still have a toxic effect when consumed by humans. Guo et al. (2014) hypothesised that Tenebrionidae might have evolved adaptations to neutralise toxic fungi encountered in their natural environment. If this is indeed the case, and mealworms successfully detoxify mycotoxins into metabolites which pose no threat to humans, this would be a beneficial trait for the insect production sector. Risk of mycotoxin contamination acquired during the production process of mealworms would be strongly diminished. However, it would have to be kept in mind that when using organic by-products as feed for mealworms, diets may contain material on which mealworms have not evolved to feed, and may thus also contain mycotoxins not normally encountered by mealworms. It is therefore important to assess the safety of mealworms, as well as other edible insects, when grown on feed contaminated with a wider range of mycotoxins than those commonly present in grains.

Other forms of contamination which could occur during the production process, such as contamination by heavy metals or pathogens, were not addressed in this thesis. These forms of contamination are less likely to occur in closed farming systems when producing for human consumption, but still do have relevance especially when producing insects on organic by-products. Special consideration is required when producing insects to be used as animal feed on manure. Heavy metals or pathogens could be harboured in the insects and subsequently end up inside the consuming livestock. This would pose a threat not only to the animals, but also to humans consuming meat of these animals. Lalander et al. (2014) used Black soldier flies (*Hermetia illucens*) to convert organic waste into larval biomass which could be fed to animals. The researchers found a reduced concentration of *Salmonella* spp. and viruses in the organic waste material after *H. illucens* had been feeding on it, but no reduced concentration of *Enterococcus* spp.. Furthermore, *H. illucens* presence did not inactivate eggs of the parasite *Ascaris suum*. In a study producing *H. illucens* on human faeces, Lalander et al. (2013) found a six log cycle reduction of *Salmonella* spp. concentration in the faeces after larvae had been feeding on it, but only a small reduction in concentration of *Enterococcus* spp. (< 1 log cycle). Larvae and prepupae of *H. illucens* were examined for presence of these pathogens after harvest. A higher concentration of

Enterococcus spp. was found in the larval gut (10^6 cfu/g) than in the prepupal gut (10^5 cfu/g), but less than 1 cfu/g and 0.5 cfu/g *Salmonella* spp. respectively. The high concentration of *Enterococcus* spp. shows that additional processing steps are required before feeding the insects to animals. Charlton et al. (2015) examined the presence of harmful chemical contaminants in several fly species suitable for animal feed, produced on organic waste substrates such as manure, fish offal and pig offal. Contaminants analysed included pesticides, heavy metals and veterinary medicines. Fly larvae contained contaminants in levels below the maximum concentration recommended by European Commission, World Health Organisation and Codex Alimentarius, with the exception of several samples of *M. domestica* in which cadmium and the medicine nicarbazin were present in levels exceeding the lowest EU limit for animal feed.

Insects produced on feed expected to be free of harmful compounds or pathogens still contain large amounts of microorganisms, due to the rich microbial flora in the insect gut. Microorganisms from the insect gut are, however, taxonomically very different from vertebrate pathogens and are therefore not expected to pose a health concern (Eilenberg et al., 2015; Giaccone, 2005). Giaccone (2005) analysed the microbial count of several species of edible insects, including *T. molitor*, *Z. atratus* and the House cricket *A. domesticus* and found over 10^5 cfu/g, mostly made up of Gram-negative bacteria. Pathogenic species such as *Salmonella* spp., *Listeria* spp., *Bacillus cereus* and *Staphylococcus aureus* were not found. Nevertheless, several commercial producers of edible insects in The Netherlands allow insects to fast for one day to void the gut content prior to harvest (Van Huis et al., 2013). Belluco et al. (2013) argue that safe storage and preservation of insect-derived products is more important from a food safety perspective than the microbial count of freshly harvested, live insects. Microbial count of fresh as well as processed *T. molitor* and *A. domesticus* under different storage conditions and durations was determined by Klunder et al. (2012). Heating steps such as boiling and roasting strongly reduced the overall microbial count, although spore forming bacteria were not fully eliminated. Fermentation of composite flour containing powdered roasted *T. molitor* reduced both Enterobacteriaceae and bacterial spores and increased shelf-life. Rumpold et al. (2014) explored the possibility of using non-thermal methods such as high hydrostatic pressure and cold plasma treatment to preserve *T. molitor*. Indirect cold plasma treatment was found most effective for surface decontamination of the larvae. This method could potentially be used to surface sterilise live mealworms, although more research is required to explore this option. Indirect cold plasma had limited penetration capacity and was therefore not suitable for reduction of overall microbial count, for which thermal treatments at 90°C were most effective. A combination of treatment is suggested for most effective decontamination.

Contamination risk of edible insects can thus be reduced both by ensuring the material used as feed for the insects is free of pathogens and toxic compounds, and by applying appropriate decontamination and processing steps after harvest of the insects. Safe storage

of the edible insect product is also essential, as Fellows (2014) stresses, dried insects can pick up moisture when stored in moist areas, which can then lead to growth of bacteria and fungi.

Allergenicity

Allergenicity is a safety concern relevant for all food products. Around 5% of the general population is affected by food allergy (Sicherer and Sampson, 2014). Symptoms range from local mild reactions to potentially fatal anaphylaxis (Perry and Pesek, 2013). Correct labelling of food products to provide information on ingredients and allergenicity risk is essential for allergic individuals in order to avoid potentially harmful food ingredients. Research on the allergenicity of edible insects is still in its infancy, and mostly concerns case descriptions of individuals experiencing allergic reaction to an edible insect. Most incidents of food allergy to edible insects concern people from countries having a tradition of insect consumption, and symptoms can be severe. For example in China, there were over 60 reported cases of anaphylaxis upon consumption of insects including Domesticated silkworm pupae (*Bombyx mori*), bee larvae and locust and cricket species in the period 1980-2007 (Ji et al., 2009). A Thai hospital reported seven cases of anaphylaxis to fried insects (grasshopper and cricket species) in the period 2005-2006 (Ji et al., 2009; Piromrat et al., 2008). One case of anaphylaxis upon consumption of *T. molitor* has been described (Freye et al., 1996). Often, it is unknown which proteins are responsible for the allergic reaction. A more widely studied case of allergy to insect extract in food in the West concerns cochineal, the natural red food dye extracted from *Dactylopius coccus*. Allergic individuals reported asthma in response to food containing cochineal (Lizaso et al., 2000; Oplatowska-Stachowiak and Elliott, 2015). Lizaso et al. (2000) identified three different allergens in cochineal, though more information than molecular weight is not yet available for these proteins. According to Oplatowska-Stachowiak and Elliott (2015), cochineal has been reported to be used unauthorized and in too high concentrations, which represents a food safety concern.

In this thesis, the allergenicity of mealworm species was determined *in vitro* (Chapter 5: Van Broekhoven et al., 2015a; Chapter 4: Verhoeckx et al., 2014). A factor complicating research on allergenicity of edible insects in the West is that at present, only few people are known to experience allergic reactions upon insect consumption, meaning very few sera are available for research purposes. For this reason, research in this thesis focussed on risk of allergic cross-reactivity for people allergic to different arthropods: crustaceans or House dust mite. Determining allergic potential through cross-reactivity is relevant because in the West, the number of people sensitized to edible insects is far fewer than in countries accustomed to insect consumption. People experiencing allergic reactions to edible insects are thus likely to have been sensitised through a different route, for example through inhalant allergy to House dust mites or cockroaches, or through food allergy to related arthropod foods, in particular crustaceans. Insects and crustaceans are more closely related

than was generally thought until recently, both groups together forming the clade Pancrustacea (Pennisi, 2015; Regier et al., 2010).

Allergic potential and cross-reactivity were determined, as well as which proteins are responsible for the observed reaction. In Chapter 4, tropomyosin and arginine kinase were identified as major cross-reactive proteins in raw *T. molitor* when using sera of people with combined crustacean and House dust mite allergy. In Chapter 5, cross-reactivity was determined for all three mealworm species using sera of people allergic to either crustaceans or House dust mite, and effects of processing and *in vitro* digestion were studied. Tropomyosin, but not arginine kinase, was identified to be the major cross-reactive protein for crustacean allergic individuals and several different proteins including α-amylase, hexamerin 1B precursor and muscle myosin cross-reacted with IgE from sera from House dust mite allergic individuals. Heat processing as well as *in vitro* digestion did reduce, but not eliminate allergenicity.

Assessing allergic potential by comparison of protein sequence with well-described allergens, which can be done with software such as Allermatch™, allows for a broad exploration of potential allergens even when at present, the number of people having experienced allergic reactions upon the consumption of insects is still scarce. However, when the complete proteome is not yet known, as is the case with the mealworm species used in this thesis, potential allergens can be missed. Identification of cross-reactive proteins obtained from different patient sera is thus important in order to obtain a representative list of allergenic proteins. This was illustrated by the fact that several different allergenic proteins were identified in Chapters 4 and 5, even though all patients were allergic to House dust mite and/or crustaceans. Whether these allergies are combined or individually present already makes a difference in the types of proteins allergic individuals respond to. To get a more complete view of potentially allergenic proteins, sera are best used in individual tests, rather than combined in a pool. However, due to the scarcity of sera and the amount used, this is not always possible.

Although *in vitro* tests are powerful as first steps in research to determine allergic potential, *in vivo* tests are important to confirm allergenicity of food proteins, with oral food challenges such as the double-blind placebo-controlled food challenge being the gold standard in allergy diagnosis. Recently, Broekman et al. (2014) further explored allergenic cross-reactivity for *T. molitor* by means of skin prick tests on shrimp allergic individuals. All experienced a positive reaction to *T. molitor* extract. Double blind placebo controlled food challenges to further confirm allergenicity are underway.

Determination of potential cross-reactive allergens is both a more readily executable and a relevant first step in allergy research to edible insects in the West. As more sera of people experiencing allergic reactions upon insect consumption become available, more different allergenic proteins are expected to be discovered, because individuals being primarily sensitized to insect protein might react to different proteins than those primarily sensitized to House dust mites or crustaceans. However, pan-allergens such as tropomyosin

and arginine kinase, which are major allergens present across different classes of organisms, will likely remain of major importance. To date, tropomyosin has not yet been described as allergen in other species of edible insects than mealworms. Arginine kinase, on the other hand, was described as major allergen in *B. mori* (Liu et al., 2009) and the Field cricket species *Gryllus bimaculatus* (Srinroch et al., 2015). In Chapters 4 and 5, non-water soluble protein was extracted in addition to water soluble protein and tropomyosin was found to be present in the non-water soluble fraction. The protein extraction procedures used by Liu et al. (2009) and Srinroch et al. (2015) might not have yielded tropomyosin. Alternatively, serum used in these studies was likely obtained from patients primarily allergic to arginine kinase. Chapters 4 and 5 showed that patients allergic to arginine kinase were not allergic to tropomyosin and vice versa. Srinroch et al. (2015) furthermore described hemocyanin, glyceraldehyde 3-phosphate dehydrogenase and hexamerin 1B precursor as allergens in *G. bimaculatus*. Hexamerin 1B precursor from *T. molitor* was found to be cross-reactive with sera from House dust mite allergic individuals. Although not found to be cross-reactive with the sera used, glyceraldehyde 3-phosphate dehydrogenase was identified in *T. molitor* protein (Verhoeckx et al., 2014) and could thus be another potential allergen in mealworms. Srinroch et al. (2015) found *G. bimaculatus* cross-reacts *in vitro* with serum from people allergic to freshwater prawn (*Macrobrachium* spp.). Cross-reactivity between allergens from insects and crustaceans remains clinically important also when people are primarily sensitised to edible insects rather than crustaceans, as was illustrated by Piromrat (2008). A woman experiencing anaphylaxis upon consuming fried insect (presumably a cricket or locust species) developed anaphylaxis caused by consumption of shrimp several months later.

Allergenicity of heat processed in addition to raw edible insects should be determined, because insects are generally not consumed raw in the West and heat processing can influence protein integrity and thus allergenicity. The effect of heat processing on allergic cross-reactivity of mealworm protein was explored in Chapter 5. Allergenicity was not eliminated by heat processing. This corresponds with a review on the effect of food processing on allergenicity by Verhoeckx et al. (2015) in which it was concluded that heat processing does not completely eliminate the allergic potential of proteins. On the other hand, fermentation, hydrolysis and potentially pressure treatments may reduce allergenicity to such an extent that the food product would be safe for consumption by allergic individuals. Allergenicity of fermented insect products might be worth investigating; for example, fermentation of mealworms as studied by Klunder et al. (2012) might provide for a product that has increased shelf life and is simultaneously less allergenic than heat processed mealworms.

Further research contributing to consumption of insects in the West

Consumer acceptance

In Western societies, the idea of eating insects is often regarded as primitive and disgusting (Deroy et al., 2015; Looy and Wood, 2006; Van Huis et al., 2013). Several authors agreed that consumer acceptance is one of the major barriers to the introduction of edible insects in the West (Halloran et al., 2014; Rumpold and Schlüter, 2015; Van Huis et al., 2013). Therefore, studies on consumer acceptance of edible insects, especially when exploring methods to effectively improve consumer acceptance, form an important contribution to research on insects as food.

Food acceptance and disgust are typically culturally bound (Martins and Pliner, 2005; Tan et al., 2015), although personal and situational factors also play a role. Positive sensory perception such as good taste and familiar and good appearance are important arguments for food acceptance, while a bad taste or appearance, as well as the belief a food product might be harmful or unhealthy, are important arguments for food rejection. Healthiness is sometimes listed as another important argument for food acceptance (Martins and Pliner, 2005), and Van Huis (2013) argues that nutritional information as well as the advantages for sustainability and animal welfare edible insects hold over conventional livestock can be motives for unaccustomed consumers to recognise the importance of edible insects. Although these arguments deserve consideration, several studies found that rational arguments alone are not sufficient in order to convince Western consumers to include insects in their diet (Deroy et al., 2015; Lensvelt and Steenbekkers, 2014; Looy et al., 2014; Tan et al., 2015; Verbeke, 2015). Deroy (2015) furthermore argues that providing information on the widespread custom of insect consumption in the world will not convince the Western consumer. Instead, insects should be prepared in such a way as to appeal to Western consumers both in taste and appearance. Processing method has a strong influence on consumer acceptance of edible insects. For example, a study by Caparros Megido (2013) on Belgian consumers willing to try edible insects showed that crispy textures were preferred. Similarly, according to Fellows (2014), restaurant chefs report that customers tend to prefer crisp insects over soft textured insects. Grinding or processing insects in order to make them less recognisable helps to lower the barrier for unaccustomed consumers (Caparros Megido et al., 2013; Van Huis et al., 2013). In a study involving Dutch and Australian consumers, Lensvelt and Steenbekkers (2014) found that participants from both countries were more willing to try insects when made unrecognisable, for example by grinding into flour. Tan et al. (2015) compared perception of edible insects in groups of Thai and Dutch participants and found that reducing the visibility of the insects, as well as incorporating them into familiar food products improved the willingness to try an unfamiliar species. Hwang et al. (2015) studied consumer preference and purchase intention of sautéed and oven roasted *T. molitor* in whole, chopped or powder form in Korea. Participants preferred the powdered form regardless of preparation method.

Initiatives are taken up to develop food products containing insects for the Western market. For example, 'Buqadilla' is a deep fried snack which contains 40% ground *A. diaperinus* and 'Crikizz', developed by Ynsect, is a cassava snack containing 10-20% ground *T. molitor* (Van Huis et al., 2013). The Nordic Food Lab (Copenhagen, Denmark) recognises taste as the most important aspect of food and aims to assess the gastronomic value of various edible insect species, including ants (*Formica rufa* and *Lasius fuliginosus*) and locusts (*Schistocerca gregaria* and *Locusta migratoria*), by including them in newly developed dishes appealing to Western consumers (Evans, 2014). Similar to exposure to insect dishes, where consumers have the opportunity to taste, information on which insect species can be used for food and how they should be prepared is key to acceptance by unaccustomed consumers (Lensvelt and Steenbekkers, 2014; Looy et al., 2014; Tan et al., 2015; Van Huis, 2013). The Insect Cookbook written by Van Huis et al. (2014) can aid in this endeavour.

Scaling up of production

The intensive production of conventional livestock has a long history in Western society and as a result, much research has contributed to increase and optimise the production process, leading to higher yield and reduction of costs and labour. In contrast, the large scale production of insects for human consumption is still in its infancy (Van Huis et al., 2013). Currently, labour costs in insect farming are high and the resulting relatively high market price of edible insects on the Dutch market might pose a barrier for consumers (Lensvelt and Steenbekkers, 2014; Van Huis et al., 2013). Research into automation of large scale production is thus recommended. Insects suitable for large scale production should among others have the ability to live in high densities, have a high rate of biomass increase and a high oviposition rate (Van Huis et al., 2013). Inexpensive feed should be used to decrease production costs. Producing insects on organic by-products might aid in the process of cost reduction, although more research is needed, as well as more food production regulation, to explore the possibilities of using organic by-products as feed for insects.

Post-harvest processing steps, such as freeze-drying commonly applied to edible insects sold in The Netherlands, further increase the market price. Similarly, while extraction of insect protein might be an effective method to increase acceptance of edible insects by Western consumers, the high production costs would currently not provide for economically feasible products (Van Huis et al., 2013). In addition, additional processing steps require use of energy, which will make production of edible insects considerably less sustainable.

Scaling up and automation of edible insect production will aid in increased production and cost reduction, but might also come with the challenge of increased disease pressure (Eilenberg et al., 2015). For example, *Acheta domesticus* densovirus (AdDNV) poses a frequent problem in the mass-production of *A. domesticus* (Liu et al., 2011) and *T. molitor*

has been found susceptible to among others *Beauveria bassiana*, especially when inbred (Rantala et al., 2011). Research on diseases within edible insect production is still scarce and protocols for diagnosis and control of diseases for edible insects are currently lacking (Eilenberg et al., 2015). Because use of antibiotics is undesirable, it is important to select for strains with high disease resistance for large scale production of edible insects, and to provide for sufficient genetic diversity.

Legislation

According to the General Food Law of the European Union, food business operators are responsible for ensuring their product and production process complies with all requirements of food law (Van der Spiegel et al., 2013). At present, no clear legislation regarding insects as food exists within the EU (Van der Spiegel et al., 2013) (Belluco et al., 2013; Van Huis et al., 2013). Van Huis et al. (2013) identified this unclear legislation as a major obstacle for investors in insect production for food and feed. Belluco et al. (2013) and Van der Spiegel et al. (2013) extensively reviewed the current state of European legislation regarding edible insects. Insects as food are currently not included in food regulations and it is not yet clear whether insects will be classified as novel foods under the Novel Food Regulation (EC, 1997). An insect-derived product which is authorized and commonly used in the EU is the red food dye E120 extracted from *D. coccus*. However, this food dye will be re-evaluated in 2015, together with other food additives (Belluco et al., 2013). If consumption of edible insects to a significant degree in the EU before May 15, 1997 cannot be proven, insects would likely be classified under category *e* of the Novel Food Regulation, which states "foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals" (Van der Spiegel et al., 2013);(Belluco et al., 2013). This would especially apply when protein isolated from insects is used in food. However, the classification might not apply when whole insects are consumed. According to Food Standard Agency of the United Kingdom, insects are exempt from the scope of the Novel Food category because they are normally consumed whole and thus do not fall under the definition of a novel food. In contrast, a batch of Domesticated silkworm pupae was recently rejected in Italy under classification as unauthorized "novel food ingredient in food supplement" (Belluco et al., 2013).

Within the context of the new Novel Food Regulation, which is at present only available in draft form (COM, (2007) 872 final), edible insects might classify as traditional food from third countries (Belluco et al., 2013). For this category, if a history of safe use in the country of origin has been demonstrated and EU member states as well as EFSA do not present safety objections based on scientific evidence, the food can be placed on the EU market (COM, (2007) 872 final). This new regulation could lead to more clear legislation regarding the use as food of already more commonly used edible insects such as *T. molitor*, *A. domesticus* and *Locusta migratoria*. While progress regarding clear legislation is still underway in the EU, individual countries can already implement regulation regarding the

production and use of whole edible insects (that is, no isolated protein or other components to be used as food ingredients) for human consumption. For example, in Belgium, a list of 10 already farmed insect species were approved for human consumption by the Federal Food Agency of Belgium in December 2013 (FAVV, 2013; Halloran et al., 2014). This list includes the three mealworm species used in this thesis, several cricket and locust species as well as *B. mori*.

Strict EU regulations apply not only to the food product to be consumed, but also to the safety of the production process. Annex III of Regulation (EC) 767/2009 lists materials which are not allowed to be used as feed material for production animals, including faeces and household waste (EC, 2009; Van der Spiegel et al., 2013). Furthermore, in order to prevent the risk of transmittable spongiform encephalopathies such as BSE, protein of animal origin is not allowed to be fed to ruminants such as cows (EC, 2001). This could be a complicating factor when using organic by-products as feed for insects to be used as human food or animal feed. Several studies have explored the possibility of producing *H. illucens* and *M. domestica* on manure (Khan et al., 2012; Lalander et al., 2014; Myers et al., 2008; Oonincx et al., 2015). Larvae of these species could be used as animal feed (Sealey et al., 2011; Zuidhof et al., 2003); however, under current EU regulations, these cannot be produced on manure. Different organic by-products such as those used in Chapter 2 to produce mealworm species would not provide a legislation problem, because the same organic by-products are currently used as feed ingredients for conventional livestock. However, the regulation has to be kept in mind when exploring the possibilities of producing edible insects on other types of organic by-products originating from the food industry, such as the organic wastes used by Ramos-Elorduy et al. to feed *T. molitor* (Ramos-Elorduy et al., 2002).

Conclusion and way forward

This thesis contributed to the research field of insects as food by demonstrating plasticity in growth and development, as well as nutritional composition of three mealworm species when grown on different diets. Although more research on effect and optimisation of insect diet is needed, results of Chapter 2 are promising in terms of possibilities to alter production yield, feed conversion efficiency and nutritional composition of mealworms. Furthermore, this thesis added to the increasingly available data on food safety aspects associated with edible insect consumption. The capacity of *T. molitor* to partially excrete mycotoxin present in the diet through faeces as shown in Chapter 3 is promising. However, more research should determine the fate of the non-excreted part in the larval body, as well as the effect of other mycotoxins and other types of contaminants that may be present in insect feed composed of organic by-products or waste material. Chapters 4 and 5 show individuals allergic to crustaceans and House dust mite may experience allergic reactions

when consuming mealworms. Products containing mealworms or mealworm protein that may appear on the food market in the future should thus be clearly labelled with allergy information in order to protect the consumer.

Diet fed to insects will have an effect on the contamination risk of the end product and should therefore be taken into account when assessing the food safety of a given edible insect species, especially when organic by-products are used as feed. Before determining the risk of contamination through feed, it is important to assess which risks are expected to be relevant depending on the feed used. For example, microbial risks would be more relevant when manure is used, while mycotoxins could pose a higher risk when dried material originating from the food industry is used. More research is needed into the effect of processing, shelf life and storage of edible insects because this influences food safety, nutrient composition and sensory parameters.

Consumer acceptance is a major factor determining the success of inclusion of edible insects into the Western diet, while legislation is a major factor influencing production of edible insects. Food technology and consumer science are vital in research determining consumer acceptance of insect-derived food products. Research determining nutritional value and food safety as well as sustainability of production and processing of different edible insect species could form the basis in development of coherent guidelines and regulations on insects as food. This is necessary not only to allow for the presence of edible insects on the EU market, but also to provide the consumer with a safe product. The above shows that a multi- and transdisciplinary approach is essential for research on insects as food.

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Summary

Insects are included in the human diet in many parts of the world, especially in the tropics and subtropics. Globally, over 2000 species of insects are consumed. In Western countries, insects no longer form part of the diet, but strong arguments exist for their reintroduction. The global demand for animal protein is increasing due to a growing world population and increasing welfare. Production of conventional livestock is associated with detrimental effects on the environment, such as global warming, land degradation and loss of biodiversity. Insect nutritional composition varies between species and life stages, but is comparable to meat. Furthermore, insect production has several benefits over the production of conventional livestock. Insects need less land area to be produced and emit less greenhouse gases per unit of growth. In addition, insects are poikilotherm, that is, they do not invest energy in keeping a constant body temperature. As a consequence, they can invest more energy in growth. This means that, compared to conventional livestock, insects require relatively less feed to gain biomass.

This thesis is part of the SUPRO-2 project for sustainable insect production for human consumption. The aims of the research presented in this thesis were to 1) explore the possibility to produce edible insects more sustainably on organic by-products and 2) to investigate potential associated food safety risks. Research focused on three species of edible beetle larvae of the family Tenebrionidae, better known as mealworms, which are commercially produced by several Dutch insect breeding companies: the Yellow mealworm (*Tenebrio molitor* L.), the Giant mealworm (*Zophobas atratus* Fab.) and the Lesser mealworm (*Alphitobius diaperinus* Panzer). An overview of the nutritional value of these mealworm species, as well as an overview of food safety risks, is provided in the first chapter.

Chapter 2 focused on production of the three mealworm species on diets composed of organic by-products originating from the food and bioethanol industry. Four diets were composed, differing from each other in protein and starch content. Larval growth and survival was monitored and compared to larvae grown on control diet as used by commercial insect breeders. Effects of dietary composition on larval feed conversion efficiency, as well as on larval protein content, fat content and fatty acid profile, were determined. Diets high in protein, which was mainly caused by brewer's yeast, allowed for shorter larval development time, increased weight gain, and increased survival compared to control diet. With respect to body composition, larval protein content did not differ between different diets, while dietary fat did affect larval fat content and fatty acid profile. Larval fatty acid profile did, however, not necessarily follow the same trend as dietary fatty acid composition. Favourable diets with respect to fast larval grown and high survival led to comparable or less favourable n6/n3 fatty acid ratios compared to control diets. Results from this study show that mealworms can by successfully produced on diets composed of organic by-products. Altered diets can be used by commercial breeders to increase production yield as well as produce more sustainably. More research is needed to determine which diet compositions lead to a more favourable n6/n3 fatty acid ratio. Furthermore, it is

yet unknown how altered diet might affect adult fecundity or viability of future insect generations.

In chapter 3, risk of contamination of *Tenebrio molitor* with a common mycotoxin, deoxynivalenol (DON), was investigated. Insect diets, especially when composed of organic by-products, can be contaminated with mycotoxins, which are stable to food processing. Larvae of *T. molitor* were grown for two weeks on three different types of wholegrain wheat flour: uncontaminated (control); naturally contaminated with several mycotoxins, including 4.9 mg/kg DON; and wheat flour spiked with 8 mg/kg DON. Larval survival and weight gain were compared on the three diets. After harvest, the presence of DON and DON derivatives in larvae as well as larval faeces was analysed by LC-MS/MS. Larvae grown on contaminated diets did not show adverse effects on survival and weight gain compared to larvae grown on control diet. No DON or DON derivatives were detected in unmetabolised form in the larvae after harvest. However, excretion of DON in was observed in faeces after feeding on naturally contaminated diet (*ca.* 14% of ingested DON) and after feeding on DON-spiked diet (*ca.* 41% of ingested DON). It is not known why the excretion of DON was lower in naturally contaminated diet. Because not all DON was excreted and none was detected in the larval bodies, it is suggested DON is partially metabolised by *Tenebrio molitor*. Metabolites could be either sequestered or excreted through faeces, but the nature of these metabolites remains to be investigated. It is promising for commercial mealworm breeders that DON is not sequestered by *T. molitor*. However, further studies are necessary to ensure possible DON metabolites produced by *T. molitor* do not pose a health risk to the consumer.

Chapters 4 and 5 explored the potential of allergic cross-reactivity of mealworm protein. In Chapter 4, the allergic potential of *T. molitor* was assessed *in vitro* using sera from individuals allergic to both House dust mites and crustaceans. Cross-reactivity was assessed by immunoblotting and basophil activation. Furthermore, protein stability was determined using an *in vitro* pepsin digestion test. Serum IgE from House dust mite and crustacean allergic individuals cross-reacted with *T. molitor* protein in both immunoblotting as well as basophil activation tests. This suggests a realistic risk of allergic reaction can be expected upon consumption of this mealworm species. The major cross-reactive proteins were identified as tropomyosin and arginine kinase, which are well known arthropod allergens. The proteins were moderately stable against *in vitro* digestion with pepsin.

Chapter 5 expands on the study of Chapter 4 by assessing the allergic potential of all three mealworm species *in vitro* using sera from individuals allergic to either House dust mites or crustaceans. Because insects are not consumed raw in the West, influence of the food processing methods boiling, frying and lyophilisation on allergenicity were determined. Furthermore, the effect of *in vitro* digestion on allergenicity was determined. Serum IgE from both House dust mite allergic and crustacean allergic individuals cross-reacted with protein from all three mealworm species in immunoblot. Proteins cross-reacting with serum from House dust mite allergic individuals were α-amylase, hexamerin

1B precursor and muscle myosin. For crustacean allergic individuals, the major cross-reacting protein was tropomyosin. Heat processing as well as *in vitro* digestion did diminish, but not eliminate cross-reactivity. This shows that heat processing of mealworms cannot eliminate allergenicity. Two studies showing allergic potential of edible mealworm species indicates food products containing mealworms may need to be labelled with allergy information to ensure consumer safety.

In Chapter 6, the results presented in this thesis were discussed in a broader context. Limitations and topics for further research are identified. By exploring sustainably production and two different food safety risks, this thesis aimed to contribute to the growing research field of insects as food. Although many follow-up studies are required to form a better understanding on the influence of diet on mealworm body composition, as well as on possible food safety risks associated with mealworm production and processing, this thesis provides a first insight. In addition, different aspects that may create barriers to the introduction of edible insects are discussed in Chapter 6, such as consumer acceptance and legislation. The discussion shows that a multi- and transdisciplinary approach is essential for research on insects as food.

Samenvatting

Insecten vormen onderdeel van het menselijk dieet in veel delen van de wereld, met name in de tropen en subtropen. Wereldwijd worden er meer dan 2000 soorten insecten geconsumeerd. In Westerse landen vormen insecten geen onderdeel meer van het dieet, echter, er bestaan sterke argumenten voor het herintroduceren van eetbare insecten. Wereldwijs is er een toenemende vraag naar dierlijk eiwit; dit komt door een groeiende wereldbevolking en toenemende welvaart. De productie van conventionele landbouwdieren wordt in verband gebracht met schadelijke effecten op het milieu, zoals het broeikaseffect, degradatie van landbouwgrond en verlies aan biodiversiteit. De voedingswaarde van eetbare insecten varieert afhankelijk van soort en levensstadium, maar is vergelijkbaar met vlees. Daarnaast heeft de productie van insecten enkele voordelen ten opzichte van de productie van conventionele landbouwdieren. Insecten kunnen worden geproduceerd op een kleiner landoppervlak en produceren minder broeikasgassen per groeieenheid. Ook zijn insecten koudbloedig, waardoor zij geen energie investeren in het behouden van een constante lichaamstemperatuur. Zij kunnen daardoor meer energie investeren in groei. Dit betekent dat insecten relatief minder voedsel nodig hebben om lichaamsmassa te produceren, vergeleken met conventionele landbouwdieren.

Dit proefschrift is onderdeel van het SUPRO-2 project over het duurzaam produceren van insecten voor menselijke consumptie. De doelen van het onderzoek in dit proefschrift waren 1) het onderzoeken van de mogelijkheid om eetbare insecten duurzamer produceren op diëten vervaardigd uit organische bijproducten en 2) het onderzoeken van het mogelijke voedselveiligheidsrisico's. Het onderzoek richtte zich op drie soorten eetbare keverlarven uit de familie Tenebrionidae, beter bekend als meelwormen, welke commercieel geproduceerd worden door verschillende Nederlandse insectenkwekers: de Gewone meelworm (*Tenebrio molitor* L.), de reuzemeelworm (*Zophobas atratus* Fab.) en de buffaloworm (*Alphitobius diaperinus* Panzer). Een overzicht van de voedingswaarde van deze meelwormsoorten, alsook een overzicht van voedselveiligheidsrisico's, is weergegeven in het eerste hoofdstuk.

Hoofstuk 2 richtte zich op de productie van de drie soorten meelwormen op diëten vervaardigd uit organische bijproducten uit de voedings- en bioethanolindustrie. Vier diëten waren geproduceerd met verschillende eiwit- en zetmeelgehalten. De groei en overleving van de larven was bijgehouden en vergeleken met larven die werden gevoerd met controledieet dat ook wordt gebruikt door commerciële insectenkwekers. Effecten van dieetsamenstelling op zowel de voederconversie, en het eiwit- en vetgehalte van de larven was bepaald. Diëten die hoog waren in eiwit, voornamelijk veroorzaakt door biergist, leidden tot een kortere ontwikkelingstijd van de larven, een toename aan gewicht en lagere mortaliteit vergeleken met controledieet. Wat betreft lichaamssamenstelling verschilde het larvale eiwitgehalte niet tussen de verschillende diëten, terwijl het vetgehalte van het dieet wel invloed had op het vetgehalte en de vetzuursamenstelling van de larven. De vetzuursamenstelling van de larven volgde echter niet noodzakelijk dezelfde trend als de vetzuursamenstelling van het dieet. Gunstige diëten met betrekking tot snellere groei en

lagere mortaliteit van de larven leidde tot vergelijkbare dan wel minder gunstige n6/n3 vetzuurverhoudingen vergeleken met controlediëten. Resultaten van deze studie laten zien dat meelwormen succesvol geproduceerd kunnen worden op diëten vervaardigd uit organische bijproducten. Aangepaste diëten kunnen door commerciële kwekers worden gebruikt om de meelwormoogst te vergroten, alsook om duurzamer te produceren. Meer onderzoek is nodig om te bepalen welke dieetsamenstellingen leiden tot een gunstiger n6/n3 vetzuurverhouding. Daarnaast is het nog onbekend welk effect aangepaste diëten hebben op de vruchtbaarheid van het volwassen insect, of op de levensvatbaarheid van latere generaties insecten.

In hoofstuk 3 werd het risico op contaminatie van *T. molitor* met een algemeen voorkomend mycotoxine, deoxynivalenol (DON) onderzocht. Insectendiëten, met name wanneer deze vervaardigd zijn uit organische bijproducten, kunnen gecontamineerd zijn met mycotoxinen, welke stabiel zijn tijdens voedselverwerking. Larven van *T. molitor* werd gedurende twee weken verschillende soorten volkoren tarwemeel als voedsel voorgezet: niet gecontamineerd (controle); natuurlijk gecontamineerd met verschillende mycotoxinen, waaronder 4.9 mg/kg DON; en meel waaraan 8 mg/kg DON was toegevoegd. Mortaliteit en gewichtstoename van de larven werd vergeleken over de drie diëten. Na de oogst werden de aanwezigheid van DON en DON derivaten bepaald in zowel de larven als de feces van de larven met behulp van LC-MS/MS. Larven op gecontamineerde diëten vertoonden geen negatieve effecten op mortaliteit en gewichtstoename vergeleken met larven op controledieet. DON en DON derivaten werden niet aangetoond in de larven in ongemetaboliseerde vorm. Echter, DON werd uitgescheiden in feces van zowel larven op natuurlijk gecontamineerd dieet (*ca.* 14% van ingenomen DON) als in feces van larven op dieet waar DON aan was toegevoegd (*ca.* 41% van ingenomen DON). Het is onbekend waarom de uitscheiding van DON lager was voor het natuurlijk gecontamineerde dieet. Omdat niet alle DON was uitgescheiden en er niets was gedetecteerd in de larven zelf, is het aannemelijk dat DON gedeeltelijk gemetaboliseerd is door *T. molitor*. Metabolieten kunnen zowel opgeslagen worden in het lichaam, als worden uitgescheiden in feces, maar de soort en eigenschappen van deze metabolieten moet nog worden onderzocht. Het is veelbelovend voor commerciële insectenkwekers dat pure DON niet wordt opgeslagen in het lichaam van *T. molitor*. Echter, meer studies zijn nodig om te bepalen of mogelijke DON-metabolieten geproduceerd door *T. molitor* een risico vormen voor de gezondheid van de consument.

In Hoofdstukken 4 en 5 werd onderzocht of meelwormeneiwit mogelijk allergische kruisreacties kan veroorzaken. In hoofdstuk 4 werd de allergeniciteit van *T. molitor*-eiwit *in vitro* bepaald met behulp van sera van mensen allergisch voor zowel huisstofmijt als schaaldieren. Kruisreactiviteit was bepaald door middel van immunoblot en basophil activatie. Daarnaast was de stabiliteit van het eiwit bepaald door middel van een *in vitro* digestietest met pepsine. Serum IgE van mensen allergisch voor huisstofmijt en schaaldieren vertoonde allergische kruisreactiviteit met *T. molitor*-eiwit in zowel

immunoblot als basophil activatietests. Dit suggereert dat er een realistische kans is op een allergische reactie na het consumeren van deze meelwormsoort. De belangrijkste kruisreagerende eiwitten waren tropomyosine en arginine kinase, welke bekende allergenen zijn in geleedpotigen. De eiwitten waren redelijk stabiel tegen *in vitro* vertering met pepsine.

Hoofdstuk 5 breidde uit op Hoofdstuk 4 door het onderzoeken van de allergeniciteit van alle drie de meelwormsoorten *in vitro* met behulp van sera van mensen die allergisch waren tegen huisstofmijt of schaaldieren. Omdat insecten niet rauw worden geconsumeerd in Westerse landen, werd het effect van de voedselverwerkingsmethoden koken, frituren en vriesdrogen op de allergeniciteit onderzocht. Daarnaast werd het effect van *in vitro* vertering op de allergeniciteit onderzocht. Serum IgE van zowel mensen allergisch tegen huisstofmijt als mensen allergisch tegen schaaldieren vertoonde kruisreacties met eiwit van alle drie de meelwormsoorten op immunoblot. Eiwitten die kruisreageerden met serum van huisstofmijt-allergische mensen waren α-amylase, hexamerin 1B precursor en spiermyosine. Zowel hittebereiding als *in vitro* vertering deden kruisreactiviteit afnemen, maar niet geheel verdwijnen. Dit toont aan dat hittebereiding van meelwormen niet voldoende is om de allergeniciteit te doen afnemen. Twee studies lieten potentiële allergeniciteit zien van eetbare meelwormen. Dit geeft aan dat voedselproducten met meelwormen mogelijk moeten worden gelabeld met allergie-informatie om de veiligheid van de consument te garanderen.

In Hoofdstuk 6 worden de resultaten uit dit proefschrift bediscussieerd in een bredere context. Beperkingen en onderwerpen voor verder onderzoek worden besproken. Door het onderzoeken van duurzame productie en twee voedselveiligheidsrisico's streeft dit proefschrift naar het bijdragen aan het groeiende onderzoeksveld van insecten als voedsel. Hoewel er veel verder onderzoek nodig is om een beter begrip te vormen van de invloed van dieet op de lichaamssamenstelling van meelwormen, alsook van de verschillende voedselveiligheidsrisico's verbonden aan de productie en verwerking van meelwormen, geeft dit proefschrift een eerste indruk. Daarnaast worden er in Hoofdstuk 6 verschillende aspecten besproken die barrières kunnen vormen tegen de introductie van eetbare insecten, zoals acceptatie door de consument en wetgeving. De discussie laat zien dat een multi- en transdisciplinaire aanpak essentieel is voor onderzoek naar insecten als voedsel.

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Curriculum Vitae

Sarah van Broekhoven was born in Arnhem, The Netherlands, on 3 December 1984. She graduated from VWO in 2003 and moved to Wageningen to study Plant Sciences. Her Bachelor essay focused on the dangerous aspects of flower pollination and its influence on the flower-pollinator mutualism. For her Masters degree, Sarah specialised in Plant Pathology and Entomology. She completed a minor thesis on the search for resistance against *Verticillium* wilt disease in Arabidopsis and tomato at the Laboratory of Phytopathology under the supervision of Dr Koste Yadeta, and a major thesis on the effect of phenidone, an inhibitor of the octadecanoid pathway, on the oviposition preference of two cabbage white butterfly species under the supervision of Dr Maaike Bruinsma. For her internship at Macquarie University, Sydney, Australia, Sarah assisted Dr Felipe Gawryszewski on a project on the effect of crab spider presence on pollinator behaviour in the field. After graduation, her broad interest in entomology motivated her to work on a PhD thesis on mealworms for human consumption, supervised by Prof. Dr Arnold van Huis, Prof. Dr Joop J.A. van Loon and Prof. Dr Martinus A.J.S. van Boekel. The PhD project was part of the larger project "Sustainable production of insect proteins for human consumption" (SUPRO2) and was financially supported by the Dutch ministry of Economic Affairs. Results of this PhD thesis are presented in this book. During her PhD thesis, Sarah organised PhD lunch meetings between 2010 and 2013, and was a member of the PhD council of the Graduate School Product Ecology and Resource Conservation between 2011 and 2014. In October 2015, Sarah joined the editorial team of Bijenhouden, the magazine of the Dutch Beekeepers Association.

Publications

Published

S. van Broekhoven, S. Bastiaan-Net, N.W. de Jong and H.J. Wichers, 2015. Influence of processing and *in vitro* digestion on the allergic cross-reactivity of three mealworm species. *Food Chemistry, in press* (DOI: 10.1016/j.foodchem.2015.10.033)

S. van Broekhoven, D.G.A.B. Oonincx, A. van Huis and J.J.A. van Loon, 2015. Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products. *Journal of Insect Physiology*, 73, 1-10

K.C.M. Verhoeckx, **S. van Broekhoven**, C.F. den Hartog-Jager, M. Gaspari, G.A.H. de Jong, H.J. Wichers, E. van Hoffen, G.F. Houben and A.C. Knulst, 2014. House dust mite (Der p 10) and crustacean allergic patients may react to food containing Yellow mealworm protein. *Food and Chemical Toxicology*, 65, 364-373

S. van Broekhoven, Q.H.T. Doan, J.J.A. van Loon and A. van Huis, 2013. Exposure of three edible mealworm species to mycotoxin contaminated feed, and methods to reduce toxin levels. *Proceedings of the Netherlands Entomological Society, 2013*

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Submitted

D.G.A.B. Oonincx, **S. van Broekhoven**, A. van Huis and J.J.A. van Loon. Feed conversion, survival and development, and composition of four insect species on diets composed of food-derived by-products.

In preparation

S. van Broekhoven, J. Mota Gutierrez, T.C. de Rijk, W.C.M. de Nijs and J.J.A. van Loon. Effect of mycotoxin contaminated diet on edible Yellow mealworm (*Tenebrio molitor* L.) and possible contamination risk.

Education Statement

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Tenebrionid beetle larvae and related food safety issues; SUPRO-2 meeting

Writing of project proposal (3 ECTS)

- The production of Tenebrionid larvae to convert feed from side streams to protein for human consumption

Post-graduate courses (4.2 ECTS)

- Advanced food analysis (2010)
- Genetics and physiology of food-associated microorganisms (2010)
- Hunger defeated? Long-term dynamics of global food security (2013)

Laboratory training and working visits (0.2 ECTS)

- Production of mealworms; commercial insect breeder (2010)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Journal of Agricultural and Environmental Sciences: Influence of irrigation methods on parameters of an insect pest in cotton (2014)
- African Journal of Biotechnology: Mineral content of two edible insect species as sold on the market in Africa (2015)

Deficiency, refresh, brush-up courses (2 ECTS)

- Food related allergies and intolerances (2010)

Competence strengthening / skills courses (4.7 ECTS)

- Interpersonal communication (2010)
- Competence assessment (2010)
- Presentation skills (2011)
- Scientific publishing (2011)
- Stress identification and management (2012)
- Communication with the media and general public (2012)
- Techniques for writing and presenting scientific papers (2012)
- Career assessment (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Day (2010-2013)
- PE&RC Weekend (2012)

Discussion groups / local seminars / other scientific meetings (7.7 ECTS)

- Mini-symposium nieuwe voedselveiligheidsrisico's (2010)
- Kick-off meeting SUPRO2 (2010)
- Entomologendag (2010-2013)
- PhD Lunch meetings entomology (2010-2014)
- FQHE Lunch meetings (2013-2014)

International symposia, workshops and conferences (7.5 ECTS)

- 3rd International conference on food digestion (2014)
- 1st International conference on insects to feed the world; presentation (2014)
- European academy of allergy and clinical immunology congress (2014)
- World mycotoxin forum 8th conference; presentation (2015)

Lecturing / supervision of practical's / tutorials (5.1 ECTS)

- Fundamentals of plant breeding, plant pathology and entomology (2010-2014)
- Ecology (2011-2012)
- Food culture and customs (2011-2013)
- Globalization and sustainability of food production and consumption (2012)
- Insects as food and feed (2015)

Supervision of 2 MSc students

- The effect of mycotoxin contaminated feed on three mealworm species
- The effect of deoxynivalenol contaminated feed on Yellow mealworm: excretion or sequestration?

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