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Exploring the chemical safety of fly larvae as a source of protein for animal feed

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RESEARCH ARTICLE

Abstract

There is an urgent need to increase the supply of sustainable protein for use in animal feed and the use of insect protein provides a potential alternative to protein crops and fishmeal. For example, fly larvae are highly compatible with use in animal feed containing much digestible protein with levels of key amino acids that are comparable with those found in high value alternatives such as soybean. However, the safety of protein from insects and subsequently the meat and fish from animals fed on such a diet requires further assessment. Here we present safety data from the larvae of the four fly species that have perhaps the greatest economic relevance in relation to their use as animal feed being: house fly (*Musca domestica*), blue bottle (*Calliphora vomitoria*), blow fly (*Chrysomya* spp.) and black soldier fly (*Hermetia illucens*). Diverse rearing methods were used to produce larvae fed on a range of waste substrates and in four geographically dispersed locations being; UK, China, Mali and Ghana. Chemical safety data were collected by a fully accredited laboratory in the UK. The levels of the main subclasses of chemical contaminants considered for animal feed were determined, being; veterinary medicines, pesticides, heavy metals, dioxins and polychlorinated biphenyls, polyaromatic hydrocarbons and mycotoxins. The larvae analysed generally possessed levels of chemical contaminants which were below recommended maximum concentrations suggested by bodies such as the European Commission, the World Health Organisation and Codex. However, the toxic heavy metal cadmium was found to be of concern in three of the *M. domestica* samples analysed.

Keywords: feed, fly larvae, hazards, protein

1. Introduction

Increasing demand for food (particularly meat, fish and eggs) has led to an urgent need for new, safe supplies of protein from sustainable sources for inclusion in animal feed. Here we use an analytical approach to measure the levels of potential chemical contaminants in the species of fly larvae that have greatest commercial potential amongst insects for the production of protein for animal feed.

More than 40 million tonnes of crop proteins, primarily soybeans and corn gluten feed, are imported annually into EU countries representing 80% of the EU's crop protein consumption (Häusling, 2011). The availability of food for human consumption at the global level is heavily impinged upon by the demands that livestock production places on land and water use. It has been estimated that around three quarters of the world's agricultural area is devoted to producing livestock either directly or indirectly (Foley *et al.*, 2011). Production of feed crops represents 24% of global crop production by mass (Cassidy *et al.*, 2013). Animal protein production is estimated to require 5-20 times more water than that required for the production of cereal protein on a per kilogram basis (Chapagain and Hoekstra, 2003), but

when the water required for forage and grain production is included in the equation this figure approaches 100 times (Pimentel and Pimentel, 2003) and this places considerable stress upon the sustainability of the global water supply.

Invertebrates contribute to the natural diet of wild fish and 'free range' monogastric livestock across the world offering the potential to be used effectively as alternatives to other animal and soy based proteins in animal feed. Insects thrive on waste products from various sources including those which have no other use; they efficiently convert nitrogenous compounds into valuable protein whilst requiring fewer valuable resources such as land and water per unit protein than protein crops (Van Huis, 2013).

The production of insects specifically with the intention of being fed to domestic animals has been the subject of evaluations for several decades (e.g. Bondari and Sheppard, 1987; Hem *et al.*, 2008; Newton *et al.*, 2005), but has not yet reached a stage that has led to any significant replacement of traditional protein used for livestock production with insect based protein. Importantly, much of the work to date has made little or no attempt to process insect protein into amenable products or to assess safety, social and acceptability issues.

The work presented here is part of an EU funded research project (PROteINSECT) and therefore the results obtained are interpreted in the context of EU legislation, however similar rules exist in other parts of the developed world e.g. North America. In the European Union, the use of insects as a source of protein for animal feed is currently prohibited for animals raised for human consumption under regulation EC 999/2001 (EC, 2001), which prohibits all processed animal protein (PAP), with the exception of hydrolysed proteins and in some cases fishmeal, from being used in animal feed. A recent amendment to this legislation (EU Regulation 56/2013; EC, 2013) allows the use of non-ruminant PAP in fish feed although this currently does not extend to the use of PAP derived from insects. Further proposed amendments such as the extended use of non-ruminant PAP (possibly including insects) are currently difficult to implement due to the lack of a clear method of species origin determination in PAP. It is highly unlikely that insects will be permitted in animal diets until thorough consideration of the safety of their use has been made and diagnostic methods for the detection of processed insect protein in animal feed are available. A key consideration for feed suppliers is the safety of raw materials and potential risks from the use of insect protein include; chemical contaminants, parasites, microbiological threats, infectious prions and allergens.

The safety of insects for food and feed has recently been reviewed (Belluco *et al.*, 2013; Van der Spiegel *et al.*, 2013) but little data is available to support risk analysis, particularly for the use of insects as feed, where only a small number of safety related studies have been undertaken (e.g. Awoniyi *et al.*, 2004). Only isolated information in relation to the chemical risks of insects has been published (e.g. Diener *et al.*, 2011) with inferences to food/feed use sometimes made.

Safety considerations of insects that can be used in food and feed will often be species specific. For example, there is an unknown risk that some insects will contain natural metabolites or proteins that are toxic to humans or animals when eaten. This may extend beyond known venoms in, for example, bees and wasps. A safe history of human consumption of several insect species has recently been reported (Van Huis *et al.*, 2013).

Current regulations that limit undesirable substances in animal feed are described in EC Directive 2002/32/EC (EC, 2002). This covers a range of contaminants and residues including heavy metals, pesticides, veterinary medicines, and environmental contaminants. The potential for insects to bio-accumulate chemical substances and pathogens present in waste streams has yet to be explored to the standards required to fulfil regulatory requirements for the use of insects as food or feed, raising significant concerns about the safe use of insects in the food chain. The persistence of chemical residues, such as antibiotics and pesticides through the food chain is of particular concern where, for example, manure or anaerobic digestate is used as feedstock, possibly leading to longer term issues such as antibiotic resistance in livestock. The use of food waste as feedstock generates further concerns over microbiological safety and the formation of natural toxins produced during food spoilage such as mycotoxins. Industrial toxins such as dioxins may also be important depending on insect rearing and preservation processes. To some extent processing insects into a protein meal will reduce the chemical risk of using insects as a protein source for animal feed. For example, highly toxic lipophilic endocrine disruptors such as dioxins could be removed as a potential issue by defatting the insects before feeding.

Similarly, there is currently an unknown risk for livestock of allergenic proteins in insects. Tropomyosin, an allergen responsible for shellfish allergy, is also present in many insect species. For example, tropomyosins from house dust mites and cockroach have sequence identities to shellfish tropomyosin of around 80% (Ayuso *et al.*, 2002; Santos *et al.*, 1999). Cross-reactivity of insect proteins to crustacean allergic individuals has been demonstrated (Leung *et al.*, 1996; Reese *et al.*, 1999; Verhoeckx *et al.*, 2013). Whilst this is clearly important in making choices in relation to entomophagy, it is also a major consideration in relation to insects for use as animal feed as allergenic response in farm animals will result in animal welfare concerns in addition to economic and nutritional implications in relation to, for example, weight gain and meat yield.

Transmissible spongiform encephalopathy caused by the transfer of infectious prions is a particularly emotive area epitomized by the bovine spongiform encephalopathy (BSE) or mad cow disease crisis. BSE is associated with the feeding of meat and bone meal or PAP to ruminants. Whilst there is no evidence to suggest infective forms of prion proteins are present in insects, there may be a greater risk that infective prion protein transmission occurs through the use of meat-containing food waste or slaughterhouse waste as a feed stock for insects, which may then act as disease vectors by retaining residual specified risk materials such as undigested spinal cord or brain in their digestive track.

Existing fly rearing methods have concentrated largely on the black soldier fly (*Hermetia illucens*) (Bondari and Sheppard, 1987; Hem *et al.*, 2008; Newton *et al.*, 2005; St-Hilaire *et al.*, 2007a,b). Recent studies have revealed high potential for the use of *H. illucens* produced on a range of agricultural wastes (e.g. pig manure, fish offal waste) and by-products in small-scale aquaculture in tropical and sub-tropical regions (e.g. Hem *et al.*, 2008). *H. illucens*, as a tropical/sub-tropical species may not be a suitable candidate for widespread adoption for animal feed in Europe. In order to make appreciable inroads into the replacement of traditional protein for animal feed in European conditions, insect production will need to occur on a very large scale, be highly efficient, and make use of a range of available waste streams. This can perhaps be best achieved primarily through the use of the house-fly (*Musca domestica*) due to its ability to inhabit a wide range of climates. *M. domestica* also has a short lifecycle resulting in efficient conversion of agricultural waste into highly digestible protein with high levels of key amino acids (Adeniji, 2007). Species of *Calliphoridae* have also been studied as a potential source of protein for animal feed (Sing *et al.*, 2012; Yehuda *et al.*, 2011)

It is believed that the most economically viable way to produce protein from insects for inclusion in animal feed is from Dipteran larvae. Here for the first time we present a comprehensive analysis of larvae from four Dipteran species: *M. domestica*, *Calliphora vomitoria*, *Chrysomya* spp. and *H. illucens*. The fly larvae have been reared using a range of methods and in dispersed geographies and subsequently tested for the main regulated classes of chemicals and elements that are routinely monitored in the feed supply chain.

2. Materials and methods

Samples

A range of species, production methods and geographical locations were examined to cover a broad spectrum of larval rearing techniques. Five samples of dried *M. domestica* larvae were produced and analysed (samples MD1-MD5): one sample from the UK, Ghana and Mali, and two samples from China representing key production geographies/environments in Asia, Africa and Europe. *C. vomitoria* (samples CV1 and CV2) larvae were produced in the UK and a mixture of *Chrysomya* spp. (sample CH) larvae and separately *H. illucens* (sample HI) larvae were produced in Ghana. Samples were chosen from worldwide institutions that already had production methods in place to rear large numbers of larvae to facilitate planned feeding trials. Pre-existing safety information was not available for any of the samples; to our knowledge this is the first time that the larvae have been screened for chemical contaminants. Larval production methods were chosen to be broadly representative of the size and scale of international maggot rearing from small field based operations in parts of Africa to the larger, industrial scale production in China. Rearing substrates were those which are used locally and represent low or zero value waste materials. The larval production methods and analytical procedures are detailed below.

Production methods

Musca domestica – Institut d'Economie rurale, Bamako, Mali

The system is based on natural oviposition on exposed substrates in rearing beds. A mixture of 10 kg of dry poultry manure (including straw) and 20 l of water was placed in 1×1 m concrete rearing beds to attract naturally-occurring *M. domestica* for oviposition. The rearing beds are outdoors, but protected from rain and direct sun by a roof. After a few hours, substrates were covered by a permeable plastic sheet to allow the development of larvae for three days. On the fourth day, larvae were separated from the substrate using colanders. The larvae were purged and dried for one day in the sun on a metallic sheet. Gut clearance can be observed to be successful when the colour of the larvae changes to white and this is achieved in less than 24 h. Since larvae were obtained through natural oviposition, it cannot be ruled out that the sample analysed in the study also contained some other fly species. However, the examination of large samples of adult flies reared from maggots obtained from this substrate showed that over 99% were *M. domestica*.

Musca domestica – Fish for Africa, University of Stirling and CABI, Accra, Ghana

Eggs of *M. domestica* were obtained from adult rearing in cages (10,000-40,000 adults in 1-2 m^3) placed in a shed at ambient temperature and humidity conditions. Adults were fed with dry milk, sugar and water (derived from collected drain and rain water), and eggs were laid on paper or fabric covering fermented chicken manure. Freshly laid eggs were collected and placed in a tray (45×76×16 cm) on a mixture of chicken manure and fish feed waste obtained from a fish feed factory, and water, in variable proportions. Approximately 3 g of eggs were placed on approximately 8 kg of wet substrate. The trays were covered by a nylon

mesh and placed in a shed for three days. On the fourth day, larvae were separated from the substrate by hand or by using colanders. The larvae were purged for one day and dried in a gas oven at 60-80 °C for a few hours. A small proportion of the larvae were allowed to pupate. Pupae were placed in the cages on a regular basis to refresh the adult stock.

Musca domestica – Guangdong Entomological Institute, Guangzhou (farm at Huizhou), China

House fly larvae from Guangdong Entomological Institute were obtained from a rearing facility at a chicken production farm in Huizhou, Guangdong Province. About 200,000 flies were reared in rooms of 18.4 m^3 (4.0×2×2.3 m) maintained at a minimum of 25 °C. There are 8 rooms for adult housefly rearing in Huizhou. The flies were fed with a mixture of 50% dry milk and 50% sugar, and water was continuously available. The oviposition substrate consisted of fermented wheat bran covered by fabric. Eggs were collected daily and placed on humid chicken manure that had been previously fermented in a solid fermenter. Larvae developed for four days at 25-30 °C. The rearing trays were placed in closed plastic tents to reduce the level of oxygen, which induces the larvae to leave the substrate. Larvae were then collected at the bottom of the cages. Larvae are usually given fresh to the poultry on the farm but, for the purpose of the analyses, they were kept for a day to clear their guts and then dried in an oven for several hours at 60-80 °C. A small amount of the eggs laid in the adult cages are used to produce larvae on wheat bran to maintain the adult rearing.

Musca domestica – Huazhong Agricultural University, Wuhan, China

House fly larvae from Huazhong Agricultural University were obtained from a rearing system set up at a farm in Liling (Hunan Province). Adult flies were reared in gauze cages (about 45,000 adults per cage of 1.80×1.70×0.85 m). Adults were fed with a dry mixture of milk powder and sugar. Water was also provided. Eggs were laid on fermented wheat bran in a cup covered by black fabric. Eggs were collected daily and placed in a batch of 15 g on approximately 150 kg of fresh pig manure in concrete beds of 2.40×1.98 m in a plastic greenhouse. When mature, larvae were collected with a brush and a shovel. Larvae are usually given fresh to the poultry but, for the purpose of the analyses, they were kept for a day to ensure gut clearance and dried in an oven for several hours at 60-80 °C. A small amount of larvae were used to produce pupae and adults to refresh the adult rearing.

Musca domestica – Grantbait and FERA, UK

Adult house flies were maintained in a room at approximately 20 °C. Sugar, milk powder and wet paper were available at all times. Poultry manure was provided for oviposition and was removed after approximately 8 h. Eggs laid on the poultry manure were transferred to plastic trays containing moistened poultry manure. The trays were covered with a thin layer of perforated plastic film and placed in an insulated but ventilated portacabin where the temperature was maintained between 15 and 30 °C. The trays were checked daily and if required, the upper layer of manure was removed and replaced with fresh manure. After approximately 5 days the larvae were removed from the poultry manure, by repeatedly removing the upper layer of the manure in the trays so that the larvae migrate to the lower layer. The layer of manure containing the larvae was transferred to a mesh and left to crawl through the mesh overnight and into sand. The collected larvae were separated from the sand and any manure debris by sieving. The larvae were left for up to 24 h to clear their guts. Larvae were killed by placing in water at approximately 80-90 °C. The larvae were left to air dry overnight and were then dried at 65 °C in a dehydrator for approximately 3 h.

Hermetia illucens – Fish for Africa and University of Stirling, Accra, Ghana

Eggs of *H. illucens* were obtained from adults kept in captivity in large gauze cages (80×80×150 cm). Up to 5,000 adults were reared in the cages, which were kept in a shed at ambient temperature and humidity conditions, but placed outside during sunny days to stimulate mating and oviposition. Water was provided through moistened paper and manual sprayers. The oviposition sites were made of a bowl of brewery solid wastes or moist poultry manure, covered with cardboard strips and dried banana leaves. Eggs were collected every two days and placed in small plastic containers until hatching, which occurred 6 to 7 days later. Then, the young larvae were transferred to trays (45×76×16 cm) prepared with a mixture of 3 kg moist spent grain (brewery solid waste), 2 kg dry fish feed factory waste, 0.5 l yeast and 4.5 l water. The trays were covered by a nylon mesh and placed in a shed for approximately a week. The larvae were separated from the substrate by hand, washed with water and placed in sawdust overnight to empty their guts. Larvae were dried in a gas oven at 60-80 °C for 2 h. A small proportion of the larvae were allowed to develop to pupae, which were placed in small cages. Emerging adults were then placed in the rearing cages to refresh the adult stock.

Chrysomya spp. – Fish for Africa and University of Stirling, Accra, Ghana

The *Chrysomya* spp. in Ghana were reared as *M. domestica* except that small pieces of raw chicken were used in the adults' cages as an oviposition attractant. At least two species were present *Chrysomya putoria* and *Chrysomya megacephala* but it cannot be ruled out that other species were also present.

Calliphora vomitoria – Grantbait, UK

C. vomitoria larvae originated from a commercial fish-bait breeding system. Adult *C. vomitoria* were maintained in a room at ambient temperature. Sugar and water were continuously available. Fresh pig offal (3 days old) was provided daily for oviposition. Egg-bearing offal was collected daily and transferred to a room at 20-25 °C overnight. Larval development took place in concrete pits (approx. 2.5×2.5×0.45 m) in a ventilated unheated shed. Pits were covered by canvas. A pile of offal was placed in each pit using a spade and inoculated with offal infested with larvae. Offal was added as required to support larval development. When the larvae reached the appropriate stage, the offal/larvae mixture was transferred to coarse mesh sheets suspended above an empty pit. The larvae move out of the offal and fall through the mesh into the pit. Some larvae were transferred to a separate room where a stock of breeding adults is maintained at ambient temperature. Larvae are usually kept fresh and sold alive for fishing bait but, for the purpose of the analyses, they were kept for a day to ensure gut clearance and dried in an oven for several hours at 60-80 °C.

Analytical materials and quality assurance

A range of analytical methods were chosen in order to determine the levels of chemical contaminants (1,140 compounds measured) in line with EU guidelines and regulation (2002/32/EC; EC, 2002). Due to the nature of the substrates that are used for the growth of the fly larvae (mainly manure and other animal waste), potential contaminants that were considered were; veterinary medicines, pesticides and mycotoxins, some of which have primary excretion routes via faeces. Animal waste could also be a source of environmental contaminants such as; heavy metals, dioxins, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs).

All laboratory materials used in the analysis were of analytical grade and sourced from reputable suppliers in the UK. All solvents used were of high-performance liquid chromatographic (HPLC) grade (VWR International Ltd., Lutterworth, UK). All analytical standards were sourced from Sigma-Aldrich (Gillingham, UK). All analytical testing was performed in a laboratory accredited to ISO9001 (The Food and Environment Research Agency, York, UK) with most methods also accredited to ISO17025 albeit not for a fly larvae matrix at present with accreditation mainly for similarly composed food products such as shellfish and meat.

Sub-sample preparation

All samples were collected and dried at each production site as described and assumed to have no remaining metabolic activity. Samples (approx. 1 kg) of each dried larvae were transported by courier at ambient temperature to Fera (York, UK). Transit times were no longer than 2 weeks. Each sample of dried larvae was ground into a fine powder (approx. 0.5-1 mm^3) using a pestle and mortar. Six 25 g aliquots of each sample were created and analysed as described below. All samples and sub-samples were stored at room temperature in the dark.

Veterinary medicines

For chloramphenicol analysis a 3 g sample was extracted by homogenising in 7 ml of acetonitrile. The extract was centrifuged, applied to a 20 ml Chem Elut cartridge (Varian; VWR International Ltd.) and eluted with 10 ml of dichloromethane. The eluent was reduced to near dryness under nitrogen, re-suspended in hexane/ethyl acetate and loaded onto a pre-conditioned NH2 SPE cartridge (Macherey Nagel; Fisher Scientific, Loughborough, UK). The cartridge was washed with ethyl acetate and the analytes eluted with 3 ml ethyl acetate/methanol. The eluent was then reduced to dryness under nitrogen and re-suspended in 1 ml HPLC grade water. Samples were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Isocratic C18 LC, Acquity UPLC HSS; Waters Corporation, Wilmslow, UK, with negative ion electrospray MS/MS using an Agilent 6490 TQ; Agilent Technologies, Santa Clara CA, USA).

A further multi-residue screen was undertaken using a 5 g sample extracted using 15 ml of 1% oxalic acid in acetonitrile. After homogenising, sodium sulphate was added and the sample shaken and centrifuged. The supernatant was applied to a dispersive C18 solid phase extraction cartridge. After further shaking and subsequent centrifugation, an aliquot of the supernatant was analysed by reverse phase HPLC (Acquity HSS; Waters Corporation) coupled to MS/MS (Agilent 6490 TQ) and time of flight mass spectrometry (Agilent 6530 qTOF; Agilent Technologies).

For both methods larvae samples were over-spiked with 175 certified analytical standards to assess recovery of the method for quality control purposes.

Quantitative data from MS/MS analysis was obtained for 68 compounds with limits of detection (LOD) in the range 0.15-200 µg/kg. Qualitative data from qToF-MS analysis was obtained for 492 compounds with approximate LODs in the range 1-100 µg/kg.

Pesticides

A 3 g sub-sample was hydrated with 20 ml of HPLC grade water and extracted in 60 ml of ethyl acetate. The extract (20 ml) was further purified using gel permeation chromatography (HPGPC; Gilson, Middleton, WI, USA, using an Envirosep-ABC column; Phenomenex, Macclesfield, UK) prior to concentration down to 0.5 ml and subsequent analysis using gas chromatography with mass spectrometric detection (Agilent 5973 Inert MSD; Agilent Technologies).

A second 1 g sub-sample was hydrated with 9 ml of HPLC grade water and extracted in 10 ml of acetonitrile, in the presence of 1.7 g sodium citrate, 1 g sodium chloride and 4 g magnesium sulphate. Analysis was carried out using liquid chromatography with mass spectrometric detection (UPLC-MS/MS) in selected reaction monitoring mode (Agilent 6490 TQ). The presence of residues was confirmed using the same technique in multiple reaction monitoring mode.

For both methods larvae samples were over-spiked with 393 certified analytical standards to assess recovery of the method for quality control purposes.

The concentrations of 393 pesticide residues were measured with approximate LODs of 10, 20 or 50 µg/kg.

Heavy metals

Deionized (18.2 MΩ cm) water, metal analysis grade reagents and acid cleaned plasticware were used throughout. Aliquots of sample (0.5 g) were weighed into allotted digestion vessels and a mixture (4:1) of nitric acid and hydrochloric acid added (5 ml). The vessels were capped and the contents digested under high temperature and pressure using a single reaction chamber microwave digester system (Ultrawave; Milestone, Shelton, CT, USA). Reagent blanks, certified reference materials and a spiked sample were also taken through the procedure. The resulting solutions were transferred to pre-marked acid-cleaned plastic test tubes (15 ml polypropylene; Yorlab, York, UK) and diluted to 10 ml with deionised water. The digest solutions together with a set of standards covering the expected concentration range were internally standardised with rhodium in dilute nitric acid (1%, v/v). Multi-element measurements were made using an Agilent 7700x ICP-MS (Agilent Technologies) with collision cell.

The concentration of 48 heavy metals and trace elements were measured with approximate LODs of 10, 20 or 50 µg/kg.

Dioxins, polychlorinated biphenyls and polyaromatic hydrocarbons

For dioxin and PCB analysis a 22 g sample was homogenised before an aliquot was fortified with known amounts of surrogate (^{13}C-labelled) analogues of target analytes and exhaustively extracted using >200 ml hexane, toluene and dichloromethane. The extract was further purified using adsorption chromatography (mixed silica based adsorbents). Ortho-PCBs, non-ortho-PCBs, polychlorinated dibenzodioxins/polychlorinated dibenzofurans and PBDEs were segregated into two separate fractions using an activated carbon column eluted with toluene. Each fraction was concentrated and further purified using a 'clean-up' column containing sulphuric acid impregnated silica gel, before the inclusion of additional surrogate standards. Final determination was by high resolution gas chromatography with high resolution mass spectrometric detection (Micromass, Autospec Ultima; Waters Corporation).

Maximum limits for dioxins and PCBs are stated as the sum of the ICES 6 for higher limit compounds (10 µg/kg, sum of PCB 28,52,101,138,153 and 180) and as the World Health Organisation toxic equivalence factor (WHO-TEF) for all lower limit compounds (1.25 ng/kg).

For polyaromatic hydrocarbon (PAH) analysis a further 3 g sample was spiked with ^{13}C labelled internal standards, saponified with 200 ml methanolic potassium hydroxide and extracted with 100 ml cyclohexane. Crude extracts were concentrated down to 50 ml and purified by partitioning into 50 ml dimethyl formamide followed by application to silica gel columns (silica gel adjusted to 10% water). Extracts were eluted with 150 ml cyclohexane before concentrating to 25 µl. The concentrations of PAHs were determined using gas chromatography with mass spectrometric detection (Thermo Trace; Thermo scientific, San Jose, CA, USA) and quantified with reference to the ^{13}C labelled internal standards.

The concentration of 28 PAHs was measured with approximate LODs in the range 0.1-0.02 µg/kg.

Mycotoxins

A 1 g sample was weighed into a plastic centrifuge tube (15 ml; Alpha Laboratories Ltd., Eastleigh, UK). The extraction solvent used was a mixture of acetonitrile : water : acetic acid (79:20:1.4 ml). Tubes were vortex mixed, then extracted for 2 h on an orbital shaker. After extraction tubes were centrifuged at 4,000 rpm, for 20 min at 4 °C. An aliquot (1 ml) of the supernatant was transferred to a glass vial, this

was diluted with a mixture of acetonitrile : water : acetic acid (20:79:1.1 ml). The vials were stored overnight in a fridge at 4-8 °C. Sample extracts were filtered by syringe filter (0.22 µm, nylon, Nalge; VWR) and collected in glass autosampler vials for analysis.

Ultra performance (UP) LC-MS/MS analysis was carried out using a Waters UPLC system with a XEVO TQ-S mass spectrometer (Waters Corporation). Two analytical runs, one using neutral mobile phase conditions and one using acidic conditions, were required to ensure optimum chromatographic performance and ionisation of analytes. The method was capable of detecting several groups of mycotoxins. In addition, masked forms of some of the mycotoxins were also included.

Larvae samples were over-spiked with 69 certified analytical standards to assess recovery of the method for quality control purposes.

The concentrations of 69 compounds were measured with approximate LODs in the range 5-500 µg/kg.

3. Results

Table 1 presents a summary of the significant results obtained from the testing undertaken. Where compounds have not been detected the approximate LOD's have been stated. LOD's are approximate as full method validation has not been undertaken for a fly larvae matrix at this time. However, data were generated using methods that are fully validated in related matrices such as shellfish and meat.

Veterinary medicines

4,4'-dinitrocarbanilide (nicarbazin) was detected in *M. domestica* sample MD1. This was identified in the veterinary medicines screen and therefore was not quantified but is present above the limit of detection (100 µg/kg) and possibly above the maximum concentration for animal feed (500 µg/kg) specified in Directive 2009/8/EC (EC, 2009a). All other veterinary medicines tested were absent from the larvae or present at concentrations below the LOD.

Pesticides

Chlorpyrifos was found in *M. domestica* larvae sample MD5 at 800 µg/kg. The concentration of this compound is not specifically regulated in animal feed. However, Codex recommend that if this organophosphate insecticide is present in animal fodder (alfalfa) the concentration should be below 5,000 µg/kg (Codex Alimentarius, 2014), indicating that this result does not pose a significant safety threat.

Piperonyl butoxide was found in *C. vomitoria* sample CV1 at 200 µg/kg. This insecticide synergist does not have a widely adopted recommended maximum concentration, but Codex advise that pea fodder should contain less than 2,000 µg/kg (Codex Alimentarius, 2014), again indicating that there is unlikely to be a significant safety concern.

No other pesticide residues were detected.

Heavy metals

The toxic heavy metal cadmium was found to be present in all samples. The level of cadmium in *M. domestica* samples 2, 4 and 5 was above the lowest EU limit for cadmium in animal feed (500 µg/kg) specified in directive 2002/32/EC (EC, 2002), being 625 (MD2), 711 (MD4) and 723 (MD5) µg/kg.

Dioxins, polychlorinated biphenyls and polyaromatic hydrocarbons

The PCB ICES6 values for all samples were below the EU regulatory limit for animal feed of 10 µg/kg (EC Directive 2002/32/EC amendment 277/2012/EC; EC, 2012). They

Table 1. Summary of positive results (all units are µg/kg).

Compound	Fly larvae species[1]								
	MD1	MD2	MD3	MD4	MD5	CV1	CV2	CH	HI
4,4'-dinitrocarbanilide (nicarbazin)	Present	<100	<100	<100	<100	<100	<100	<100	<100
Chlorpyrifos	<50	<50	<50	<50	800	<50	<50	<50	<50
Cadmium	334	625	348	711	723	20	18	370	120
Beauvericin	<5	<5	6.9	<5	<5	<5	<5	<5	<5
Enniatin A	12.5	<5	<5	<5	<5	<5	<5	<5	<5
Enniatin A1	7.3	<5	<5	<5	<5	<5	<5	<5	<5
Piperonyl butoxide	<10	<10	<10	<10	<10	200	<10	<10	<10

[1] MD = *Musca domestica*; CV = *Calliphora vomitoria*; CH = *Chrysomya spp.*; HI = *Hermetia illucens.*

ranged from 0.05 to 4.28 µg/kg. Similarly, the WHO-TEF was calculated for all samples analysed and values of 0.14 to 0.44 ng/kg were obtained, which are below the EU regulatory limit of 0.75 ng/kg.

PAH4 values were calculated for all samples analysed and these were between 0.28 and 9.82 µg/kg. No limits for PAHs in animal feed are specified in EU regulations. Regulatory limits in food for PAH4 range from 1 (lowest limit, baby food) to 35 (highest limit, smoked seafood) µg/kg (EC regulation 1881/2006 as amended by 835/2011; EC, 2011).

Mycotoxins

Several naturally occurring mycotoxins were found. Beauvericin was present at 6.9 µg/kg in MD3. Enniatin A (12.5 µg/kg) and Enniantin A1 (7.3 µg/kg) were found in MD1. These compounds were not present at levels that are believed to pose a safety risk and they are not amongst those mycotoxins (e.g. aflatoxin) for which the European Commission has set maximum concentrations for feed in Directive 2002/32.

4. Discussion and conclusions

Extensive analysis of a wide range of chemical contamination risks which may arise from the use of the fly larvae species that can realistically be expected to be farmed as main stream animal feed has been undertaken. The geographical sampling locations were designed to include areas with varying intensities of industry (and therefore environmental pollution). Nine samples of larvae were analysed for over 1000 of the chemical risks that are routinely monitored to ensure the safety of animal feed (and also food). A significant and recurring risk is the potential bioaccumulation of metals in insects and in particular cadmium. Our results support previously published initial findings (Diener *et al.*, 2011). Further studies are required to determine the source of cadmium and if this is found to relate to transfer from feedstock then maximum limits for cadmium in feedstock for insects may be required to ensure control when entering the food chain. Data indicate the presence of higher than permissible levels of cadmium in *M. domestica* produced in specific and potentially more polluted locations and it may transpire that location and local environment impact on the level of cadmium in both feedstock and farmed insects. Further work is required to determine if cadmium and other potential risks in insects used as feed are transferred into farm animals and until this has been undertaken careful supply chain and environmental management could ameliorate potential risks.

The presence of the veterinary medicine nicarbazin in one sample of *M. domestica* which was fed on manure highlights the need to carefully consider the feedstock that is used to feed the fly larvae, as this may be contaminated with chemical residues. Nicarbazin is a coccidiostat antiprotozoal agent that acts upon coccidia parasites and is frequently used to treat chickens through their feed. The UK Food Standards Agency has recently issued guidance (FSA, 2014) in relation to the accumulation of nicarbazin in poultry. This result most probably arose from the use of contaminated poultry manure as a growth medium and related to the permitted use of veterinary medicines in poultry production with subsequent use of contaminated poultry faeces used as feedstock for fly larvae. As a primary excretion route for many medicines, egested material if used as feedstock may introduce chemical risks into the feed chain. However, these are manageable risks which can be monitored through an understanding of the history of the feedstock or by analytical testing for potential risks prior to feeding.

Other potential chemical hazards such as pesticides and mycotoxins were found to be of limited concern in this study. However, whilst we present for the first time an extensive chemical safety dataset for farmed insects to be used as animal feed, it is important to acknowledge that each combination of insect and feedstock may present different risk potential and therefore routine monitoring is required to ensure the continued safety of an insect containing animal feed supply chain. There is also a requirement for enabling legislative change should insects be introduced as a new feedstock for farm animals. For example, currently it is not permitted to feed animals on most waste materials under EC Regulation 1069/2009 and EC Regulation 767/2009 (EC, 2009b,c). These rules were largely designed to prevent, for example, the feeding of poultry faeces to cattle. Similarly, EU Regulation 1099/2009 relates to slaughterhouse and on-farm slaughter procedures which clearly were not designed to apply to insects (EC, 2009d).

Microbiological risks were not explored in this study and the view of the authors is that microbial and parasitic risks can largely be minimised by introducing appropriate processing methodologies such as heating and protein extraction techniques within a commercial setting. However, further work is necessary to assess the biological safety of insects for food and for feed produced for local use and without access to industrial processing technologies. Furthermore, safety assessment at the point of human consumption through e.g. eggs, milk, fish or meat has yet to be undertaken and it is not unreasonable to anticipate bioaccumulation of, for example, dioxins or metals from permissible levels in insect containing feed to higher levels in resultant food.

These results show that from 1,140 compounds analysed only 7 have been observed to be present or present above regulatory limits (specifically Cd). These data set a platform for further analysis and with the exception of the samples contaminated with cadmium would be cleared for use as animal feed in terms of chemical contaminant levels.

An improved understanding of the impact of variations in feedstock and the natural environment would help establish robust conclusions about the safety of fly larvae and other insects as an animal feedstock. However, with appropriate quality assurance mechanisms and testing regimes in place to monitor for chemicals in the larvae then we can conclude that it is feasible to produce fly larvae that are free from chemicals of concern to the animal feed sector. Notwithstanding, processing of the insects into a protein rich meal could also remove chemical contaminants prior to feeding.

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