Allergenic risks of mealworm and other insects

An approach to assess the risks of new food proteins in allergic patients

Allergene risico's van meelworm en andere insecten

Een aanpak voor de benadering van de risico's van nieuwe voedingseiwitten in allergische patiënten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 23 mei 2017 des middags te 2.30 uur

door

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geboren op 28 november 1980 te Utrecht

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ISBN 9789462956087
Drukker: Proefschriftmaken

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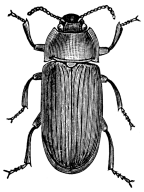

General Introduction

Chapter 1:

General Introduction

Innovations in food supply and allergy risks

The growing world population and the increased impact on the environment creates the demand for changes in agricultural practices and food supply. New or improved (climate or pest resistant) crops (e.g. genetically modified maize or soy), currently unused by-products (e.g. beet leaves) and alternative sources of food protein (e.g. insects, algae) can help make our food supply much more sustainable. Insects are suggested as a more sustainable alternative for meat and fish, due to shorter production duration, compared to other animal protein sources, and a more favorable biomass conversion rate. This is combined with a high nutritional and energy content. Insect production has additional advantages of innovations in agricultural practices. However, innovations in food supply will only succeed if they are healthy and safe. Solving one problem should not create a new one. Therefore, when new foods are introduced on the marked, precaution is needed to avoid food safety issues. Legislation in the form of the General Food Law is in place to ensure safety and hold the producer responsible for a high safety standard of the product [1]. Additionally, food safety aspects should be assessed before a new food can commercially enter the European market. Guidelines are present to help in the safety assessment of food additives, flavorings, food enzymes, foods based on genetically modified organisms (GMO's) and infant formula [2, 3]. When a new food is introduced that has not been consumed regularly in Europe before May 1997, and is not regulated by any specific regulation, it is considered a 'Novel food'. The EU Novel Food Law prescribes specific procedures and requirements for marketing approval of new foods, ingredients and processes [4].

Aspects of food safety to be addressed according to the Novel Food Law are nutritional, microbiological, toxicological and allergenic safety. When it comes to products that are based on – or contain – new or modified proteins, allergenicity especially poses a potential health risk. To prevent the emergence of food allergies to new foods introduced into our diet, adequate and accepted methods and standards for assessing the consumers' expected health response to new or modified products are needed. Such methods and standards are currently lacking.

Food allergy, and particularly Immunoglobulin E (IgE)-mediated food allergy, is a major health and food safety problem worldwide, and affects 2-4% of adults and 5-8% of young children [5, 6]. Food allergy prevalences in Europe vary between the different foods, and have been estimated around percentages of 0.2% for peanut, 0.6% for cow's milk, 0.5% for tree nuts and 0.1% for shrimp, based on positive double blind placebo controlled food challenge (DBPCFC) [7]. IgE-mediated food allergy is an immunologic, non-toxic adverse reaction to otherwise harmless substances (allergens) in food, generally food proteins. The mechanisms underlying IgE-mediated food allergy consist of a sensitization and an elicitation phase. Sensitization may occur upon contact with the food allergen, and results in the generation of allergen-specific IgE (sIgE). Elicitation of symptoms may occur upon subsequent contact with the allergen leading to symptoms. Symptoms occur within minutes to hours (usually less than two) after allergen ingestion [8], and involve one or more of the following systems; the skin (pruritus, urticaria, or angioedema), the gastro-intestinal tract (diarrhea, vomiting, contractions, increased bowel movement), the respiratory tract (asthma attack, hoarseness, stridor/laryngeal angioedema) or the cardiovascular system (dizziness, drop in blood pressure, loss of consciousness) [9]. Some foods are more likely to cause mild symptoms (e.g. apple) than others (e.g. peanut). Labeling legislation is in place in most regions of the world that prescribe that the presence of specific major allergenic ingredients in food products is to be declared to inform allergic consumers [10,11].

Assessment of cross-reactivity and primary sensitization and allergy by new food proteins: the goal and contents of this thesis

Allergies to new food proteins can result from cross-reactivity in existing sensitized or allergic individuals and thereby thus immediately manifest themselves in elicitation of allergic reactions upon consumption (elicitation phase). However, allergies to new food proteins may also result from *de novo* sensitization of - and development of new allergies in - susceptible individuals, in this case thus first requiring these individuals to become sensitized (sensitization phase) before subsequent exposure or intake may elicit allergic reactions (elicitation phase). The aim of this thesis was to develop and test a structured approach to assess the cross-reactive and *de novo* allergenicity of potential new food proteins, and examine the allergenic risks of insects as potential new food protein sources as a case study for the structured approach, with specific focus on mealworm as one

of the candidate insects as new food protein source. There is no substantial history of use of insects in food in Europe. However, in some other regions of the world, consumption of insects is more common [12]. Reports of food allergy to insects are known, mostly from Asia, where insects are high on the list of causing anaphylactic symptoms [13]. Detailed descriptions and assessment of the relevance with respect to a possible introduction of insects as a new food protein source in Europe were not available prior to the start of our study.

In Chapter 2, the background and proposed structured approach for allergenicity assessment of new food proteins and protein sources is presented and studies on mealworm and other insects are presented and discussed in the following chapters. Both in cross-reactive allergy, such as apple allergy in pollen-related-food allergy [14], and primary allergy, such as generally is the case for peanut [15], changes in allergenicity caused by heating have been described. Different forms of processing therefore need to be considered in allergenicity assessment. In Chapter 3, this is illustrated for the case for mealworm. Chapters 4 and 5 address risks of cross-reactivity of mealworm proteins in patients with allergies to phylogenetically related allergenic sources (shrimp and house dust mite) and risks of sensitization in patients with unrelated allergies. Primary sensitization and allergy caused by mealworm exposure is described in Chapter 6. In Chapter 7 it is addressed whether and to what extend risks identified for mealworm may also apply to other insects that may candidate as new food protein source, with emphasis on two identified at-risk populations: shrimp and primary mealworm allergic individuals.

The results of this thesis are summarized and discussed in the final chapter, in light of the risks of insect proteins for different risk groups in the general population.

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Chapter 2:

Allergenicity assessment strategy for novel food proteins and protein sources

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(Regul Toxicol Pharmacol. 2016 Aug;79:118-24).

Chapter 2:

Allergenicity assessment strategy for novel food proteins and protein sources

Abstract
To solve the future food insecurity problem, alternative and sustainable protein sources (e.g. insects, rapeseed, fava bean and algae) are now being explored for the production of food and feed. To approve these novel protein sources for future food a comprehensive risk assessment is needed according to the European food legislation. Allergenicity risk assessment might pose some major difficulties, since detailed guidance on how to assess the allergenic potential of novel foods is not available. At present, the approach relies mostly on the guidance of allergenicity assessment for genetically modified (GM) plant foods. The most recent one was proposed by EFSA [1,2]; "weight-of-evidence approach". However, this guidance is difficult to interpret, not completely applicable or validated for novel foods and therefore needs some adjustments. In this paper, we propose a conceptual strategy which is based on the "weight-of-evidence approach" for food derived from GM plants and other strategies that were previously published in the literature. This strategy will give more guidance on how to assess the allergenicity of novel food proteins and protein sources.

1. Introduction

Strategies are being developed to change the current agricultural practices by creating more sustainable and new climate resistant crops and to ensure an adequate, safe, sustainable and nutritious food supply (e.g. alternative protein sources) in the near future. Before novel food proteins or protein containing products can be brought to market, we need to take precautions to avoid that novel products will add to the burden of food allergy. At least we have to take care that we will not introduce allergens as potent as the major allergenic foods such as peanut. On the other hand, we also have to be aware that any (novel) protein might have some risk of allergenicity. Therefore, we need to take care that we will not exclude promising new protein sources with low or virtually absent allergenic potential from the market. The EU novel food law requires that a comprehensive food safety assessment (addressing nutritional value, microbiological, toxicological, and allergenic risks) has to be performed for

all novel foods or food ingredients that were not commonly consumed in the EU before May 1997, before they can be launched onto the food market (EC regulation No 258/97 and EU recommendation 97/618 EC; http://eur-lex.europa. eu/). For the assessment of nutritional, microbiological and toxicological risks, standard and well defined methods do exist. The assessment of allergy risks for a novel protein source is less straight forward. At present, the approach relies mostly on the guidance of allergenicity assessment for genetically modified (GM) plant foods. The most recent one was proposed by EFSA [1,2]: the so-called "weight-of-evidence approach". The purpose of these guidelines was to prevent the introduction of an allergenic protein into a food source, which might pose a risk for consumers allergic for this protein or to prevent the introduction of a protein that is similar to an allergenic protein, so that cross reactivity might occur. The applicability of these guidelines for the assessment of new and modified proteins or protein containing products (e.g. insects, algae, alternatively processed products) is hampered, since there is no generally accepted, validated and broadly applicable method available for allergenicity hazard and risk assessment. The shortcomings of the current guidelines for this latter purpose will be discussed in this paper. Food allergy is an adverse reaction of the human immune system to an otherwise harmless food component and the prevalence of food allergy in Europe is up to 3% according to the EAACI food allergy and anaphylaxis guidelines group [3]. Food allergy develops in two phases. In the first phase, susceptible subjects become sensitized to specific food proteins after dietary exposure, or possibly via other routes of exposure (inhalation and/ or skin contact). This may result in the production of specific IgE to the food protein [4,5]. When sensitized subjects subsequently encounter the respective allergen(s) again, cellular bound specific IgE will recognize the allergens and an allergic reaction may be elicited. Allergic symptoms may vary considerably and can range from mild, local and transient effects to potential fatal reactions like systemic anaphylaxis [6,7]. Generally, food allergens are proteins but the vast majority of food proteins are weak or virtually non-allergenic [8,9]. Most cases (90%) of food allergic reactions are caused by a limited range of products; milk, egg, peanut, tree nuts, fish, soy, wheat and crustaceans [10-12]. Furthermore, the manifestations of food allergies can be dependent on geography, dietary habits, food preparation and age at which food is first consumed [13]. It is therefore possible that a food product that was not reported to be common or known as allergenic in Asia can be an allergenic food in Europe, for

example kiwi fruit [13]. Another example is the allergy to peach, a member of the Rosacea family which is attributed to birch pollen in Central and Northern Europe (Pru p 1, the Bet v 1 homologue, PR-10) and leads to mild reactions (oral allergy syndrome), while in the Mediterranean areas where birch trees are less common, peach allergy may result from sensitization to Pru p 3 (lipid transfer protein, LTP) and/or Pru p 4 (profilin) which more commonly leads to severe allergic reactions [14]. At the moment, novel foods such as insects and rapeseed are entering the market without a proper allergenicity risk assessment. For mealworms, larval stage of the yellow mealworm beetle, it was recently demonstrated in a double-blind placebo controlled food challenge (DBPCFC) that 87% of a shrimp allergic patient population showed allergic reaction upon eating Yellow mealworm and that *de novo* sensitization to Yellow mealworm proteins is possible [15 Broekman et al., 2015a] [Broekman, JACI, in press]. In case of rapeseed, which was formally in use in the EU only in the form of rapeseed oil, the EFSA panel concluded, that a risk of sensitization to rapeseed protein isolate cannot be excluded and that it is likely that rapeseed will trigger allergic reactions in mustard allergic subjects [16 EFSA NDA Panel (EFSA NDA Panel, 2013)]. This conclusion was based on a food challenge and a skin prick tests with crushed rapeseed (not protein isolate) in atopic Finnish children with atopic dermatitis and suspected food allergies. 10.9% of the children showed sensitivity in the SPT and 89% of these children reacted positive in the food challenge. Cross reactivity with mustard seeds was demonstrated using IgE binding tests with serum form mustard allergic patients. Furthermore, structural homology of 95% of seed storage proteins of various members of the brassicaceae, incl. mustard was shown. In this assessment, clinically relevant studies were performed with crushed rapeseed but not with rapeseed protein isolate. In the latter, a higher protein concentration can be expected and furthermore, the effect of processing was not taken into account. Other novel food dossiers submitted in the last five years for approval by the EFSA (e.g. Chia seed, *Lentinus edodos* and alfalfa) were lacking properly conducted clinically relevant tests (e.g. SPT, or basophil activation tests (BAT)) and in most cases no formal proof of absence of allergenicity using double-blind placebo controlled food challenge (DBPCFC) was given, nor was the effect of processing or the sensitizing potency tested [17,8,19]. Food challenges are essential for determining if IgE binding measured with techniques such as immunoblot, BAT and SPT is clinically relevant. IgE binding or IgE cross reactivity does not automatically indicate that an allergic reaction will

occur. For instance, some proteins have cross reactive carbohydrate determinants (mostly found in plants) that bind to IgE but do not elicit an allergic reaction [20]. Furthermore, cross reactivity between taxonomically related foods, such as the legume family (peanut, soy, lupine, white bean) does not automatically indicate clinical cross reactivity [21]. Ibañez et al. showed that white bean and overall green bean are well tolerated by children allergic to other legumes [22]. It is in the interest of the producer of novel food products to predict allergenicity in an early stage of product development to avoid withdrawal of the novel food from the food market after introduction. For this reason, it is necessary to assess the allergenic potential of novel foods before a well-informed decision can be made on the allergenic potential of a novel food and to guide the implementation of risk managements tools such as labelling. Risk management aspects are not addressed in this paper. In this paper, the current risk assessment strategy and guidelines will be discussed and a conceptual strategy is suggested, aimed to give better guidance in how to assess the allergenicity of novel food proteins and protein sources.

2. Current strategy and guidelines

As already mentioned above there is no predictive and validated method to assess the allergenicity of novel proteins (sources) or protein containing products. In most recently filed novel food dossiers, parts of the allergenicity risk assessment guideline for Genetically Modified Organisms (GMO) which was drafted in 2010 by the EFSA's Genetically Modified Organisms (GMO) Panel [1] and updated in 2011 [2], were used. The in this guideline suggested weight-of evidence approach (Fig. 1) involves an integrated case-by-case approach to be used in the allergenicity risk assessment of newly expressed proteins in genetically modified (GM) feed and foods.

The safety evaluation mainly focusses on:

1) Evaluation of the source of the gene
2) Sequence homology with known allergens
3) Binding to IgE from allergic individuals
4) Stability of the protein in a pepsin resistance test.

2.1. Source of the gene

Allergenicity assessment of GM food starts with the evaluation of the source of the gene. If the source of the gene has a proven allergenic potential then a careful assessment is mandatory to ensure that the gene

of interest does not encode for an allergen. The relevance of this evaluation is apparent from the incidence that a GM soybean was produced that contained a gene from Brazil nut. This GM soybean showed allergenic reactions in Brazil nut sensitive individuals [11].

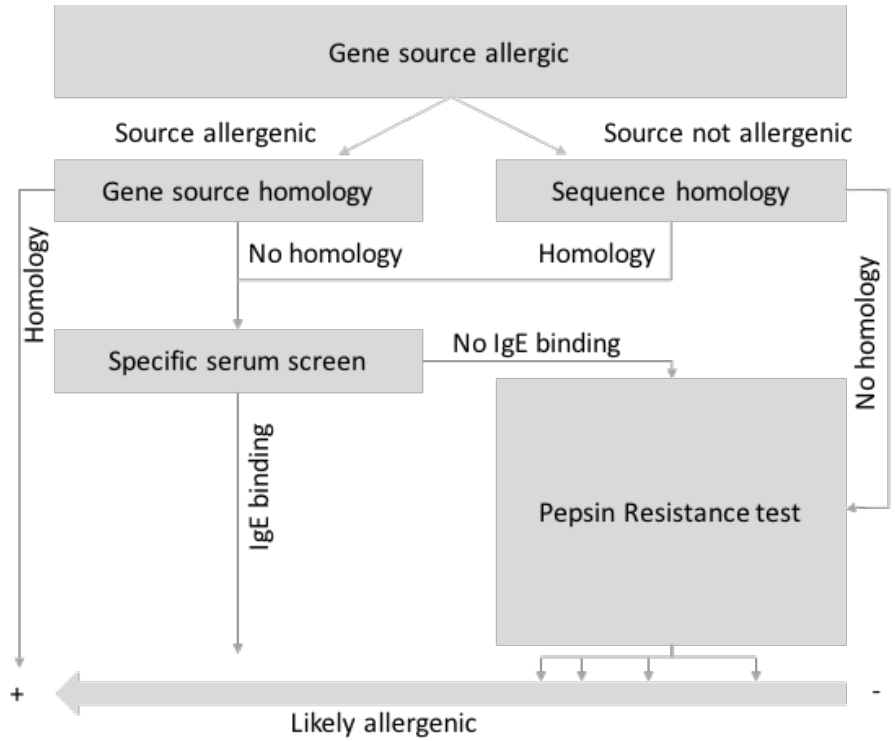

Figure 1. Flow chart summarizing the Weight-of-evidence approach for allergenicity assessment of newly expressed proteins in GMO.

2.2. Sequence homology to known allergen(s)

Bioinformatic tools are used to compare the amino acid sequence of the newly expressed protein with the sequences of known allergens to determine sequence homology. High sequence homology is associated with a high risk of a cross-reactive allergic reaction. FAO/WHO 2001 and Codex 2003 recommended that 35% sequence identity to a known allergen over a window of at least 80 amino acids is considered a minimal requirement to regard a protein allergenic in nature [23]. This criterion is still supported by the EFSA GMO panel [2].

2.3. IgE binding tests

Specific serum screening is recommended by Codex and EFSA guidelines in cases where the source of the gene/protein commonly causes allergies, or when there is a high degree of sequence homology of the protein (>35% homology, see 2.2) to a known allergen. In a specific serum screen binding of the transgenic protein with sera from patients with a clinical food allergy to a specific allergen/food is tested to determine whether the transgenic protein is not cross reactive with a known food allergen.

2.4. Pepsin resistance test

Resistance to pepsin is proposed as a criterion for a protein to be considered as a potential allergen. However, it has been established that no absolute correlation exists [24-26] between pepsin resistance and allergenicity and there is no internationally accepted protocol available to perform such in vitro digestibility tests. Improvement and good guidance for the interpretation of pepsin resistance test and validation of the test with allergens and (virtually) non-allergens is currently under review of the EFSA GMO panel (EFSA workshop June 17th Brussels, www.efsa.europa.eu/en/events/ event/150617).

2.5. Additional test

The EFSA guidelines suggest additional test such as T cell epitope screens and animal models to be applied, once developed and validated. Unfortunately, no validated and predictive models have been developed up till now and no further guidance is given on how to use these models. The tests and interpretation of outcomes of the aforementioned strategy are difficult to apply to novel food proteins and proteins sources. Especially the elements “source of the gene” and “homology testing” are difficult to perform when assessing the allergenicity of complex protein mixtures from new organisms. Then we are not talking about an insertion of just one gene with a known sequence but about new complex mixtures of hundreds or thousands of proteins for which, in most cases, gene sequences are not known. This makes comparison with known allergen sequences a very complex, time consuming and most likely a non-feasible process. IgE binding tests and pepsin resistance tests are parts of the assessment that might be useful in a strategy for novel food proteins, however, more guidance on procedures and interpretation of outcomes, particularly where “non-negative” findings are observed (e.g. some degree of pepsin digestion) is needed. Furthermore, IgE binding or IgE cross reactivity with

cross reactive carbohydrate determinants or taxonomic related allergens does not automatically mean that an allergic reaction will occur [20-22]. In addition, other factors that might influence allergenicity, such as processing and matrix, should be taken in to account as well as history of safe use and where possible food challenges. These items are lacking in the current EFSA strategy.
Below, a framework is suggested for allergenicity assessment of novel and modified food proteins. Cross-reactivity can mostly be assessed using currently available techniques and tests. Risks associated with *de novo* sensitization can partly be assessed using currently available techniques and tests but will also require new approaches yet to be developed.

3. Conceptual strategy

The need for an allergenicity assessment strategy for novel protein (sources) was already mentioned by Gubesch and coworkers, who assessed the allergenicity of three novel vegetables, namely water spinach, hyacinth bean and Ethiopian eggplant [27]. For this assessment, they used a three-step strategy. The first step was to analyze the presence of pan-allergens by immunoblot with specific animal antibodies. In the second step IgE binding to the extracts of these vegetables was tested by EAST (Enzyme-allergo-sorbent test) and immunoblot analysis using sera with IgE-reactivity to known pan-allergens or to phylogenetically related foods.

In the final third step the clinical relevance of the IgE binding was tested using SPT and open oral food challenge (OFC). This stepwise procedure seemed successful to confirm the presence of allergenic proteins in the vegetable extracts and their IgE binding capacity. Also, the in vivo studies showed the potential of the vegetables to elicit a clinically relevant allergic reaction. Another important example is the allergenicity assessment of Nangai nuts [28]. In this study, the relevance of a food challenge became very clear since none of the 12 patients who showed Nangai sensitization (RAST, SPT or histamine release), had a positive food challenge with Nangai nuts. The aforementioned strategies together with the weight-of evidence approach for foods derived from GM plants was a good starting point for the development of a generic food allergy assessment strategy for novel proteins (Fig. 2). The different aspects of the strategy are described in more detail below. It should be noted that elements of the scheme may be omitted on a case by case basis when this element is not applicable.

3.1. Product information

3.1.1. History of exposure

A thorough investigation on exposure history can provide more information on previous adverse effects of (dermal, respiratory and/or oral) exposure to the protein (source), for instance in an occupational setting. Also, history of safe use in other parts of the world can be helpful, however it should be kept in mind that in food allergy, environmental and geographical dependent factors are important and that safe use in some parts of the world does not exclude allergenicity in others. (e.g. pollen related Rosacea fruits allergy) [14]. However, further guidance may be needed on how much and which information on history of safe use is needed and how to use and interpret this information.

3.1.2. Taxonomy and relationship

Since knowledge on the allergenic potential of the novel protein source might be scarce or not available at all, gathering information on the possible allergenicity of biologically related species is requisite. For this purpose, the phylogenetic tree or evolutionary tree can be used. The phylogenetic tree is a branching diagram showing the inferred evolutionary relationships among various biological species or other entities and is based upon similarities and differences in their physical or genetic characteristics. Relationships to known allergenic sources might give indications for allergenic risks, based on cross reactivity, and thus the allergic populations at risk. However, taxonomic relationship only does not lead to conclusive evidence. For instance, serological cross reactivity between fish species is frequent, but in a significant proportion of patients, clinical relevance appeared to be limited to only certain species [29].

3.1.3. Protein identification

The identification of proteins in the novel protein source can be helpful to assigns putative allergens in the novel protein source and thus provide useful information to define the population at risk. For instance, identification of a tropomyosin protein in an insect extract might indicate that shrimp allergic patients might be at risk when eating insects, since tropomyosin is the major allergen in shrimp and other crustaceans. Identification of proteins is typically performed by LC-MS/MS analysis and database searches after digestion of the proteins with trypsin. The proteins are separated by reversed phase chromatography prior to in-line analysis of their masses and fragmentation patterns in the mass spectrometer. The

masses of the parent ions and their fragments are used to search databases of known protein sequences to match to in silico digestion patterns of these proteins. Identification of novel proteins (not present in the database) is based on homologies with known proteins present in the database and the more peptide masses that match the predicted masses, the more certain one is of the likelihood that the protein is identified.

3.1.4. Information on usage

Information on how the novel protein (source) is intended to be used in a food product is important for determining which extracts and (processed) forms of the product have to be tested in the allergenicity assessment. Is the novel protein (source) embedded in a certain matrix (high fat or high sugar) and is it processed (e.g. backing, frying)? So, what is the final product and form that will be consumed. This information is needed, since matrix and processing may have an effect on solubility, digestibility and allergenicity [30,31]. We know, for instance, that heated apple has lower allergenic properties than raw apple [32] and heat processing has a significant impact on the digestibility of ovalbumin from egg [33]. Another important issue is the intended level of use. What is the expected amount of proteins that will be present in the food product and how often will this product be consumed? This is meaningful information that can be used to determine how many protein should be used in a food challenge to determine dose-response curves and thus the allergenic risk.

3.1.5. Research material: Extract(s)

All information obtained in the previous sections is needed to make the correct choices concerning the preparation of extracts to be tested (e.g. processed or not) in in vitro and in vivo assays, but also which food product has to be used in food challenge studies. The extracts and challenge food should be a good representation of the substances that will be present in the commercial food product. Matrix and processing may have an impact on the solubility of proteins, which may change due to aggregation or unfolding of proteins. Special attention has to be paid to the extraction buffers to ensure that a relevant protein set is tested in the assessment. In most studies only one buffer, mostly a TRIS or phosphate buffer is used. As a consequence, only readily soluble proteins will be extracted from the food product and in this way an incomplete protein panel is tested for allergenicity. It is therefore recommended to use for instance a sequential extraction procedure using in succession TRIS buffer, urea, and SDS/DTT

buffer as was used by Broekman et al. [34] and mentioned by the ILSI panel on Processing and allergenicity [31]. Furthermore, identification of the proteins present in the different extracts is requisite.

3.2. Cross-reactivity testing

3.2.1. IgE binding studies

Targeted IgE binding screens can be used to identify putative allergen(s). Serum from well-characterized allergic patients is needed for this targeted serum screen. Based on the previously obtained information a smart selection of allergic patients that might be at risk can be made. For instance, sera from individuals previously sensitized against phylogenetically related foods, (e.g. serum from shrimp allergic patients, when testing insects) can be used. Also, negative control group(s), sera from non-phylogenetically related allergies should be used (e.g. peanut, when testing insects) to exclude non-specific IgE binding. When it is not clear which allergic individuals have to be tested, a panel of sera obtained from patients with different allergies/allergy profile can be used. For example; pollen (birch/grass, mite), plant (e.g. peanut, soy, tree nuts, wheat), animal (egg, milk, fish, crustaceans). Preferably individual sera from patients with a well-documented allergy should be used rather than (pooled) sera to improve the sensitivity of the test. Depending on the study design it is important to consider the amount of patient sera to be used and the selection of sera should be critically evaluated, since patient selection can have a big impact on the outcome of the test. IgE binding can be tested using different techniques; ELISA, RAST, immunoblot etc. Immunoblot has the advantage over the other techniques, because with this test more than one protein can be visualized simultaneously. Moreover, this technique gives more information on the presence of different allergenic proteins and differences between patients. The disadvantage is, that with SDS-PAGE, proteins will lose their natural structure (denaturing buffers) and thus false negative results can be obtained. Using another IgE binding test, for instance ELISA or RAST, simultaneously is therefore preferred.

3.2.2. Functional IgE testing

Binding of proteins with IgE from an allergic patient may indicate that the novel protein (source) can induce an allergic reaction in the allergic patient under investigation. However, IgE binding as such does not automatically indicate that a clinically relevant reaction will take place. Therefore, it is

necessary to test the clinical relevance of the in vitro IgE binding with functional IgE testing strategies; such as basophil activation test (BAT) and skin prick tests (SPT). However, a DBPCFC, which is regarded worldwide as the 'gold standard' in food allergy diagnosis, is preferable. For all these test an approval of a medical ethical committee is needed and the test has to be performed in a specialized clinical and safe setting. Alternatively, to the DBPCFC or as a pre-screening, SPT and BAT can be used. The SPT is often used in clinical diagnosis of food allergy and widely accepted. The BAT is not used in routine diagnostics and the predictive value is not proven yet, however the advantage over the SPT is that different protein extracts, including stringent buffers such as Urea and SDS/DTT can be used. An alternative for the BAT with human cells is the Rat Basophilic Leukaemia cells (RBL) assay. This in vitro assay uses rat basophilic leukaemia cells transfected with the Fcε receptor type I, which is primed with human IgE. However, some disadvantages of this assay have been reported such as low IgE receptor expression on RBL with respect to human basophils, which lead to low sensitivity of the assay and the need for sera with a high ratio of specific/non-specific IgE. Unfortunately, only a minority of the sera from food allergic patients will meet this specificity criterion.

3.2.3. Identification IgE binding proteins

The IgE binding proteins can be identified as described under protein identification after isolation of the proteins using for instance magnetic beads immobilized with IgE or excision of protein bands from a SDS-PAGE gel after detecting the IgE binding bands using immunoblotting.

3.3. Sensitizing potency (in case of history of sensitization)

3.3.1. History of use

Another important aspect in the allergenicity assessment of novel protein (sources) is the potency to sensitize an individual *de novo*. Information on the history of use and exposure can be a starting point for this. Identification of work related allergic symptoms (inhalant or dermal) should be assessed using for instance special questionnaires when visiting facilities where the novel protein (source) is produced. Individuals sensitized or allergic to the source under investigation can be included in further studies. Furthermore, investigation of the literature on case reports and use of the novel protein (source) in other geographically situated countries is important.

3.3.2. Identification IgE binding proteins

Sera from the sensitized or allergic individuals can be used to identify putative sensitizing proteins. This identification might help to determine if *de novo* sensitization may lead to new allergies or can lead to cross-reactive reactions with already known allergies. For instance, when an individual is primary sensitized to insect proteins, is it possible to become allergic for house dust mite or shrimp as well?

3.3.3. Cross reactivity with known allergies

This cross reactivity can be tested in the same manner as described above using IgE serum screens and IgE functionality testing. The use of inhibition studies is requisite to determine primary sensitization to the novel protein or cross reactivity to already known allergens.

3.3.4. New allergy

Whether *de novo* sensitization may lead to a new food allergy can be tested using functional IgE testing (as described above), preferably a DBPCFC with the novel protein (source).

3.4. Sensitizing potency (no history of sensitization)

When no information on a history of sensitization is available, the prediction of *de novo* sensitization will be difficult, since there are currently no tests available that can predict for *de novo* sensitization [35]. However, some animal models that determine immunogenicity and allergenicity of proteins do exist and these models have been used to try to identify the allergenic potential of proteins. Unfortunately, these models have not been validated using the appropriate number of allergens and (virtually) non-allergenic proteins. The same is true for in vitro cell based assays such as DC activation or cytokine release from T cells. Hence, it is not possible to predict, using methodologies available to date, the sensitizing potency of novel protein (sources).

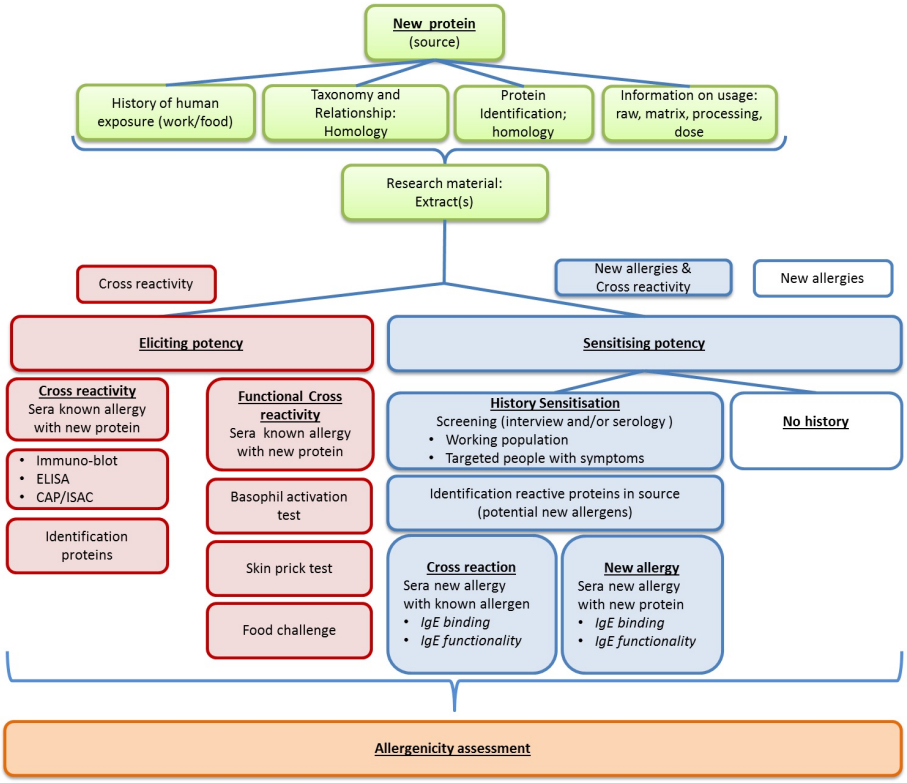

Figure 2. Schematic overview of suggested allergenicity assessment strategy of novel proteins and protein containing sources.

4. The way forward

In this paper, a generic strategy is suggested to assess the allergenicity of novel protein (sources), based on tools and tests that are currently available. With this strategy, it is possible to determine cross-reactivity/co-sensitization and thus whether and which part of the existing allergic population is at risk. The strategy can also address *de novo* sensitization in case a history of sensitization to the novel protein source is known. However, the strategy is not applicable for the assessment of *de novo* sensitization when no subjects can be found with a history of sensitization to the novel protein (source). In allergenicity assessment it cannot be ruled out that novel or modified proteins in food may induce *de novo* sensitization which may eventually give rise to new allergies. The assessment of *de novo* sensitization is hardly or not covered in any of the

previously mentioned guidelines and for this reason many safety assessment dossiers submitted to the EFSA are lacking relevant information on this aspect. Therefore, there is an urgent need for a strategy that is capable of predicting the sensitizing potency of proteins. For the development of this strategy, the following key determinants for the development of an allergic reaction may need to be considered:
a) the timing, dose and route of protein exposure (e.g. mucosal or dermal)
b) the intrinsic properties of a protein (e.g. physical/chemical and biological properties)
c) the context (e.g. lipids) in which the protein is seen by the individual's immune system (e.g. matrix/processing).
It can be envisaged that no single test is available that is able to predict the *de novo* sensitizing potency of a protein (source) and thus a set of assays should be considered and used in the assessment. The parameters such as mentioned under a, b and c or combinations thereof can be used to find correlations between properties of proteins and their allergenic potential. For this strategy, it is important to define how allergens can be ranked based on their allergenic potential. To this end, one should decide which criteria should be used to scale a panel of low/intermediate/high allergenic proteins. Currently an ILSI Europe Expert Group and TNO are developing an allergenicity scaling system Prioritizing of allergenic foods according to their public health importance [36]. Furthermore, a COST Action network (ImpARAS, www.imparas.eu) has recently started to discuss with an out-of-the-box view, new ideas and more predictive in vivo, in vitro and in silico models and approaches to improve the current allergenicity risk assessment strategy, with the focus on sensitization. A third initiative is the shared research program (SRP) Food allergy, which is initiated by TNO and is a collaboration between industry, universities and TNO. In this program, the focus is on the development of a predictive allergenicity assessment strategy which is based on an allergenic scaling as described above and predictive physical chemical and biological markers. This new strategy must help regulatory bodies to assess novel protein (sources) and improve allergy management.

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Chapter 3:

Effect of thermal processing on mealworm allergenicity

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(Mol Nutr Food Res. 2015. 59(9):1855-64.)

Chapter 3:

Effect of thermal processing on mealworm allergenicity

Abstract
Scope: The growing world population requires the exploration of new sustainable protein sources to ensure food security. Insects such as mealworm are promising candidates. For safety reasons, a risk assessment, including allergy risks, is needed. Since allergenicity can be influenced by thermal processing, it is highly important to take this into account.
Methods and results: Fresh mealworm was heat processed and extracted by a sequential extraction method using in succession Tris, urea, and a combined SDS/DTT buffer. Extracts were tested using immunoblot, basophil activation test and skin prick test in 15 shrimp allergic patients, previously indicated as population at risk for mealworm allergy. Immunoblots showed a difference in IgE binding between processed and unprocessed mealworm extracts. However, this was due to change in solubility. Some allergens were soluble in urea buffer, but became more soluble in Tris buffer and vice versa. IgE binding was seen for all extracts in blot and basophil activation test. The results from 13 skin prick tests showed a skin reaction similar between processed and unprocessed mealworm.
Conclusion: Thermal processing did not lower allergenicity but clearly changed solubility of mealworm allergens. A sequential extraction method allowed for assessment of a broader protein panel.

Introduction
A huge shortage of protein sources for human food consumption is expected in the near future due to the growing world population [1]. Sustainable protein sources are being explored to solve the coming food insecurity problem. The larvae of the yellow mealworm beetle *(Tenebrio molitor*) is a good candidate and is already for sale in Great Britain, the US, and in the major supermarkets in the Netherlands and Belgium [2,3]. However, a thorough safety assessment, and in particular an allergenicity risk assessment, is yet to be performed [4]. Allergenicity is not only a theoretical threat, since 0.1–5.7% of the pediatric and 0.1–3.2% of the adult European population has a food allergy [5]. Moreover, previously we [6] found that IgE from patients sensitized to shrimp and house dust mite (Der p 10; closely related species), binds to mealworm proteins. The

relevant proteins were identified as the pan allergens tropomyosin and arginine kinase, which are major allergens in shellfish (e.g. shrimp and lobster). Allergenicity can be influenced by factors such as matrix [7] and processing—for instance, by changing protein structure and thus IgE-binding epitopes [8]. This was previously reported for other foods such as peanut, tree nuts, and apple [9–12]. Thermal processing by dry roasting enhanced allergenicity of peanut [12], while for tree nuts the allergenic properties changed in such a way that most pollen allergic patients reacting to tree nuts had no clinical reaction after eating the heat processed food [12,13]. Thus, processing may have an impact on the risk of getting an allergic reaction for mealworm. Since mealworm is closely related to shellfish one might expect that processing may alter the allergenicity of mealworm proteins in a comparable manner to shellfish. For instance, Nakamura et al. [14] reported that thermal processing resulted in an enhanced IgE-binding capacity of scallop tropomyosin in dot blot and competitive ELISA using serum from scallop allergic patients. This enhanced capacity was suggested to be a result of glycation between free amino acids and aldehyde or ketone groups of sugars during heating. The same group found an opposite result after Maillard reaction with squid tropomyosin [15]. Samson et al. [16] found no significant difference between boiled and raw shrimp extract using immunoblot with serum from shrimp allergic patients. However, inter individual differences in protein recognition were observed. Carnes et al. [17] reported that boiled extracts of shrimp and lobster had higher IgE-binding capacity in ELISA and recorded greater skin reactivity in skin prick test (SPT). A similar finding was observed by Liu et al., [18] when testing shrimp tropomyosin. Taken together, the results in the above-mentioned papers are to some extent contradicting. This could be due to solubility issues. Therefore, more attention should be paid to the preparation of extracts to ensure the presence of a representative set of proteins for allergenicity assessment. Most studies reported the effect of processing using immunoblot and ELISA. Unfortunately, these methods lack information on the functionality of IgE binding, which canbe measured using SPT and basophil activation test (BAT). These tests are therefore preferred over immunoblot and ELISA in allergenicity assessment. However, they cannot replace food challenges—the “gold” standard. Immunoblot, BAT, and SPT were used in this study to test the effect of processing on mealworm allergenicity. Shrimp allergic patients were tested due to the lack of a sufficient number of mealworm allergic patients. To ensure that most relevant proteins were

covered, a sequential protein extraction method was used and the presence of allergens was confirmed, using nanoLC–MS.

Materials and Methods

Patient selection and screening

Three sera from patients diagnosed with shrimp allergy at the University Medical Centre Utrecht, the Netherlands, were used to test the effect of processing on protein solubility using immunoblot. For allergenicity testing, 15 adult patients diagnosed with shrimp allergy, based on suggestive history and sensitization were included. All patients reacted positive to mealworm protein in SPT and serology. All patients gave informed consent before answering the questions and for the performance of SPT and blood collection. The study was approved by the local ethics committee.

Thermal processing of mealworm

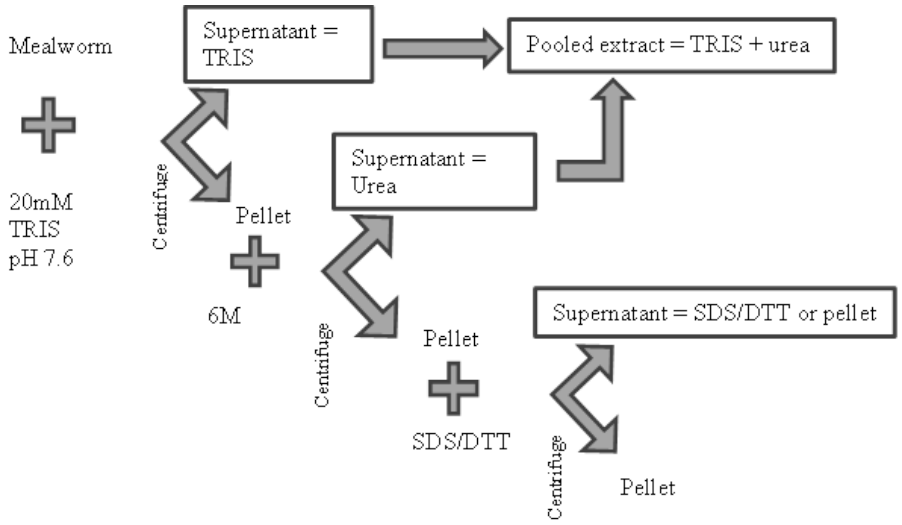
Raw and freeze dried Yellow mealworms in final larval stage were kindly provided by Dutch insect farm Kreca (Ermelo, the Netherlands). Raw mealworms (50 g) were heat processed by various methods: Blanching for 1 min at 100°C, boiling in 300 mL water for 10 min at 100°C, baking for 3.5 min at 1000 Watt on an induction cooker (Prima Donna Donnatsi-199k), or frying for 30 s at 180°C in peanut oil. All processed and unprocessed mealworms were stored at −20°C until further use.

Mealworm extract preparation

Five grams of raw, freeze-dried, and processed mealworms were extracted using a sequential protein extraction method (see Fig. 1). First, the mealworms were mixed with 25 mL ice-cold Tris buffer (20 mM Tris buffer pH 7.6 containing 1 mM phenylthiocarbamide (Sigma Aldrich) and Halt Protease Inhibitor Cocktail (Thermo Scientific)). The amount of mealworm was corrected for weight gain or weight loss due to processing. Subsequently, the mealworms were disrupted, using an ultraturrax (3 × 10 s) under continuous cooling. After centrifugation (30 min, 15 000 × g at 4°C), the supernatant was recovered. The insoluble residue was washed once with 5 mL Tris buffer (as described above). The 30 and 5 mL supernatants were combined. Twentyfive milliliter was used for sample cleanup and concentration using TCA precipitation. Second, the remaining pellet was extracted overnight at 4°C with 30 mL urea buffer (6 M urea in 20 mM Tris buffer pH 7.6 containing 1 mM phenylthiocarbamide and Halt Protease Inhibitor Cocktail). The sample was subsequently centrifuged and

the supernatant was collected. The pellet was washed once more with 5 mL urea buffer, centrifuged, and the supernatant was combined with the 30 mL urea supernatant. Twentyfive milliliters of the extract was TCA precipitated. Tris and urea extracts were combined (1:1) for the BAT. Finally, the insoluble residue was almost completely dissolved at room temperature in 20 mL SDS/DTT buffer (20 mM Tris pH 7.6, 2% SDS, and 1% DTT) and the supernatant was collected after centrifugation. All TCA precipitated samples were redissolved in 6 M urea buffer and stored at −20°C before further use. Protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA).

Figure 1.



Preparation scheme of mealworm extracts

SDS-PAGE gel of processed mealworm extracts

For SDS-PAGE, the Criterion system with a 10–20% Ready Gel Tris-HCl gel (Bio-Rad) was used according to the manufacturer's instructions. All mealworm extracts (10 μg per sample) were loaded on the gel under reducing conditions (Laemmli buffer). After protein separation, the proteins were visualized using Coomassie-staining (Instant Blue, Expediton, UK).

Immunoblot with serum of shrimp allergic patients

All mealworm extracts were applied on the SDS-PAGE as described above and transferred to a polyvinyldifluoride membrane using the Criterion Blotter system (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked overnight with 3% BSA and incubated for 1 h with serum from a shrimp allergic patient (1:50) in PBS with 0.1% Tween 20 containing 3% BSA (PBST). After thorough washing, the membranes were incubated for 1 h with Goat anti human IgE (KPL, Gaithersburg, MD, USA) 1:100 000in PBST. After washing, the bands were visualized using a chemi-luminescent peroxidase substrate kit ECL (Sigma) according to the manufacturer's instructions. Blots were scanned using the Chemidoc XRS+ image scanner with Imagelab software (Bio-Rad).

Protein identification and quantification using Nano LC-MS/MS

Trypsin Digestion extracts (TRIS and urea)

Extracts (50 µg protein) were subjected to conventional in-solution tryptic digestion as previously described [6]. After reduction and alkylation, the proteins were digested with trypsin (enzyme: substrate ratio of 1:25 w/w) overnight at 37°C with agitation. Peptide mixtures were desalted by C18 Stage Tips, fabricated by using C18 disks (3M, Neuss, Germany), and used according to the original protocol [19]. Briefly, 1 of 10 of each tryptic digest solution was diluted fivefold in 0.1% TFA (solution A), and applied onto Stage Tips, which were previously conditioned with 10 µL of solution B (0.1% formic acid, 50% acetonitrile) followed by 10 µL of solution A. After sample loading, Stage Tips were washed with 10 µL of solution A. Peptide elution was achieved by adding 8 µL of solution B. Purified peptide eluates were diluted tenfold in mobile phase A (see below) and used for mass spectrometric analysis (0.5% of the original sample for each preparation). Three technical replicates of C18 purification and mass spectrometric analysis were injected for each sample.

Trypsin digestion pellets (SDS/DTT)

Pellets obtained after urea extraction and centrifugation were dissolved in 200 µL lysis buffer (100 mM Tris pH 7.6 containing 4% SDS and 0.1 M DTT), incubated for 5 min at 95°C and sonicated. Once clarified, each sample for mass spectrometric analysis was subjected to filter-aided sample preparation [20], using a 30 kDa Microcon filtration unit (Millipore). Peptides were recovered by centrifugation at 14 000 g; followed by an additional washing step to mobilize the peptides retained by the

membrane in the filtration unit, using 50 µL NaCl 0.5 M. Flow-through was pooled, desalted by C18 Stage Tips (as described previously) and subsequently injected for massspectrometric analysis (0.5% of the original sample, corresponding to 400 ng of proteins). Three technical replicates were injected for each sample.

Nano LC-MS/MS analysis and database search

The peptide mixture was analyzed according to Verhoeckx et al. [6], with small changes. Chromatography was performed on an Easy LC 1000 nano scale liquid chromatography (nanoLC) system (Thermo Fisher Scientific, Odense, Denmark). The analytical nanoLC column was a pulled fused silica capillary, 75 µm id, in-house packed to a length of 10 cm with 3 µm C18 silica particles from Dr. Maisch (Entringen, Germany). Four microliters of the peptide mixtures was loaded at 500 nL/min directly onto the analytical column. A binary gradient was used for peptide elution. Mobile phase A was 0.1% formic acid, 2% acetonitrile, whereas mobile phase B was 0.1% formic acid, 80% acetonitrile. For both types of analysis, that of extracts and that of pellets, gradient elution was achieved at 350 nL/min flow rate, and ramped from 8 to 35% B in 60 min, and from 30 to 100% B in additional 8 min; after 5 min at 100% B, the column was re-equilibrated at 0% B for 2 min before the following injection. MS detection was performed on a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) operating in positive ion mode, with nano-electrospray (nESI) potential at 1800 V applied on the column front end via a tee piece. Data-dependent acquisition was performed by using a top-12 method with resolution (FWHM), AGC target, and maximum injection time (ms) for full MS and MS/MS of, respectively, 70 000/17 500, 106/105, 50/60. Mass window for precursor ion isolation was 1.6 m/z, whereas normalized collision energy was 25. Ion threshold for triggering MS/MS events was 2 × 104. Dynamic exclusion was 30 s. Data was processed using Proteome Discoverer1.3 (Thermo Fisher Scientific), using Sequest as search engine, and the Swiss Prot database accessed on February 2013 as sequence database (3 123 840 sequences for Metazoataxonomy). The following search parameters were used: MS tolerance 15 ppm; MS/MS tolerance 0.02 Da; fixed modifications carbamidomethyl cysteine; enzyme trypsin; maximum missed cleavages 1; taxonomy Metazoa. Search results were filtered by q values using Percolator integrated in Proteome Discoverer, to achieve a peptide level FDR of less than 1%.

Relative protein Quantification
A label-free approach was adopted for relative quantification of allergens, using five unique peptides for arginine kinase and three unique peptides for tropomyosin. Peak areas for each peptide were calculated using extracted ion chromatograms (XICs) via the Xcalibur software (Thermo Fisher Scientific). Peak areas for each peptide were subsequently normalized using the total peptide-spectrum matches (TPSM) of the corresponding LC–MS/MS analysis. The Tris freeze-dried sample, one with the highest TPSM, was chosen to confirm linearity between injected amount and TPSM. Triplicate measurements of peak area were averaged for each peptide and expressed as relative value compared to the average area of the same peptide in the Tris unprocessed sample. Relative quantification at the protein level was achieved for all proteins by taking the median value of all associated peptides.

Basophil activation test (BAT) using shrimp allergic patient serum
BAT was performed as described by Meulenbroek et al. [21] with minor modifications. Cells were incubated with a dilution series ($1{:}10^7$–$1{:}10^2$) of processed and unprocessed mealworm extracts (combined TRIS and urea extracts (5 mg/mL) and SDS/DTT extracts (no concentration determined)). Shrimp extract (ALK), 2 mg/mL, and shrimp tropomyosin Pen a 1 (Indoor Biotechnologies), 1 mg/mL, were used as positive controls. CD63, CD123, and CD203c expression was analyzed by flow cytometry using FACS Canto II and FACS Diva software (BD Bioscience, USA). The results were expressed as a percentage of CD63+ basophils. Basophils of two patients did not respond in repetition to any of the extracts, nor to the positive control. Basophils of a third patient showed spontaneous release of CD63 on the negative control. These three patients were therefore excluded.

Skin Prick test (SPT) with processed mealworm extracts
SPT solutions of the processed and unprocessed mealworms (0.4 mg/mL) were kindly provided by ALK (ALK-Abello, Spain). These solutions were prepared in PBS, which has more or less the same extraction characteristics as the Tris buffer mentioned above. The solutions were applied on the flexor aspect of the forearm using 1 mm tip lancets (ALK). Histamine dihydrochloride 10 mg/mL and glycerol diluent were used as positive and negative controls, respectively. SPT reactivity was recorded after 15 min and measured as the ratio of the mean of the wheal elicited by the tested extract and histamine control. When the ratio was 0.5 or greater, the

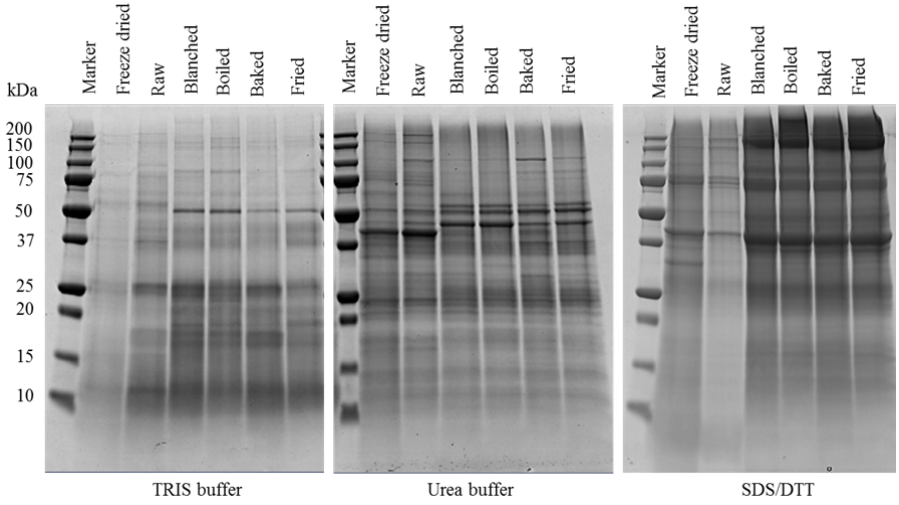
reaction was regarded as positive. No statistical tests were performed due to the limited size of the group.

Results

Heat processing changes solubility

It can be concluded from Fig. 2 that protein profiles significantly changed after heat processing in all tested extracts (Tris, urea, and SDS/DTT). Bands of proteins from the Tris extracts with MW < 25 kDa and at ±50 kDa were more intense in all heat processed extracts compared to the unprocessed extracts (raw and freeze-dried). In case of the urea extract, protein bands with a MW of ±40kDa were more pronounced in unprocessed extracts, whereas bands near 45 and 50 kDa were more pronounced in all heat processed extracts. In the SDS/DTT extract the same band at ±45 kDa diminishes after heating, while a band appears at ±37 kDa. In addition, high molecular weight proteins (70–200 kDa) were detected in the SDS/DTT buffer extracts after heating. These changes in protein profiles were the result of changes in solubility, as shown by the LC–MS analysis.

Figure 2.



Coomassie stained SDS-PAGE gels of extracts from processed mealworms. TRIS, urea and SDS-DTT extracts were prepared from raw, freeze-dried, blanched, boiled, baked and fried mealworm.

LC–MS analysis of processed and unprocessed Tris, urea, and SDS/DTT extracts identified a wide range of proteins in mealworm. Putative

mealworm allergens (e.g. tropomyosin, arginine kinase, myosin light chain, and triosephosphate isomerase), identified in Tris and urea extract were previously reported by us [6]. However, in this study we also identified putative allergens in the SDS/DTT extract (Table 1).
The most dominant protein in the SDS/DTT extract was arginine kinase after heat processing. The concentrations of these putative mealworm allergens were different in the tested extracts. It can be concluded from Fig. 3 that processing causes a shift in solubility from Tris to urea and vice versa. For instance, arginine kinase, which was abundant in raw and freeze dried mealworm Tris extracts, was almost undetectable in heat processed mealworm Tris extract.
However, it became detectable in urea extracts after heat processing. For tropomyosin, the opposite effect was found. The solubility of tropomyosin in Tris buffer improved after heat processing.
The same phenomenon was seen for other allergens such as myosin light chain, which behaved in a similar manner to tropomyosin on processing. Triosephosphate isomerase, another allergen, behaved similar to arginine kinase. However, quantification was difficult, due to detection limits (data not shown). In conclusion, solubility of mealworm allergens changed after heat processing.

Heat processing does not obviously change IgE binding capacity
The above-mentioned change in allergen solubility was confirmed by the immunoblot of the three shrimp allergic patients (Fig. 4). The immunoblots showed IgE binding tproteins in all tested mealworm extracts, Tris, urea, and SDS/DTT. In the lane of raw and freeze-dried Tris extract a protein band at ±40 kDa can be seen, which was previously identified as arginine kinase [6]. After processing, this band becomes more pronounced at a slightly higher MW. Comparing these results with the LC–MS analysis (Fig. 3), this band is most likely tropomyosin (±37 kDa). In the lane of raw and freeze-dried urea extract, a ±37 kDa band (previously identified after in-gel digestion, as tropomyosin [6]) was detected. The intensity of this band diminished after processing. The decline of tropomyosin band intensity in the urea extract is in accordance with the LC–MS data (Fig. 3). Estimation of the overall effect of processing on IgE-binding capacity is difficult due to this shift in solubility. For this reason, a pool of Tris and urea extract was used for IgE cross-linking functionality testing.

Heat processing does not change IgE cross-linking functionality.

From the 12 useful BATs, 11 showed activation after incubation with both the mixed Tris /urea extracts as well as the SDS/DTT extracts, indicated by an elevation in the percentage of CD63+ cells (Fig. 5). Basophils of one patient reacted solely to proteins in the SDS/DTT extract. Overall, activity of basophils to processed or unprocessed mealworm proteins was not clearly different. However, basophils from three patients were somewhat more strongly activated by the processed Tris /urea mealworm extracts, than by the unprocessed mealworm extracts.

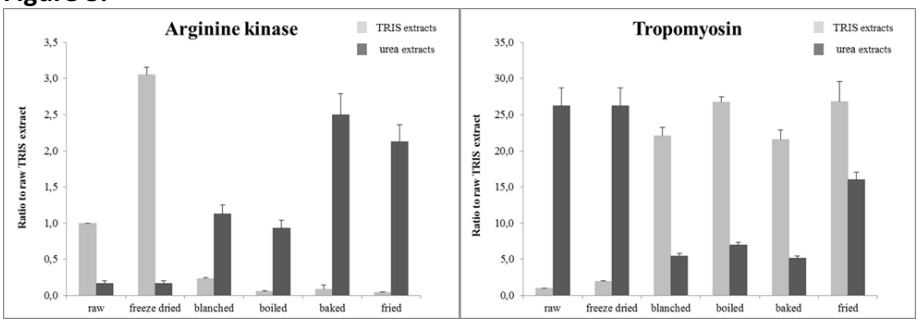
Table 1: Proteins identified in SDS/DTT extract using LC-MS-MS

Protein (source)	Accession	Score	Sequence Coverage (%)	Peptides identified	PSM	Mass (kDa)
Unprocessed						
Myosin heavy chain-like (*Triobolium castaneum*)	D6WVJ3	401	33	85	158	262,1
Actin-87E (*Drosophila melanogaster*)	P10981	188	42	20	79	41,8
Actin-2 (*Diphyllobothrium dendriticum*)	P53456	146	15	18	54	41,7
Actin, cytoskeletal 1 (*Lytechinus pictus*)	P53465	128	19	10	60	41,8
Fibronectin_type3 (*Tribolium castaneum*)	D6W7B4	112	7	50	53	989,6
Actinin (*Apis mellifera*)	H9K1K1	88	10	26	37	101,8
Hemocyanin (*Tenebrio molitor)*	Q9Y1W5	67	30	20	31	90,6
Ca-transporting ATPase sarcoplasmic/ endoplasmic reticulum type (*Drosophila pseudoobscura)*	Q292Q0	65	17	17	26	109,1
ATPase_P-type_Ca-transporter (*Triobolium castaneum*)	D6WZH8	63	11	17	26	109,8
Hexamerin 2 (*Tenebrio molitor*)	Q95PI7	59	18	11	25	84,5
Larval cuticle protein A2B (*Tenebrio molitor*)	P80682	52	43	6	15	12,3
Cuticle protein (*Tenebrio molitor*)	Q9TXE4	46	27	3	15	23,2
ATP synthase subunit alpha (*Triobolium castaneum*)	D6WSI9	42	23	12	19	59,5
Tubulin beta-1 chain (*Manduca sexta*)	O17449	40	26	11	19	50,2
Troponin T-like (*Triobolium castaneum*)	D6W953	39	14	5	16	45,7

Protein (source)	Accession	Score	Sequence coverage (%)	Peptides identified	PSM	Mass (kDa)
Processed						
Myosin heavy chain-like (*Triobolium castaneum*)	D6WVJ3	418	34	89	155	262,1
Actin-87E (*Drosophila melanogaster*)	P10981	218	47	23	275	41,8
Actin, cytaloplasmic (*Xenopuslaevis)*	O93400	211	48	22	175	41,7
Kinase tranferase (*Triobolium castaneum*)	D6W7B4	147	13	97	183	989,6
Alpha-actin like (*Triobolium castaneum*)	D2A2X1	122	45	46	147	106,7
Actinin (*Triobolium castaneum*)	B3P8U6	86	29	27	66	106,7
Hemocyanin (*Tenebrio molitor)*	Q9Y1W5	82	36	29	106	90,6
Ca-transporting ATPase sarcoplasmic/ endoplasmic reticulum type (*Drosophila pseudoobscura)*	Q292Q0	76	21	24	91	109,1
Arginine kinase *(Xylosandrus crassiusculus)*	D5L6P4	75	48	15	93	27,0
Alpha tubulin (*Drosophila melanogaster*)	K7WKV5	64	43	16	60	46,1
Tubulin beta-1 chain (*Manduca sexta*)	O17449	57	35	17	77	50,2
Filamin-B like (*Triobolium castaneum*)	D6W7G0	56	12	26	65	267,3
ATP synthase subunit alpha (*Triobolium castaneum*)	D6WSI9	54	35	20	62	59,5
Prophenoloxidase (*Tenebrio molitor)*	O97047	51	36	25	63	79,1
Hexamerin 2 (*Tenebrio molitor*)	Q95PI7	47	27	18	58	84,5

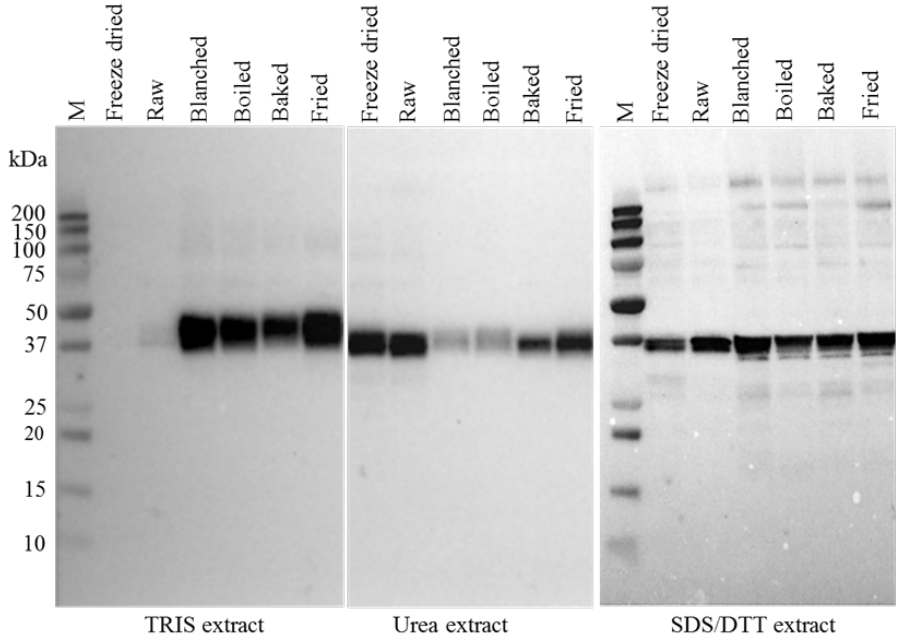
Top 15 proteins identified by LC-MS/MS in the SDS/DTT fraction in the unprocessed and processed extracts. Arranged on highest mean score of 3 measurements. Sequence coverage, Peptides identified and PSM are given as a mean of 3 measurements. Identification was based on homology with metazoan proteins in the Swiss Prot database. Proteins are noted in bold when assigned as allergen by the IUIS allergen nomenclature subcommittee. PSM = peptide-spectrum matches, value that represents the number of MS/MS spectra that matched peptide sequences assigned to that particular protein. Score = the sum of individual Sequest scores of all the identified peptides which were assigned to the protein itself. The score is the probability that the observed match is not a random event.

Figure 3.



LC-MS analysis of tropomyosin and arginine kinase in processed mealworm extracts (raw, freeze-dried, blanched, boiled, baked and fried, respectively). The results are presented as mean of three LC-MS analyses and calculated as ratio relative to the amount in the raw TRIS extract.

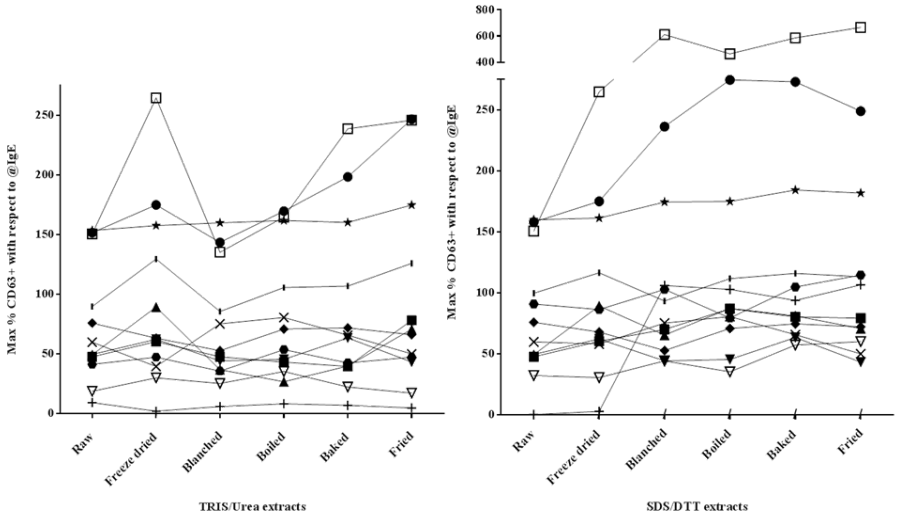
Figure 4.



Immunoblot of processed mealworms with serum from one of the three shrimp allergic patients. The mealworms were extracted with a TRIS, urea and SDS-DTT buffer respectively

All 15 patients showed a positive skin reaction to shrimp, house dust mite, and mealworm extract. In addition to unprocessed mealworm, all showed a positive skin reaction to blanched, boiled, baked, and fried extracts (Table 2). However, some inter individual differences were seen in skin reactivity. Two patients had an increased skin reaction to processed extracts. The wheal size increased from 2+ to 3+ from unprocessed to processed. Skin reaction of one patient decreased by blanching and of one patient by frying. Overall, SPT reactions were comparable between all processed extracts in 13 of 15 patients.

Figure 5.



Basophil activation test with extracts (left: pooled TRIS and urea extract, right: SDS/DTT extract) from processed mealworms (freeze-dried, fresh, blanched, cooked, baked and fried respectively). Maximum % CD63+ basophils were calculated with respect to @IgE positive control. Each line represents one patient.

Table 2. Skin prick test results, expressed as a ratio of histamine control, using extracts from the different processed mealworms (raw, blanched, boiled, baked and fried) in 15 shrimp allergic patients.

No.	Sex	Age (y)	Raw	Blanched	Boiled	Baked	Fried
1	F	46	3+	3+	3+	3+	2+
2	F	23	2+	3+	3+	3+	3+
3	M	69	2+	2+	3+	2+	2+
4	M	45	2+	2+	2+	3+	2+
5	F	27	3+	2+	3+	3+	3+
6	M	19	2+	3+	2+	2+	2+
7	F	60	2+	2+	2+	2+	2+
8	M	30	2+	3+	3+	3+	2+
9	M	27	2+	2+	2+	2+	2+
10	F	47	2+	2+	2+	2+	2+
11	F	52	0	0	1+	1+	1+
12	M	26	2+	2+	2+	2+	2+
13	M	34	2+	2+	3+	2+	3+
14	F	23	2+	2+	2+	2+	2+
15	M	46	2+	2+	3+	3+	3+

M male, F female, Mean SPT as a ratio of histamine control (3+)

Discussion

From the results obtained in this study it can be concluded that heat processing influences protein solubility. Some proteins became less soluble in Tris buffer due to heat-induced denaturation but these proteins could still be solubilized in a chaotropic reagent such as urea (arginine kinase). Other proteins that under natural conditions were insoluble in Tris buffer became more soluble after heating (tropomyosin). Furthermore, processing did not lower IgE-binding capacity and IgE cross-linking functionality of mealworm allergens (e.g. tropomyosin, arginine kinase). A representative panel of proteins was assessed due to the use of a sequential extraction method. To the best of our knowledge, this is the first study to assess the effect of thermal processing on mealworm allergenicity. Furthermore, the sequential protein extraction method used in this study has, as far as we know, never been used to assess the effect of processing on allergenicity.

Heat processing strongly changed the solubility characteristics of mealworm proteins. Change in allergen solubility, might be caused by

changes in 3D structure of the proteins after heat treatment. Some proteins will lose, irreversibly, their functional properties and solubility and form aggregates due to denaturation, while others may have increased solubility. Tropomyosin is a muscle protein, which forms a complex with the insoluble actin and troponin and is heat stable as a result of its coiled coil helical construction [22]. The improved solubility is most probably due to breakage of interactions with these other difficult to solubilize proteins. However, no evidence could be found in literature to corroborate this. Another possibility is the formation of soluble aggregates, which was also demonstrated for the Japanese cedar pollen allergen Cry j 1 by Aoki et al. [23]. Moreover, Usui et al. [24] showed that heat processing of purified tropomyosin from shrimp did not induce the formation of insoluble aggregates. However, difference in solubility in PBS buffer between heated and unheated tropomyosin was not in agreement with our results. This might be due to fact that heat processing of tropomyosin was not tested in its natural environment (complex with actin) and thus breakage of interactions with other proteins cannot be demonstrated. In contrast, arginine kinase is a globular protein, which tends to unfold during heating, exposing hydrophobic amino acids, which are normally inside the protein. The exposed hydrophobic amino acids from different molecules will interact in such a way, that formation of larger protein aggregates will occur [25]. Cross-linking of arginine kinase may also be caused by polyphenol oxidase-mediated cross-linking. In most cases these aggregates become insoluble. Further aggregation of globular proteins during heating is favored through the formation of disulphide bridges. To solubilize these aggregates a more stringent buffer is needed, which confirms our finding that arginine kinase was not detected in the Tris buffer after heating. Another possibility for the LC–MS detection of tropomyosin in Tris buffer after heat processing is the improved digestibility after heating. This effect was also seen in a study from Takagi et al. who showed that thermal treatment markedly increased the digestibility of ovalbumin [26]. This is because ovalbumin is a globular protein that unfolds during heating, which exposes amino acid sequences that can be hydrolyzed by trypsin. However, for mealworm tropomyosin this improved digestibility after heating was not confirmed by the immunoblot. Furthermore, enhancement of trypsin digestion is not expected for tropomyosin because of its helical structure that upon heating will not suddenly expose different amino acid sequences. Therefore, it is more likely that extractability and thus solubility is the main reason for the difference between the unprocessed and heat

processed samples instead of improved digestibility. When testing only protein extracts prepared in Tris buffer, which is the routine procedure, one could wrongly conclude that IgE-binding capacity to tropomyosin would be elevated due to heat processing [17]. However, the correction is that possibly important allergens are overlooked when using just one buffer type. This was demonstrated by our immunoblot data and might also be the case in some studies [16–18] where induction of IgE binding after heat processing was observed. In these studies, only PBS extracts, which is a nondenaturing extraction buffer similar to the Tris buffer, were used. Solubility issues are often encountered when proteins are processed. In most cases this phenomenon is not recognized since the composition of the protein extracts is not identified [27]. IgE binding on the immunoblot was detected in all tested extracts, indicating that in mealworm there are more allergens present than the ones identified in Tris buffer. The most dominant IgE-binding proteins in the Tris extract and inthe urea extract were identified as tropomyosin and arginine kinase, respectively, which confirms our previous findings [6]. In the SDS/DTT extract, arginine kinase (±40 kDa band) was also identified and in addition IgE binding to proteins with a higher molecular weight was detected. The high MW proteins that were identified in the SDS/DTT extracts by LC–MS/MS, were myosin heavy chain, paramyosin, and hemocyanin. It is not clear if these are the same IgE-binding proteins as detected in the immunoblot. However, myosin heavychain and paramyosin were recently identified as shrimp allergens [28–30]. Moreover, paramyosin and hemocyanin are included in the IUIS database as arthropod allergens. Since mealworm and shrimp are closely related it can be envisioned that paramyosin and myosin heavy chain could be potential mealworm allergens. Another option is that the high molecular weight proteins are the result of arginine kinase cross-linking. According to LC–MS identification arginine kinase was also detected in the SDS/DTT extracts especially after heat processing. Processing did not change IgE functionality in BAT and SPT. The advantage of BAT over SPT is that BAT allows testing of extracts prepared with stringent buffers such as urea and SDS/DTT, while due to patient safety SPT only allows protein extracts prepared according to clinical guidelines in sterile PBS buffers [31]. The results from the BAT indicate, besides some inter individual variability in basophil activation, no significant effect due to heat processing. Processing showed only induced basophil activation in three patients. In SPT, 13 of 15 patients, did not react differently to the processed extracts. Only two patients (other than those in BAT) showed a trend of increased

skin reaction. This might be caused by the increased solubility of some allergens in PBS after heat processing, which might also be the case in the study of Nowak-Wegrzyn et al. [32]. The authors reported that boiled shrimp extract induced larger skin response compared to raw shrimp extract in some shrimp allergic patients. Together these results strengthen the need for different extraction buffers to assess the allergenicity of a broad representative protein panel. The strength of this study was the combined use of clinical, *ex vivo* and *in vitro* tests in combination with a sequential extraction method and LC–MS analysis. This allowed inclusion of a broader panel of mealworm proteins in the allergenicity assessment, than usually studied. In conclusion, heat processing did not lower the allergenicity of mealworm proteins, but clearly changed the solubility of these proteins. A sequential extraction method allowed for inclusion of a broader protein panel in the allergenicity assessment of mealworm.

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Chapter 4:

Majority of shrimp allergic patients are allergic to mealworm

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(Published as letter to the editor J Allergy Clin Immunol. 2016. 137(4):1261-3.)

Chapter 4:

Majority of shrimp allergic patients are allergic to mealworm

Abstract
Background: The growing world population motivates the exploration of new sustainable protein sources to ensure food security. Insects like mealworm are promising candidates, with active ongoing marketing efforts within America and Europe. This warrants further risk assessment, specifically of potentially allergic responses. On the basis of pilot results, we hypothesized that patients allergic to shrimp and/or house dust mite are at risk for mealworm allergy.
Objective: To investigate the allergic potential of mealworm in the shrimp allergic population.
Methods: We included fifteen shrimp allergic patients in a double-blind placebo controlled challenge trial, performing diagnostic ImmunoCAP, skin prick test (SPT), Basophil activation test (BAT) and immunoblot in all patients to characterize our patient population.
Results: 13 out of 15 patients had a positive response to mealworm, starting at doses of 0.1 g of ingested mealworm. Positive ImmunoCAP and/or SPT, confirmed that all subjects were sensitized to mealworm. Ten patients recognized tropomyosin, either in combination with or without arginine kinase in standard diagnostic tests. The range of allergens inferred from the immunoblot assay suggests that known and unknown allergens are involved.
Conclusion: The majority of our shrimp allergic population has a DBPCFC proven food allergy to mealworm, reacting to known and unknown arthropod allergens at a relevant dose of mealworm.

Background
The development of alternative and sustainable food sources is considered a top priority on the innovation agenda of many national and international bodies [1,2]. Various alternative protein sources such as algae and insects are being investigated as possible candidates. *Tenebrio mollitor*, the larvae of the yellow mealworm beetle (mealworm) was identified to have great potential for its protein content, sustainability and low maintenance in rearing [3,4]. Mealworm as an ingredient in burgers or dried crispy snack is

already available through delicatessen shops and major supermarkets in Europe, North America and Australia.
Mealworm proteins may induce respiratory allergy [5,6] and one case report described a systemic reaction after eating mealworm [7]. Unfortunately, confirmation of an allergy with a food challenge is still lacking. Other insects, such as *Bombyx mori* (silkworm), tettigonid or acridid (grasshopper or cricket) and *Gonimbrasia belina* (mopane worm) have been reported to cause food allergy [8-10]. Although toxicological and microbiological risk of such insect-based foods has been assessed [11-12], the potentially allergenic risks have not been systematically studied yet.
Up to 5% of the Western European population have a food allergy, with shellfish among the ten most prevalent eliciting sources [13]. Shellfish is the common name to describe both crustaceans (including shrimp, crab and lobster) and mollusks. The proteins tropomyosin and arginine kinase are the major shellfish allergens, whereas sarcoplasmic calcium-binding protein, myosin light chain, hemocyanin, troponin c and alpha-actin are considered to be minor allergens [14-20]. Because shellfish belong to the same phylum (arthropoda) as insects, a key question is whether cross-reactivity or co-sensitization [21] may lead to clinically relevant allergic responses. Therefore, the aim of this study was to assess if a mealworm food challenge could trigger a food allergic reaction in patients allergic to shrimp.

Methods

Study design

Medical histories were obtained and sensitization to food allergens (shrimp, other shellfish, and wheat), inhalant allergens (house dust mite (HDM), cat, dog, birch- and grass pollens) and insect allergens (mealworm, cockroach and silkworm) determined. Patients sensitized to mealworm participated in a double-blind placebo controlled food challenge (DBPCFC) with mealworm. Patients were excluded when pregnant, diagnosed with severe asthma (FEV1 <70%), or using systemic immunosuppressants or beta-blockers.

Patient population

Adult patients (n=60) from the University Medical Center Utrecht, all diagnosed with shrimp allergy on the basis of specialist opinion and diagnostic testing, were found eligible. Patients were ranked according to sIgE titers to shrimp and those with the highest titers were invited first,

since for further serology, higher titers were preferred. Because most of these invited patients had a pollen allergy, we made sure that subsequently patients without pollen allergy were invited, to avoid selection bias. All subjects gave written informed consent before participation. The study was approved by the local ethics committee (NL43731.041.13).

Specific IgE
Specific IgE using ImmunoCAP (HDM, shrimp and mealworm) and ImmunoCAP ISAC was tested according to the manufacturer's recommendations (Thermo Fisher Scientific, Uppsala, Sweden), and expressed in kU/L and ISU respectively. Tests were considered positive with a value of 0.35 or higher in ImmunoCAP and 0.3 or higher in ImmunoCAP ISAC.

Mealworm extract
Fresh and freeze dried Yellow mealworms in final larval stage, were kindly provided by Dutch insect farm Kreca (Ermelo, the Netherlands). Tris/urea and SDS/DTT extracts were prepared as described previously [22] and were used for immunoblot and BAT. SPT solution of mealworm (0.4 mg/mL), was kindly provided by ALK (ALK-Abelló, Spain).

Immunoblot
For SDS-PAGE, the Criterion system with a 10-20 % Ready Gel® Tris-HCl gel (Bio-Rad, Hercules, (CA, USA) was used according to the manufacturer's instructions. Mealworm, shrimp (ALK) and shrimp tropomyosin Pen a 1, (Indoor Biotechnologies) extracts (5 µg) were loaded on the gel, under reducing conditions (Laemmli buffer). After protein separation, immunoblot was performed as described previously [22].
Basophil activation test (BAT)
BAT was performed as described previously with minor modifications. Cells were incubated with a dilution series ($1{:}10^7$ to $1{:}10^2$) of mealworm extracts (Tris/urea (5 mg/mL) and SDS/DTT). Shrimp extract (ALK, 2 mg/mL), and shrimp tropomyosin Pen a 1 (Indoor Biotechnologies, 1mg/mL), were used as positive controls [22]. The results were expressed as a percentage of CD63+ basophils.

Skin Prick test (SPT)
Commercial SPT solutions for HDM, shrimp, common inhalant allergens and wheat (ALK), crab, lobster, cockroach and silkworm (Greer) were used.

SPT was performed according to the procedure described previously [23]. SPT reactivity was measured as the ratio of the mean of the wheal elicited by the tested extract and histamine control (3+). When the ratio was 0.5 or greater, the reaction was regarded as positive.

Food challenge
Blanched mealworm was added to chicken meat with added salt, pepper and 0.3% nutmeg. In the placebo, the mealworm was replaced with chicken meat. Patients received 7 portions on each challenge day, starting at 2.16 mg mealworm protein (corresponding with 10 mg total mealworm), followed by 21.6 mg, 216 mg, 648 mg, 2.16 g, 6.48 g, and 13.0 g mealworm protein.
The challenge was discontinued and considered positive in case of objective symptoms or if a suggestive moderate to severe subjective symptom lasted for > 45 minutes, and scored according to Mueller and Sampson [24,25].
Four patients had an open food challenge to boiled Dutch shrimp, prior to participating in this study, consisting of 7 dosing steps (0.1 g, 0.3 g, 1 g, 3 g, 10 g,30 g and 100 g shrimp).

Analysis
Population size analyses using binominal distribution, indicated that 15 patients were needed to be included in the DBPCFC with mealworm, to calculate the possibility of allergy with a statistical confidence of 90%, if 15% of the sensitized patients would react to DBPCFC. This 15% was (based on the 30% prediction value of the SPT from a recent shrimp study [26]) used to prevent too limited power, since sensitization testing methods for mealworm were used without any knowledge on the positive predicting values and due to the absence of experience with the mealworm extracts. Log-logistic, log-normal, and Weibull eliciting dose (ED) with ED5, ED10, indicating where 5% and 10% of the allergic population is predicted to react were estimated for mealworm using cumulative dose probability distribution models. (supplementary data)

All results were performed using SPSS Inc, Chicago, version 21.0.

Results
Clinical characteristics of shrimp allergic patients
We enrolled 18 patients in this study to include 15 patients in the DBPCFC.

This amount was based on binominal distribution for minimal inclusion to find at least one at risk patient. But three patients were excluded from further analysis due to an exercise induced allergy in one, one for lack of evidence for mealworm sensitization and in one an inconclusive mealworm food challenge result. Sensitization was 94.4% within the invited shrimp allergic patients. In total, we completed our study with 15 patients.
Median age of the 15 patients was 38 years (range 19-69) and 47% was male.
Symptoms by history, ranged from oral symptoms to anaphylactic shock. Four patients had a positive diagnostic shrimp challenge in the past (Table 1). None of the patients knowingly consumed mealworm proteins. The majority had inhalant allergies to HDM (11/15) and pollen (11/15, of which 7 were mild, with no need of medication during the season)) and 9 patients had one or more other food allergies. All patients avoided eating other shellfish (e.g. crustaceans and mollusks).

Sensitization pattern of shrimp allergic patients
Mean sIgE to shrimp was 13.0 kU/L (range 0.37 kU/l to 53 kU/L) and all patients had a positive shrimp SPT (Table 1). ImmunoCap ISAC analysis showed that 10 patients were sensitized to tropomyosin from *Peneus monodon* shrimp (Pen m 1) and only one patient was additionally sensitized to Pen m 4 (sarcoplasmic calcium binding protein). Six patients were sensitized to Pen m 2 (arginine kinase). Three were not sensitized to any of the shrimp components, although shrimp ImmunoCAP was positive. Furthermore, sensitization to tropomyosins from other species (HSM, Cockroach and Anisakis) was found and SPT to crab, lobster, cockroach and silkworm were positive (Table 2) in most patients.

Shrimp allergic patients showed (functional) sIgE to different mealworm proteins
ImmunoCAP to mealworm was positive (> 0.35 kU/L) in 9 patients with a mean of 1.8 kU/L (range 0.66 to 6.0 kU/L), table 3. Molecular weight of the recognized protein bands ranged from 10 to 200 kDa. The majority (14/15) recognized tropomyosin and arginine kinase (10/15). Additionally, 4 patients recognized bands with a molecular weight > 60 kDa, including a band at 200 kDa and 4 mainly recognized proteins < 25kDa. Interestingly, IgE from one patient bound only to a 200 kDa protein (Supplementary Figure 1).

Table 1. Demographics, history, SPT, threshold (if performed) to shrimp and sIgE to relevant components

Pt	Sex	Age (y)	AA/ AD/ AR	Mueller shrimp history	SPT shrimp	Shrimp CAP (kU/L)	Pen m 1 (ISU)	Pen m 2 (ISU)	Pen m 4 (ISU)	Diagnostic shrimp challenge
1	M	27	n/y/y	1	2+	1.3	2.7	0	0	-
2	M	69	y/y/y	1	2+	35	19	0	0	-
3	F	60	y/y/y	1	3+	4.3	0	0.80	0	**10 g** (2.16 g) obj
4	M	34	n/n/n	2	3+	5.5	12	0	0	-
5	F	46	y/y/y	2	3+	1.9	0	4.2	0	-
6	M	30	y/y/y	3	3+	26	58	0	0	**0.3 g** (65 mg) subj **1 g** (216 mg) obj
7	M	46	y/n/y	3	3+	15	18	0	1.2	-
8	F	23	n/n/y	3	2+	4.6	7.6	1.6	0	**0.1 g** (21.6 g) subj
9	F	23	y/y/y	3	3+	53	44	33	0	-
10	F	52	n/n/n	4	1+	0.37	0.60	0	0	-
11	M	19	n/n/n	4	1+	6.1	0	0	0	-
12	M	45	n/y/y	4	2+	18	0	0	0	-
13	M	26	n/n/n	4	2+	1.9	3.3	0.60	0	**1 g** (216 mg) obj
14	F	27	y/y/y	4	4+	18	16	4.1	0	-
15	F	47	y/n/y	4	1+	2.0	0	0	0	-

AA Allergic Asthma, AD Allergic dermatitis, AR allergic rhinitis; n no, y yes; M = male, F = female; SPT in respect to histamine 3+; Pen m 1 shrimp tropomyosin; Pen m 2 shrimp arginine kinase; Pen m 4 shrimp Sarcoplasmic calcium binding protein; ISU ISAC Standardized Units; Shrimp challenge eliciting dose of shrimp in bold, protein between brackets. subj subjective symptoms; obj objective symptoms; ED unknown, eliciting dose not documented.

Mealworm SPT was positive in all patients and reactivity was comparable to shrimp and HDM. Mealworm BAT was positive for all patients with an approved test. Three patients were non-responders and one showed spontaneous release, therefore, in total 11 BATs were interpretable. (Table 3).

Table 2. Sensitization to tropomyosin, crustaceans, cockroach and silkworm by ImmunoCAP ISAC and/or SPT.

Pt.	Der p 10 (ISU)	Ani s 3 (ISU)	Bla g 7 (ISU)	SPT crab	SPT lobster	SPT cockroach	SPT silkworm
1	3.6	1.6	2.2	2+	2+	2+	1+
2	12	20	14	nt	nt	nt	nt
3	0	0	0	2+	2+	2+	2+
4	9.3	13	11	3+	3+	2+	0
5	0	0	0	nt	nt	nt	nt
6	53	41	44	2+	2+	2+	2+
7	16	7.4	19	3+	3+	3+	2+
8	4.7	6.5	7.5	2+	2+	2+	2+
9	50	40	46	nt	nt	nt	nt
10	0.90	0.60	0.60	1+	0	2+	1+
11	0	0	0	2+	2+	3+	2+
12	0	0	0	2+	2+	3+	3+
13	2.7	4.7	2.7	2+	3+	2+	2+
14	20	17	15	4+	4+	3+	2+
15	0	0	0	1+	2+	2+	2+

Der p 10 house dust mite tropomyosin; Ani s 3 anisakis tropomyosin; Bla g 7 cockroach tropomyosin; ISU ISAC Standardized Units; nt not tested

Majority of shrimp allergic patients reacted with allergic symptoms to mealworm challenge

DBPCFC confirmed mealworm allergy in 13 out of 15 shrimp allergic patients (Table 4). Symptoms ranged from oral allergy (7/13), and symptoms such as urticaria (6/13), nausea (8/13) abdominal cramping (4/13), vomiting (1/13), to respiratory: dyspnea (2/13). Subjective symptoms started at a dose of 21.6 mg mealworm protein, and objective symptoms at a 10-fold higher dose (216 mg). One patient reacted four hours after the final dose, showing both subjective and objective symptoms. Two patients did not complete the DBPCFC due to the severity of their reaction on the first day. Since they both reacted to the active dose and therefore only the results of the placebo day were missing, it was decided to keep them in the analysis. Patient characteristics from the two

patients with a negative challenge, were similar to those that reacted positive. Mealworm allergy was scored between 0 and 3 according to Mueller classification or between 0 and 4 according to Sampson classification. (Table 4).

Table 3. Sensitization patterns to mealworm (n=15): SPT, ImmunoCAP, BAT and immunoblot.

Pt.	SPT mealworm	Mealworm CAP (kU/l)	CD63 release mealworm	CD63 release shrimp	CD63 release Pen a 1	blot trop. mealworm	blot a-k. mealworm	blot shrimp	blot Pen a 1
1	2+	0.16	+	+	+	+	-	+	+
2	2+	3.1	NR	NR	NR	+	+	+	+
3*	2+	0.20	+	-	+	+	+	+	-
4	2+	1.2	+	+	+	+	+	+	+
5	3+	0.31	+	+	+	+	+	+	+
6	2+	4.0	SR	SR	SR	+	+	+	+
7	2+	2.5	+	+	+	+	-	+	+
8	2+	0.80	NR	NR	NR	+	-	+	-
9	4+	6.0	+	+	+	+	+	+	+
10	1+	0.07	NR	NR	NR	+	+	+	+
11	2+	0.30	+	+	+	+	-	+	-
12	2+	1.6	+	+	-	-	-	+	-
13	2+	0.21	+	+	+	+	+	+	+
14	4+	0.66	+	+	+	+	+	+	+
15	3+	2.3	+	+	+	+	+	+	-

Pen a 1 = shrimp tropomyosin NR = non-responder (control negative) SR = spontaneous release (spontaneous release on negative control) s.trop = shrimp tropomyosin pen a 1, trop = tropomyosin, a-k = arginine kinase

*suspected switch in BAT assay shrimp and tropomyosin results.

Table 4. Mealworm DBPCFC in 15 patients.

Pt	**0.01g** (2.16 mg)	**0.1g** (21.6 mg)	**1g** (216 mg)	**3g** (648 mg)	**10g** (2.16 g)	**30g** (6.48 g)	**60g** (13.0 g)	**Mueller mw**	**Sampson mw**	**Diagnostic shrimp challenge**
1	-	-	-	-	-	-	-	Neg	Neg	-
2	-	-	-	OA	OA	-	OA	0	1	-
3	-	-	-	P,U	-	-	P,U,NC,F	1	**2**	**10 g**
4	-	-	-	-	OA	OA	OA	0	1	-
5	-	-	-	-	-	-	OA,N,Sw,U	1	3	-
6	-	-	-	-	-	-	N,AP	2	**2**	**0.3 g** / **1 g**
7	-	OA	OA	-	OA,AP	OA,AP	ND	2	2	-
8	-	-	-	OA,U	OA	OA	OA,U,AR,Cj	1	**2**	**0.1 g**
9	-	-	-	-	OA,Dy,NC	OA,N,S	OA,N,P,Dy	3	4	-
10	-	-	-	-	-	-	-	Neg	Neg	-
11	-	-	-	-	U	N,AP	ND	2	2	-
12	-	-	-	-	-	N,AP,Cj	ND	2	2	-
13	-	-	-	-	-	-	U,N	2	**2**	**1 g**
14	-	-	OA	-	-	OA	OA,Cj,P,N,V	2	2	-
15	-	-	U	U,C	U,C,Dy,W,F,N	ND	ND	3	4	-

Symptoms during mealworm challenge. Light grey indicates subjective threshold doses, dark grey represents objective threshold doses. Mealworm dose in bold, amount of protein between brackets. AP abdominal cramp or pain; AR, allergic rhinitis; C, cough; Cj, conjunctivitis; Dy, dyspnea; F, flushing; NC nasal congestion; N, nausea; OA, oral allergy; P, pruritus; S, sneezing; Sw, difficulty swallowing; U, urticaria; V, vomiting; W, wheezing. ND = No dose given; mw, mealworm.

When comparing the mealworm challenge outcome of four patients that also had a shrimp challenge, eliciting doses (ED5 and ED10) as well as severity were in the same range. To place these eliciting doses in a broader scope, population threshold dose distribution information for mealworm was compared with population threshold data for shrimp and other allergenic foods from the VITAL- panel [27] (Supplementary Table 1 and Figure 2 and 3). These were comparable to those of shrimp.

Conclusion / Discussion

The most important conclusion from our study is that mealworm allergy is highly likely to be present in shrimp allergic patients with potentially severe outcome when exposed to mealworm. Out of 15 total, 14 shrimp allergic patients were sensitized to either mealworm tropomyosin and/or arginine kinase, and 13 out of 15 shrimp allergic patients reacted positive in a DBPCFC with mealworm with 11 developing moderate to severe symptoms, requiring immediate treatment with anti-histamines, corticosteroids or epinephrine. IgE binding was found not only to tropomyosin and arginine kinase, well-known shrimp and HDM allergens, but also to other as yet unidentified proteins. We note that the thresholds for mealworm were comparable to those from shrimp.

Although the sample size of 15 patients was limiting power, the observed magnitude of effect is nearly fully penetrant for this particular patient group, which is a representation of the shrimp allergic population in a tertiary centre. Even though a slight underrepresentation of the lower sIgE titers for shrimp was seen in the study population in comparison to the total available shrimp allergic population in our hospital (n=60), the statistical and graphical comparison of the severity score of the patient-histories showed no clear evidence of deviations from the shrimp allergic population at our hospital. Therefore, we believe that the study group was representative for the shrimp allergic population at our hospital. The strength of this study was the use of DBPCFC, the gold standard in food allergy diagnosis, in addition to a broad panel of in vitro and in vivo sensitization tests. To the best of our knowledge, no other study has assessed allergenicity of insect-based food to this extent.

The majority of the shrimp allergic patients that participated in the DBPCFC were not only sensitized to mealworm but to other crustaceans (14/15) or insects (11/12) as well. This might well be due to the homology between tropomyosins within the crustacean family and arthropods (sequence identity ranging 95-98%) [28]. In addition, these patients were also

sensitized to tropomyosins from other species: Ani s 3 (anisakis), Der p 10 (HDM), Bla g 7 (cockroach). Ten out of 15 patients were sensitized to arginine kinase according to immunoblot, which is confirmed by the fact that 11 out of 12 patients tested had a positive SPT to silkworm, with arginine kinase as the major allergen. The sequence identity between shrimp and silkworm (*Bombyx mori*) arginine kinase was reported to be 84% [29]. Additionally, many other protein bands, besides tropomyosin and arginine kinase, with varying molecular weights, (10 kDa - > 200 kDa) were observed. These bands could represent other shellfish allergens, such as troponin c, alpha-actinin [19, 30], vitellogen, paramyosin or chitinase from other insects or house dust mites [31,32] [E. Weber IUIS database].

Finding such a high percentage of shrimp allergic patients with a probably cross-reactive allergy to mealworm (87%) is striking. One open challenge study with two shrimp species (*P.monodon* and *M.rosenbergii*), found only half of patients reacting to both species [33]. Another open challenge study found that less than 50% of the shrimp allergic patients reacted to two shrimp species (*L.vannamei* and *P.monodon*) [34]. These shrimp species were phylogenetically more closely related than shrimp and mealworm. There are no prior DBPCFC studies on cross-reactivity/co-sensitization of shrimp allergic patients with arthropods, but cross-reactivity within crustacean appeared to be as high as 75% between different species, based on studies on sequence homology and serology [35]. A high degree of co-sensitization (50-100%) to crab, crayfish and lobster using SPT was found in shrimp allergic patients [36]. Our data indicate a high degree of cross-reaction/co-sensitization even between less related species.

Two patients had recurrence of symptoms 2 to 5 hours after an initially favorable response upon treatment. In one patient the symptoms were long-lasting, despite treatment. Another patient started to react only 4 hours after the last dose, which is unusual with the exception of meat allergy due to alpha galactose sensitization [37]. Perhaps this delayed response can be explained by matrix effects as high-fat or protein-rich matrices were found to lower gastric emptying rates and delay uptake [38,39]. A role for chitin from the mealworm, estimated 19.6 mg/kg by Finke et al. [5] seems unlikely, since the cumulative dosage of chitin during the DBPCFC never exceeded the 45 grams, which was reported for shellfish chitin to be without problems [40].

The severity of mealworm allergy varied between mild (oral allergy) and moderate (urticaria and gastro-intestinal symptoms) to severe (dyspnea). Five of the patients showed oral or skin/mucosal symptoms (Mueller score 0-1). An open shrimp challenge study reported 80% oral or skin/mucosal symptoms [34]. A similar profile was found in a DBPCFC study with shrimp where the majority 70% experienced oral or skin/mucosal symptoms with Mueller score 0-1[41]. Together these data indicate that mealworm is at least as allergenic as shrimp. Furthermore, it must be mentioned that symptoms during DBPCFC are always an underestimation of severity since the reaction is treated when stopping criteria are met.

Comparison of the preliminary population ED10 estimate for mealworm from our study with the ED10 value for shrimp from recent population threshold data from the EuroPrevall project and the Vital Scientific Expert Panel (VSEP) [41,27] indicates a comparable potency for effect elicitation for mealworm and shrimp protein. More mealworm challenge data is needed to confirm this initial analysis. These findings are highly relevant for consumption of insect containing foods, as the individual thresholds for objective symptoms (216 mg of mealworm) are equal to or lower than the amount of mealworm protein that is currently being used in insect snacks. For instance, in the Belgian brand Damhert, that is already on the market in Belgium and the Netherlands, 259 mg of mealworm protein is used in a 20-gram snack, where a serving is about three of these snacks. This indicates that shrimp allergic patients are already at risk when consuming these insect snacks.

In conclusion, shrimp allergic patients are at risk when eating mealworm as an alternate source of dietary protein. Since mealworm containing products are increasingly being consumed in America and European countries, it might be prudent to consider notifying shrimp and shellfish allergic patients about this risk.

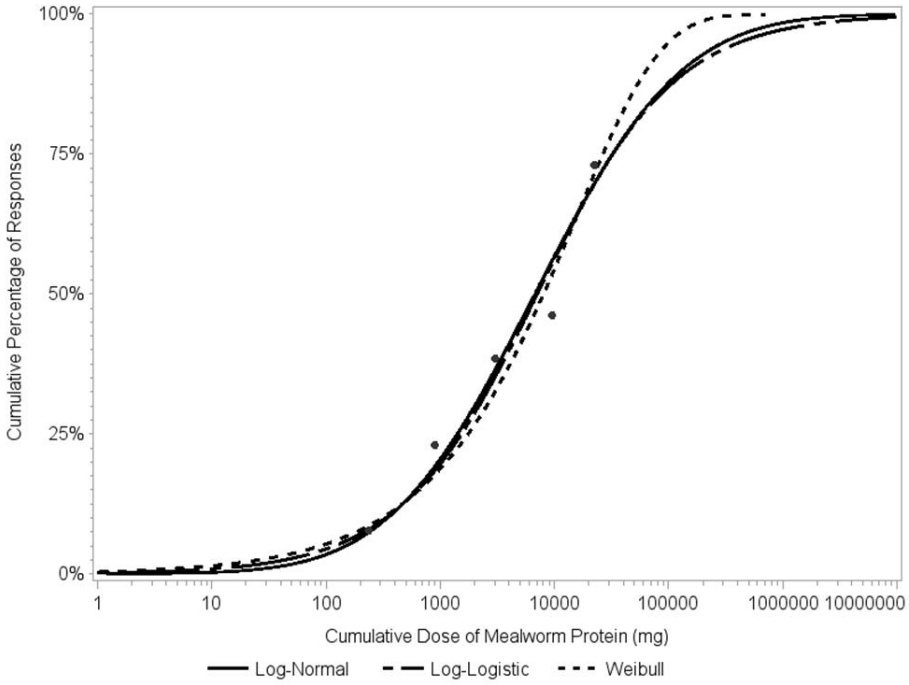
Supplementary Material

Supplementary Table 1. Log-logistic, log-normal, and Weibull ED estimates from the cumulative dose mealworm probability distribution models.

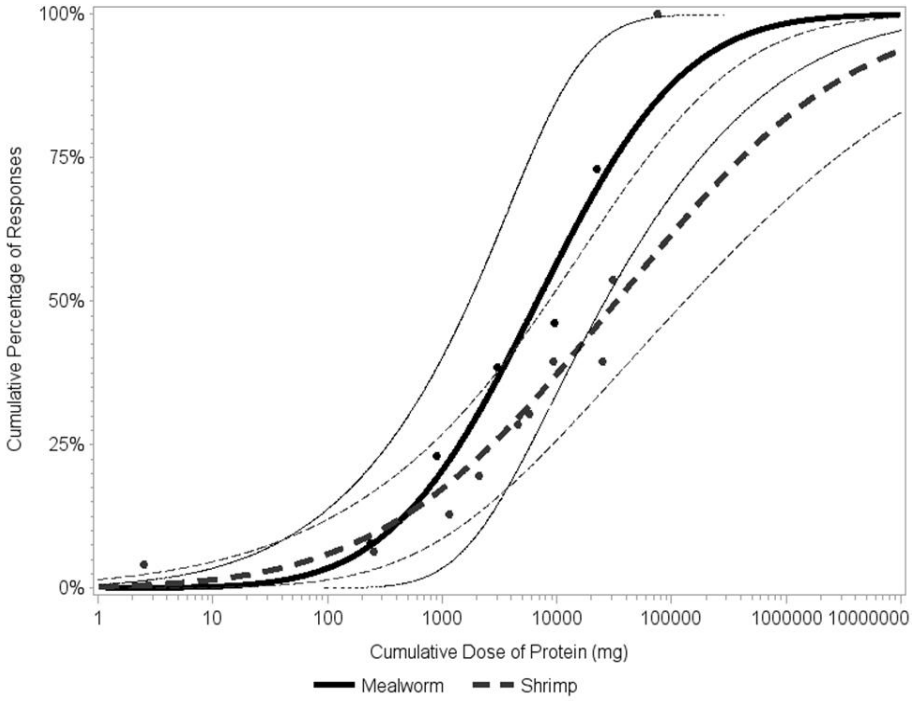
	ED05 (95% CI)	ED10 (95% CI)	ED50 (95% CI)
Log-logistic	118 (9.2, 1522)	334 (41.4, 2693)	7058 (1808, 27550)
Log-normal	149 (17.5, 1270)	346 (55.9, 2145)	6754 (1735, 26296)
Weibull	89.2 (4.1, 1954)	309 (27.3, 3513)	8032 (2511, 25696)

CI confidence intervals expressed as mg mealworm protein

Supplementary Figure 1. Log-logistic, log-normal, or Weibull probability distribution models of mealworm (expressed as cumulative mg mealworm protein). Predicted distributions and actual challenge data points (•) are displayed.



Supplementary Figure 2. Log-normal probability distribution models of mealworm and shrimp (expressed as cumulative mg protein) and corresponding 95% confidence intervals.



Population threshold data for shrimp analyzed by the Vital Scientific Expert Panel (VSEP)* and the EuroPrevall project**.

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Chapter 5:

Not only shrimp allergic, but possibly all atopic populations are at risk for mealworm allergy.

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(In preparation)

Chapter 5:

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Abstract

Background: In previous work, we showed that the majority of shrimp allergic patients from our study population (n=13/15) were food allergic to mealworm, probably due to cross-reactivity. Arthropod pan-allergens tropomyosin and arginine kinase were involved. In this study, we broadened the study population, to get more insight in the size of the populations at risk.

Methods: Mealworm sensitization was examined in shrimp allergic patients (n=67), HDM allergic rhinitis patients without tropomyosin and arginine kinase sensitization or shrimp allergy or sensitization (n=58), patients with seasonal rhinitis (n=20), and non-atopic controls (n=62). The patients were further characterized using clinical history, and ImmunoCAP for HDM and shrimp. ImmunoCAP ISAC was performed in the HDM population, to specify the sensitization to major HDM allergens Der p 1, and 2 and to ensure the absence of sensitization to Der p 10, and potentially shrimp and cockroach allergens (check) to avoid any overlap with the shrimp allergic population. A new, specifically for this study prepared ImmunoCAP with our mealworm extract, was used in this study. ImmunoCAP values equal to or higher than 0.35 kU/L were considered positive.

Results: Mealworm sensitization in the shrimp allergic population was 88%, with a median of 4.8 kU/L. Mealworm sensitization in the HDM allergic subpopulation was 22%, with a median of 0.62 kU/L, and in seasonal rhinitis patients 15%, with a median of 1.11 kU/L. None of the 62 non-atopic controls were sensitized to mealworm. Because the HDM patients with cross-reactive allergens between shrimp and HDM were excluded, most likely the allergens involved are different from the ones in shrimp-mealworm cross-reactivity.

Conclusion: Shrimp allergic patients are most at risk for food allergy to mealworm. However, there might be a risk also in HDM allergic and other atopic populations. Although the percentages of patients sensitized to mealworm in these latter groups are lower than in the shrimp allergic group, on a population level these groups may concern substantially larger at risk populations.

Background

Introduction of novel foods may pose risks of development of new allergies. This is illustrated by the example of lupin, which was introduced as a new protein source and replacement for soy and caused food allergy in peanut allergic patients [1]. Mealworm is another food recently proposed and introduced as protein source. We previously studied potential allergenic risks of mealworm. Shrimp allergic patients were shown to be at high risk for food allergy to mealworm [2]. Both shrimp and mealworm belong to the arthropod phylum and we showed cross-reactivity to at least tropomyosin and arginine kinase [2]. Some other studies reported insect sensitization in patients with shrimp and house dust mite (HDM) allergy, ranging from 30.7 to 100% [3,4]. Since HDM also belongs to the arthropod phylum, HDM allergic patients might also be at risk when eating mealworm/insects. Since many more people suffer from house dust mite allergy, than from shrimp allergy (lifetime prevalence for HDM induced rhinitis is about 17.1% in Europe and around 0.1% for shellfish allergy [5,6]), this could be an even larger patient population at risk for food allergy to insect proteins. Within the HDM population, sensitization to many different HDM allergens is seen, which differs depending on location and age [7]. Most recognize Der p 1 (fecal allergen) and/or Der p 2 (allergen from the mite intestine). Smaller parts of the HDM allergic population recognize Der p 10, a tropomyosin that originates in the muscle of the HDM, or arginine kinase (Pen m2), that derives from shrimp muscle [8]. Some patients recognize for instance Der p 23 (also fecal) [9]. To study the potential risk for the HDM population, we selected HDM allergic patients sensitized to HDM (e.g. Der p 1 and/or 2), but not tropomyosin (Der p 10, Bla g 7 or Pen m 1), arginine kinase (Pen m 2) or any shrimp allergens, to study the sensitization to mealworm proteins. Patients with sensitization to tropomyosin, arginine kinase or shrimp were excluded to avoid any overlap with the shrimp allergic group. The shrimp group from our previous study was expanded, to determine more precisely the percentage of mealworm sensitization in the total shrimp allergic group. We further studied seasonal rhinitis patients without HDM sensitization and non-atopic controls. With this study, we aimed to increase our insight into the risk for shrimp allergic patients and to identify other potential at-risk populations.

Methods

Study design and patient selection

In this study four patient groups were included:
1. Shrimp allergic patients (n=67) were diagnosed with shrimp allergy by specialist opinion, based on careful medical history and sensitization in either skin prick test (SPT) and/or ImmunoCAP.
2. HDM allergic rhinitis patients (n=58) were diagnosed with HDM allergy based on specialist opinion by medical history (perennial or autumn/winter seasonal symptoms of allergic rhino-conjunctivitis) and positive SPT or, when SPT was absent, positive ImmunoCAP HDM. Patients with sensitization to tropomyosin (Der p 10, Pen m 1 and Bla g 7), arginine kinase (Pen m 2) or shrimp, based on data obtained by ImmunoCAP and ImmunoCAP ISAC, were excluded to avoid any overlap with the shrimp allergic group. This HDM allergic subpopulation will be further referred to as HDM allergic patients.
3. Allergic rhinitis patients (n=20) with seasonal rhino-conjunctivitis during spring and/or summer and sensitization to inhalant allergens other than HDM were a 3rd group studied. Patients with concomitant HDM or shrimp sensitization were excluded to avoid any overlap with the shrimp and HDM allergic group. This 3rd group will be referred to as seasonal rhinitis patients from here on.
4. Non-atopic control subjects (n=62) were randomly selected from the EuroPrevall study. These patients had no atopic history and were not sensitized to any common inhalant or food allergens.
All serum samples were from adult patients from the University Medical Center Utrecht who provided informed consent.

Specific IgE
HDM and shrimp sensitization was determined using ImmunoCAP (Thermo Fisher Scientific, Uppsala Sweden). A new, specifically for this study prepared ImmunoCAP with our mealworm extract, was used in this study.
ImmunoCAP ISAC (Thermo Fisher Scientific, Uppsala Sweden) was performed in the HDM population, to gather information on general sensitization is this group. Both ImmunoCAP (HDM, shrimp and mealworm) and ImmunoCAP ISAC were tested according to the manufacturer's recommendations (Thermo Fisher Scientific, Uppsala, Sweden), and expressed in kU/L and ISU respectively. Tests were considered positive with a value of 0.35 or higher in ImmunoCAP and 0.3 or higher in ImmunoCAP ISAC.

Preparation of the mealworm extract for ImmunoCAP

For the ImmunoCAP mealworm, a mealworm extract was prepared by extracting 5 grams of freeze-dried mealworms using a sequential protein extraction method [10].
First the insects or shrimp were mixed with 25 mL ice-cold Tris buffer (20 mM Tris buffer pH 7.6 containing 1 mM phenylthiocarbamide (Sigma Aldrich) and Halt Protease Inhibitor Cocktail (Thermo Scientific). Subsequently the mealworms were disrupted, using an ultraturrax (3 x 10 sec) under continuous cooling. The ultraturrax was washed with 5 ml cold Tris buffer and the wash liquid was added to the sample suspension. After centrifugation (30 min, 15 000 x g at 4 °C), the supernatant was recovered. The insoluble residue was washed once with 5 mL Tris buffer. The 30 mL and 5 mL supernatant were combined. 25 mL was used for sample cleanup and concentration using TCA precipitation. Secondly, the remaining pellet was extracted overnight at 4 °C with 30 mL urea buffer (6 M urea in 20 mM Tris buffer pH 7.6 containing 1 mM phenylthiocarbamide and Halt Protease Inhibitor Cocktail). The sample was subsequently centrifuged and the supernatant was collected. The pellet was washed once more with 5 mL urea buffer, centrifuged and the supernatant was combined with the 30 mL urea supernatant. 25 mL of the extract was TCA precipitated. Tris and urea extracts were combined (1:1).
The Tris/urea mealworm extract was coupled to ImmunoCAP beads by Thermo Fisher Scientific.

Statistical analysis
Principal component analysis (PCA) was conducted as general screening tool. In partial least squares discriminant analysis (PLSDA) the data were autoscaled (each variable scaled to mean of zero and variance of 1) because different units are involved and we don't want that variables with large values will dominate in the analysis. PLSDA was used to investigate if positive mealworm CAP/negative mealworm CAP differences were present (classification model including jackknife based variable selection and 10-fold double cross validation).

Results
The majority of the shrimp allergic population was sensitized to mealworm
In the shrimp allergic patient group, 69% were female and median age was 42 years. 81% had a history of rhinitis, 57% of asthma and 66% of atopic dermatitis. Median ImmunoCAP shrimp was 4.3 kU/L, with an inter quartile range (IQR) of 0.93-17. The large majority, 65 of 67, was also sensitized to

HDM (97%), with a median CAP value of 40.3 kU/L and a IQR of 6.9->100kU/L.
Mealworm IgE was positive in 59 patients (88%). Median sIgE to mealworm for the positive 59 patients was 4.8 kU/L with an IQR of 1.3-14.2 (Table 1, Figure 1). In the patients with and without asthma the percentages of mealworm sensitization were similar, as were the percentages in patients with or without atopic dermatitis. 15 patients had a DBPCFC with mealworm in our previous study. These patients are shown in figure 1 as black downward triangles. Filled when the challenge was considered positive, open in the two with a negative outcome. In these 15 patients, 73% had a history of rhinitis, 53% of asthma and 53% of atopic dermatitis. Median ImmunoCAP shrimp was 5.5 kU/L, with an inter quartile range (IQR) of 1.9-18.8 kU/L. The large majority, 14 of 15, was also sensitized to HDM (93%), with a median CAP value of 12.3 kU/L and a IQR of 6.9-55 kU/L. Median sIgE to mealworm in this shrimp allergic subgroup was 5.8 kU/L with an IQR of 3.5-15.0 (Table 1).

A significant part of the HDM allergic population was sensitized to mealworm

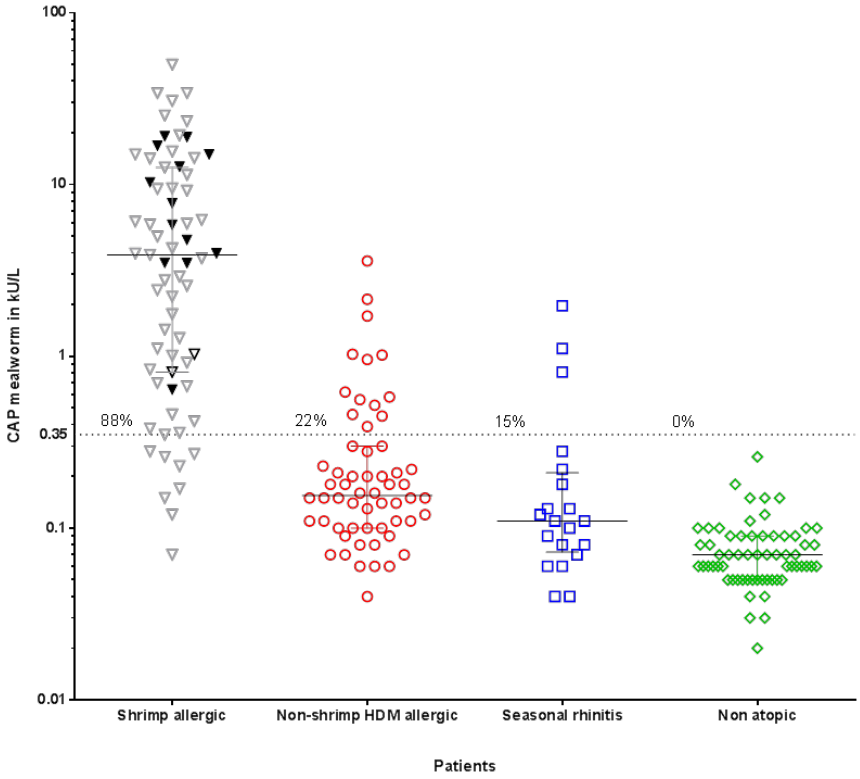
In the HDM allergic patient group (n=58), 81% was female and median age was 40 years. 52% had asthma, 41% had atopic dermatitis and 69% had food allergy, but not to shellfish. The median HDM CAP value (in the patients that were tested 54/58, due to limited serum) was 8.5 kU/L and an IQR of 1.5-34.9 kU/L. This is significantly lower than in the shrimp allergic group (Table 1). In the patients with and without asthma the percentages of mealworm sensitization were similar, as were the percentages in patients with or without atopic dermatitis and with or without food allergy. Within the HDM population 2 different groups can be made; 1) patients recognizing Der p 1 and/or Der p 2, 2) patients not recognizing Der p 1, Der p 2 or Der p 10 but other HDM allergens not on ImmunoCAP ISAC. The sIgE levels for mealworm in these groups are shown in table 1. In the Der p 1 and/or Der p 2 group, 7 patients showed sensitization to mealworm. In the group recognizing other proteins than Der p 1, 2, and 10, 6 patients had sIgE for mealworm.

Table 1. Patient characteristics of the four diagnostic groups

	Shrimp N = 67	**HDM N = 58**	**Rhinitis N = 20**	**Non-atopic N = 62**
Female	46 (69%)	47 (81%)	14 (70%)	34 (55%)
Age (years)	42 (29-51)	40 (30.5-49.5)	43 (35.8-49.3)	49 (39-57.3)
Patients with positive Mealworm CAP	59 (88%)	13 (23%)	3 (15%)	0 (0%)
sIgE Shrimp (kUa/L)	4.3 (0.93-17)	0.07 (0.03-0.11)	0.03 (0.03-0.17)	0
sIgE HDM (kUa/L)	40.3 (6.9->100)	8.5 (1.5-34.9)	0.07 (0.02-0.12)	0
Positive sIgE Mealworm (kUa/L)	4.8 (1.3-14.2)	0.62 (0.49-1.4)	1.11 (0.81-.)	0
Allergic Asthma	38 (57%)	31 (53%)	7 (35%)	0 (0%)
Atopic Dermatitis	44 (66%)	24 (41%)	5 (25%)	0 (0%)
Food allergy	67 (100%)	40 (69%)	15 (75%)	0 (0%)
HDM sensitization	65 (97%)	58 (100%)	0 (0%)	0 (0%)
Der p/f 1&2	- -	29/6 + [#] (50%)	- -	- -
Der p/f 1 only	- -	4 (7%)	- -	- -
Der p/f 2 only	- -	9/1+ (15%)	- -	- -
No HDM allergens on ImmunoCAP ISAC	- -	15/5+ (26%)	- -	- -

All data represent medians and inter quartile ranges or numbers and percentages. * ISAC not performed in one patient. # 29/6+ means 29 patients recognized components 6 were mealworm positive, per allergen recognition pattern, number of patients sensitized to mealworm

Figure 1: ImmunoCAP (kU/L) mealworm results in log 10 in shrimp, HDM, seasonal rhinitis and non-atopic populations. Sensitization to mealworm 88% of shrimp allergic patients (previously in DBPCFC in black) and 22% of HDM allergic patients, convincingly higher than 15% in rhinitis and 0% of non-atopic control subjects.



Black downward triangles are the shrimp allergic patients with mealworm allergy, open black triangles are 2 shrimp allergic patients with a negative DBPCFC with mealworm from our previous study.

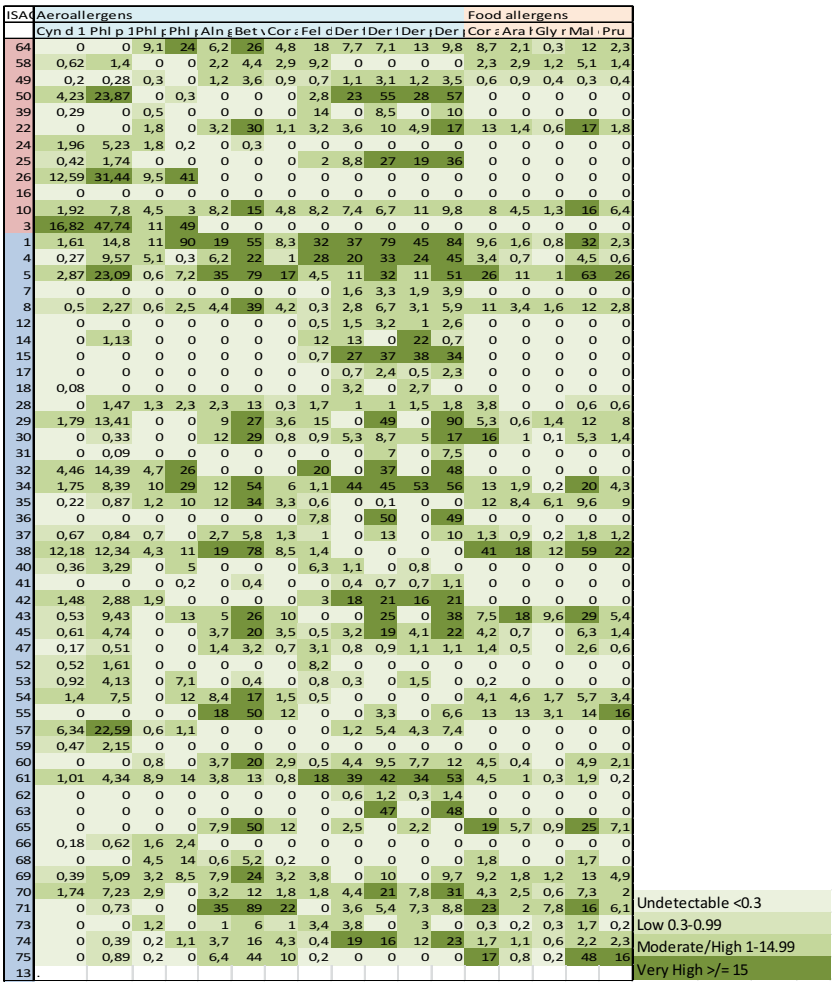
Mealworm sIgE was positive in 13 patients (22%). Median sIgE to mealworm for the positive subgroup was 0.62 kU/L with an IQR of 0.49-1.4
In Figure 2a the ISAC results of allergens mostly recognized by the HDM allergic patient group are shown. 50% of these patients recognized the inhalant allergen components Phl p 1 (grass) and Fel d 1 (cat). Bet v 1 was recognized by almost 50% of the patients. Among the food allergens, Cor a 1.0401 (Bet v 1 related hazelnut protein) and Mal d 1 (Bet v 1 related apple protein) were the most recognized. The percentage of birch-pollen related food allergy in this group is unknown.

Principal component analyses showed no clear separation between the groups with mealworm positive or mealworm negative CAP. However, in PLSDA from the 112 components tested in ImmunoCAP ISAC, four components showed to discriminate between HDM patients with a positive mealworm and negative mealworm CAP (Pla a 2, Cyn d 1, Jug r 1, MUXF3). The plane tree Pla a 2 showed the best discrimination between the two groups in the multivariate analysis. Biological explanation for this discriminative factor could not be found (Figure 2b).
The percentage of positive mealworm ImmunoCAP's in the HDM allergic group was 4 times lower than the percentage in the shrimp allergic group. Median sIgE to mealworm was 7.7 times lower in the HDM than the shrimp allergic group. Almost the same difference (4.7 times) was seen for HDM sensitization between the two groups.

In the seasonal rhinitis population mealworm sensitization was found
In the seasonal rhinitis group 78% was female, median age was 43 years, 35% had asthma, 25% had atopic dermatitis and 75% had food allergy. All were sensitized to inhalant allergens, 90% to pollen and 15% to animal dander (one patient was sensitized to both).
Mealworm sIgE was positive in 3 patients (15%). Median sIgE to mealworm for the positive subgroup was 1.11 kU/L with a range from 0.81-1.97 (Table 1). In the patients with and without asthma, the percentages of mealworm sensitization were similar, as were the percentages in patients with or without atopic dermatitis and with or without food allergy.
The percentage positive mealworm ImmunoCAP's was 1.5 times higher in the HDM group compared to the seasonal rhinitis group.

No mealworm sensitization in the non-atopic population
In the non-atopic subjects group, 55% was female and median age was 49 years. All sIgE tests including mealworm were negative in the 62 non-atopic subjects.

Figure 2a: ImmunoCAP ISAC-results in the HDM population as heat-map.



ISAC	Aeroallergens												Food allergens				
	Cyn d 1	Phl p 1	Phl p	Phl p	Aln g	Bet v	Cor a	Fel d	Der f	Der f	Der p	Der p	Cor a	Ara h	Gly m	Mal d	Pru
64	0	0	9,1	24	6,2	26	4,8	18	7,7	7,1	13	9,8	8,7	2,1	0,3	12	2,3
58	0,62	1,4	0	0	2,2	4,4	2,9	9,2	0	0	0	0	2,3	2,9	1,2	5,1	1,4
49	0,2	0,28	0,3	0	1,2	3,6	0,9	0,7	1,1	3,1	1,2	3,5	0,6	0,9	0,4	0,3	0,4
50	4,23	23,87	0	0,3	0	0	0	2,8	23	55	28	57	0	0	0	0	0
39	0,29	0	0,5	0	0	0	0	14	0	8,5	0	10	0	0	0	0	0
22	0	0	1,8	0	3,2	30	1,1	3,2	3,6	10	4,9	17	13	1,4	0,6	17	1,8
24	1,96	5,23	1,8	0,2	0	0,3	0	0	0	0	0	0	0	0	0	0	0
25	0,42	1,74	0	0	0	0	0	2	8,8	27	19	36	0	0	0	0	0
26	12,59	31,44	9,5	41	0	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	1,92	7,8	4,5	3	8,2	15	4,8	8,2	7,4	6,7	11	9,8	8	4,5	1,3	16	6,4
3	16,82	47,74	11	49	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1,61	14,8	11	90	19	55	8,3	32	37	79	45	84	9,6	1,6	0,8	32	2,3
4	0,27	9,57	5,1	0,3	6,2	22	1	28	20	33	24	45	3,4	0,7	0	4,5	0,6
5	2,87	23,09	0,6	7,2	35	79	17	4,5	11	32	11	51	26	11	1	63	26
7	0	0	0	0	0	0	0	0	1,6	3,3	1,9	3,9	0	0	0	0	0
8	0,5	2,27	0,6	2,5	4,4	39	4,2	0,3	2,8	6,7	3,1	5,9	11	3,4	1,6	12	2,8
12	0	0	0	0	0	0	0	0,5	1,5	3,2	1	2,6	0	0	0	0	0
14	0	1,13	0	0	0	0	0	12	13	0	22	0,7	0	0	0	0	0
15	0	0	0	0	0	0	0	0,7	27	37	38	34	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0,7	2,4	0,5	2,3	0	0	0	0	0
18	0,08	0	0	0	0	0	0	0	3,2	0	2,7	0	0	0	0	0	0
28	0	1,47	1,3	2,3	2,3	13	0,3	1,7	1	1	1,5	1,8	3,8	0	0	0,6	0,6
29	1,79	13,41	0	0	9	27	3,6	15	0	49	0	90	5,3	0,6	1,4	12	8
30	0	0,33	0	0	12	29	0,8	0,9	5,3	8,7	5	17	16	1	0,1	5,3	1,4
31	0	0,09	0	0	0	0	0	0	0	7	0	7,5	0	0	0	0	0
32	4,46	14,39	4,7	26	0	0	0	20	0	37	0	48	0	0	0	0	0
34	1,75	8,39	10	29	12	54	6	1,1	44	45	53	56	13	1,9	0,2	20	4,3
35	0,22	0,87	1,2	10	12	34	3,3	0,6	0	0,1	0	0	12	8,4	6,1	9,6	9
36	0	0	0	0	0	0	0	7,8	0	50	0	49	0	0	0	0	0
37	0,67	0,84	0,7	0	2,7	5,8	1,3	1	0	13	0	10	1,3	0,9	0,2	1,8	1,2
38	12,18	12,34	4,3	11	19	78	8,5	1,4	0	0	0	0	41	18	12	59	22
40	0,36	3,29	0	5	0	0	0	6,3	1,1	0	0,8	0	0	0	0	0	0
41	0	0	0	0,2	0	0,4	0	0	0,4	0,7	0,7	1,1	0	0	0	0	0
42	1,48	2,88	1,9	0	0	0	0	3	18	21	16	21	0	0	0	0	0
43	0,53	9,43	0	13	5	26	10	0	0	25	0	38	7,5	18	9,6	29	5,4
45	0,61	4,74	0	0	3,7	20	3,5	0,5	3,2	19	4,1	22	4,2	0,7	0	6,3	1,4
47	0,17	0,51	0	0	1,4	3,2	0,7	3,1	0,8	0,9	1,1	1,1	1,4	0,5	0	2,6	0,6
52	0,52	1,61	0	0	0	0	0	8,2	0	0	0	0	0	0	0	0	0
53	0,92	4,13	0	7,1	0	0,4	0	0,8	0,3	0	1,5	0	0,2	0	0	0	0
54	1,4	7,5	0	12	8,4	17	1,5	0,5	0	0	0	0	4,1	4,6	1,7	5,7	3,4
55	0	0	0	0	18	50	12	0	0	3,3	0	6,6	13	13	3,1	14	16
57	6,34	22,59	0,6	1,1	0	0	0	0	1,2	5,4	4,3	7,4	0	0	0	0	0
59	0,47	2,15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0,8	0	3,7	20	2,9	0,5	4,4	9,5	7,7	12	4,5	0,4	0	4,9	2,1
61	1,01	4,34	8,9	14	3,8	13	0,8	18	39	42	34	53	4,5	1	0,3	1,9	0,2
62	0	0	0	0	0	0	0	0	0,6	1,2	0,3	1,4	0	0	0	0	0
63	0	0	0	0	0	0	0	0	0	47	0	48	0	0	0	0	0
65	0	0	0	0	7,9	50	12	0	2,5	0	2,2	0	19	5,7	0,9	25	7,1
66	0,18	0,62	1,6	2,4	0	0	0	0	0	0	0	0	0	0	0	0	0
68	0	0	4,5	14	0,6	5,2	0,2	0	0	0	0	0	1,8	0	0	1,7	0
69	0,39	5,09	3,2	8,5	7,9	24	3,2	3,8	0	10	0	9,7	9,2	1,8	1,2	13	4,9
70	1,74	7,23	2,9	0	3,2	12	1,8	1,8	4,4	21	7,8	31	4,3	2,5	0,6	7,3	2
71	0	0,73	0	0	35	89	22	0	3,6	5,4	7,3	8,8	23	2	7,8	16	6,1
73	0	0	1,2	0	1	6	1	3,4	3,8	0	3	0	0,3	0,2	0,3	1,7	0,2
74	0	0,39	0,2	1,1	3,7	16	4,3	0,4	19	16	12	23	1,7	1,1	0,6	2,2	2,3
75	0	0,89	0,2	0	6,4	44	10	0,2	0	0	0	0	17	0,8	0,2	48	16
13	.																

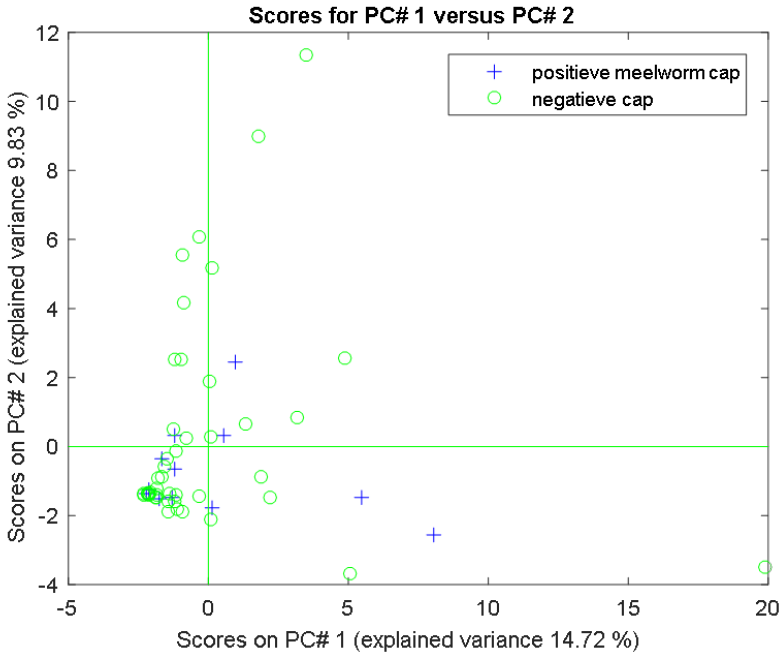
On the top left are mealworm sensitized patients. The rows are from left to right the aeroallergens, food allergens and 'others'.

Discussion

Mealworm sensitization was found in the shrimp allergic (88%), HDM allergic (22%) and seasonal rhinitis population (15%). sIgE levels for mealworm were higher in the shrimp allergic group than the HDM group. In the patients with and without asthma the percentages of mealworm

sensitization were similar, as were the percentages in patients with or without atopic dermatitis and with or without food allergy.

Figure 2b: Sensitisation profile from HDM patients with a positive mealworm CAP was comparable with sensitisation pattern of HDM patients with a negative mealworm CAP according to principle component analysis



We previously found that 87% shrimp-allergic patients that were mealworm sensitized had a DBPCFC confirmed mealworm food allergy. If we combine this information with the current finding that 88% of shrimp allergic patients was mealworm sensitized, it is estimated that approximately 75% of the total shrimp allergic population might be mealworm allergic. Given the high degree of cross-reactivity between shrimp and other shellfish [11], also other shellfish allergic patients, e.g. for crab and lobster might have a high risk of mealworm allergy.

This sensitization is probably mostly caused by cross-reaction of the well-known arthropod pan allergens tropomyosin and arginine kinase, based on the results others and we have shown on cross-reactivity between shrimp and insects [12-14]. We cannot exclude the possibility that also other

arthropod allergens play a role. In our previous study, we found besides tropomyosin and arginine kinase also other IgE binding protein bands [2]. These allergens could for instance be paramyosin, chitinase or serine protease [15-17].
In the HDM group, mealworm sensitization was present in 22%. Recently one other study reported on mealworm sensitization in HDM allergic patients. Mealworm sensitization was shown in 10/11 HDM patients, 4 of whom were not shrimp sensitized [4]. Rudolf et al. reported 30.7% sensitization, with SPT and intradermal test to the grain pest confused flour beetle in HDM allergic patients [18]. From those data, it cannot be extracted if the found reactivity was based on (known) major allergens (tropomyosin and arginine kinase. Since our HDM group consisted of patients not sensitized to tropomyosin, arginine kinase or shrimp, HDM allergic patients probably recognize other mealworm allergens.
Sensitization to mealworm in HDM patients with Der p 1 sensitization is unlikely to be caused by cross-reaction with allergens homolog to allergens from the C1 cysteine protease family. Der p 1 (C1 cathepsin) belongs to the C1 cysteine protease family and is for only 33% homologous to C1 cathepsin identified in mealworm. This was based on a homology calculation with the Uniprot database (data not shown). So far, no mealworm homologue for Der p 2 was found.

A large part (42%) of the HDM group, sensitized to mealworm, did not recognize Der p 1 or 2 (and not Der p 10 as this was an exclusion criterion) on the ImmunoCAP ISAC (Table 1). Since all patients were sensitized to HDM by either SPT or ImmunoCAP, this indicates they recognize other HDM allergens. The variation in recognition of (major) allergens by the HDM population was also described by others, although the percentage of Der p 1 and 2 negative patients in our study was somewhat higher than the 30% reported by others [8]. It was suggested that HDM allergic patients without IgE binding to Der p 1, 2 or 10 recognize a peritrophin-like protein, Der p 23, possibly chitin binding protein [9]. Der p 23 has shown homology to so far not completely characterized allergens from cockroach [19] and might therefore be a cross-reactive allergen between HDM and insects. To the best of our knowledge, Der p 23 -like allergens were not reported as cross-reactive allergens between shellfish and insects, so these allergens might be specific for cross reactivity between HDM and insects.

Remarkably, the seasonal rhinitis patients without HDM or shrimp sensitization (and therefore without suspected cross-reactivity via allergens involved in these allergies) also showed sensitization to mealworm, although at a lower percentage (15%). Insect sensitization in seasonal rhinitis patients was described in countries such as India and Iran [20, 21]. Research has been performed on insect sensitization to mosquitos or moths in atopic patients. Sensitization to those insect species was shown in percentages that reach or even exceed those of house dust mite in some regions [21]. Insect sensitization may result from respiratory exposure, but other routes of sensitization might be involved. Exposure by insects contaminating our food is not rare [22]. Armentia et al. showed allergy to *Bruchus lentis*, a lentil pest, in 16 patients [23]. Skin prick test (SPT) was negative for pure lentil extract, but positive for *Bruchus lentis* and infested lentil in all sixteen patients. Bronchial Provocation Test, Double blind, placebo-controlled food challenge (DBPCFC), and immunoblotting were positive in the majority of patients.

Although the prevalence of sensitization to mealworm is much higher in the shrimp allergic population, HDM allergy and allergy other than shrimp and HDM are much more prevalent (lifetime prevalence for shellfish allergy is around 0.1% in Europe and about 17.1% for HDM induced rhinitis [5, 6]; allergy prevalence is estimated around thought to be around 10%-20% of the general population [24]. Therefore, even though the percentages of patients sensitized to mealworm in these latter groups are lower than in the shrimp allergic group, on a population level these groups may concern substantially larger at risk populations. The clinical relevance of the sensitization to mealworm in the HDM and other allergic populations remains to be confirmed, preferably by double blind food challenges.

Conclusion: Not only shrimp allergic patients but also other atopic populations are sensitized to mealworm and potentially at risk for mealworm allergy. The allergens involved in the shrimp allergic population vs the other atopic populations are probably different.

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Chapter 6:

Primary respiratory and food allergy to mealworm

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(Published as letter to the editor J Allergy Clin Immunol. 2017. Mar 5. pii: S0091-6749(17)30340-8.)

Chapter 6:

Primary respiratory and food allergy to mealworm

Abstract
Background: Allergenicity of insects newly introduced, as food is one of the major risks to consider before marketing. Co-sensitization/cross-reactivity with shrimp was previously demonstrated, raising the issue of whether the risk might be broader and that primary allergy to these insects might be possible.
Objective: To elucidate the possibility of *de novo*/primary mealworm sensitization and allergy.
Methods: We conducted a study in mice to determine the sensitizing capacity of mealworm. Furthermore, we also conducted a clinical study with subjects (n=4) having a history of symptoms that started after exposure to mealworm during domestic or professional breeding. Sensitization was determined by measuring IgE binding to mealworm proteins, using ImmunoCAP, skin prick test (SPT), immunoblot and Basophil Activation Test (BAT). All four subjects underwent double blind placebo controlled food challenge (DBPCFC) with mealworm and open food challenge with shrimp. Subsequently, using LC-MS, we identified mealworm proteins that bound to IgE in sera from mice and human subjects.
Results: Mealworm induced IgE against mealworm proteins in mice and in all 4 human subjects. IgE from mealworm-exposed mice and humans recognized known mealworm allergens i.e. tropomyosin, arginine kinase and myosin heavy chain. Two human subjects had food allergy to mealworm confirmed by DBPCFC and were not shrimp allergic. The other two patients had a suspected respiratory allergy to mealworm. Furthermore, a new putative allergen was identified: larval cuticle protein.
Conclusion: Exposure to mealworm can lead to primary sensitization in mice and humans. In humans, this can lead to both inhalant and food allergy.

Background
Given their predicted world population of 9 billion people by 2050, the FAO is stimulating the investigation of insects as a new sustainable protein candidate for feed and food [1]. Mealworm (larvae of the yellow mealworm beetle, (*Tenebrio mollitor L.*) has great potential, due to its

sustainability and nutritional value and is currently introduced as a protein ingredient in commercially available burgers in a number of European countries [2,3]. Its effect on food allergy prevalence is not known. Depending on age, food allergy prevalence ranges within the European population from 0 to 5.7% [4]. Food allergy to insects is not regularly reported in Westernized countries. However, some reports from anaphylaxis due to insect ingestion are available from Asia, where insects are more commonly served [5,6]. For example, anaphylaxis upon ingestion has been reported for a regularly eaten insect in Asia, the larvae of the silk worm (*Bombyx mori*) [6]. Although prevalence of food allergy to insects is not described, the silk worm is estimated to cause anaphylactic shock over 1000 times a year in China compared to an estimated 1080 to 30,000 cases of anaphylaxis to any food in the US each year [6,7,8].

Food allergy to mealworm has so far been reported only once. This involved a systemic reaction including pruritis, generalized urticaria and diarrhea [9]. There are also a few reports of allergies to insects in an occupational setting [10,11]. Occupational allergy to mealworm in combination with waxmoth (*Galleria mellonella*) and greenbottle (*Lucilia caesar*) was already described in 1994 in 14 amateur and professional anglers who used these larvae as live fish bait [12]. The 14 subjects described mealworm-handling symptoms ranging from asthma and rhino-conjunctivitis to contact urticaria. These symptoms manifested after 2.3 to 6.3 years of exposure.

In a recent study, we showed that consumption of mealworm forms a risk for the majority of shrimp allergic patients [13]. We demonstrated that all studied shrimp allergic patients were sensitized/ co-sensitized to multiple mealworm allergens such as tropomyosin and arginine kinase [13]. In insects (e.g. cockroach and cricket) and arthropods (e.g. shrimp and crab), tropomyosin and arginine kinase have been described as major allergens with high sequence homology, which may contribute to cross-reactivity within the arthropod phylum [14-16]. Cross-reactivity was also indicated in the same study where it showed that 87% of these shrimp allergic patients reacted positively to mealworm in a DBPCFC, indicating a food allergy to mealworm. The risk of primary sensitization or allergy to insects or proteins thereof, however, has not yet been characterized.

To test the sensitizing potential of food proteins, several rodent and non-rodent animal models have been developed [17-19]. Recently we used a model previously described by Bowman et al. [20] to successfully distinguish a panel of five known allergenic proteins from five known low

allergenic proteins in C3H/HeOuJ mice (manuscript submitted). In the current study, we used this model to determine whether mealworm and shrimp can induce primary sensitization.
Moreover, we searched for evidence of primary sensitization and allergy in humans, studying domestic and professional mealworm breeders and their sensitization profiles, the allergens involved and the development of clinical allergy.

Methods

Study design

This study was designed to assess whether mealworm could cause primary sensitization and food allergy.
For the clinical trial, through insect farms and internet blogs, 4 subjects were located with histories of symptoms after handling, eating or other exposure to mealworm. During a screening visit, their medical histories were obtained and, using SPT, subjects were examined for sensitization to food allergens (various shellfish and wheat, the latter being an ingredient of mealworm feed), inhalant allergens (HDM, cat, dog, birch- and grass pollens) and insect allergens (mealworm, cockroach and silkworm). In addition, BAT and Westernblot were performed using mealworm, shrimp and shrimp-tropomyosin extracts. Specific IgE (sIgE) for shrimp, HDM and mealworm was determined using ImmunoCAP, and sIgE to allergen components was tested using ImmunoCAP ISAC. Immunoprecipitation in combination with LC-MS was used to identify proteins that bound to IgE in serum from mice and human subjects. All subjects participated in a double blind, placebo-controlled food challenge (DBPCFC) with mealworm and in an open food challenge with Dutch shrimp (*Crangon crangon*) to exclude shrimp allergy as a cross-reactive source of the mealworm allergy [21].

Shrimp and mealworm extract preparation

Fresh and freeze dried yellow mealworm (*Tenebrio mollitor L.*), in final larval stage, were kindly provided by Dutch insect farm Kreca (Ermelo, the Netherlands). Extracts for human trial (SPT from ALK) and in vitro testing (Tris and urea extract for immunoblotting and combined Tris/urea extract for BAT) were prepared as described previously [21]. For the animal study, to compare sensitization with a known and possibly similar allergen, shrimps (*Pandalus borealis*) were obtained from a local supermarket. Peeled shrimp (10 g) were freeze-dried and the protein content measured using the Kjeldahl method. A shrimp extract (20% protein) was prepared by

homogenizing the shrimp with water using an Ultra Turrax. Mealworms were washed several times with demineralized water and using Kjeldahl the protein content was measured. To inactivate endogenous digestion enzymes, dry mealworms were heated at 95 °C for 5 minutes. After careful removal of the skin, the inner part of the mealworm was mixed with water using Ultra Turrax to obtain a mealworm extract (20% protein). The shrimp and mealworm suspensions were frozen until further use.

Sensitization of mice with mealworm and shrimp extract
The mouse study was conducted with female C3H/HeOuJ mice obtained from a colony maintained under SPF conditions at Charles River, Sulzfeld, Germany. At commencement of the sensitization (day 0), the mice were 7 weeks old. Mice were allowed access to food (cereal-based VRF1 diet; SDS Special Diets Services, Whitham, England; certified free of mealworms and shrimp) and water ad libitum. Mice were gavaged two times at a weekly interval with shrimp/mealworm extract in PBS (20 mg protein) with 10 µg cholera toxin (CT; List Biological Laboratories, Inc., Campbell, CA). Control animals received PBS and CT only. At day 16, antigen-specific IgG1 (immunogenicity) and IgE (allergenicity) was determined as described by Smit et al. (submitted manuscript). The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 2010/63/EU) and Dutch legislation (The Experiments on Animals Act, 1997). This included approval of the study by the Netherlands Organisation for Applied Scientific Research's (TNO's) Animal Experimental Committee (DEC-number 3640).

Primary sensitization to mealworm in humans
Four adult domestic or professional mealworm farmers, each of whom displayed symptoms upon occupational mealworm exposure by inhalation or mealworm ingestion, were included in this study. The subjects were selected for suspected mealworm allergy based on suggestive history and sensitization and gave informed consent before participation.
The study was approved by the local ethics committee (NL43731.041.13).
Immunoblot, BAT, SPT (mealworm, ALK), shrimp (ALK, Stallergen and Greer), HDM (ALK and Greer) and ImmunoCAP mealworm (kindly prepared for us by Thermo Fisher), shrimp and HDM (Thermo Fisher) and ImmunoCAP ISAC (Thermo Fisher) tests were performed as described previously [13].

A DBPCFC was performed in all four human mealworm sensitized subjects. This challenge was performed as described previously [13]. An open food challenge with boiled Dutch shrimp (*Crangon crangon*) was performed according to the challenge protocol for shrimp used at our clinic, using 7 servings, dosing from 1 mg of shrimp to 100 g of shrimp.
Both the DBPCFC and the open shrimp challenge were discontinued and considered positive in case of objective symptoms or if a suggestive moderate to severe subjective symptom lasted for > 45 minutes. The days of the DBPCFC were de-blinded, after a panel review by three clinical experts on the outcome.

Identification of IgE binding proteins
For immunoprecipitation, Dynabeads M-280 Tosylactivated (10 mg, Invitrogen) were used according to the manufacturer's instructions. After coating with 0.2 mg Goat anti-Hu IgE (AP175 Upstate, Milipore) the separate beads were incubated for one hour at 37 °C with 1 mL human or mouse serum. Conjugated beads were cross-linked with 5 mM BS^3 (Pierce) according to the manufacturer's instructions 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 100 µL Tris/urea mealworm mixture diluted with 900 µL PBS. After washing 3 times, proteins were eluted with 2 times 100 µL 0.1 M glycine and the pH of the solution was neutralized using 30 µL of 1 M Tris-HCL pH 8.5. Incubation with mealworm extract was repeated 3 times and all eluates (800 µL) were pooled. Before analysis, samples were freeze dried, reconstituted in 250 µL 0.05% SDS, reduced with 10 mM DTT (1 h, 37°C), alkylated with 24 mM iodoacetamide (1h, 37 °C) and digested with 600 ng proteomics-grade trypsin after quenching with 2 mM DTT (20 min, 37 °C). Peptides were purified by strong cation exchange stage tips and subsequently injected for mass spectrometric analysis. Of this peptide mixture, 4 µL was analyzed according to Verhoeckx et al. [21], with the following minor modifications: The gradient elution was achieved at 350 nL/min flow rate, ramped from 8 % B to 35 % B in 60 min, and from 30 % B to 100 % B in an additional 8 min; after 5 min at 100 % B, the column was re-equilibrated at 0 % B for 2 min before the subsequent injection. MS detection was performed on a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) using a top-12 method with resolution (FWHM). Data was processed using Proteome Discoverer 1.3 (Thermo Fisher Scientific, Bremen, Germany), using Sequest as search engine, and

the Swiss Prot database accessed on February 2013 as sequence database (3,123,840 sequences for Metazoa taxonomy). The following search parameters were used: MS tolerance 15 ppm; MS/MS tolerance 0.02 Da; fixed modifications carbamidomethyl cysteine; enzyme trypsin; max. missed cleavages 1; taxonomy Metazoa. High confidence peptides (confidence > 99 %) were filtered out, using Percolator, integrated in Proteome Discoverer. Protein hits based on two successful peptide identifications were considered valid.

Statistical analysis
Data were collected in IBM SPSS Statistics 21. Mice data are presented as means and analyzed using GraphPad Prism software. Antibody levels were analyzed using Mann Whitney U test or by Kruskall- Wallis, followed by Dunn's Multiple comparison test.

Results
Sensitization to mealworm and shrimp in the mouse model
Mice were dosed by gavage with mealworm or shrimp extract in PBS (20 mg protein) with 10 µg cholera toxin (CT; List Biological Laboratories, Inc., Campbell, CA). Control animals received PBS and CT only. Mealworm extract induced extract-specific IgG1 in 3/6 animals and extract-specific IgE in 2/6 animals (Figure 1). Shrimp extract led to the induction of extract-specific IgG1 and IgE in 5/6 animals. Together these data show the potency of both extracts to induce primary sensitization.

Development of mealworm allergy in four human subjects
All four human subjects (between 22 and 46 years of age, one female, three male) developed mealworm allergy during either professional or domestic mealworm breeding, potentially caused by a combination of dermal and respiratory exposure. Two subjects, who worked in a room where mealworms were kept at a professional scale, would develop, minutes after entering the room, symptoms such as conjunctivitis and rhinitis, which disappeared gradually, without medication, after leaving the room. Neither subject had any other inhalant or food allergies. Since starting to work at the farm, they had sporadically consumed mealworms in small amounts (several worms at a time) without symptoms. The other two subjects reared mealworms in a separate room at home on a small scale. One of the two (subject 4) began to develop rhino-conjunctivitis after about two years of exposure, which progressed to dyspnea and wheezing

after he started the domestic rearing. Their oral intake of the mealworm was higher than that consumed by the professional farmers: up to 50 grams of mealworm each on around 10 occasions. After having eaten mealworm several times, both domestic farmers encountered progressive symptoms, from pruritis of the lips to a feeling of swelling, no anaphylaxis occurred. Neither subject had a shrimp allergy or any other food allergy. One of them (subject 4) had mild conjunctivitis, rhinitis, nasal congestion and sneezing related to HDM or birch pollen exposure. For a detailed overview of the patient characteristics see Table 1.

Sensitization profile of mealworm allergic subjects

All four subjects were sensitized to mealworm according to ImmunoCAP (0.75 kU/L, 2.32 kU/L, 2.25 kU/L and 14.6 kU/L respectively) and SPT (Table 1). Their sensitization profiles, to shrimp (SPT, CAP, Pen m 1, Pen m 2 and Pen m 4) and house dust mite (SPT, CAP, Der p 1, Der p 2 and Der p 10), are shown in Table 1. Only subject 2 showed sensitization to shrimp by IgE binding to tropomyosin and a positive skin reaction to shrimp. However, this subject shows no clinical symptoms when eating shrimp. Furthermore, all subjects were sensitized to some common inhalant allergens, such as tree and grass pollen and animal dander, although titers were very low as shown by ISAC (see supplemental material). Although sensitized, only one subject had rhinitis symptoms, possibly caused by HDM.

As Figure 1 shows, IgE from all subjects bind to more or less the same proteins in the mealworm extracts, however with different intensities. Comparing the subjects with a reported respiratory allergy to mealworm (1 and 2) to the subjects with food allergy (3 and 4), no clear convincing differences were observed. The exceptions are a protein at around 15 kDa in the Tris extract on the immunoblot of subject 3 and a protein of approximately 10 kDa in the immunoblot of subject 4. The basophils of all 4 subjects were activated by mealworm extracts (Figure 2). The percentage of CD63+ basophils ranged between 18 and 65. No basophil activation was seen after incubation with shrimp tropomyosin (Pen m 1). However, the basophils of subjects 3 and 4 were slightly activated after incubation with shrimp extract (% CD63+ basophils between 5 and 11). These sensitization results were not fully corroborated by SPT, where only subjects 2 and 3 showed a minor reaction to shrimp.

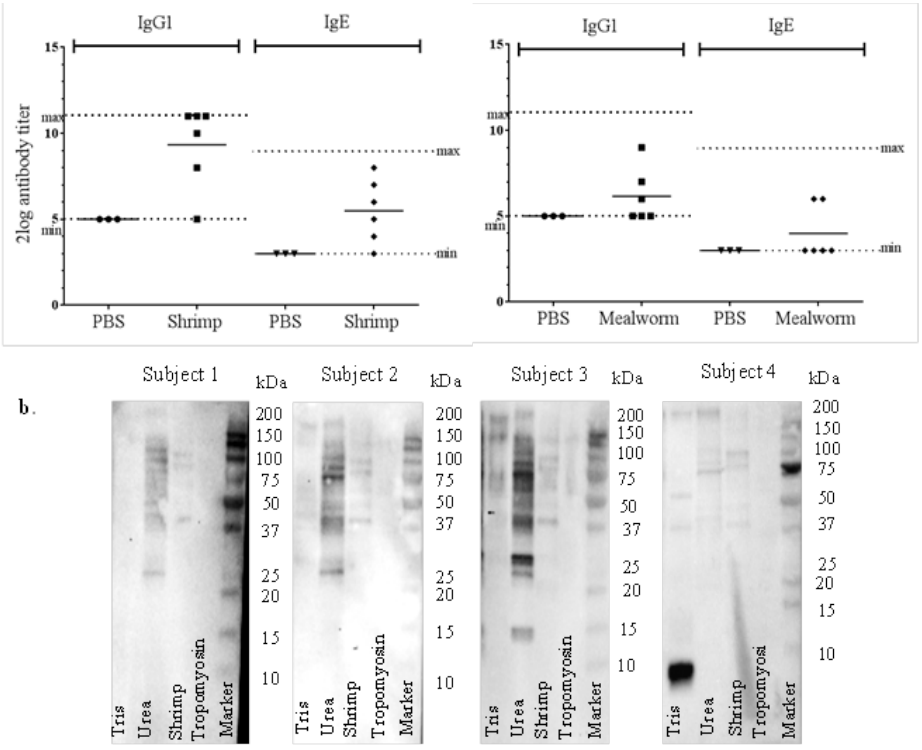
Table 1a. Patient characteristics

[a] Subject	Sex	Age (years)	AA/ AD/ AR	Professional/ Domestic	Years of exposure	Direct inhalant exposure (h/w)	Oral exposure (times consumed)	Average intake (in grams)	Symptoms inhalation	Symptoms ingestion	HDM symptoms	Shrimp symptoms	[c] DBPCFC mealworm
1	M	46	n/y/n	Prof	2	0.5	< 10	~ 1	C,R	n	n	n	neg
2	F	22	n/y/n	Prof	5	6	< 5	~ 1	C,N,R	n	n	n	neg
3	M	28	n/n/n	Dom	7	2-4	< 10	~ 50	n	OA, U	n	n	pos
4	M	32	n/n/y	Dom	10	2-4	5-10	~ 50	C,N,S,D, W	OA, U	C, N, S	n	pos

[b]	Mealworm		HDM						Shrimp					
Subject	**CAP (kU/L)**	**SPT ALK**	**CAP (kU/L)**	**SPT Greer**	**SPT ALK**	**Der p 1 (ISU)**	**Der p 2 (ISU)**	**Der p 10 (ISU)**	**CAP (kU/L)**	**SPT Greer**	**SPT ALK**	**SPT Stallergen**	**Pen m 1 (ISU)**	**OFC shrimp**
1	0.75	3+	0	2+	0	0	1.1	0	0	0	0	0	0	neg
2	2.32	3+	0.4	2+	0	0	0	0.6	0.7	2+	2+	2+	0.6	neg
3	2.26	3+	0	2+	0	0	0	0	0	0	0	1+	0	neg
4	14.6	2+	28	3+	2+	9.7	13	0	0	0	0	0	0	neg

a. Mealworm exposure and clinical history to direct inhalation (while working with the animal) and ingestion of mealworm, HDM and shrimp. M = male, F = female AA allergic asthma; AD atopic dermatitis; AR allergic rhinitis; n = no; y = yes; Prof = professional farmer; Dom = domestic farmer; h/w hours per week; GI = gastro-intestinal; C, conjunctivitis; D, dyspnea; N nasal congestion; OA, oral allergy; R, rhinitis; S, sneezing; U, general urticaria; W, wheezing. **b.** Sensitization in SPT, CAP for mealworm, HDM and shrimp and ISAC (with at least one subject <0.3 ISU) were included in the table. Der p 1, house dust mite peptidase C1; Der p 2, house dust mite NPC2 family; Der p 10, house dust mite tropomyosin; Pen m 1, shrimp tropomyosin **c.** Challenge results. neg = negative pos= positive

Figure 1. IgE binding profile in mice and humans



a. Antibody responses (IgE and IgG1) to mealworm extract, shrimp extracts and control (PBS) in mice model on day 16. Data represent the mean 2log antibody titer ± SEM. Horizontal dotted lines represent the detection limits of the assays (min/max).

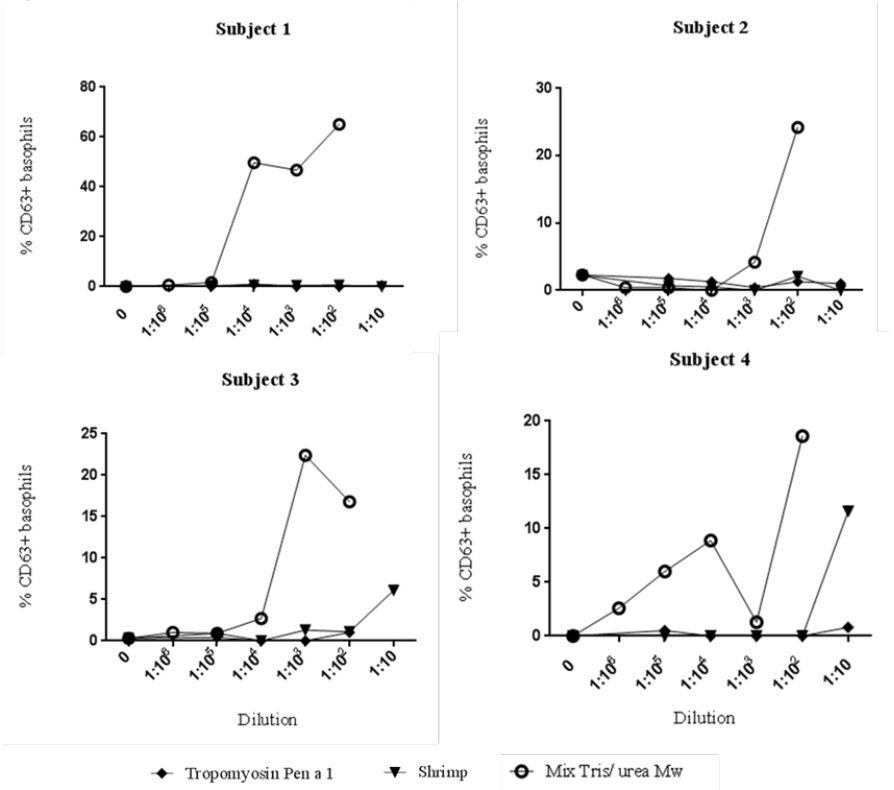
b. Subjects show IgE binding to allergens in both the mealworm and the shrimp extract. No binding is seen to Pen a 1 (shrimp tropomyosin) IgE from mealworm food allergic subjects 3 and 4 additionally bind to proteins with low molecular weight.

IgE binding proteins identified for sensitized mice and human subjects

Table 2 shows the top 6 IgE binding mealworm proteins we identified based on highest mean score (which is the probability that the observed match is not a random event) for mice and table 3 provides the same for humans. These tables also show the top 5 known allergens (not in the

protein top 6). For both mice and humans, IgE binding to known allergens such as tropomyosin, arginine kinase, myosin light and heavy chain was shown. Both mouse and human IgE recognized three proteins with convincing high scores (5 to 10 times higher compared to the others) and with high sequence coverage (with respect to our previous and current results). These were identified as three highly homologous (> 88% sequence identity) mealworm larval cuticle proteins (LCP).

Figure 2. Functional IgE binding



Basophil activation tests showing basophil activation to mealworm proteins (Tris/Urea extract) and shrimp (SPT ALK). No reactivity to Pen a 1 (shrimp tropomyosin) was seen. Clinically subject 1 and 2 showed respiratory symptoms to mealworm; subjects 3 and 4 showed food allergic symptoms.

Discussion

In mice, mealworm extract was able to induce primary sensitization: induction of mealworm specific IgG1 and IgE. Shrimp extract used as a control showed IgG1 and IgE to shrimp. The percentage of sensitization was higher for shrimp than for mealworm, which might suggest stronger potency of shrimp than mealworm. The used mouse model [20] has the ability to differentiate between allergens (e.g. Ara h 1, beta-lacto globulin) and low/non-allergens (e.g. gelatin, beef tropomyosin), but it is not known whether the model can measure differences in sensitizing potency. It might not, given previous results from a DBPCFC study with mealworm and shrimp, which showed that eliciting thresholds for shrimp and mealworm were comparable, suggesting that the allergenicity is similar.

Mealworm exposure in humans by domestic or professional breeding and ingestion resulted in inhalant and/or food allergy to mealworm. Primary insect allergies have been documented in case studies in insect raising laboratory workers, bait handlers or grain workers as occupational allergies [11,23,24]. The development of insect-based occupational allergies was documented for blowfly (*Lucilia cuprina*) and other species of adult flies, grasshopper and mealworm [11, 23, 24]. Reports of anaphylaxis due to ingestion of the larvae of the silk worm (*Bombyx mori*), from Asia, lack history of prior occupational exposure. These reports disclosed neither shrimp sensitization nor allergy, therefore a cross-reactive result cannot be excluded [8]. Only one case of food allergy to mealworm has been reported so far, with a systemic reaction including pruritis, generalized urticaria and diarrhea after eating mealworm, but this was not confirmed by DBPCFC. In this case, neither occupational exposure nor shrimp allergy was mentioned [9].

Although based on a very limited number of cases, the results of this study suggest the occurrence of 3 different phenotypes of primary allergy due to exposure to mealworm allergens. The 2 occupational workers who had limited oral exposure developed occupational respiratory allergy whereas the domestic breeders who reportedly had more frequent and higher oral intakes developed food allergy. One of the two never experienced respiratory symptoms during the work with mealworm. The data suggest that the differences in exposure patterns might result in a different phenotype. Most food related occupational allergens have not been proven to induce symptoms after ingestion.

Table 2. IgE binding proteins identified in mice

Protein (source)	Accession	Score	Sequence coverage (%)	Peptides identified	PSM	Mass (kDa)
Beads Mice						
Larval cuticle protein A1A (*Tenebrio molitor*)	P80681	519	97	15	114	17.7
Larval cuticle protein A2B *(Tenebrio molitor)*	P80682	300	97	10	70	12.3
Larval cuticle protein A3A (*Tenebrio molitor*)	P80683	292	97	15	70	14.0
Tropomyosin like *(Tribolium castaneum)*	D6X4X3	85	38	12	23	32.3
Actin-87E *(Drosophila melanogaster)*	P10981	60	22	9	14	41.8
TM-E1A=Cuticular protein (*Tenebrio molitor*)	Q9TXE4	32	27	3	9	23.2
Other known allergens identified						
Chitin binding protein *(Nasonia vitripennis)*	K7IX02	29	8	4	7	23.0
Myosin heavy like *(Tribolium castaneum)*	D6WVJ3	27	15	9	10	262.1
Troponin T like *(Tribolium castaneum)*	D6W953	17	14	5	6	45.7
Myosin light chain 2 *(Tribolium castaneum)*	D6WZU7	12	9	3	4	31.3
Arginine kinase (Fragment) *(Lasippa tiga)*	D1L9H9	11	19	2	2	12.0

Proteins identified on beads with IgE from the mealworm sensitized mice after incubation with Tris/urea extract using LC-MS/MS Top 6 IgE binding proteins identified using LC-MS/MS based on highest mean score (pooled sensitized mouse sera (n =6)) and top 5 known allergen (not in protein top 6). Arranged on highest score. Identification was based on homology with metazoan proteins in the Swiss-Prot database. Known allergens are noted in bold, based on arthropod nomenclature. PSM = peptide-spectrum matches, value that represents the number of MS/MS spectra that matched peptide sequences assigned to that particular protein. Score= the sum of individual Sequest scores of all the identified

peptides which were assigned to the protein itself. The score is the probability that the observed match is not a random event.

Table 3. IgE binding proteins identified in four human subjects

Protein (source)	Accession	Score	Sequence coverage (%)	Peptides identified	PSM	Mass (kDa)
Beads Subjects 1,2,3 & 4						
Larval cuticle protein A1A (*Tenebrio molitor*)	P80681	521	84	15	115	17.7
Larval cuticle protein A2B (Tenebrio molitor)	P80682	436	90	10	97	12.3
Larval cuticle protein A3A (*Tenebrio molitor*)	P80683	378	97	15	88	14.0
Tropomyosin like (Tribolium castaneum)	D6X4X3	78	37	12	21	32.3
TM-E1A=Cuticular protein (*Tenebrio molitor*)	Q9TXE4	56	43	6	14	23.2
Myosin heavy chain like (*Triobolium castaneum*)	D6WVJ3	55	21	17	20	262
Known allergens identified						
Troponin T like *(Triobolium castaneum)*	D6W953	32	19	8	12	45.7
Chitin binding protein *(Nasonia vitripennis)*	K7IX02	27	6	3	8	23.0
Arginine kinase (Fragment) *(Stibochiona nicea)*	B3TFY5	12	11	3	4	22.6
Myosin light chain 2 (*Triobolium castaneum*)	D6WZU7	10	9	3	3	31.3
Allergen Ale o 10 *(Aleuroglyphus ovatus)*	A1KYZ1	6	5	2	3	33.0

Proteins identified on beads with IgE from the 4 subjects after incubation with Tris/urea extract using LC-MS/MS Top 6 IgE binding proteins identified using LC-MS/MS based on highest mean score (4 subjects) and top 5 known allergen (not in protein top 6) Sequence coverage, Peptides identified and PSM are given as the mean of the 4 sera. Identification was based on homology with metazoan proteins in the Swiss-Prot database. PSM = peptide-spectrum matches, value that

represents the number of MS/MS spectra that matched peptide sequences assigned to that particular protein. Score= the sum of individual Sequest scores of all the identified peptides which were assigned to the protein itself. The score is the probability that the observed match is not a random event.

A few isolated cases have been reported on occupational allergy and the subsequent development of food allergy to the same source of protein. These individuals were reported in garlic, buckwheat, shrimp and snow crab industry [25-28].

Whether this lack of evidence is due to underreporting or due to actual absence of food allergic symptoms in occupational allergy is unclear, data on intake of the work-related food are missing.

In line with our study, insect allergy, developing generally after several years of exposure, has been described in various papers [12,29,30]. The manifestation of occupational allergic symptoms, seen in 14 subjects after 2.3 to 6.3 years of exposure to mealworm, greenbottle and waxmoth [12]. Allergic symptoms in another case developed after occupational exposure to mealworm for 5.4 years [30].

From the four mealworm-sensitized human subjects, two developed food allergy. These two showed higher sIgE to mealworm, consumed larger amounts of mealworm (~50 g) and were exposed for a longer period of time (7-9 years) than the two with respiratory allergy (~1 g mealworm and 2-5 years of exposure). This might suggest that occupational exposure for a longer period of time or oral exposure with high doses are required to develop food allergy to mealworm. Both food allergic subjects were exposed to mealworm through the respiratory, dermal and oral route and therefore we cannot pinpoint the exact route(s) of exposure responsible.

The risk for mealworm allergy in shrimp allergic patients through cross-reactivity was shown in our previous study. The present study shows that primary respiratory and food allergy to mealworm can develop due to dermal, respiratory and/or oral exposure. The relative contribution of the oral exposure route cannot be established, although it is remarkable that the 2 subjects that developed food allergy to mealworm had a more frequent and higher preceding consumption of mealworm. All 4 subjects that developed primary allergy to mealworm were atopic patients, but without any food allergy. So far there are no indications that also non-

atopic individuals could be at relevant risk for development of primary allergy due to exposure to or consumption of mealworms.

Our data suggest that, since three of our subjects produced higher levels of sIgE to mealworm than to any other food or inhalant allergen (including other arthropods allergens), mealworm might be the primary sensitizer. Although the fourth subject showed slightly higher HDM sensitization compared to mealworm, this subject had IgE to Der p 1 and 2 (fecal components), but not to Der p 10 (tropomyosin) and arginine kinase, which makes cross-reactivity to HDM less likely. Furthermore, immunoblots from shrimp allergic patients showed different binding patterns compared to the four primary mealworm allergic subjects [13]. Since the sIgE to shrimp and HDM from the four mealworm allergic subjects was low, inhibition studies were impossible. However, a role for shrimp was unlikely given that food challenge with shrimp was negative in all mealworm allergic patients.

In all four human subjects, the mealworm proteins involved were the previously undiscovered allergen larval cuticle protein and, to a seemingly lesser extent, the well-known tropomyosin and myosin heavy chain. Some of these proteins (e.g. tropomyosin, arginine kinase, myosin heavy chain) were also involved in cross-reactive food allergy to mealworm in shrimp allergic patients [22]. The most abundant IgE binding proteins identified in this study, larval cuticle proteins (LCP), were not identified with the same high score and high sequence coverage (not even in the top 20 proteins) in shrimp allergic patients of our first study [22]. This might indicate that LCP is a less dominant allergen in shrimp allergy compared to mealworm allergy. These larval cuticle proteins have not been previously identified as allergens in insects or crustaceans, so we have discovered a novel arthropod allergen in mealworm.
LCP is a mealworm specific protein, having a conserved domain in arthropod cuticles known as R&R consensus and binds chitin [32]. The chitin-binding complex links the soft internal tissue to the exoskeleton of the larvae. The LCPs identified are highly homologous, with a molecular weight varying from 12.3 to 17.7 kDa. The lack of bands on the immunoblot at 17 kDa might be accounted for by migration on gel to a higher molecular weight. This discrepancy between migration on gel and molecular weight is characteristic of many cuticle proteins. Aggregated forms of cuticle proteins exist and can appear at higher MW on the immunoblot, depending on use of urea, temperature and pH [28]. Given the differences

between the shrimp allergic, cross-reactive mealworm allergy (low score and sequence coverage) and the four subjects in this study (high score and sequence coverage), we hypothesize that LPC could be the principle sensitizing allergen in primary mealworm allergy. LCPs from mealworm are most likely both inhalant and food allergens, since they were identified in subjects with respiratory as well as food allergy to mealworm.

Conclusion

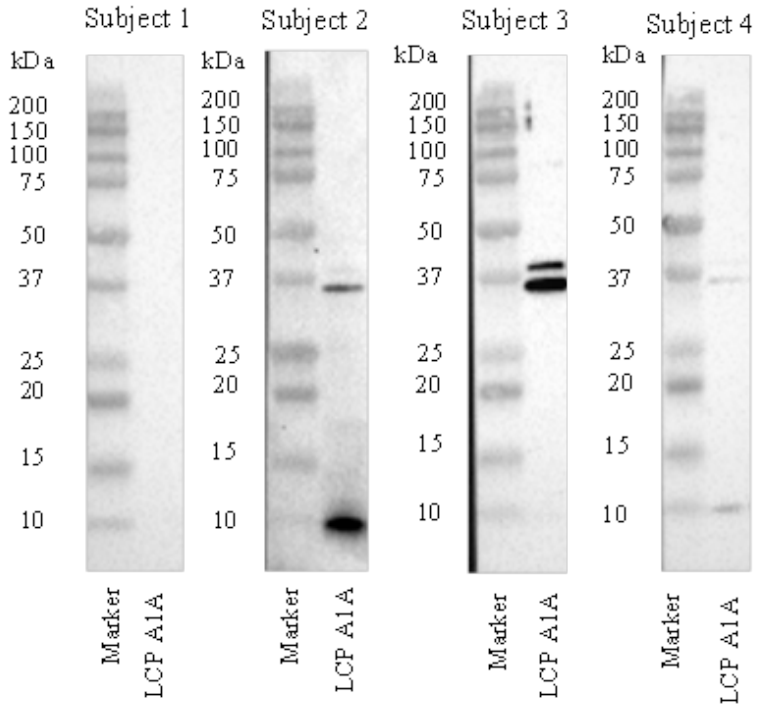
Exposure to mealworm can lead to primary sensitization in a mouse model and in humans and can result in food allergy. The risk of primary sensitization and allergy should be incorporated in the risk assessment of new proteins.

Supplementary Table E1. ImmunoCAP ISAC results

Subj.	Pen m 1	Der p 1	Der p 2	Der p 10	Bla g 7	Ani s 3	Cyn d 1	Phl p 1	Phl p 2	Phl p 4	Phl p 5
1	0	0	1.1	0	0	0	0	0	0	0	0
2	0.6	0	0	0.6	0.6	0.8	2.3	0	0	2.0	0
3	0	0	0	0	0	0	2.0	0	0	2.8	2.1
4	0	9.7	13	0	0	0	14	20	1.2	0	3.6
Subj.	**Cry j 1**	**Cup a 1**	**Pla a 2**	**Can f 5**	**Fel d 1**	**Der f 1**	**Der f 2**	**Lep d 2**	**Jug r 2**	**Jug r 3**	**MUXF 3**
1	0	0	0	0	0	0	2.1	5.4	0	0	0
2	0	0	0.4	0	0	0	0	0	2.4	0	1.7
3	0.5	1.0	1.5	0	0	0	0	0	1.9	0	2.0
4	0	0	0	1.0	6.2	10	24	0	0	0.3	0

All patients show some sensitization to both common inhalant and food allergens: values (>0.3) expressed in ISU. Only scores that were positive in at least one subject are shown.Pen m 1, 2, 4 from shrimp; Der p 1, 2, 10 from house dust mite; Bla g 7 from cockroach; Ani s 3 from anisakis; Cyn d 1 from Bermuda grass; Phl p 1,2,4,5 from timothy grass; Cry j 1 from Japanese cedar; Cup a 1 from Arizona cypress; Pla a 2 from plane tree; Can f 5 from dog; Fel d 1 from Cat; Der f 1,2 from house dust mite; Lep d 2 from storage mite; Jug r 2, 3 from walnut; MUXF3 bromelain.

Figure E1 Three mealworm allergic subjects sensitized to larval cuticle protein A1A on immunoblot



Both food allergic subjects 3 and 4 show IgE binding to mealworm larval cuticle protein A1A. IgE from one inhalant allergic subject bound to LCP A1A.

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Chapter 7:

Is mealworm or shrimp allergy indicative for food allergy to insects?

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(*Provisionally accepted, Molecular Nutrition and Food Research*)

Chapter 7:

Is mealworm or shrimp allergy indicative for food allergy to insects?

Abstract

Scope: The growing world population is a key driver for the exploration of sustainable protein sources to ensure food security. Mealworm and other insects are promising candidates. Previously we found that shrimp allergic patients are at risk for mealworm allergy, and that mealworm can induce a primary allergy [3].

This study set out to investigate the allergenic potential of edible insects, suggested for human consumption by agencies such as WHO/FAO, in both the shrimp (potentially cross-reactive) and primary mealworm allergic population. The following insects were studied: mealworm, house cricket, giant mealworm, lesser mealworm, African grasshopper, large wax moth and black soldier fly.

Methods and results: 15 shrimp (mealworm sensitized or allergic) patients and four primary mealworm allergic subjects, who participated in previous studies, were included. All shrimp allergic patients were sensitized to multiple insects with similar response profiles for all insects tested. Primary mealworm allergic patients, showed IgE binding to proteins from only a few insects on immunoblot, although basophil activation test was positive for all tested insects.

Conclusion: Shrimp allergic patients are at risk of food allergy to mealworm and other insects. Primary mealworm allergic subjects are suspected not to react to all insects.

Introduction

In order to preserve agricultural land, energy and water and reduce CO^2 footprint, changes to our current systems of food production must be made. Therefore, on recommendation of the FAO, governments are sanctioning investigations aimed at determining if certain insects can be consumed as a new sustainable protein candidate for feed and food [1, 2]. In two recent studies, we showed that the majority of shrimp food allergic patients also had mealworm food allergy [3]. In addition, we found that primary mealworm allergy can develop in professional and hobby insect breeders [4].

Prevalence of food allergy ranges in Europe up to 5.7% depending on age, with shellfish among the ten most prevalent foods [5]. Prevalence of insect food allergy in Europe is not known. At present insects are not commonly consumed in Western countries. There are some reports on food allergy to insects in Asia, which are especially focused on the pupal stage of the silkworm (*Bombyx mori*), which is regularly consumed in Asia. As a result of the cultural influences of traditional Chinese food and medicine, Chinese people often eat oil-fried, water-boiled or ground pupa powder of the silkworm and anaphylaxis has been described upon ingestion [6]. In China, it was estimated from literature (1980 -2007) that around 17 % of food related anaphylaxis was caused by eating insects (locust, grasshopper and silkworm) [7]. Food allergy to mealworm was reported previously in the US by Frye et al. [8]. Frye et al., presented a case of anaphylaxis in a patient after ingestion of mealworm. In a previous study, we demonstrated that shrimp allergic patients were food allergic to mealworm in a double-blind placebo controlled food challenge (DBPCFC). This allergy was based on cross- or co-sensitization to multiple mealworm allergens such as tropomyosin and arginine kinase [3]. In insects (e.g. cockroach and cricket) and arthropods (e.g. shrimp and crab) homologous proteins such as tropomyosin and arginine kinase have been described, which may contribute to cross-reactivity between shrimp, mealworm and other insect species [9, 10]. This could suggest that shrimp and mealworm allergy might be indicative for allergy to other insects. In our previous study, we also showed that primary mealworm sensitized subjects were not allergic to shrimp. The study suggested that primary sensitization for mealworm was not caused by tropomyosin and arginine kinase, but that other proteins, such as larval cuticle protein (LCP) might play a role [4]. This assertion is at conflict with the shrimp cross-reactivity theory and might indicate that primary mealworm sensitization is not indicative for allergy to other insects.

Before insects can become a substantial part of the diet, potential risks of food allergic reactions should be explored. In this paper, the risk of food allergy to insects other than mealworm is addressed. Seven insects from 4 different orders (Coleoptera, Lepidoptera, Diptera and Orthoptera) and different life stages (larvae and adult) were investigated. These insects are described in the EFSA report as possible novel food or feed [11]. Sensitization and functional IgE binding to *Tenebrio molitor*; mealworm (larvae), *Acheta domesticus*; house cricket (bug), *Zophobas morio*; giant mealworm (larvae), *Alphitobius diaperinus*; lesser mealworm (larvae),

Locusta migratoria; African grasshopper (bug), which we will call grasshopper, *Galleria mellonella*; large wax moth (larvae) and *Hermetia illucens*; black soldier fly (larvae) was tested in blood from patients with shrimp allergy or a primary mealworm allergy from our previous studies [3, 4]. Proteins were identified, using LC-MS-MS, in the different insect extracts for comparative analysis.

Materials and methods

Study population

15 shrimp allergic patients and four primary mealworm allergic subjects showing sensitization to mealworm from our previous studies, were included in the study [3, 4]. 13 out of 15 shrimp allergic patients had a food allergy to mealworm as indicated by a positive DBPCFC. Two primary mealworm allergic subjects had a positive DBPCFC to mealworm and the other two had an inhalant allergy to mealworm.

All subjects gave written informed consent before participation. The study was approved by the local ethics committee (NL43731.041.13).

Specific IgE

ImmunoCAP mealworm was specifically produced for this project by Thermo Fisher Scientific, Uppsala, Sweden and was tested according to the manufacturer's recommendations. IgE is expressed in kU/L. Tests were considered positive with a value of 0.35 or higher.

Insect extracts

Fresh Yellow mealworms (*Tenebrio molitor*), giant mealworms (*Zophobas morio*), lesser mealworms (*Alphitobius diaperinus*), large wax moths (*Galleria mellonella*) and black soldier flies (*Hermetia illucens*) all in final larval stage, and crickets (*Acheta domesticus*) and grasshoppers (*Locusta migratoria migratorioides*) in adult form were kindly provided by Dutch insect farm Kreca (Ermelo, the Netherlands). Dutch shrimp (*Crangon crangon*) were bought from a local store.

Five grams of insects or boiled shrimp were extracted using a sequential protein extraction method [12]. First the insects or shrimp were mixed with 25 mL ice-cold Tris buffer (20 mM Tris buffer pH 7.6 containing 1 mM phenylthiocarbamide (Sigma Aldrich) and Halt Protease Inhibitor Cocktail (Thermo Scientific). Subsequently the insects or shrimp were disrupted, using an ultraturrax (3 x 10 sec) under continuous cooling. The ultraturrax was washed with 5 ml cold Tris buffer and the wash liquid was added to

the sample suspension. After centrifugation (30 min, 15 000 x g at 4 °C), the supernatant was recovered. The insoluble residue was washed once with 5 mL Tris buffer. The 30 mL and 5 mL supernatant were combined. 25 mL was used for sample cleanup and concentration using TCA precipitation. Secondly, the remaining pellet was extracted overnight at 4 °C with 30 mL urea buffer (6 M urea in 20 mM Tris buffer pH 7.6 containing 1 mM phenylthiocarbamide and Halt Protease Inhibitor Cocktail). The sample was subsequently centrifuged and the supernatant was collected. The pellet was washed once more with 5 mL urea buffer, centrifuged and the supernatant was combined with the 30 mL urea supernatant. 25 mL of the extract was TCA precipitated. Tris and urea extracts were combined (1:1).

Immunoblot and dot blot

For SDS-PAGE, the Criterion system with an Any kD Ready Gel® Tris-HCl gel (Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions. Insect extracts (5 µg) were loaded on the gel, under reducing conditions (Laemmli buffer) together with 5 µg shrimp control. After separation, proteins were transferred to a polyvinyldifluoride membrane using the Criterion Blotter system (Bio-Rad) according to the manufacturer's instructions.

The membrane was blocked overnight with 5% dried milk powder in phosphate buffered saline with 0.1% Tween 20 (PBST) and incubated overnight with serum from a shrimp allergic patient or primary mealworm allergic subject (1:50) in 1 % dried milk powder in PBST. After thorough washing, the membranes were incubated for 1 hour with HRP-labeled Goat anti human IgE (KPL, Gaithersburg, MD, USA) 1:50.000 in PBST. After washing, the bands were visualized using a chemi-luminescent peroxidase substrate kit ECL (Sigma) according to the manufacturer's instructions. Control blots were performed to exclude a-specific binding. Results are shown in figure E1. Blots were scanned using the Chemidoc XRS+ image scanner with Imagelab software (Bio-Rad).

Dot blots were performed on a PVDF membrane, using a Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Insect extracts (5 µg of protein) were loaded on the membrane, under reducing conditions (Laemmli buffer) together with 5 µg shrimp control. Reducing conditions were used on dot blot to keep the binding potential similar to the proteins on Westernblot. After

loading the protein on the membrane, the same procedure described for the immunoblot was performed.

Basophil activation test (BAT)

BAT's were performed with blood from shrimp allergic patients and primary mealworm allergic subjects from our previous studies [3, 4]. Five shrimp allergic patients were excluded from the BAT because they were non-responders, showing only IgE binding to freeze dried mealworm or spontaneous release in previous BAT. One shrimp allergic patient was not able to participate as a result of emigration. BAT's were performed as described previously by Meulenbroek et al. [13] with minor modifications. Cells were incubated with a dilution series (1:107 to 1:102) of insect extracts (5 mg/mL). Mealworm or shrimp extract (5 mg/mL) was used as positive control. CD63, CD123 and CD203c expression was analyzed by flow cytometry using FACSCanto II and FACSDiva software (BD Bioscience, USA). The results were expressed as a percentage of CD63+ basophils. The BAT was considered positive when the percentage of CD63+ cells was at least 5% and no spontaneous expression of CD63 on the cells was measured. Individuals with basophil response of 0-5% CD63, after anti-FcεRI stimulation, i.e. the positive control, were regarded as non-responders. In total 13 BATs were approved.

Nano LC-MS/MS analysis and protein identification

Proteins from the Tris/urea mixtures of each insect (20 µg) were digested and analyzed according to Verhoeckx et al. [14], with small changes. Chromatography was performed on an Easy LC 1000 nanoscale liquid chromatography (nanoLC) system (Thermo Fisher Scientific, Odense, Denmark). The analytical nanoLC column was a pulled fused silica capillary, 75 µm i.d., in-house packed to a length of 10 cm with 3 µm C18 silica particles from Dr. Maisch (Entringen, Germany). Tryptic peptides (125 ng) were loaded at 500 nL/min directly onto the analytical column. A binary gradient was used for peptide elution. Mobile phase A was 0.1% formic acid, 2% acetonitrile, whereas mobile phase B was 0.1% formic acid, 80% acetonitrile. Gradient elution was achieved at 350 nL/min flow rate, and ramped from 8% B to 35% B in 60 min, and from 30% B to 100% B in additional 8 min. After 5 min at 100% B, the column was re-equilibrated at 0% B for 2 min before the following injection. MS detection was performed on a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) operating in positive ion mode, with

nanoelectrospray (nESI) potential at 1800 V applied on the column front-end via a tee piece. Data-dependent acquisition was performed by using a top-12 method with resolution (FWHM), AGC target and maximum injection time (ms) for full MS and MS/MS of, respectively, 70,000/17,500, 10e6/10e5, 50/60. Mass window for precursor ion isolation was 1.6 m/z, whereas normalized collision energy was 25. Ion threshold for triggering MS/MS events was 2e4. Dynamic exclusion was 30 s. Data was processed using Proteome Discoverer 1.3 (Thermo Fisher Scientific, Bremen, Germany), using Sequest as search engine, and the Swiss Prot database accessed on September 2016 as sequence database (8,109,403 sequences for Metazoa taxonomy). The following search parameters were used: MS tolerance 15 ppm; MS/MS tolerance 0.02 Da; fixed modifications carbamidomethyl cysteine; enzyme trypsin; max. missed cleavages 1. Search results were filtered by q-values using Percolator integrated in Proteome Discoverer, to achieve a peptide-level FDR of less than 1%. Minimum peptide count was 2.

Analysis
Descriptive analyses were performed using SPSS Inc, Chicago, version 21.0.

Results
Shrimp and primary mealworm allergic patients
The 15 shrimp allergic patients, of whom 13 had a food allergy to mealworm, had a median age of 38 years (range 19-69) and 47% was male. Sensitization to shrimp was shown by a positive ImmunoCAP (> 0.35 kU/L) with a median of 5.5 kU/L (range 0.37 to 53.3 kU/L). The majority had inhalant allergies to HDM (11/15) and pollen (11/15). 8 had atopic dermatitis and 9 patients had one or more other food allergies. None of the patients had knowingly consumed mealworm or other insects [3]. Four primary mealworm allergic subjects regularly exposed to some of the tested insects were also studied. In addition to mealworm, cricket was consumed by subjects 1, 3 and 4, grasshopper by subjects 2, 3 and 4 and lesser mealworm by subjects 1, 2 and 4. Wax moth was consumed only by subject 4 and giant mealworm only by subject 3. The super worm and black soldier fly had never been consumed by any of the primary mealworm allergic subjects. Oral allergy symptoms were reported for grasshopper in subjects 2 and 3, and cricket in subject 4; however, this was not confirmed with a food challenge.

Sensitization to all insects in shrimp allergic patients
Sensitization to mealworm was shown by a positive ImmunoCAP to mealworm (> 0.35 kU/L) in all shrimp allergic patients with a median of 5.8 kU/L (range 0.64 to 19.1 kU/L), see Table 1. IgE binding on dot blot to extracts of different insects, was found for all patients. This IgE binding was comparable to that of mealworm extract, except for two patients. These patients showed only IgE binding to proteins of a few insects, as shown in Table 1 (representative dot-blots can be found in figure E2). Basophil reactivity to all insect species was highly similar, see Table 2. The BAT results corroborated the dot blot findings, and showed to be somewhat more sensitive than CAP.

Cross-reactive proteins were identified using immunoblot. Different protein profiles were recognized by the shrimp allergic patients. The majority (9/15) recognized a band at approximately 40 kDa. Identification of this band in mealworm previously showed that it contained both tropomyosin and arginine kinase. Patient 12 showed also IgE binding to a 50 kDa proteins and patients 4, 8, and 10 mainly recognized bands with a molecular weight > 100 kDa, including a band at 200 kDa. Patients 9 and 11, who had no food allergy to mealworm, recognized proteins with different molecular weights compared to the other 13 patients. Figure 1a shows the coomassie stained protein gel of the insect extracts and figure 1b shows representative immunoblots of patients 2, 4, 9 and 12.

Insect sensitization of primary mealworm allergic subjects
The primary mealworm allergic patients were all sensitized to mealworm and subjects 3 and 4 had a food allergy to mealworm. Only subject 2 showed minor sensitization to shrimp but had no food allergy to shrimp, which was tested in a food challenge [4]. Although all subjects were sensitized to some common inhalant allergens (tree and grass pollen and animal dander), only subject 4 had mild rhinoconjuctivitis to HDM and birch pollen. Subjects 1 and 2 had atopic dermatitis and none had any other food allergy [4]. In contrast to the shrimp allergic group, primary mealworm allergic subjects did not show sensitization to all tested insect extracts on dot blot (Table 1). However, sensitization was shown in the BAT to all insects, with differences in reactivity. Subjects 2, 3 and 4, who had experienced clinical symptoms after eating grasshopper and cricket, showed a positive BAT for these insects (Table 2). Dot blot, immunoblot and basophil reactivity were different for all insects. All primary mealworm

allergic subjects recognized proteins between 10-200 kDa depending on the insect tested. Protein binding was mainly seen for black soldier fly, giant mealworm, cricket and wax moth, which confirms the dot blot data. Striking is that IgE from these subjects did not recognize bands with an MW of tropomyosin or arginine kinase. See Figure 1 for two representative immunoblots of the food allergic subjects 3 and 4.

Table 1. CAP mealworm and insect extract recognition on dot blot by shrimp allergic patients and mealworm allergic subjects. Cap IgE values are expressed in kU/L, + indicates a positive signal and - a negative signal on the dot blot.

Shrimp Patient	CAP mw	Dot mw	Dot cricket	Dot giant mw	Dot lesser mw	Dot grasshop-per	Dot wax moth	Dot black soldier fly
1	0.64	+	+	+	+	+	+	+
2	19.1	+	+	+	+	+	+	+
3	18.9	+	+	+	+	+	+	+
4	15.0	+	+	+	+	+	+	+
5	7.78	+	+	+	+	+	+	+
6	5.83	+	+	+	+	+	+	+
7	3.97	+	+	+	+	+	+	+
8	16.8	+	-	+	+	+	-	-
9	1.0	+	+	+	+	+	+	-
10	10.3	+	+	+	+	+	+	+
11	0.81	+	+	-	-	+	-	-
12	3.49	+	+	+	+	+	+	+
13	4.76	+	+	+	+	-	-	-
14	3.50	+	+	+	+	+	+	+
15	12.7	+	+	+	+	+	+	+
Mealworm Subject	**CAP mw**	**Dot mw**	**Dot cricket**	**Dot giant mw**	**Dot lesser mw**	**Dot grasshop-per**	**Dot wax moth**	**Dot black soldier fly**
1	0.75*	+	-	-	-	-	-	-
2	2.32*	+	+	+	+	-	-	-
3	2.26*	+	+	-	-	-	+	-
4	14.6*	+	+	-	-	-	+	-

* previously published [4] ; mw = mealworm

Table 2. BAT sensitization patterns to different insects, for shrimp patients and mealworm allergic subjects.

Shrimp Patient	@IgE	mw	cricket	giant mw	lesser mw	grass hopper	wax moth	black soldier fly
4	67.6	64.9	68.3	60.8	48.0	76.6	60.7	41.6
5	28.5	25.4	45.3	45.2	41.1	71.3	41.0	59.2
6	55.7	60.1	63.5	48.0	54.7	69.9	65.8	23.2
7	49.3	30.5	50.5	32.5	30.8	56.4	44.7	66.1
9	74.6	41.4	36.4	41.6	48.7	49.5	34.9	43.3
10	41.7	51	48.5	35.2	54.7	62.1	60.7	63.9
12	26.6	5.6	19.8	3.2	2.9	38.2	9.3	9.8
13	39.5	51.2	40.7	44.4	41.6	42.9	42.5	53.0
15	57	69.9	60.3	65.8	63.4	75.4	58	65.1
Mw Subject	**@IgE**	**mw**	**cricket**	**giant mw**	**lesser mw**	**grasshopper**	**wax moth**	**black soldier fly**
1	54.9	77.1	57.0	71.6	71.7	62.5	64.8	67.2
2	26.9	58.9	10.9	34.8	17.8	58.7	16.1	43.9
3	6.8	27.8	14.4	16.6	8.9	13.6	5.1	12.3
4	18.8	28.9	51.8	5.4	21.9	18.8	20.2	41.2

The results are expressed as a percentage of CD63+ basophils. The grey color codes represent the ratio of maximum % of CD63 up-regulation with respect to mealworm. mw = melaworm

<0.25	0.25-0.5	0.5-0.75	0.75-1.25	1.25<

Nano LC-MS/MS analysis

Proteins in the different insect extracts were identified and analyzed using LC-MS to clarify the sensitization results to the different insects. It can be concluded from Table 3 that both tropomyosin and arginine kinase were present in all insect extracts. A wide range of other proteins and putative allergens (e.g., myosin light chain and triosephosphate isomerase) were also identified in the different insect species (Table 3). The previously described new allergen, mealworm larval cuticle protein (LCP) was also

present in the giant mealworm and cricket extract, but was not found in the other insect species tested.

Discussion

Shrimp allergic patients with food allergy to mealworm showed IgE reactivity (blot and BAT) to all insect extracts. The main IgE binding proteins were tropomyosin and/or arginine kinase (9/13). Binding to other proteins with a MW of ~50 kDa and MW > 100 KDa was also seen. Primary mealworm allergic subjects showed sensitization to some tested insect extracts.

No clear similarities between insect sensitization patterns on the immunoblot were seen for the primary mealworm allergic subjects. Furthermore, basophil reactivity to the insect extracts tested was different for these subjects.

Figure 1a Coomassie stained gel of the insect extracts used in immunoblot

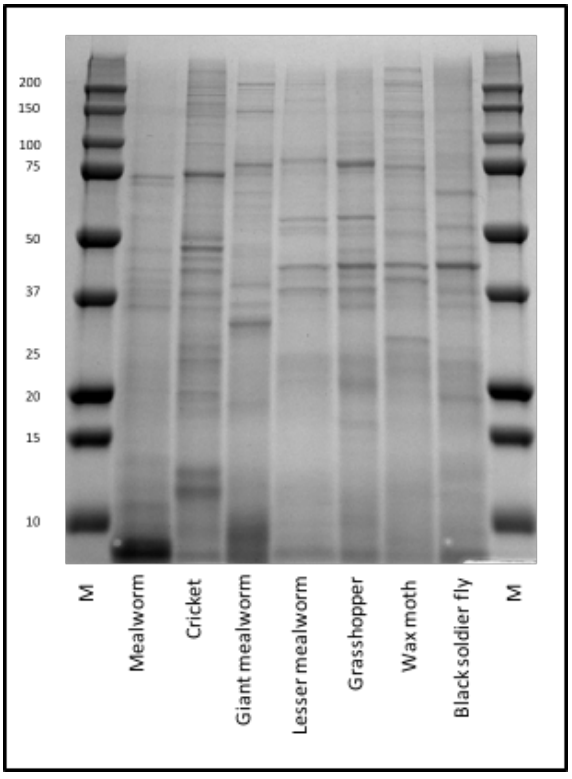
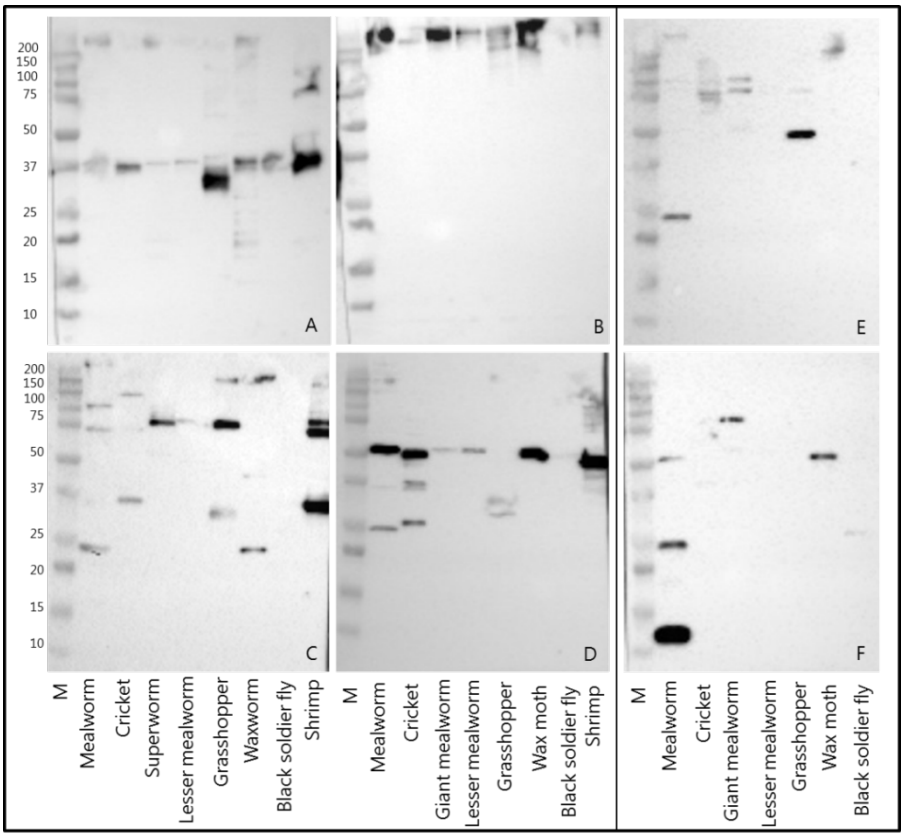


Figure 1b. Four representative immunoblots of two shrimp allergic patients 2, 4, 9 and 12 (A, B, C and D) and two primary mealworm allergic subjects 3 and 4 (E and F) with tris/urea insect extracts.



That IgE from shrimp allergic patients binds to proteins from different insects is not surprising because crustaceans and insects both belong to the same phylum (Arthropoda). Within the clade Pancrustacea, shrimp belong to the sub-phylum crustacea and insects belong to the sub-phylum Hexapoda [15]. As a result of this phylogenetic relation, homology between proteins of shrimp and different insects can be expected and has been previously documented. Sequence identity between arginine kinase from shrimp (*Litopenaeus Vannamei*) and silkworm (*Bombyx mori*) was reported to be 83% [16] and for tropomyosin from house fly and Dutch shrimp 76%

and up to 86% between cockroach and non-biting midge [17, 18]. High sequence homology is indicative for cross-reactivity, but must be confirmed by additional tests such as IgE binding (immunoblot), functional IgE binding (basophil activation test or skin prick test) or by a food challenge - the gold standard in food allergy research.

Only a few reports show cross reactivity between shrimp and insects. Srinroch et al. used pooled serum from 16 prawn allergic patients to show cross reactivity, on immunoblot, with arginine kinase from de African field cricket (*Gryllus bimaculatus*) and Lanchester's freshwater prawn (*Macrobrachium lanchesteri*) [19]. Another paper showed cross-reactivity, also using immunoblotting, between arginine kinase of Bombay locust (*Patanga succincta*) and IgE from pooled prawn allergic patient sera (n=16) [20]. Cross reactivity is also supported by the simultaneous IgE reactivity to different tropomyosins (e.g. cockroach, HDM, anasakis) on ImmunoCAP ISAC, a diagnostic tool used in food allergy diagnosis. To our knowledge, this is the first study to investigate cross-reactivity between IgE from shrimp and mealworm allergic patients with house cricket, giant mealworm, lesser mealworm, African grasshopper, large wax moth and black soldier fly.

A considerable number of arthropod allergens (e.g. tropomyosin, arginine kinase, myosin light chain and triosephosphate isomerase) were identified in the insect extracts using LC-MS/MS, which could also be involved in the cross-reactivity. Tropomyosin and arginine kinase were identified in all insect extracts, and most patients showed reactivity to these allergens.

Table 3. Allergens identified in Tris/urea insect extracts using LC-MS-MS

Allergen	mw	giant mw	lesser mw	cricket	grass hopper	wax moth	black soldier fly
Myosin light chain	x	x	x	x	x	x	x
Tropomyosin	x	x	x	x	x	x	x
Arginine kinase	x	x	x	x	x	x	x
Paramyosin	x	x	x	x	x	x	-
Troponin T	x	x	-	x	x	x	x
GAPDH	x	x	x	x	-	x	x
Triosephosphate isomerase	x	x	x	-	-	-	x
Apolipophorin	x	-	-	x	x	x	-
Profilin	x	-	x	x	x	-	-
Troponin C	-	x	x	-	x	-	-
Glutathione S-transferase	x	x	-	-	x	-	-
Hexamerin	x	-	-	-	x	x	-
Fatty acid binding protein	-	-	-	-	x	x	-
Hemolymph	x	-	-	-	-	x	-
Larval cuticle protein A1A	x	x	-	-	-	-	-
Larval cuticle protein A2B	x	-	-	x	-	-	-
Larval cuticle protein A3A	x						

Details on protein identification such as score, sequence coverage, peptides identified etc. can be found in the supporting information.

This supports the possibility of cross-reactivity to insects and confirms the comparable IgE binding and BAT reactivity results for the shrimp allergic patients. We therefore speculate that tropomyosin and arginine kinase were the most dominant allergens responsible for cross reactivity between shrimp and insects, but cross-reactivity to the other allergens cannot be excluded. In contrast to the shrimp allergic group, where similar reactivity's were seen between the different insect extracts, primary mealworm sensitized subjects showed variability in the degree and percentage of sensitization to the different insects. They showed lack of sensitization to some tested insect extracts (e.g. grasshopper, black soldier fly) in immunoblot and variable activation intensities in the BAT. Primary mealworm allergic subjects hardly recognized the pan allergens tropomyosin and arginine kinase, which is in line with our previous study where we showed that mealworm larval cuticle proteins instead of tropomyosin and arginine kinase seem to play a principal role in primary mealworm allergy [4].

Comparison of sequences from insect cuticle proteins was previously performed by Andersen et al. [21]. Some homology was found between cuticle proteins from different insect orders, while most have quite dissimilar sequences [21]. This is strengthened by the fact that LCP was only identified in two other insects in our study. A common peptide sequence was found in giant mealworm and cricket extracts, which was also documented for their clades by Andersen et al. [21]. This could explain the possible allergic reaction one of our primary mealworm allergic subjects experienced when consuming cricket and not with other insects. The low homology of inter-species larval cuticle proteins, the different protein binding profiles seen on immunoblot, and variation in BAT, might suggest primary sensitization to the different insects could be caused by different proteins. This indicates mealworm allergy is not indicative for insect allergy and suggests the possibility of species-specific insect allergy when primarily sensitized to insect-specific proteins. Species specific insect allergy has been described previously for housefly (*Musca domestica*) and cockroach [22, 23]. Binding of IgE from a person solely allergic to housefly was inhibited by housefly in ELISA, but only mildly by the closely related lesser housefly. No inhibition was seen with blowfly (*Lucilia spp*.), fruit fly (*Drosophila spp*.), horsefly (*Haematopota pluvialis*) and mosquito (*Culex pipiens*) [22]. Specific insect allergy was also described by Lopata et al, who demonstrated mono-sensitivity to American cockroach (*Periplaneta Americana*) or German cockroach (*Blattella Germanica*) in 17 out of 38

subjects [23]. Siracusa et al. showed inhibition neither from wax moth (*Galleria mellonella*) and mealworm (*Tenebrio molitor*) on greenbottle (*Lucilla caesar*), nor from greenbottle and mealworm on wax moth, nor from greenbottle and wax moth on mealworm [24]. These results are in line with our data, where the primary sensitized subjects can consume many other insects without symptoms.

Because of their quite homogenous reaction to all tested insect extracts, combined with the allergic reaction to mealworm in 87% of our previously studied shrimp allergic patients [3], we expect that shrimp allergic patients could likely have a clinical reaction when eating other insects.

The clinical relevance of mealworm sensitization in shrimp allergic patients was high (87%). Information on predictive values of insect sensitization and food allergy are scarce. Some information can be found on insect sensitization and inhalant allergies. Siracusa et al. found sensitization in 31.6% of 76 workers with live fish bait (e.g. mealworm and wax moth) to at least one insect. Work related asthma and rhinitis was seen in 29% of those workers [25]. Another study investigating occupational allergy to grasshopper (*Locusta migratoria*) [26] could not differentiate between symptomatic and asymptomatic patients, based on sensitization results. From eight sensitized subjects that worked in a breeding facility, five had clinical symptoms when working in the grasshopper facility. From the four primary mealworm sensitized subjects in our study, three developed respiratory symptoms when working with mealworm [4]. No other research has been performed studying food allergy to insects. Therefore, it is difficult to draw any conclusions on the clinical relevance of the insect sensitization in primary mealworm allergic subjects. DBPCFC is needed to translate sensitization to clinical outcome.

Based on the information presented in this paper we conclude that shrimp allergic patients are probably at risk not only for food allergy to mealworm but also to other insects. Given the variability in sensitization to other insects, primary mealworm allergic subjects are suspected not to react to all insects.

Supplementary Material

Figure E1 A-specific binding control immunoblots with serum from A: non-atopic person B; Grass-pollen allergic patient C: no serum (2e antibody only) and D: Cod-allergic patient with tris/urea insect extracts of different insects.

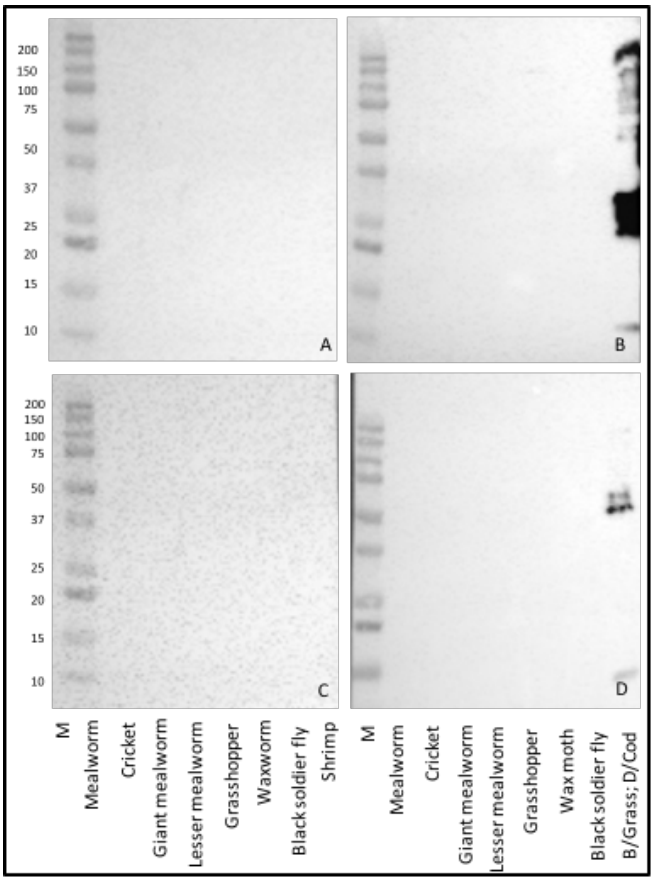
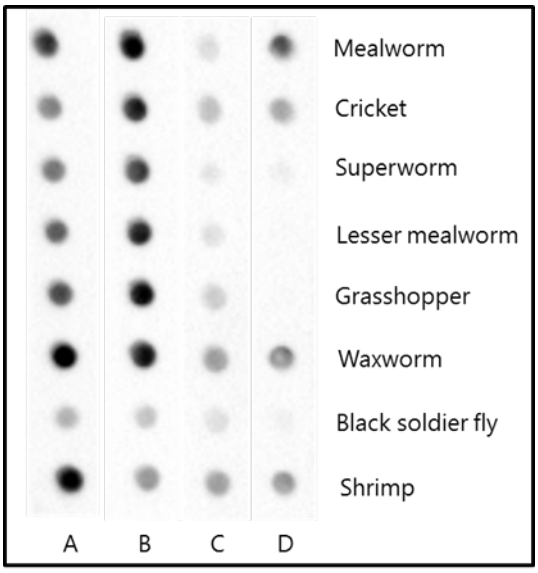


Figure E2 Four representative dot blots of 3
shrimp (A=2; B=4; C=8) and 1 mealworm allergic patient (D=Mw4).



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Chapter 8:

Summary and general discussion

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Summary and general discussion

Allergenicity assessment of new food protein sources and case studies on insects, with particular focus on mealworm

New and more sustainable food production is needed to face environmental decay and challenges such as the growing world population. When new foods are introduced onto the marked, precaution is needed to avoid food safety issues. When precaution for health and safety is taken, allergenicity is one of the items on the program. For novel foods, this is mandatory [1]. Structured approaches for allergenicity assessment were not available, which is why we have developed a structure approach to test allergenicity of novel foods (Chapter 2). In Chapters 3-7, the proposed approach was applied on insects as a potential novel food protein source, with particular focus on mealworm.

The proposed structured approach to asses allergenicity for novel protein sources is a step-wise approach (Chapter 2). The first step is the collection of general information on the product, comprising the possible history of exposure, possible phylogenetic relationships to known allergenic sources, protein identification, and information on intended use, such as the form of processing and levels of intake. For the successive steps these findings are of major importance, as these determine the identification of potential risk populations and the extracts and form of processing for assessment.

Chapter 3 explores different forms of processing for mealworm. The data showed that processing changed the solubility of the major allergens (tropomyosin and arginine kinase), which ended up in other fractions than expected. This emphasized the necessity of using different buffers in allergenicity assessment to allow for studying all proteins potentially involved in IgE reactivity. For instance, if only water soluble fractions would be used, a decrease in IgE binding due to processing could give the impression of a decreased allergenicity, while actually, allergenic proteins may have gotten other solubility characteristics and may be absent in water soluble fractions while still being present in the processed food and thus being able of inducing allergic reactions.

Using different extracts, the second part of the assessment was performed. In several steps, cross-reactivity of mealworm proteins was tested in a potential at-risk population: shrimp allergic patients. Shrimp is phylogenetically closely related to mealworm and shrimp allergic subjects could be allergic to homologous allergens possibly present in mealworm. Building on positive serological data from this potential risk group, clinical challenges were performed to proof or exclude actual allergy. The majority of the shrimp allergic patients (88%) showed IgE that cross-reacts with mealworm proteins (Chapters 4 and 5). Most (87% in 15 patients challenged) of these mealworm sensitized, shrimp allergic patients proved to be allergic to mealworm in a double blind placebo controlled food challenge (Chapter 4).

Based on phylogeny, another group potentially being at risk for mealworm allergy was identified: house dust mite (HDM) sensitized and allergic individuals. Part of the HDM sensitized patients shows sensitization to cross-reactive shrimp and HDM allergens (e.g. tropomyosin and arginine kinase). These patients were already included in the population studied in Chapter 4 and who showed a high prevalence of sensitization and allergy to mealworm. However, HDM sensitized and allergic individuals that are not sensitized to these cross-reactive shrimp-HDM allergens also showed IgE reactivity to mealworm proteins (22% of the study population) (Chapter 5). A seasonal rhinitis population was used as representative of another atopic population without known cross-reactive allergies and also showed, yet at a lower prevalence (16%), IgE reactive to mealworm proteins (Chapter 5). Non-atopic subjects showed no mealworm sensitization.

Besides cross-reactivity, a risk of potential primary sensitization to new proteins sources has to be considered. Information from a history of exposure may provide important information in this respect. Insect sensitization and allergy were previously described to occur upon consumption and in occupational and other settings [2,3]. With a search among professionals with a history of occupational exposure to insects and amateur breeders of mealworm, subjects with previous experience of allergic symptoms to mealworm were recruited for the study described in Chapter 6. In this study, the existence of mealworm (food) allergy without shrimp allergy was demonstrated. Mealworm larval cuticle proteins (LCP's) were shown to be involved in this primary sensitization to mealworm and not the pan allergens tropomyosin and arginine kinase. Because the included subjects all had a history of mixed respiratory, dermal and oral

exposure, a direct extrapolation in terms of risks upon introduction of mealworm proteins as food protein source cannot be made, but the results prove a primary sensitizing and allergy inducing potency of mealworm proteins.

Chapter 3-6 addressed the allergenicity of mealworm. Other insects could also be a potential new source of food protein, as is suggested by scientists and governmental bodies as FAO/WHO and EFSA [4]. Chapter 7 evaluates whether and to what extend the results for mealworm allergenicity can be extrapolated or applied to other insect species. Results show that, based on cross-reactivity patterns to proteins from other insects in shrimp allergic patients, similar risks as for mealworm should be expected if other insects, i.e. *Tenebrio molitor*; mealworm (larvae), *Acheta domesticus*; house cricket (bug), *Zophobas morio*; giant mealworm (larvae), *Alphitobius diaperinus*; lesser mealworm (larvae), *Locusta migratoria*; African grasshopper (bug), *Galleria mellonella*; large wax moth (larvae) and *Hermetia illucens*; black soldier fly (larvae) would be consumed by these patients. Recently other studies in shrimp allergic populations have shown similar *in vitro* results for *Gryllus bimaculatus*, *Zophobas morio*, *Alphitobius diaperinus*, *Locusta migratoria* [5-7]. Yet, primary mealworm allergic patients showed variable patterns of sensitization to the insects, and might therefore have a variable risk of allergy to other insects. Other studies seem to corroborate these findings, as some insect proteins show intra-class or intra-order cross-reactivity, but others mono-sensitization, without even inhibition from family-members [8-10].

Overall, the stepwise approach as proposed in Chapter 2 proved useful to assess mealworm allergenicity. These steps however do not necessarily elucidate a potential *de novo* sensitizing or allergy inducing potency. In case of a known history of exposure and sensitization to a novel protein source, aspects of *de novo* sensitization can be studied and be used for risk assessment. However, absence of proof of sensitization or allergy is not a proof of absence of a *de novo* sensitizing and allergy inducing potency, particularly if a sufficient history of relevant exposure is lacking. Also, because differences of sensitization patterns and allergy have been shown between cultures and geographical regions, safe use in one place might not automatically imply safety in another. Mustard for instance is reported to be a prevalent source of food allergy in France, but is far less prevalent in other European countries [11]. And, when kiwi was introduced to Europe, there was no proof of history of allergenicity in the countries it originated

from [12]. In Europe, it is nowadays reported as being one of the most common causes of food allergy [13]. These data illustrate, that prevalence can differ largely between cultures or regions and lack of proof of allergenicity, is not proof of lack of allergenicity. Therefore, irrespective of the opportunities described in this thesis, independent research into the possible *de novo* sensitizing and allergy induction potency of new proteins should be performed. Unfortunately, reliable predicting methodology is lacking for this. New approaches for testing and predicting the *de novo* sensitizing and allergy inducing potency of novel protein sources are needed to supplement our proposed approach. A similar conclusion was recently also drawn by a working group from the EU Cost Action project *Improving Allergy Risk Assessment Strategy for New Food Proteins* (ImpARAS) [14].

Ideally, a simple *in vitro* test would be capable of predicting the potential allergenicity of a new protein (source). However, in the assessment of the allergenicity the multifactorial nature of allergy should be considered. Theoretically, all proteins may be capable of inducing *de novo* sensitization and food allergy, but many interacting factors will determine whether or not a protein will be or become a major allergen [15]. These factors include the exposure to the protein (the amount of protein per serving and the frequency), the influence of processing, digestion and the matrix, and absorption and presentation to and by cells of the immune system [15,16]. It is therefore unlikely that a single *in vitro* assay will ever be able to have a sufficient predictive power. *In vivo* approaches automatically provide opportunities to include many of the involved influential factors. Animal models have been capable of demonstrating *de novo* sensitization and can be used for studying mechanisms in allergy [17], but despite many past attempts, so far none showed a sufficient predictive value for assessing the safety or risks of new proteins or protein sources [18]. Crevel et al. applied a study protocol with human volunteers to investigate whether the intended new protein additive Ice Structuring Protein (ISP) would cause sensitization when ingested at the expected dose [19]. One of the limitations of such an approach is the limitation in time. The volunteers were only exposed to the protein on a daily base for two months, in which period; IgG levels against the protein could be measured, but no IgE. This leaves room to speculate whether the IgE levels would rise when exposed for a longer period of time. Further, such studies can only be conducted with a limited number of healthy test persons under relatively controlled conditions and will not necessarily predict the outcome of consumption by

large populations, including atopic and allergic subjects, under many different circumstances and with ranging influential factors. The limited predictive value of a human study would pose additional ethical constraints on top of the general ethical considerations human testing poses. To account for the role of the most important factors, a combination of tests and parameters will possibly in future provide the best approach for assessing the allergenicity of new proteins or protein sources.

Interpretation, extrapolation and discussion of the results in terms of population risks of insects as new food protein source

We showed that insects as potential new food protein sources pose significant risks to shrimp allergic patients. Shrimp belongs to the crustaceans. High sequence homology of the major allergens from different crustaceans has been proven [20]. Serological and functional tests such as SPT and basophil activation show cross-reactivity between the various crustacean species. Unfortunately, most data on this cross-reactivity stem from *in vitro* results and not clinical evidence. Nevertheless, based on the presented results on the risks of insect allergy and the strong intra-crustacean cross reactivity, we expect the risks of insects for patients allergic to other crustaceans, such as crab or lobster, to be comparable as for shrimp allergic patients.

The results from our studies cannot be as easily used to assess the risk of mealworm allergy for patients with a mollusk allergy. The intra-class homology for mollusks is similarly high for tropomyosins as for crustaceans (ranging from 82% to 100% between the different mollusks, comparable to the intra-crustacean homology). But even though the same major allergens are involved in the two, there is less cross-reactivity between crustaceans and mollusks (56-65%) [21,22]. Sicherer et al. showed from data of a telephone survey that only 14% of patients report both crustacean and mollusk allergy [23].

Our studies demonstrated that most shrimp allergic patients are sensitized and allergic to mealworm. We did not investigate whether and therefore cannot exclude that shrimp sensitized patients without shrimp allergy might also be at risk of mealworm allergy. Further studies would be needed to address this question. We also showed that 22% of a HDM allergic population without tropomyosin, arginine kinase or shrimp sensitization and 16% of a seasonal rhinitis population showed mealworm-protein

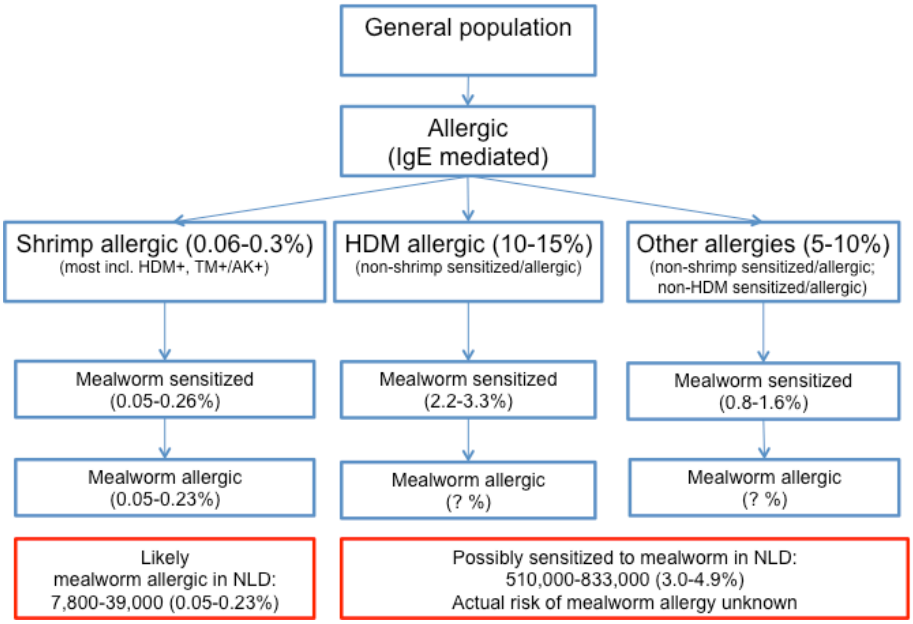
reactive IgE. Although a higher prevalence of sensitization to mealworm was found in the population of shrimp allergic patients, HDM allergy and seasonal rhinitis are much more prevalent. Lifetime prevalence for shellfish allergy is around 0.1% in Europe, to 2% in the US. Prevalence of HDM induced rhinitis is estimated at about 17.1% for by some and 17.6% and 9.7% for grass and tree pollen sensitized seasonal rhinitis [24-26]. Others report 12.7 % in the Netherlands affected by perennial allergic rhinitis and 6.6% of seasonal rhinitis [27]. This could mean that, even with lower percentages of patients with mealworm-reactive IgE, the number of patients at risk of mealworm allergy for these two groups may be higher than the number of shrimp allergic patients at risk. It should be noted that sensitization to mealworm in the seasonal rhinitis and HDM allergic populations could be caused by cross-reactivity to other insects, particularly because primary sensitization to insect proteins in the seasonal rhinitis population could be an explanation of the IgE reactivity to mealworm proteins. It therefore seems likely that atopic individuals in general may be expected to show some prevalence of (primary) sensitization to insect proteins. Such primary sensitization may result from historical exposure to insect proteins. We are all exposed to insect allergens, both aerosolized and as food contamination [28,29]. In the US one of the major causes for respiratory allergies is the cockroach, and in other climates, other insects thrive. Allergens from those insects, such as moths or mosquitoes have shown to cause respiratory allergies [30].

Further, we demonstrated that primary allergy to insects may develop due to insect exposure. In theory, besides the development of such primary insect allergy, cross-reactive allergies might develop due to insect-protein sensitization. Reasoning the other way around, development of shrimp or HDM allergy cross-reactive to mealworm allergy could be potential risks. In the four primary mealworm sensitized subjects, shrimp allergy was excluded and HDM sensitization and allergy was only present in one subject, but it cannot be excluded that cross reactive allergies might be demonstrated if larger populations would be studied or with prolonged exposure to insects. In patients with both HDM and shrimp allergy, many studies have tried to elucidate the primary sensitization route; did shrimp sensitization cause HDM allergy or did HDM sensitization cause shrimp allergy? The outcome of these studies differs. Most inhibition studies find HDM as a primary sensitizer, and not only caused by cross-reaction to tropomyosin [31]. A recent study from Spain demonstrated, using inhibition studies, that HDM is the primary sensitizer in shrimp allergic

subjects in humid climates, while in dry climates shrimp was the primary sensitizer of shrimp allergy [32]. These data indicate that the development of cross reactive allergies such as shrimp allergy or HDM allergy due to insect sensitization cannot be excluded but further studies would be needed to assess this possible risk.

The results obtained in this thesis can be extrapolated to general populations, e.g. the Dutch general population, to assess the potential health impact of a possible introduction of insect proteins, e.g. mealworm protein, as a new food protein source. We showed that 88% of shrimp allergic patients are sensitized to mealworm and 87% of these mealworm-sensitized patients are actually mealworm food allergic. The prevalence of shrimp allergy is estimated at about 0.1% (0.06-0.3) of the general population [25]. Based on these numbers, it can be estimated that 0.05-0.23% of the general population may be mealworm allergic due to a shrimp allergy, which equals to 7,800-39,000 individuals in the Netherlands. This is a likely estimate of the minimal size of the population with mealworm food allergy, since also the HDM allergic and other atopic populations might be at-risk. Although the percentages of patients sensitized to mealworm in these latter groups are lower than in the shrimp allergic group (presumably 16% to 22% versus 88%), on a general population level these groups may concern substantially larger potential at-risk populations. Based on the WAO White Book on Allergy (WAO 2013), a prevalence of about 10-15% may be assumed for HDM-allergy and 5-10% for other IgE-mediated allergies (in absence of shrimp or HDM allergy) [33]. A calculation of the potential sizes of the various at-risk populations is given in Figure 1. Based on these numbers, the percentage of the population possibly sensitized to mealworm could be up to 4.9%, corresponding to up to 833,000 people in the Dutch general population. With the data available, we cannot predict which percentage of mealworm sensitized non-shrimp allergic individuals might actually have mealworm food allergy. Further studies would be needed to characterize the actual risks in these populations. The numbers discussed above and in Figure 1 only address risks of potential existing sensitization in the population. We showed that exposure to mealworm may also induce primary sensitization and allergy. No data are available to characterize the risks of such primary sensitization and resulting primary allergies and possibly resulting cross-reactive allergies. These potential risks therefore were not taken into consideration in the numbers discussed above and in Figure 1. Further studies would also be needed to characterize these risks.

Figure 1. Schedule of potential at-risk populations for mealworm food allergy due to existing sensitization in the general Dutch population

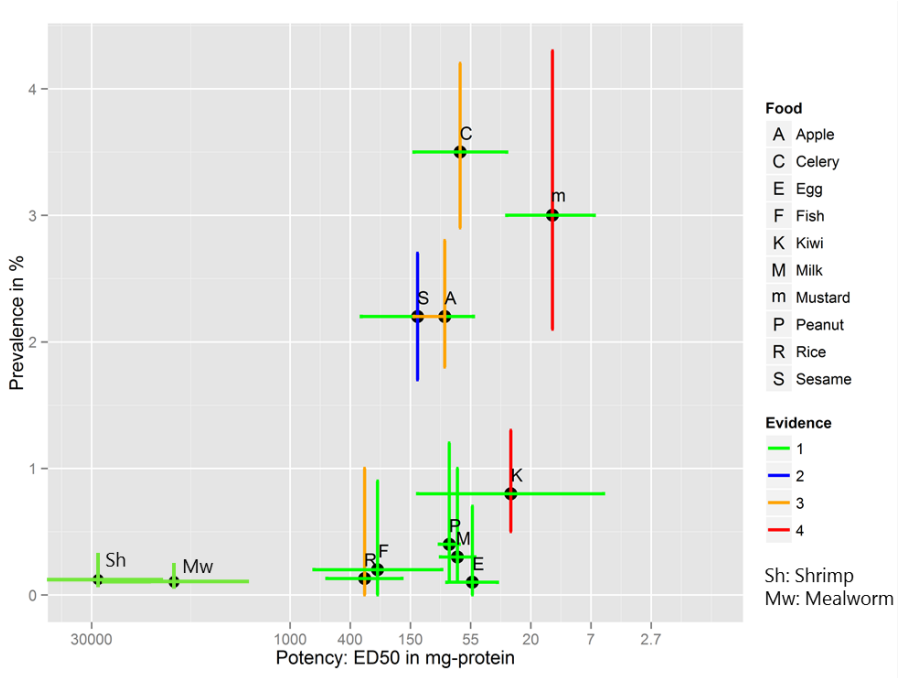


* : based on White book on allergy, WAO 2013
** : based on Nwaru 2014
*** : based on this thesis

The relevance of the risks of mealworm allergy as assessed in this thesis in terms of the potential relative health impact of mealworm sensitization can be assessed by using an approach for scaling and comparing the population's allergies to different foods as recently proposed as a proof of principle by Houben et al. [34]. The scaling approach is based on the allergy elicitation thresholds in allergy sufferers to foods and the prevalence of allergy for foods. Information on these thresholds in individuals allergic to mealworm is available from the studies in Chapter 4 and 6 from the shrimp allergic population and several primary sensitized subjects. The ED50 value for the mealworm allergic responders in de DBPCFC studies was 7 grams of mealworm protein using log-logistic distribution (95% CI: 1.8 - 27.5) as was

shown in Chapter 4. The ED50 value for shrimp was 28 grams (95% CI: 9.6 - 82) [35]. The percentage of the population affected by mealworm food allergy can be assumed to be minimally 0.05% to 0.23% if only 77% of the shrimp allergic population would be allergic to mealworm (see Figure 1). The minimal health impact of the allergenicity of mealworm relative to that of other allergenic foods is visualized in Figure 2.

Figure 2. The potential health impact of mealworm food allergy relative to that of other allergenic foods, based on a likely estimate of the minimal size of the population with mealworm food allergy (see text) figure adapted from Houben *et al.* 2016. Evidence levels in order of quality of data.



Evidence level 4 is Self-reported to evidence level 1 (for shrimp and mealworm): highest level of reliability of data (prevalence and potency based on DBPCFC-confirmed allergy)

Mealworm allergy could have a similar impact on the general health of the population as shrimp allergy. However, if (parts of) other populations with mealworm-reactive IgE are also mealworm allergic, a higher prevalence would apply. For these other populations, the potency of mealworm

should be assessed further, as a potency difference to the populations from Chapters 4 and 6 was not studied.

This thesis provides information of use to various stakeholder groups. The most important are the allergic patients and their health care providers. Both groups should be aware of the risk the introduction of insect proteins might pose. Medical doctors should be informed of the cross-reactive risk, to educate the patients in their care, and make the link when a patient presents with symptoms. Depending on legislation, it might be considered to label products containing insect proteins to warn the crustacean allergic population of the risk these might pose to them. However, such labeling would not necessarily sufficiently inform or warn other populations potentially at risk, such as HDM allergic or other atopic populations. Risk managers, both public and private, and regulators should carefully consider the various (potential) risks of new food proteins sources and assess the possible risk management options and based on this, assure a careful risk-benefit decision-making and implementation of appropriate risk management measures. Other legitimate factors such as the need for improving the sustainability of our growing food supply may play a role in the risk-benefit decision-making and may force us to accept a certain level of risk of new food protein sources. The approaches we propose and tested can in this case be used to assess and compare the allergenic health risks of various alternatives to each other as well as to existing foods.

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***Nederlandse samenvatting*:**

Nederlandse samenvatting

Nieuwe duurzame eiwitbronnen zijn nodig om de groeiende wereldbevolking te voeden en te zorgen voor zo min mogelijke belasting van het milieu. Wanneer nieuwe voedingsmiddelen op de markt worden gebracht voor consumptie door de mens, moeten ze voldoen aan diverse veiligheidseisen. Een van de veiligheidseisen is dat mogelijke allergeniciteit onderzocht moet worden. Dit staat in de richtlijnen voor nieuwe voedingsmiddelen, geregeld onder de zgn. 'Novel food law'. Voedingsmiddelen vallen onder deze regeling wanneer ze voor mei 1997 nog niet in substantiële mate in Europa werden genuttigd. Richtlijnen over hoe de beoordeling van allergeniciteit uitgevoerd moet worden ontbraken echter. Daarom is als onderdeel van dit proefschrift een gestructureerde aanpak opgezet die vervolgens is toegepast op een potentiële nieuwe eiwitbron, nl. meelworm (*Tenebrio molitor*). Meelworm behoort fylogenetisch tot de klasse insecten en zijn een voedzame en, in vergelijking met vlees, meer duurzame eiwitbron.

De eerste stap van de voorgestelde, gestructureerde aanpak voor allergeniciteit beoordeling bestaat uit het verzamelen van algemene informatie over de (potentiele) eiwitbron: de eventuele geschiedenis van blootstelling van de mens aan het product (in Nederland of daarbuiten). Daarnaast wordt mogelijke fylogenetische verwantschap (bv. zelfde fylum of klasse) met bekende allergeen bronnen in kaart gebracht en vindt identificatie van de eiwitten en de vorm van toekomstig gebruik (zoals de manier van verwerken of bereiding en de hoeveelheid product die zal worden genuttigd) plaats. Uit het onderzoek bleek dat meelworm eiwitten bevat die veel overeenkomst vertonen met allergenen in o.a. garnaal, andere schaaldieren en huisstofmijt (HSM). Ook werd duidelijk dat meelworm in meerdere vormen geconsumeerd zou kunnen gaan worden: gefrituurd, gebakken, maar ook geblancheerd.

Voor de vervolgstappen waren deze bevindingen van belang, omdat daaruit kon worden afgeleid wat de evt. risico populaties (garnaal/HSM allergische mensen) zijn. Dit hielp om de belangrijkste studie populaties voor het onderzoek naar allergeniciteit vast te stellen. Daarnaast kon worden afgeleid aan welke vorm de mensen blootgesteld zullen gaan worden, wat belangrijk is om de test extracten te definiëren (mogelijk zijn de verschillende eiwitten niet in iedere buffer oplosbaar).

In dit proefschrift is het effect van verschillende vormen van bereiding van meelworm op allergeniciteit onderzocht. Het onderzoek liet zien dat de mate van oplosbaarheid van verschillende belangrijke eiwitten (zoals tropomyosine en arginine kinase), door de bereidingswijze werd beïnvloed, maar de allergeniciteit niet. Uit het onderzoek kwam duidelijk naar voren dat verschillende buffers nodig waren om zoveel mogelijk eiwitten en daarmee potentiele allergenen in oplossing te brengen om deze vervolgens te kunnen bestuderen. Het gebruik van alleen een waterige buffer kan leiden tot verkeerde conclusies, omdat na processing eiwitten onoplosbaar kunnen worden, maar nog steeds allergeen zouden kunnen zijn.

De volgende stap in de gestructureerde aanpak is het bepalen van mogelijke kruisreactiviteit. Omdat meelworm en garnaal fylogenetisch nauw verwant zijn en analogen van bekende allergenen in garnaal (tropomyosine en arginine kinase) ook in meelworm werden aangetoond, lag het voor de hand om de mogelijke allergeniciteit van meelworm te onderzoeken bij garnaalallergische patiënten.

Ook hierbij werd een stapsgewijze aanpak gevolgd. Eerst werd nagegaan of garnaalallergische patiënten gesensibiliseerd waren voor meelworm, dat wil zeggen of er IgE antistoffen die meelwormeiwitten herkenden, aanwezig waren in hun bloed. Dit werd gedaan met 4 verschillende testmethoden (BAT, blot, ISAC, ImmunoCAP). Bij de meeste garnaalallergische patiënten (88% van 60 patiënten) werden IgE antistoffen tegen meelworm aangetoond. Om te onderzoeken of deze sensibilisatie klinisch relevant was, werd als volgende stap een dubbelblinde voedselprovocatie met meelworm uitgevoerd. Bij deze voedselprovocatietest vertoonde de meerderheid (13 van 15) van de patiënten met IgE tegen meelworm, allergische klachten bij het eten van meelworm. Deze klachten varieerden van orale allergieklachten tot gastro-intestinale klachten en benauwdheid en waren te classificeren als mild tot ernstig.

Behalve garnaalallergische patiënten, lopen ook HSM allergische patiënten een mogelijk risico op meelworm allergie, vanwege de eerdergenoemde fylogenetische verwantschap. Een deel van de HSM allergische patiënten reageert op allergenen van HSM (tropomyosine en arginine kinase) die ook door garnaalallergische patiënten worden herkend. Deze groep patiënten was ook geïncludeerd in de garnaalallergische groep. Het overgrote deel van de HSM allergische groep (~90%) herkent echter geen tropomyosine of

arginine kinase, maar andere allergenen van HSM. In deze HSM allergische patiëntengroep werd bij 22% eveneens meelwormsensibilisatie aangetoond. Tevens zijn ook patiënten met seizoensgebonden neusklachten, maar zonder sensibilisatie of allergie voor huisstofmijt getest. Ook daarbij werd in 16 % van de patiënten sensibilisatie voor meelworm gevonden. In een controlegroep van mensen zonder aanleg voor allergische ziekten werd geen sensibilisatie voor meelworm aangetoond.

Deze bevindingen geven aan dat de groep die risico loopt op een allergie voor meelworm mogelijk breder is dan alleen de garnaal (schaaldier)-allergische populatie. Echter, de klinische implicaties van deze bevindingen moeten nog verder onderzocht worden.

Behalve een risico op sensibilisatie/allergie voor meelworm bij patiënten met sensibilisatie of allergie voor vergelijkbare allergenen (co-sensibilisatie of kruisrectie), moet ook rekening worden gehouden met de mogelijkheid dat nieuwe eiwitten een nieuwe (tot nu toe niet bekende) allergie kunnen veroorzaken. Dit wordt ook wel een 'primaire allergie' genoemd. Of meelworm een nieuwe (voedsel)allergie zou kunnen veroorzaken, werd onderzocht in een volgende stap. Mensen die t.g.v. hun hobby of beroepsmatig in aanraking komen met meelworm kunnen mogelijk gesensibiliseerd raken en dus allergisch worden. In dit onderzoek is daarom nagegaan of professionele en hobbymatige meelwormkwekers mogelijk allergische klachten hebben ontwikkeld. Vier personen die klachten hadden bij contact met en/of het eten van meelworm, waarbij ook IgE antistoffen tegen meelworm in het bloed waren aangetoond, zijn aan een nader onderzoek onderworpen. Bij twee personen werd een voedselallergie voor meelworm aangetoond. De andere twee waren op basis van anamnese en serologie zeer verdacht voor een inhalatieallergie voor meelworm. Geen van deze vier personen had een allergie voor garnaal en slechts één van de vier had een HSM allergie, zodat kruisreactiviteit als verklaring hiervoor onwaarschijnlijk was. Dit werd bevestigd door het ontbreken van sensibilisatie tegen de bekende allergenen tropomyosine of arginine kinase in drie van de vier personen (waaronder ook de HSM allergische persoon). Een ander meelwormallergeen, nl. het Larval Cuticle Protein (LCP), lijkt deels of volledig verantwoordelijk voor deze primaire meelwormallergie.

Omdat de personen met een primaire meelwormallergie op meerdere manieren (via inhalatie, huid en voeding) blootgesteld zijn aan meelworm kan er geen conclusie getrokken worden over de risico's op primaire allergie wanneer insecten als voedingsbron gebruikt gaan worden in de algemene bevolking. Het risico hierop is in elk geval niet uitgesloten.

Omdat naast meelworm, ook andere insecten als mogelijke nieuwe eiwitbron gebruikt kunnen worden, is in dit project ook de allergeniciteit van enkele andere insecten bestudeerd. Hiervoor is serum van dezelfde patiëntengroepen (garnaal- en primair meelwormallergische patiënten) gebruikt.

De garnaalallergische patiënten bleken naast *Tenebrio molitor*; (meelworm), voor het overgrote deel gesensibiliseerd voor *Acheta domesticus*; (huiskrekel), *Zophobas morio*; (morio- of super-worm), *Alphitobius diaperinus*; (buffalo- of lesser-meelworm), *Locusta migratoria*; (Afrikaanse treksprinkhaan), *Galleria mellonella*; (grote wasmot) en *Hermetia illucens*; (zwarte soldatenvlieg). Hoewel de klinische relevantie van deze resultaten (nog) niet onderzocht is d.m.v. dubbelblinde provocatie, geven deze resultaten aan dat er een risico is dat ook deze insecten allergene risico's met zich meebrengen voor garnaal- en waarschijnlijk alle schaaldier-allergische patiënten.

De mensen met een primaire meelwormallergie lieten wisselende patronen van sensibilisatie voor de verschillende andere insecten zien. Dit wijst erop dat het risico op allergie voor insecten bij deze personen anders is dan bij de garnaal- of schaaldier allergische personen. Andere allergenen dan tropomyosine en arginine kinase lijken hierbij betrokken.

Samengevat blijkt de gestructureerde stapsgewijze aanpak een goede methode om de risico's van nieuwe eiwitbronnen in kaart te brengen. Het op de markt brengen van insecten brengt een duidelijk risico op allergie met zich mee voor garnaalallergische patiënten en waarschijnlijk voor alle schaaldier allergische patiënten. Een risico voor patiënten met andere allergieën kan niet worden uitgesloten. Insecten kunnen ook een primaire allergie veroorzaken. Onderzoek, zoals in dit proefschrift beschreven is van belang om de veiligheid van nieuwe voedingsmiddelen te kunnen waarborgen. Ook zal dit besluitvorming ten aanzien van de toelating van nieuwe voedingsmiddelen en/of de eventueel te nemen risicomanagementmaatregelen ondersteunen.

Abbreviations, List of publications and contributing authors

Abbreviations:

BAT basophil activation test,

CT Cholera toxin,

DBPCFC double blind placebo controlled food challenge,

EFSA European food and safety authority,

GM Genetically Modified,

GMO Genetically Modified Organism,

HDM house dust mite,

HSM huisstof mijt,

LC-MS/MS Liquid chromatography-tandem mass spectrometry,

LCP Larval Cuticle Protein,

OFC oral food challenge,

PBST PBS containing 1 % Tween 20,

SCP sarcoplasmic calcium-binding protein,

SPT skin prick test

WHO world health organization

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Acknowledgements (Dankwoord) and Curriculum vitae:

Acknowledgements (Dankwoord)

Zonder de hulp en het enthousiasme van alle patiënten was dit proefschrift er niet geweest. Zij hebben het mogelijk gemaakt door hun enorme bijdrage dat we mooie resultaten hebben weten vast te leggen.

Daarbij kunnen ook niet ontbreken alle werknemers van Kreca en dan speciaal ook de hele familie Calis die enorm geholpen hebben bij het leren kennen van de insecten industrie.

Van Venik: Marian Peters, door ons met de insecten industrie kennis te laten maken.

En voor het maken van alle meelworm snacks: Ruig. Hierbij hebben Eelco Botter en Jan Ruig ons erg geholpen met een lekkere snack om dubbelblind mee te kunnen testen.

Binnen het UMC:
Ongelooflijk leuk dat naar het einde toe, ik twee promotoren heb gekregen: André en Carla. Ik ben bijzonder vereerd dat ik jullie beide als voorbeeld heb gehad en het voorrecht heb deze twee grote namen in de Dermatologie en Allergologie op mijn proefschrift te mogen hebben staan.

Stans, Judith, Lieneke, Florine, Mary-Ann, Floor, Wouter, Rob, Jorien mijn benchmaatje en al mijn fijne kamergenootjes, met jullie zou ik zo opnieuw zo'n traject starten, het is dus maar goed dat we met een groot deel verder mogen en makkelijk even samen kunnen lunchen met Stans!

Ook voelde ik me altijd gesteund op het lab, speciaal door Edward, Simone, Jan, Helma en Laura. Laura en Helma zijn ook doorgegroeid naar andere locaties, maar zullen voor mij toch op het lab horen. Simone, zet m op je laatste jaartje!

Ischa, bedankt voor al je goede zorgen! Verder was het zonder Ans, Astrid, Jos, Ellen, Marieke, Mignon, Mark, Francine, Els, Laury, Henny en groep Otten, Rowena, Sharrida, Astrid, Nienke, Barbara, Thuy-My, Margaret, Anna en Hannah nooit zo'n geslaagde periode geweest, door al jullie hulp aan aanmoedigingen zijn de jaren voorbijgevlogen!

Op de poli: Elly, Linda, Doreen, Hayat, jullie hulp en enthousiasme heeft me altijd vrolijk gemaakt. Gelukkig zie ik jullie nog lekker veel!

Drie super diëtistes: Dieuwke, Anouska en Anne, altijd fijn om met jullie te mogen samenwerken en altijd met een goed gevoel alle patiënten door te kunnen sturen of te kunnen bespreken. Jullie zijn bijzonder en waardevol voor onze afdeling, fijn dat ik dat van zo dichtbij heb mogen zien!

Samenwerking met het AMI-lab was zeer fijn door Coby, Willy en Saskia. Ik mocht altijd weer een nieuwe portie meelworm serum aanleveren, hoe fijn jullie altijd reageerden en hoe snel alles altijd kon worden gedaan, ideaal!

Natuurlijk zijn Kitty en Geert ook UMC, maar er zit toch meer TNO in de basis. Dat heeft voor hele fijn discussies gezorgd en het hele boek aangescherpt tot wat het is geworden. Hopelijk zullen er nog veel van deze mooie projecten worden gedaan. Heel veel dank voor nog meer Zeist gevestigde TNO-ers. Zonder Aard, Astrid, Ben, Jolanda, Marty, Emmeke, Sabina was alles minder mooi geworden. Zij hebben allen erg geholpen en mij veel geleerd.

Van de Nvwa: fijne samenwerking met Jaqueline Castenmiller en Hub Noteborn, hartelijk bedankt hiervoor.

Special thanks to Jonas Lidholm, Måns Österberg Widerstrand, Jucia Jimeno Nogales, Marco Gaspari, Caterina Gabriele, Francesca Monteleone for their much-appreciated help with extracts, immunoCAP and LC-MS/MS.

From the collaboration in ImpARAS, it's been an honour to have gotten to know and work with Annabelle Capt, Anne Constable, René W.R. Crevel, Ivan Dimitrov, Christiane K. Faeste, Rocío Fernandez-Canton, Stavroula Giavi, Kevin C. Glenn, Charlotte B. Madsen. And the special work together with Katrine L. Bøgh, Julia Upton and Thomas Eiwegger.

Mijn schoonfamilie voor al de steun die ik voelde en de heerlijke dinertjes om me even af te leiden.

Mijn ouders, Mie en I Wen, Vesper en Fletcher en natuurlijk speciaal Pip voor de mooie pen die ik begin dit jaar kreeg om dit boek mee te kunnen

schrijven, bedankt voor het er zijn en af en toe klankbord voor m'n gedachten willen zijn.

Maart, omdat je bent.

Curriculun vitae

Henrike Broekman was born on the 28th of November 1980 in Utrecht. She graduated secondary school in 1999 from Utrechts Stedelijk Gymnasium. During medical school, she developed an interest in research when she did a researchproject in the children's hospital in Boston. The interest for allergy became apparent when studying peanut allergy in her final year at the department of Dermatology and Allergology at the UMC Utrecht.
After graduation, she started a PhD program under Prof. Bruijnzeel-Koomen and Prof Knulst, leading to this thesis. At the start of 2017 she started the Dermalology recidency program at the UMC Utrecht.
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