Rearing of the black soldier fly towards application in piglet feed

by

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List of Abbreviations

49CP: 49 % crude protein

AACC: American Association for Clinical Chemistry

ADFI: average daily feed intake

ADG: average daily gain

AID: apparent ileal digestible

AIDC: apparent ileal digestibility

ANOVA: analysis of variance

As: arsenic

ATP: adenosine triphosphate

BAF: bioaccumulation factor

BSF: black soldier fly

BSF4: 4% full-fat BSF

BSF8: 8% full-fat BSF

Cd: cadmium

CFU: colony-forming unit

CON: control

df: degrees of freedom

DF-BSF: defatted BSF

DM: dry matter

EE: ether extract

EFSA: European Food Safety Authority

ESI: electrospray ionization

EU: European union

FAME: fatty acid methyl esters

F:G: feed to gain ratio

GC: gas chromatography

GLM: general linear model

HCl: hydrogen chloride

HPLC: high-performance liquid chromatography

IAS: invasive alien species

ICP-OES: inductively coupled plasma optical emission spectroscopy

IPIFF: International Platform of Insects for Food and Feed

ISO: International Organization for Standardization

LC–MS/MS: liquid chromatography-tandem mass spectrometry

LOD: limit of detection

LOQ: limit of quantification

LTime: lethal time

MCFA: medium chain fatty acid

MRL: maximum residu level

MUFA: mono-unsaturated fatty acid

N: number of sampled individuals

NaCl: sodium chloride

Nd: not determined

P: significance of statistical test

PAP: processed animal protein

Pb: lead

PUFA: poly-unsaturated fatty acid

R^2: coefficient of determination

RH: relative humidity

SCP: supercooling point

SD: standard deviation

SE: standard error

SFA: saturated fatty acid

SPSS: Statistical Product and Service Solution

t: t-statistic

TDF: total dietary fiber

TSE: Transmissible Spongiform Encephalopathies

χ^2: chi-square statistic

Chapter 1

Introduction, objectives and thesis outline

1.1 Introduction

1.1.1 Insects for food and feed

Due to the growing world population, the increasing urbanization and the rising income in emerging economies, the demand for animal-based food products is on the rise (Alexandratos and Bruinsma, 2012). Approximately 26% of the world's ice-free surface, which equals 75% of the total agricultural surface, is occupied for livestock production as pastures and crops cultured for feed. According to Gerber et al. (2013), about 14.5% of all anthropogenic greenhouse gas emissions could be allocated to livestock production. In addition, 8% of global human water use can be allocated to the livestock sector (Foley et al., 2011). Consequently, concerns about the environmental sustainability of the current animal-based food production are emerging (van Huis et al., 2013). Therefore, in order to meet the rising demand for agricultural products, alternative solutions need to be introduced. Entomophagy (the consumption of insects by humans) might contribute to meeting these requirements. Several insect species are nutritious (i.e. rich in animal protein and other nutrients), have a short life cycle compared to livestock and can be fed on agricultural by-products that are currently undervalued (van Huis et al., 2013; Dobermann et al., 2017).

Besides a food source for humans, insects can also be used as an alternative feedstuff for livestock production. The scope of this thesis will be insects as a feedstuff and therefore, insects as food for direct human consumption will not be further discussed. Several interesting reviews and reports addressing this matter have been published during the last few years (van Huis et al., 2013; Dossey et al., 2016; Dobermann et al., 2017).

1.1.2 Insects as a feedstuff

As mentioned in 1.1.1, the livestock sector currently uses 75% of all agricultural land worldwide (Foley, 2011). Land availability, however, is an important limiting factor of agricultural production (de Vries and de Boer, 2010). Cultivation of crops allocated to livestock, like soybean, puts pressure on

land availability, particularly in tropical areas. Consequently, these areas are subjected to deforestation, threatening tropical forests that are reservoirs of biodiversity and provide key ecosystem services (Foley et al., 2011). The intensive monoculture of soybean in South-American countries like Brazil and Argentina has a devastating effect on habitats and biodiversity. In addition, mechanical weeding increases soil erosion, while intensive cultivation is responsible for severe mining of soil fertility (Steinfeld et al., 2006). Crop production for animal feed also largely contributes to the high water use of the livestock sector (Rumpold and Schlüter, 2013).

In Europe, the most important protein rich ingredient for terrestrial animal feeds is soybean meal, which is the by-product of oil extracted soybeans. European livestock production highly depends on the import of this protein resource. The world soybean production for 2016/17 was 351.8 million metric tons of which only 2.6 million metric tons (i.e. 0.74% of the world production) was produced in the EU (EU, 2017a). As a result, 14.6 million metric tons of soybeans and 19.6 million metric tons of soybean meal was imported in the EU in 2017 (IndexMundi, 2017). Therefore, the European Parliament is concerned that this dependency could make the livestock sector in the EU vulnerable to price volatility and trade distortions (Figure 1). Consequently, feed prices may rise and as a result farmers' production costs would increase, while the profitability of the sector would decrease (Van Krimpen et al., 2013). Moreover, feed costs already represent 60-70% of total animal production costs (van Huis, 2013).

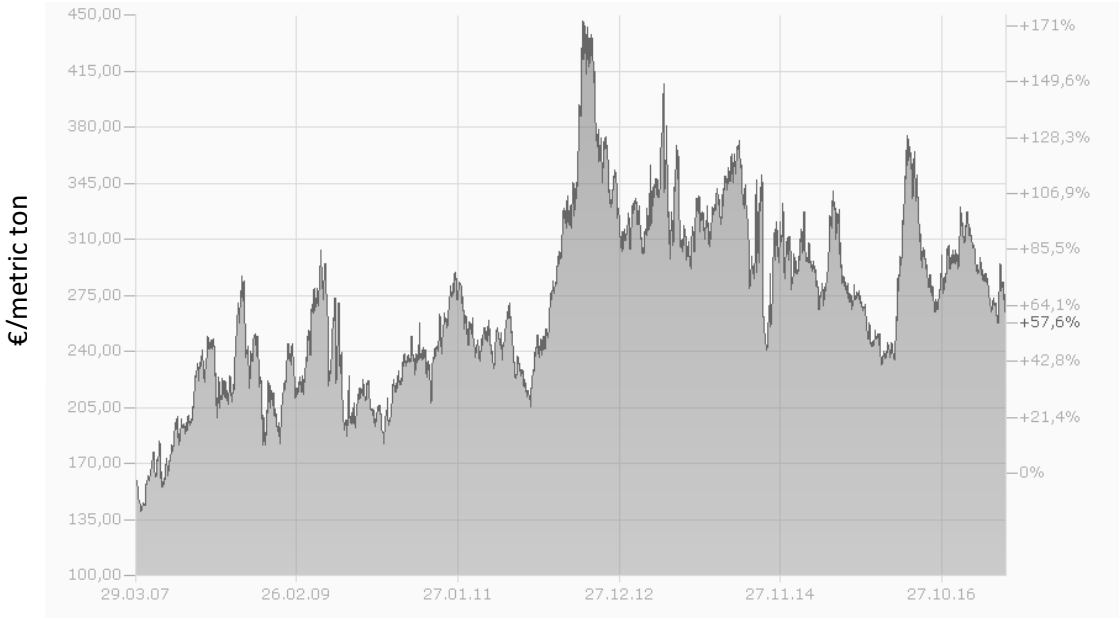

Figure 1: Evolution of soybean meal market prices during the last decade (€/metric ton) (Finanzen, 2017)

Therefore, for the economic and ecological reasons mentioned above, the need for sustainable alternative protein sources for livestock, which could partly substitute soybean meal, is becoming increasingly urgent. According to FAO (2017a), the sustainability of the agricultural systems producing these alternative protein sources is based on 5 principles:

- improvement of the efficiency in the use of resources
- conservation, protection and enhancement of natural resources
- protection and improvement of rural livelihoods, equity and social well-being
- enhancement of the resilience of people, communities and ecosystems
- effective and responsible governance mechanisms

1.1.2.1 Alternative protein-rich feed ingredients

Alternative protein sources for soybean meal are either of plant origin (e.g. grain legumes such as lupines and chick peas, and leaf proteins such as grass and sugar beet leaves), aquatic origin (e.g.

microalgae, seaweed and duckweed), microbial origin or derived from insects (van Krimpen et al., 2013). In what follows, some of these alternatives will be briefly discussed.

Grain legumes have the unique ability, like soybean, to fixate nitrogen. Most of the legumes contain approximately 21–25% crude protein. However, lupine has been reported to have a comparable protein content as soybean, up to 45–50% crude protein (Henchion et al., 2017). A disadvantage of lupine is that it contains anti-nutritional compounds, such as toxic, bitter tasting alkaloids which may reduce food intake. Cultivars with a lower alkaloid level have been developed, however, these breeds are more sensitive to several plant pests, such as the fungus *Colletotrichum gloeosporiodes* (Helsper et al., 2006). Other anti-nutritional compounds possibly present in grain legumes are protease inhibitors and lectins, which constitute part of the defensive mechanism of the seed and reduce the digestibility (Mikić et al., 2009). Consequently, in order to reduce these anti-nutritional compounds, energy consuming heat and pressure treatments (e.g. toasting, flaking, extrusion, expansion, pelletizing) will have to be applied, as is the case for soybean (Heuzé et al., 2017).

Besides the cultivation of protein crops, plant proteins can also be resourced from left-over materials such as sugar beet leaves. The valorization of these side streams might increase the sustainability of protein production, however, due to the high water contents (90% moisture or more), energy consuming drying is necessary in order to concentrate the protein content and for purposes of preservation, storage and transport (van Krimpen et al., 2013). In addition, the fiber content of these plant materials is high and, therefore, an extraction step separating proteins from fibers, might increase their applicability in the diets of monogastrics (Chiesa and Gnansounou, 2011). Many plant protein sources, compared to protein sources from animal origin, do not contain all the essential amino acids in the required proportions. However, this could be compensated by feeding blends with cereals and by supplementation of synthetic amino acids, as is done for soybean based feeds (Henchion et al., 2017).

Duckweeds, free-floating vascular plants distributed around the world on fresh (or brackish) waters, can be high in protein and minerals, while the fiber content is low compared to terrestrial plants (Hasan et al., 2009). The crude protein content is very variable and can range from 7 up to 45% on DM basis, depending on the nitrogen availability in the water used for duckweed culture (Culley et al., 1981). Thus, under optimal conditions, protein-rich duckweeds could be produced which could compete with high quality protein sources such as soybean meal. However, given the high impact of the quality of the water used to culture duckweeds, contamination with pathogens, heavy metals and organic toxins are concerns related to duckweed farming (Iqbal, 1999). In addition, duckweeds may contain anti-nutritional factors such as oxalic acid (Goopy et al., 2003), phenolic compounds, tannins and saponins (Negesse et al., 2009). Since duckweeds are very high in moisture (92-95%), energy costs and practical aspects of drying should be considered (Holshof et al., 2009).

Another group of aquatic organisms with potential as feedstuff are algae, a heterogeneous group with a complex taxonomy. Algae can be divided in two main types: the macroalgae (i.e. seaweeds), occupying the littoral zone, which can be of very large size, and the microalgae, small organisms which can be found in benthic and littoral habitats, throughout the oceans (i.e. phytoplankton) and fresh water (Hasan et al., 2009). Macroalgae have a highly variable composition, dependent of species, habitat, harvest time and environmental conditions such as light intensity time, temperature and nutrient concentration in water. Another important feature of fresh seaweeds is that the moisture content is high (70-90%) and, therefore, they need to be dried or consumed quickly (Mišurcová, 2012). Regarding feed safety, there could be the problem of concentration of inorganic elements from seawater which may contain heavy metals and other mineral contaminants. Therefore, concerning their content in trace elements, seaweeds for food and feed are subjected to national and international regulations (EU, 2011).

Microalgae, like the other aquatic plant sources mentioned above, can vary substantially in composition. This could be due to genetic factors, culture conditions and the growth stage at harvest

(Becker, 2013a). It has been recommended that for applications in animal feed, mixtures of carefully selected microalgae are made to guarantee optimal growth because a particular alga may lack the nutrients that other species may contain (Yamaguchi, 1997; Becker, 2013a). Microalgae, of which the cyanobacterium spirulina (*Arthrospira* sp.) has the broadest range of applications, are already employed in commercial aquaculture (Becker, 2013a). They are usually rich in protein, with a content that can exceed 60% of DM (Garofalo, 2011). The amino acid composition is quite similar between species, and largely unaffected by the intrinsic and extrinsic factors mentioned above. In general, aspartic acid and glutamic acid occur in the highest concentrations, whereas cysteine, methionine, tryptophan, and histidine occur in the lowest concentrations (Becker, 2013a). Spiruline protein contains all essential amino acids and is superior to a typical plant protein, including that of legumes. Compared to animal proteins, however, the contents of lysine and the sulphur containing methionine and cysteine are lower (Garofalo, 2011). In addition, most microalgal species have a relatively thick cellulosic cell wall, which complicates digesting by monogastric species. Therefore, in order to make the algal protein accessible, treatments (e.g. boiling, high temperature drying or breaking of hydrogen bonds by phenol which requires detoxification afterwards) are necessary. However, the cell wall of spirulina does not represent a barrier to proteolytic enzymes, and this alga can be digested by monogastrics without previous physical or chemical rupture of the cell wall (Becker, 2013b).

From the above mentioned alternatives, the majority had already been studied or was under investigation at the time this research started. According to FAO (van Huis et al., 2013), another alternative protein source, this time from animal origin, could be provided by insects. However, given that the number of studies concerning the rearing, nutritional value and feed safety hazards of insects as a feedstuff was limited at the time, more research was warranted. Therefore, our research focused on the potential of insects as an alternative feedstuff. However, we postulate that none of the mentioned alternative protein sources (including insects) is superior to any of the others and in the future a scenario of combining different alternatives will probably be necessary.

Insects generally have a high nutritional value (Table 1). They contain high amounts of protein, essential amino acids, fatty acids and micronutrients (e.g. copper, iron, zinc) (Verkerk et al., 2007; Rumpold and Schlüter, 2013; van Huis, 2013). The majority of the insects has a protein content exceeding 30% on dry matter (DM) basis (Verkerk et al., 2007; Premalatha et al., 2011; Veldkamp et al., 2012). Veldkamp et al. (2012) pointed out that the crude protein content of insects is in the same range as that of soybean meal. They also found that the amino acid profile of insects matches with the required essential amino acid profiles of growing pigs and broiler chickens. In general, insects contain high amounts of lysine, threonine and methionine, which are major limiting amino acids in cereal- and legume-based diets (Verkerk et al., 2007; van Huis, 2013). The protein digestibility of insects is influenced by the presence of an exoskeleton, as this contains non-digestible chitin (DeFoliart, 1992; Verkerk et al., 2007). However, recent studies conducted by De Marco et al. (2015) and Schiavone et al. (2017) showed that insect meals derived from the yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), and the black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae), are valuable sources of apparent metabolizable energy and digestible amino acids for broiler chickens. Although insects are primarily seen as an alternative protein source, they are also high in fat (Verkerk et al., 2007; Veldkamp et al., 2012). Several studies indicate that traditional protein and fat sources commonly used in feed formulation can be replaced by insects without adverse effects on animal performance and product quality (Teotia and Miller, 1973; Newton et al., 1977; Anand et al., 2008; Sealey et al., 2011).

Table 1: Nutritional composition of 2 insect species (*T. molitor* and *H. illucens*) compared to whole soybeans

	T. molitor	*H. illucens*	Soybean
Dry matter (g/kg)	422 ± 63	400 ± 50	800
Gross energy (MJ/kg DM)	28.7 ± 0.8	22.1	23.6 ± 0.4
Crude protein (N x 6.25) (g/kg DM)	482 ± 47	421 ± 10	396 ± 14
Alanine (g/kg DM)	38.5 ± 5.3	32.4 ± 3.4	17.0 ± 0.8
Arginine (g/kg DM)	25.3 ± 5.3	23.6 ± 1.3	28.5 ± 1.2
Aspartic acid (g/kg DM)	39.6 ± 9.0	46.3 ± 7.5	44.0 ± 1.6
Cystine (g/kg DM)	4.2	0.4	5.9 ± 0.8
Glutamic acid (g/kg DM)	59.7 ± 5.8	45.9 ± 10.1	70.5 ± 2.0
Glycine (g/kg DM)	25.9 ± 4.8	24.0 ± 3.4	16.6 ± 0.4
Histidine (g/kg DM)	18.0 ± 1.1	12.6 ± 4.2	10.3 ± 0.8
Isoleucine (g/kg DM)	24.3 ± 2.6	21.5 ± 2.1	17.8 ± 0.8
Leucine (g/kg DM)	45.4 ± 9.5	33.3 ± 2.5	29.7 ± 0.8
Lysine (g/kg DM)	28.5 ± 4.2	27.8 ± 3.8	24.6 ± 0.8
Methionine (g/kg DM)	7.9 ± 2.1	8.8 ± 1.3	5.5 ± 0.4
Phenylalanine (g/kg DM)	21.1 ± 2.1	21.9 ± 1.7	19.8 ± 0.4
Proline (g/kg DM)	35.9 ± 1.1	27.8	19.8 ± 1.2
Serine (g/kg DM)	37.0 ± 18.5	13.1 ± 8.0	19.8 ± 0.8
Threonine (g/kg DM)	21.1 ± 2.6	15.6 ± 7.2	15.4 ± 0.8
Tryptophan (g/kg DM)	3.2 ± 2.6	2.1	5.1 ± 0.0
Valine (g/kg DM)	39.1 ± 3.2	29.0 ± 5.5	18.6 ± 0.8
Fat (g/kg DM)	402 ± 49	260 ± 83	214 ± 17
C12:0 (g/kg Fatty acids)	5 ± 5	338 ± 108*	
C16:0 (g/kg Fatty acids)	211 ± 67	161 ± 36*	113 ± 11
C18:0 (g/kg Fatty acids)	27 ± 4	40 ± 18*	36 ± 3
C18:1 (g/kg Fatty acids)	377 ± 87	208 ± 58*	229 ± 16
C18:2 (g/kg Fatty acids)	274 ± 40	52 ± 23*	536 ± 17
C18:3 (g/kg Fatty acids)	13	3 ± 3*	78 ± 10
Ash (g/kg DM)	31 ± 7	206 ± 60	57 ± 4
Calcium (g/kg DM)	2.7 ± 1.9	75.6 ± 17.1	3.2 ± 0.8
Phosphorus (g/kg DM)	7.8 ± 3.7	9.0 ± 4.0	6.1 ± 0.6
Crude fiber (g/kg DM)	51 ± 7	70	62 ± 13

Means ± SD (values without SD originate from a single study); data from Feedipedia (2017) unless otherwise indicated; the applied analytical methods might differ among studies, however, given the limited amount of data for insects, the mean of all values is presented; *Mean value from St-Hilaire et al., 2007; Li et al., 2011; Sealey et al., 2011; Zheng et al., 2012

The rearing of insects for feed purposes could also be interesting from a sustainability point of view. Insects may contribute to the improvement in efficiency of protein production (see sustainability principles according to FAO, mentioned above in section 1.1.2). The potential of insects lies in the high reproduction capacity (hundreds of eggs/female), growth rates (life cycles completed in several weeks), the potential to convert low-value biomass into high-value protein and the high feed conversion efficiency (van Huis et al., 2013). Regarding the latter, for BSF reared on chicken feed, 1.8 kg of feed is needed to obtain 1 kg of weight increase (Oonincx et al., 2015). This feed conversion ratio is somewhat higher than the feed conversion ratio of modern broilers, though the comparison is not straightforward. During the present research, BSF will be reared on side streams, currently not considered as feed for livestock, whereas broilers are fed highly digestible feeds containing over 40% starch. A possible explanation for the high feed conversion efficiency of insects could be the fact that they do not have to spend energy to maintain their body temperature as they are poikilothermic animals (Premalatha et al., 2011; van Huis, 2013). Also given their better potential than farm animals to derive moisture from food, insects are expected to use low amounts of water (Rumpold and Schlüter, 2013). Further, insects have the potential to change the chemical composition and reduce the odor and total mass of animal manure and organic waste (Liu et al., 2008; Diener et al., 2009). However, the sustainability of insect production will to some extent depend on the applied rearing substrates (Lundy and Parella, 2015; Smetana et al., 2016). The most promising substrates, in terms of sustainability, consist of low value by-products from the agri-food chain with a good nutritional profile (e.g. dried distillers grain with solubles) or are based on the utilization of waste products with high environmental impact (e.g. manure or municipal organic waste) (Smetana et al., 2016).

1.1.2.2 Feed safety and processing

Inherent to the variety of substrates used for insect rearing, certain feed safety risks can emerge. These risks could be from microbiological origin (pathogenic bacteria, viruses, parasites, fungi and prions) or chemical origin (heavy metals, pesticides, mycotoxins, toxins produced or accumulated in insects, veterinary drugs, dioxins and dioxin-like PCBs, polycyclic aromatic hydrocarbons and

packaging migration contaminants) (EFSA, 2015). Most of the chemical risk factors could be contained by providing safe substrates to rear the insects, however, certain pathogens can develop during storage. Since insects are rich in nutrients and moisture, providing a favorable environment for microbial growth, processing will be a necessary step (Klunder et al., 2012). Processing is an important step in order to improve preservation and guarantee the safety of feedstuffs.

To allow storage and transport, harvested insects must be stabilized in some way. In most cases, this is achieved by drying (van Huis et al., 2013). In order to prevent possible loss of quality, products should be freeze dried. Freeze-drying, however, is an energy consuming process. Van Campenhout et al. (2017) investigated the possibilities of industrial microwave drying as an alternative to freeze-drying. They concluded that microwave drying is a valuable alternative to freeze-drying with a similar end product quality. Moreover, for microwave drying the treatment time is shorter than for freeze drying and browning of the product does not occur during storage afterwards.

Raw insect materials, to be used as feed ingredient, may require heat treatment as is described in the EU legislation on animal by-products (EC 1069/2009, see 1.1.2.3). In the case of *T. molitor*, larvae are killed by blanching, which is a pasteurization treatment that kills vegetative cells whereas the number of spores is not affected (Vandeweyer et al., 2017). For BSF, harvested larvae are subjected to a heat treatment at 80 °C for 30 minutes (Personal communication of Heinrich Katz from the Hermetia Gruppe, Germany). Besides heat treatment, other technologies such as high hydrostatic pressure could be used in controlling pathogenic microorganisms. A study from Kashiri et al. (2017) investigated the usefulness of this technology for BSF larvae. Using a pressure of 400 MPa for 2.5 minutes at 25°C, yeast and mold did not survive in the larval samples whereas total aerobic mesophilic microorganisms were reduced.

The Hazard Analysis Critical Control Points (HACCP) system is recognized worldwide for quality assurance and controlling physical, chemical and biological hazards throughout the production

process. The implementation of HACCP throughout the insect supply chain could be crucial in the development of the insect production sector (van Huis et al., 2013).

1.1.2.3 Legislation

The insect sector, specifically for feed production, has to comply with several types of legislation in the EU. In what follows, a short overview of the legislative status in the summer of 2017 is given.

- EC 999/2001: Transmissible Spongiform Encephalopathies (TSE) regulation. This regulation prohibits the feeding of all animal proteins, processed (PAP) or not, with the exception of hydrolyzed protein. Consequently, insect proteins are not allowed as animal feed in the EU. In contrast, living insects, insect derived oils and hydrolyzed insect protein are allowed in animal feed. Since pet food is out of scope for this regulation, insect proteins are allowed for pet food (EU, 2001).
- Directive 2013/56/EU has allowed the use of non-ruminant processed animal products (PAP) in aquaculture, including invertebrate material. However, registration of slaughterhouses for insect slaughter was required and therefore insects were still not allowed in fish feed (EU, 2013).
- Commission Regulation EU 2017/893 authorizes the use of insect proteins as fish feed. This permission is limited to 7 species (i.e. BSF, common housefly, yellow mealworm, lesser mealworm, house cricket, banded cricket and field cricket) (EU, 2017c).
- According to Regulation EC 1069/2009 the insects can only be fed with 'feed grade' substrates which are allowed to be fed directly to farm animals. These substrates consist of vegetal origin materials or a limited number of animal origin materials, including fishmeal, blood products from non-ruminants, egg and eggs products, milk and milk based products, honey, rendered fats. In addition, for the production of protein, fat/oil and chitin, to be used in feed, raw insect materials are required to undergo processing in order to sufficiently reduce biological hazards (EU, 2009a).

The International Platform of Insects for Food and Feed (IPIFF) states that on the medium-long term (2020), insect protein for poultry and pigs will be allowed. The main hurdle is that, for the moment, it cannot be proven that insect protein intended for pig or poultry feed, is not contaminated with pig or

poultry protein, respectively. The legislation might change, as soon as a reliable analytical method is available. The European Reference Laboratory for animal proteins in feedingstuffs has been working on the development of such a method since several years (IPIFF, 2017).

1.1.3 The black soldier fly, Hermetia illucens

The insect species with the highest potential for large-scale production are BSF, common housefly, *Musca domestica* (Diptera: Muscidae), and the mealworm species *T. molitor* and *Alphitobus diaperinus* (Coleoptera: Tenebrionidae). These species can potentially be used to upgrade low value side streams, of which globally an approximate amount of 1.3 billion metric tons per year are produced, into high value protein (Veldkamp et al., 2012; ABN AMRO, 2016). BSF larvae have already been formulated as a component of complete diets for poultry (Hale, 1973; De Marco et al., 2015, Schiavone et al., 2017), swine (Newton et al., 1977), and for several commercial fish species (Newton et al., 2005; St-Hilaire et al., 2007; Magalhães et al., 2017). They were found to support good growth and, therefore, it was generally concluded that BSF larvae can be a suitable protein source for animal feed.

BSF naturally inhabits (sub)tropical and temperate regions of the Americas between 45° N and 40° S and has established in several areas outside of its geographical origin (May, 1961; Callan, 1974; Kim, 1997; Üstüner et al., 2003). During its lifecycle, this holometabolous insect passes through several developmental stages (egg, 6 larval instars with the last being indicated as the “prepupal stage”, pupa and adult). Egg clusters are typically deposited in crevices or on surfaces above or adjacent to decaying organic matter, like manure. Hatching of eggs occurs in approximately 4 days (Tomberlin, 2001). At optimal temperatures of 27.0-30.0 °C for larvae (Tomberlin et al., 2009) and 27.5-37.5 °C for adults (Booth and Sheppard, 1984), the larvae reach their maximum weight (dependent of the rearing substrate) in 2 to 3 weeks and the adult females show high fecundity (600-700 eggs/female) (Tomberlin, 2001). The last larval stage (prepupa) is brown in color and at this stage larvae stop feeding and empty their digestive tract. Then, the prepupae migrate in search of a dry and protected

site in preparation of metamorphosis. Under optimal conditions, the pupation (i.e. combined prepupal and pupal stage) takes about 2 weeks (Sheppard et al., 1994). The adults do not need to feed, are not known to transmit diseases, and rely on the nutrients stored from the larval stage (Diener, 2010). In Figure 2, the life cycle of BSF from the colony maintained at the Faculty of Bioscience Engineering of Ghent University is shown. Larvae were reared in a climate chamber on chicken feed at a temperature of 27 ± 1 °C and a relative humidity of 65 ± 5%, hatching adults were removed from the chamber and placed near a sunlit window at ambient conditions (25 ± 5 °C and 50 ± 10% RH). Table 2 presents an overview of the insect's taxonomic placement.

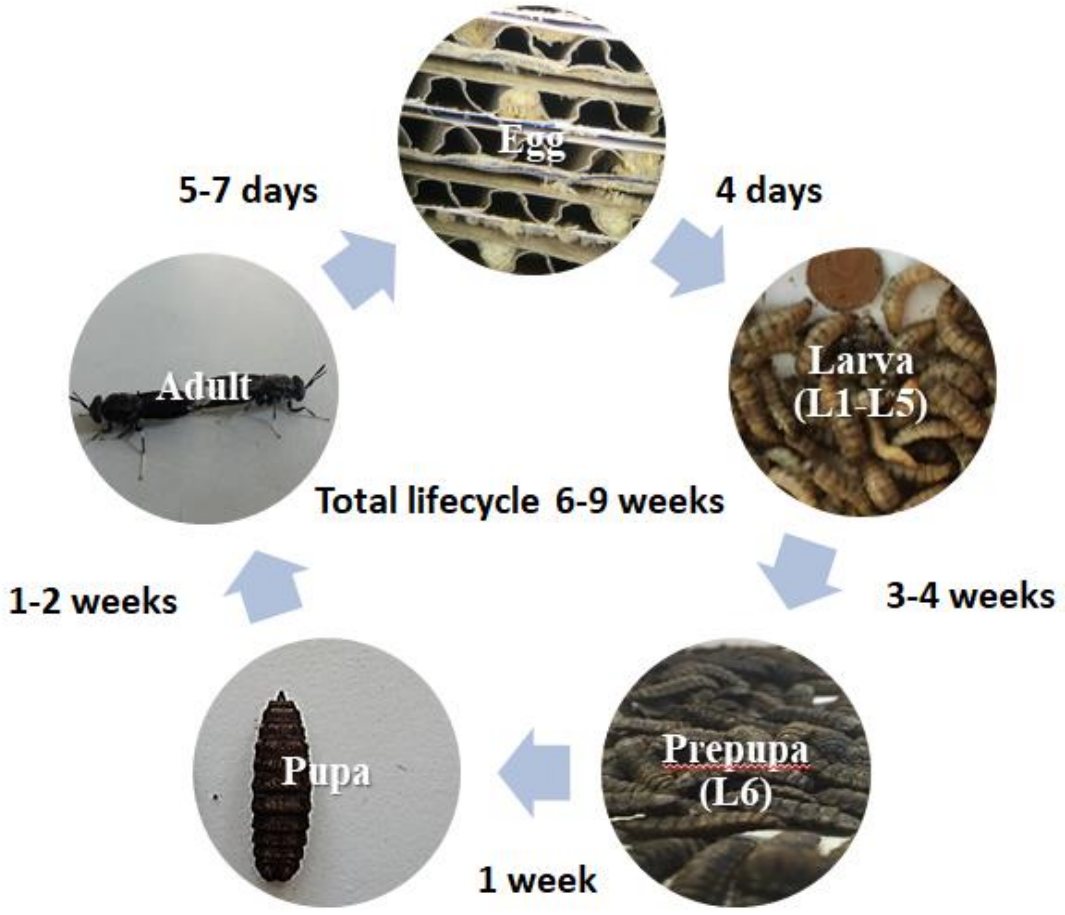

Figure 2: Life cycle of BSF

Table 2: Taxonomic placement of BSF

Kingdom	Animalia
Subkingdom	Bilateria
Infrakingdom	Protostomia
Superphylum	Ecdysozoa
Phylum	Arthropoda
Subphylum	Hexapoda
Class	Insecta
Subclass	Pterygota
Infraclass	Neoptera
Superorder	Holometabola
Order	Diptera
Suborder	Brachycera
Infraorder	Stratiomyomorpha
Family	Stratiomyidae
Subfamily	Hermetiinae
Genus	*Hermetia* Linnaeus
Species	*Hermetia illucens* Linnaeus

1.2 Objectives and thesis outline

In this Chapter 1, information is provided on the need for alternative ingredients for livestock production, and the potential of insects as a feedstuff, in particular BSF.

In Chapter 2, aspects of the rearing and reproductive biology of the BSF are described. Detailed information on the small scale laboratory rearing of BSF in literature is limited (Sheppard et al., 2002, Nakamura et al., 2016) and, therefore, a thorough description of the rearing procedures for our colony at Ghent University is included. The rearing of BSF could be divided in two specific phases being, on one hand, the rearing of larvae in order to obtain larval biomass to be harvested and, on

the other hand, the production of fertile eggs in order to provide larvae. The latter phase involves the rather complicated, and understudied, mating and oviposition behavior of BSF adult females. Therefore, in order to provide more information, which could contribute to the optimization of the egg production, an experiment evaluating the fecundity of females of different weights was performed.

The specific objective of this experiment was:

- To assess the effect of prepupal weight on the fecundity of the resulting females.
    - Hypothesis (H1): larger prepupae will develop into heavier flies with higher fecundity than their lighter counterparts.

Concerning the production of larval biomass for animal feed, a wide variety of rearing substrates could be used. In order to be sustainable, the applied substrates should be side streams. The efficiency of insect production systems, their contribution to global protein supply and the associated environmental impact will largely depend on the quality of the insect diet (Lundy and Parrella, 2015; Smetana, 2016). However, the chemical composition of these substrates may have substantial effects on the growth and quality of the resulting larval/prepupae. In Chapters 3, 4 and 5 the effects of rearing substrate on the biology (Chapter 3) and chemical composition (Chapters 4 and 5) of BSF were assessed.

In addition, given that information about possible risks associated with BSF production in Europe is limited and warranted (EFSA, 2015), environmental risks linked to the introduction of an exotic species (Chapter 3) and chemical feed safety hazards (Chapter 4) were evaluated.

The objectives for the respective chapters were:

- To determine the cold hardiness of BSF under different circumstances and implications towards overwintering potential.
    - Hypothesis (H2): the (sub)tropical BSF is not able to survive northwestern European winters.
    - Hypothesis (H3): the cold tolerance of BSF will depend on developmental stage, rearing substrate and acclimation.
- To assess the potential uptake and accumulation of heavy metals and pesticides from the rearing substrate by different larval stages of BSF (fifth instars and prepupae) and implications towards feed safety.
    - Hypothesis (H4): cadmium and pesticides with high log(K_{ow}) value are most likely to accumulate.
    - Hypothesis (H5): lower concentrations of heavy metals and pesticides are present in the post-feeding prepupae compared to fifth instar larvae.

The choice for heavy metals and pesticides was motivated by the specific nature of some of the substrates applied in our research (details provided in Chapters 4 and 5). In addition, for pesticides, to our knowledge, the number of studies is limited. Only Purschke et al. (2017) investigated 3 active substances.

In Chapters 5 and 6, nutritional aspects of BSF prepupae as a feedstuff for terrestrial monogastric farm animals were assessed, with focus on piglets in Chapter 6. Chapter 5 presents a thorough nutritional analysis of BSF prepupae reared on various commercially available side stream substrates (i.e. catering waste, vegetable waste and biogas digestate). The objective of this chapter was:

- To Evaluate the production and nutritional composition of BSF prepupae reared on side streams of different composition.
    - Hypothesis (H6): the composition of the substrate will substantially affect the growth and composition of the resulting prepupae.
    - Hypothesis (H7): the effect of substrate on the composition of the prepupae will differ according to the nutrient considered.

In Chapter 6, the nutritional value of BSF prepupae was further assessed using *in vitro* and *in vivo* experiments. Besides being a potential source of high value protein, BSF prepupae are also rich in fat, with levels ranging between 15 and 49% on DM basis. Notably, the fatty acid profile is, in general, high in the medium-chain fatty acid (MCFA) lauric acid (C12:0) (Makkar et al., 2014). MCFAs are well known for their antimicrobial effects on gut microbiota. Since in-feed antibiotics are banned in the EU and anticipating the withdrawal of zinc oxide at pharmacological doses and copper as growth promoter, there is an increasing need for reliable in-feed alternatives (EMA, 2017). Because of its high value protein and presumed antimicrobial effects, the weaner diets for piglets might be an interesting niche market for full-fat BSF products. The added value provided by the antimicrobial properties of the BSF fat could justify higher prices for BSF compared to soybean. Therefore, the objectives for this chapter were:

- To assess the possible anti-microbial effects of BSF fat extracts *in vitro.*
    - Hypothesis (H8): BSF fat will inhibit the growth of gram positive bacteria given their richness in lauric acid.
- To validate the results from the *in vitro* study by an *in vivo* experiment with weaned piglets and to validate the nutritional (protein and fat) value of this alternative feedstuff.
    - Hypothesis (H9): diets containing BSF fat will have an inhibitory effect on gram positive bacteria in the proximate small intestine of piglets.

- Hypothesis (H10): piglets reared on diets containing BSF will show no differences in performance compared to the control.

In the concluding *in vivo* experiment, BSF prepupae, full-fat and defatted batches purchased from a commercial breeder (Hermetia Gruppe, Germany), were incorporated in the feed of early weaned piglets. The nutritional value of the prepupae was assessed by recording the piglets' performance, evaluating the gut health and determining the digestibility.

In the final chapter, the research findings are discussed, conclusions are drawn and future perspectives for the commercial rearing of BSF as a feed ingredient are formulated.

Chapter 2

Rearing and reproductive biology of black soldier fly

2.1 Laboratory rearing system

An important advantage of BSF with regard to industrial rearing is that this species is relatively easy to rear (van Huis et al., 2013). This is definitely the case when BSF is compared to species like crickets or locusts and the larval development of BSF is faster than that of other interesting species like *Tenebrio molitor*. Several researchers have studied certain aspects of BSF rearing (Tomberlin, 2001; Barry, 2004; Diener, 2010; Holmes, 2010; Gobbi, 2012; Oonincx, 2015) and practical information on BSF rearing systems is provided by various authors (Newton et al., 2005, Alvarez, 2009; Dortmans et al., 2017). However, at the onset of our research, the only detailed information on BSF rearing at a laboratory scale was provided by Sheppard et al. (2002). Therefore, the laboratory colony established in order to conduct our experiments is described in the first part of this chapter.

A stock colony of BSF was established in May 2013 at the Department of Crop Protection of Ghent University, Belgium, using insects supplied by Millibeter BVBA, Antwerp, Belgium. The rearing methods were similar to those described by Sheppard et al. (2002) with several alterations. The immature life stages (eggs (Figure 3), larvae (Figure 4) and (pre)pupae (Figure 5)) were reared in the controlled environment of a climate chamber with a temperature of 27 ± 1 °C and 65 ± 5% relative humidity (RH) under complete darkness. The larvae were reared on a diet composed of 1/3 ground chicken feed pellets (Legkorrel TOTAL 77, Aveve, Belgium) and 2/3 distilled water, on weight basis, similar to the feed used by Diener et al. (2009). The emerging prepupae were allowed to self-harvest and settle in potting soil (Perspotgrond Groententeelt, STRUCTURAL Type 0, Belgium), which is a superior pupation substrate according to Holmes et al. (2013).

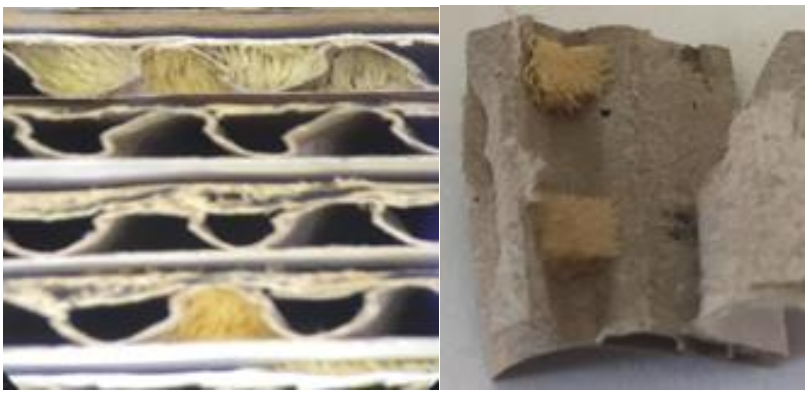

Figure 3: BSF egg clusters deposited in cardboard strips

Figure 4: BSF larvae reared on moistened chicken feed

Figure 5: BSF prepupae; left: self-harvesting; right: settling in potting soil

When flies started to emerge, they were transferred to 30 x 30 x 30 cm cages at a density of about 150-200 flies per cage (BugDorm-1 Insect Rearing Cage, South-Korea). The cages were placed near a sunlit, south-oriented window, to induce mating and oviposition, at a temperature of 25 ± 5 °C and 50 ± 10% RH (Figure 6). For mating, the flies from our laboratory colony mostly relied on sunlight. However, from November until April extra light was provided in the morning and evening using a quartz iodine lamp of 500 Watt (QVF415, Phillips), based on the findings of Zhang et al. (2010). In countries where sunlight is abundant throughout the whole year, cages could be positioned strategically in order to achieve maximum mating activity, without having to provide artificial lighting. According to Zhang et al. (2010), from Wuhan, Hubei, China where sunrise was at approximately 05:30 h, and sunset at 18:00 h during the experiments, 85% of mating was observed in the morning starting from 08:30 h and peaking at 10:00 h. The mating activity peaked at a light intensity of approximately 110 µmol m^{-2} s^{-1}, while mating almost completely ceased during midday when the light intensity was 200 µmol m^{-2} s^{-1}. Interestingly, it could be observed that most of the mating activity of flies kept under artificial light, at constant light intensities and completely deprived of sunlight, could also be perceived in the morning.

Figure 6: BSF adult rearing cages

During our research, preliminary experiments were carried out in a climate chamber with fluorescent tube light (FL40W T12, Philips) at different intensities (0-20,000 lx); however, the number of fertilized egg clusters was substantially lower than when direct sunlight was provided. Nakamura et al. (2016) observed that sunlight promoted greater fertility and hatchability than fluorescent lighting (40 Watt) supplemented with a 20 Watt LED lamp. However, Oonincx et al. (2016) showed that rearing systems with LED lamps emitting light of 365 nm, 450 nm and 515 nm in wavelength yielded more larvae than fluorescent lighting. In addition, different light intensities were tested and no differences were observed in terms of fertile egg production, which indicated that wavelength is a more important factor than light intensity.

BSF flies do not need to feed since they rely on fat reserves built up during the larval stages (Sheppard et al., 2002). However, once every day, water was sprayed on the cages in order to allow uptake by the flies. It was shown by Tomberlin (2002) that the longevity of the flies prolonged substantially when water was offered. Contrarily to the findings of Sheppard et al. (2002) who recommended the use of large cages of 200 x 200 x 400 cm, satisfactory mating and oviposition was achieved in our laboratory colony using much smaller cages (30 x 30 x 30 cm). Similar findings were reported by Nakamura et al. (2016) who kept 100 flies in cages of 27 x 27 x 27 cm. The latter authors speculated that high density is an important factor in achieving mating and oviposition in a limited space. The mating behavior of BSF is still a complex phenomenon that needs further investigation in order to optimize indoor production systems.

Different phases can be distinguished in the culture of BSF: the augmentation phase with emphasis on the reproduction (i.e. the production of eggs or young larvae) and the rearing phase of the larvae with emphasis on the production of larvae or prepupae to be harvested. Each phase has its own specific requirements and therefore separate facilities could be established (ABN AMRO, 2016). From our colony, it was observed that the nursery of young larvae is an important factor with a major influence on the further development of the larvae. Larvae that received biogas digestate, a poor

substrate in terms of nutritional composition (Chapter 5), needed 41 days from egg hatching to prepupae, while larvae that received chicken feed during the first week prior to digestate feeding only needed 24 days in total. In addition, flies that originated from larvae reared on digestate showed a high mortality and were, besides being smaller, less successful in reproducing.

2.2 Characteristics of larval and pupal stages

2.2.1 Introduction

During immature development, the exoskeletons of insect larvae are replaced regularly. This process is called molting and divides the larval period into several discrete stages. The period between two successive molts is known as an instar. The total number of larval instars is widely variable across insect species (Esperk et al., 2007). According to the literature, BSF larvae go through 6 molting stages (Hall and Gerhardt, 2002). In addition, three studies determining the morphological characteristics of the developmental stages of BSF larvae have been conducted (May, 1961; Kim et al., 2010; Gobbi, 2012). However, the results differ substantially among the studies as well as from observations of our own laboratory colony. Therefore, since identification and discrimination of larval instars is important in order to conduct specific experiments (Chapter 3), morphological characteristics of the larval and pupal stages from our colony were determined

2.2.2 Materials and methods

Kim et al. (2010) identified larval instars based on the exuviae observed during the development of a cohort of larvae. These larvae were reared on Drosophila standard medium containing 62.4 g dry yeast, 84 g dextrose anhydrous, 40.8 g corn meal, 9.2 g agar, 5 ml honey, 14.6 ml of mold inhibitor (i.e. 50 ml of 10% p-hydroxybenzoic acid methyl ester in ethanol and 23 ml of propionic acid), and 1 L of distilled water. However, since BSF larvae are inhabiting a wet food source, this method is not practical, especially for the first instars of which the exuviae are hard to distinguish from the

substrate (own observations). According to Dyar (1890) there is a significant relation between the head capsule measurements (i.e. length and width) of the larvae and their developmental stage. For BSF, head capsule measurements were already described by May (1961), Kim et al. (2010) and Gobbi (2012). However, the obtained values differ substantially from each other and, therefore, head capsule measurements were performed on larval and pupal stages from our colony.

Every day, from egg hatching until pupal development, samples were taken from the stock colony, weighed (Sartorius H110, ± 0.0001 g) and the head capsule widths and lengths were measured using a stereomicroscope (Leica S8AP0) (Figure 7). From these measurements, abrupt changes could be observed in time indicating transition to the following instar. For every instar, the mean of 30 measurements was taken at the moment that every larva reached the specific instar (the appearance of more than 2 consecutive instars at the same time was never observed). Consequently, a development table with relevant morphological characteristics of the larval and pupal stages from our colony could be constructed (Table 3).

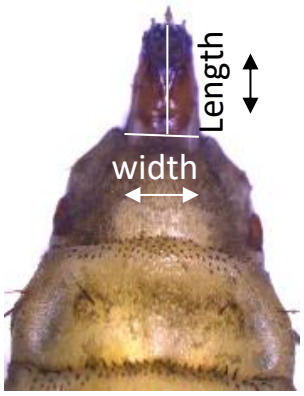

Figure 7: head capsule measurements

2.1.2 Results and discussion

Although instar number is frequently considered to be fixed within species, variability in the number of instars is not exceptional. Common factors influencing the number of instars are temperature, humidity, photoperiod, quality and quantity of the diet, rearing density, physical condition,

inheritance, and gender (Esperk et al., 2007). In our colony, however, 6 larval instars were observed using head capsule measurements. Based on the findings by other authors (May, 1961: Kim et al., 2010; Gobbi, 2012) this confirms that BSF larvae go through a fixed number of 6 molting stages.

When the data from Table 3 are compared to those in the literature, differences could be observed. Head capsule length measurements obtained by Gobbi et al. (2012) are somewhat comparable to our data. However, these measurements differ from those obtained by May (1961). In addition, the head capsule width measurements observed by Kim et al. (2010) differ from our data, with the biggest differences situated in the first 4 instars. These differences suggest that head capsule measurements of the different instars of BSF larvae are dependent on the observed BSF population. For the mosquito *Anopheles merus* Donitz seasonal variations in larval head capsule widths could be observed. Mean head capsule widths were, for all larval instars, 4.8-7.9% smaller in the summer population compared to the winter population (Sueur and Sharp, 1991).

Table 3: Characteristics (means ± SE) of larval and pupal stages of BSF (N=30)

	Larval instar						
	L1	L2	L3	L4	L5	L6/Prepupa	Pupa
Duration (days)	1 ± 0.2	3 ± 0.2	6 ± 0.4	7 ± 0.4	8 ± 0.5	9 ± 0.4	10 ± 0.5
Weight* (mg)	0.2 ± 0.01	3.8 ± 0.1	66.1 ± 0.3	134.2 ± 0.7	307.1 ± 0.9	229.2 ± 0.8	209.4 ± 0.6
Head capsule width (mm)	0.155 ± 0.007	0.418 ± 0.001	0.745 ± 0.003	0.858 ± 0.003	1.015 ± 0.004	1.157 ± 0.006	0.888 ± 0.008
Head capsule length (mm)	0.183 ± 0.008	0.557 ± 0.003	1.340 ± 0.004	1.679 ± 0.006	1.968 ± 0.007	2.052 ± 0.009	1.550 ± 0.010
							

*The fresh weights are those recorded at the moment that all insects of the sampled batches were situated in the respective instar

2.3 Reproductive performance as affected by body size

2.3.1 Introduction

Ensuring good mating and reproduction of BSF adults is much more complicated than larval rearing. Mating aspects have been investigated by Tomberlin (2001), Zhang et al. (2010) and Oonincx et al. (2016), but the reproductive capacity of BSF females currently remains understudied. Fecundity, which is expressed as the number of eggs oviposited per female, can be influenced by various factors (e.g. diet and temperature) (Vandekerkhove, 2010). Darwin (1874) was the first to describe a positive correlation between female body size/weight and fecundity in animals: larger females have a higher egg production than smaller females of the same species. For many invertebrates this phenomenon has also been reported (Shine, 1988; Honěk, 1993; Preziosi and Fairbairn, 1996). For some species, however, there is no or even a negative correlation between body size/weight and fecundity (Jiménez-Pérez and Wang, 2004). For the grass moth *Parapediasia teterella* the reproduction is positively correlated with the weight of the females as long the weight is situated under the mean of the population. Once above this mean the correlation turns to negative (Marshall, 1990). A positive correlation between female body size/weight and fecundity has been shown for various Diptera species (Honěk, 1993). For *Musca domestica*, however, no significant differences were observed between the number of eggs laid by females belonging to different weight classes (Pastor et al., 2011). Whether or not there is a correlation between female body size/weight and fecundity for BSF has not yet been reported. Therefore, in the second part of this chapter, possible differences in fecundity between BSF females belonging to different weight classes are assessed.

For BSF it has not been documented whether or not there is a correlation between female body size and fecundity. Therefore, in the following experiment, prepupae of our colony were divided into three classes according to their body weight. For all classes the fecundity of the resulting adults was assessed and, in addition, the viability of their offspring was monitored until the prepupal stage.

Whereas many studies have investigated the influence of weight or size on the fecundity of insects, different methods have been applied to determine the fecundity. Some authors estimated the lifetime fecundity by dissecting females at a certain age. This method, however, has mostly been applied for species where the oogenesis is terminated in the early adult stage (i.e. pro-ovigenic species). For other species (i.e. synovigenic species), the female needs to be monitored during the complete lifespan in order to determine the lifetime fecundity (Leather, 1988). There are species for which the lifetime fecundity can be estimated by monitoring ovipositing females only during a certain period instead of their complete lifespan (Honěk, 1993). The eggs of BSF females are deposited in one cluster during a single oviposition event (Tomberlin, 2001). Moreover, the eggs are easy to collect and, therefore, in this study the fecundity was determined without the need to perform dissections of the adult females. In addition, such dissections would not allow to discriminate fertilized from unfertilized eggs.

2.3.2 Materials and methods

Four hundred prepupae were collected from the stock colony, weighed individually (Sartorius H110, ± 0.0001) ranked from light to heavy and subsequently divided into three classes of fresh body weight (with equal numbers of individuals). The prepupae were transferred to plastic containers (SPL Life Sciences, Type Insect Breeding Dish; 10 x 4 cm) filled with potting soil (Perspotgrond Groententeelt, STRUCTURAL TYPE 0) and placed in a climate chamber at 27 °C and 65% RH. After 15 days, the first adults emerged and within 48 hours, for each weight class, 3 cages (BugDorm-1 Insect Rearing Cage; 30 x 30 x 30 cm) containing 9 or 10 adult females and 6 males per cage were set up. From colony observations it was deduced that one male could mate with multiple females and that the presence of too many males resulted in enhanced competition and thus might have a negative influence on the fertilization rate. The weight of the males was not considered and males were distributed randomly over the different female weight classes. The cages were placed in the proximity of a large sunlit window, in order to receive sufficient sunlight to induce mating. Room

temperature and RH were monitored using data loggers and the flies were provided with water sprayed on the cages twice every day. After three days, in each cage a dish was placed containing moistened compost, originating from decomposing garden waste, as an oviposition attractant; the dish was covered with cardboard strips to collect eggs. Two days later the first eggs were collected and fresh dishes with cardboard strips and compost were provided. This was repeated every 48 h and all females were monitored until they died.

Complete egg clusters were removed from the cardboard strips and weighed (Sartorius H110, ± 0.0001 g). Subsequently, 50 eggs were isolated from each cluster, hand counted and based on their joint weight the individual egg weight was determined. In this way, the total amount of eggs per cluster could be estimated. Based on this estimated individual egg weight, another group of about 50 eggs was sampled from each egg cluster (in this case the eggs were not hand counted to prevent possible damage to the eggs) and placed in a climate chamber at 27 ± 1 °C and 65 ± 5% RH in order to monitor hatching. Finally, a third sample of about 200 eggs was taken from the remaining eggs of each cluster in order to determine prepupal weight of the offspring. One week after hatching of the latter egg sample, 50 larvae were randomly collected per cluster and reared on stock colony diet (50 g of fresh diet every week) until they reached the prepupal stage. For every weight class, the emerging prepupae were pooled and the weights of the 75 first emerging prepupae were recorded. All data were analyzed using SPSS 22.0 (IBM Corp, 2013). One-Way ANOVA, followed by post hoc Tuckey (equal variances) or Tamhane (unequal variances) tests, was used when the data (i.e. prepupal weights of the first (Table 4) and second (Table 6) generations and the fecundity characteristics (Table 5)) were normally distributed, while otherwise Kruskal-Wallis tests followed by Mann-Whitney U tests were applied to compare the means. P-values below 0.05 were considered significant.

2.3.3 Results and discussion

In order to assess the influence of the fresh body weight on the fecundity of the females, prepupae were divided into 3 weight classes: light, medium and heavy. There were significant differences in prepupal body weight between the different classes (χ^2=354,667; df=2; P<0.001) (Table 4).

Table 4: Mean body weight of BSF prepupae belonging to different weight classes

Class	N	Weight (mg)	Weight range (mg)
Light	133	164.3 ± 0.1 c	111 – 185
Medium	134	201.4 ± 0.1 b	185 – 219
Heavy	134	249.4 ± 0.2 a	219 – 319

Mean ± SE; values within the same column followed by a different letter are significantly different (P<0.05; Kruskal-Wallis followed by Mann-Whitney U tests); N= no. of sampled individuals

The fecundity characteristics of BSF females for the 3 weight classes are displayed in Table 5. Only 46% of the light females were able to produce viable eggs, while this number increased to 68% for the medium sized females and even reached 88% for the heavy females. The individual egg weights were not statistically different (F=0.698; df=2, 46; P=0.503) while the mean weights of the egg clusters displayed significant differences (F=3.615; df=2, 46; P=0.035). The mean cluster weights were substantially higher for medium sized females compared to light females (P=0.035). However, the heavy females did not lay heavier egg clusters compared to those of the medium (P=0.903) and even the light (P=0.118) group. The number of eggs per cluster was the highest for the medium sized group (F= 4.695; df=2, 46; P=0.014). The difference with the heavy group was not significant (P=0.623) but that with the light group was (P=0.011). From the obtained results it could be concluded that the lightest prepupae yielded adults with a substantially lower fecundity than those of the medium and heavy group. If the latter groups were compared, there were no differences in the total amount of eggs deposited per cluster and number of hatched larvae per fertile egg cluster.

However, the substantially higher number of fertile egg clusters deposited by the heaviest females indicated that they had a higher mating success than their lighter counterparts.

Ovipositing BSF females of our population from all weight classes combined, laid on average 647 eggs. Each egg cluster weighed on average 16 mg and the mean individual egg weight was 0.024 mg. These findings are in agreement with the data obtained by Tomberlin (2001). In addition, the longevity of the females in the latter study was also comparable to that of our colony with an average value of 9-10 days, while the largest females survived the longest. Since BSF adults from our colony did not receive additional food besides water, they relied on the fat reserves built up during the larval stages. The correlation between the weight of the flies and their fat content was not assessed during this study, but from the findings in Chapter 5 it can be deduced that heavier prepupae have a higher fat content. According to Oonincx et al. (2016) and Nakamura et al. (2016) providing a sugar solution substantially prolongs the longevity of BSF flies. However, since providing only water already maintained BSF adults long enough for successful reproduction, a sugar solution was not applied in our colony.

Table 5: Fecundity characteristics of BSF females belonging to different weight classes

Weight	No. of females	No. of egg clusters	No. of hatched clusters	No. of eggs/cluster*[1]	Cluster weight (mg)*[1]	Egg weight (mg)*[1]	No. of hatched larvae/cluster*[2]
Light	28	19	13	532 ± 49 b	12.9 ± 1.1 b	0.0243 ± 0.0007 a	314 ± 29 b
Medium	28	26	19	739 ± 47 a	17.1 ± 1.2 a	0.0231 ± 0.0010 a	333 ± 21 ab
Heavy	18	16	15	669 ± 55 ab	16.4 ± 1.2 ab	0.0245 ± 0.0010 a	375 ± 31 a

*Mean ± SE; values within the same column followed by a different letter are significantly different ($P<0.05$; [1]One-Way ANOVA followed by Tukey tests; [2]Kruskal-Wallis followed by Mann-Whitney U tests)

Larvae resulting from the collected egg clusters were reared until they reached the prepupal stage. Differences between development time and mortality among the weight classes were not observed. The weights of the prepupae originating from females of different weight classes are displayed in Table 6. There were significant differences between the prepupal weights (χ^2= 42.225; df= 2; P<0.001). The heavy flies produced the heaviest prepupal offspring while the medium group produced prepupae which were significantly heavier than those originating from the light group.

Table 6: Mean body weight of BSF prepupae originating from females of different weight classes

Class	N	Weight (mg)*	Weight range (g)
Light	75	162.1 ± 3.0 c	110 - 210
Medium	75	189.4 ± 3.2 b	129 – 288
Heavy	75	206.3 ± 4.0 a	131 – 295

*Mean ± SE; values within the same column followed by a different letter are significantly different (P<0.05; Kruskal-Wallis followed by Mann-Whitney U tests); N= no. of tested individuals

The findings of this experiment could have interesting implications towards the commercial breeding of BSF. Based on our results, selective breeding programs in which the heaviest females are selected to reproduce in order to ameliorate the population performance, both for reproduction and larval production, might become a standard practice. However, since only one generation of females was tested, clear conclusions about the feasibility of selective breeding in BSF production cannot be drawn. Whether or not the prepupae originating from the heaviest flies would in turn develop into the most fertile flies is uncertain. In addition, selecting females solely based on one phenotypic trait (i.e. their weight) may overlook certain genetic factors which might also have an important influence on their fitness (e.g. resistance to diseases). In conclusion, more research is warranted in order to optimize future breeding programs for BSF.

Chapter 3

Cold hardiness of black soldier fly

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3.1 Introduction

Companies in several industrialized countries are interested in developing mass production facilities for the black soldier fly (van Huis et al., 2013). However, in many of these countries (e.g. western European countries) BSF is considered a non-native species (Leclercq, 1997). Large scale production of this insect would thus increase the probability of escape and establishment of this exotic species in the natural ecosystems of western Europe. The (un)intentional introduction and subsequent establishment of an exotic insect has been reported multiple times in western Europe. Over the last decade, examples include several accidentally imported plant pests (e.g. *Anoplophora* spp., *Epitrix* spp., *Tuta absoluta* (Meyrick)), but also carnivorous species introduced for biological control purposes (e.g. *Harmonia axyridis* (Pallas)) (DAISIE, 2006; Brown et al., 2011; EPPO Reporting Service, 2017). When such exotic species become invasive, there may be negative effects on agricultural as well as natural ecosystems. Due to such mechanisms as competition, predation, herbivory and spread of diseases and parasites, invasive alien species (IAS) are presently considered to be one of the main causes of global diversity loss, alongside climate change, habitat destruction, pollution and overexploitation (Roy et al., 2011). It is worth noting that although BSF has established in several areas outside of its geographical origin in the Americas (May, 1961; Callan, 1974; Kim, 1997; Üstüner et al., 2003), the species has not been reported to become invasive so far.

In order to assess the likelihood of a species to become invasive, an environmental risk assessment can be performed. An environmental risk assessment characterizes the probability and severity of possible harmful effects on the ecosystem, when exposed to stressors (Andersen et al., 2004). One part of this assessment for the introduction of species in temperate areas consists of the determination of the cold tolerance of the species, i.e. the ability of the species to survive winter conditions (van Lenteren et al., 2006; Bale, 2011; Maes et al., 2015). Since BSF naturally inhabits (sub)tropical and temperate regions of the Americas between 45° N and 40° S characterized by mild winters (Üstüner et al., 2003), it may seem unlikely for this species to survive the winters of

northwestern Europe (e.g. northern France, Belgium, The Netherlands, Germany, United Kingdom and Ireland). In the southern United States, the fly reportedly has only three generations per year (Sheppard et al., 1994), suggesting that the species uses some mechanisms to bridge unfavorable periods. Holmes et al. (2016) estimated that the lower temperature threshold for larval development is situated between 16 and 19 °C. However, populations of BSF were recently observed in the wild in areas with cold winters, including Germany (47° 33’ N) (Ssymank and Doczkal, 2010) and even the Czech republic (49° 55' N) (Rohacek and Hora, 2013) where larvae were discovered in the early spring, but it is not clear whether these populations have effectively established.

So far, little is known about the capability of BSF to adapt to severe winter conditions. Insect species are traditionally divided in two main groups in terms of their ability to cope with subzero temperatures: freeze-tolerant and freeze-avoiding species. The major difference between these strategies is that freeze-tolerant species synthesize ice forming agents (proteins) to allow a safe extracellular freezing, whereas freeze-avoiding species remove all potential ice forming material, such as gut content, and produce polyol or protein antifreeze agents (Bale and Hayward, 2010). In many cases, however, insects perish due to prolonged exposure to above zero cold temperatures, rather than freezing, due to the accumulation of chilling injury. The associated thermotropic damage to the cell membranes may lead to metabolic imbalances and ultimately result in death (Lee, 2010). However, changes in membrane lipid composition can protect certain insect species from damage to their cells (Bale and Hayward, 2010). Thus, a diversity of cold tolerance mechanisms has evolved in insects to cope with both temperatures below and above 0°C (Bale, 1993). In addition, many insects have developed diapause mechanisms to deal with the unfavorable winter conditions (Bale and Hayward, 2010). Although there is evidence of diapause in the Stratiomyidae family (Rozkosny and Kovac, 1998), it is not known whether diapause occurs in BSF, and if so, what are the factors triggering the process. Further, overwintering in protected environments may allow insects to survive the cold, which is the case for *H. axyridis* (Berkvens et al., 2010). Populations of BSF might also overwinter indoors, like in heated stables, and larvae have been hypothesized to survive northern

winters in livestock manure (Rohacek and Hora, 2013). Finally, insects may migrate over longer distances to avoid cold winters (Urquhart and Urquhart, 1978), but to our knowledge there are no published data on such migratory behaviors in BSF.

In a first attempt to investigate the potential of BSF to survive winters in northwestern Europe, the present study examined the cold tolerance of different developmental stages using the methodology developed by Hart et al. (2002). The cold tolerance of early instar larvae (second-third instars), late instar larvae (fourth-fifth instars), prepupae (sixth instars), pupae and adults was estimated in the laboratory, using the supercooling point (SCP) and lethal time (LTime) at 5 °C as parameters. The SCP is the temperature at which the body liquids freeze, ultimately leading to death, and therefore it is the absolute lowest limit of cold tolerance of a freeze avoiding insect (Koch et al., 2004). In order to assess the mortality due to chilling injury, lethal times (e.g. $LTime_{50}$ or the time point at which 50% of the population is expected to die) can be calculated (Berkvens et al., 2010). Diet may significantly affect the physiology of insects and may alter their responses to climatic conditions, including cold temperatures (Maes et al., 2015). Larvae of the black soldier fly can be reared on a wide variety of organic substrates (Diener et al., 2009) and the insect's life history traits (Nguyen et al., 2013) as well as chemical composition (Chapter 5) are influenced by the rearing substrate. In addition, many species become acclimatized to winter conditions due to gradually decreasing temperatures during the autumn months (Hatherly et al., 2005, Maes et al., 2015). Therefore, the effects of larval rearing substrate and acclimation on the cold tolerance of BSF prepupae were assessed in separate experiments.

3.2 Materials and methods

3.2.1 Insects

The stock colony population, provided by Millibeter BVBA to Ghent University in 2013 (Chapter 2), originated from South Georgia, USA, and was shipped to Europe around 2010.

3.2.2 Experiments

In a first experiment the SCPs and the lethal times at 5 °C of different life stages were examined. A detailed description of the methods to determine SCP- and LTime-values is given below. Larvae of different instars, prepupae, pupae and adults used in this experiment were taken from the stock colony. Larval batches were the progeny of at least 30 females. Early instar (second-third instars) and late instar (fourth-fifth instars) larvae were distinguished based on head capsule measurements (Chapter 2). Prepupae were transferred to potting soil (Holmes et al., 2013) at 27 ± 1 °C and allowed to settle for 24 h before the experiment. Preliminary observations indicated that at 27 ± 1 °C prepupae start turning into pupae from the 6^{th} day on after having settled in potting soil, and after 9 days more than 90% have reached the pupal stage. The pupae used in the experiment were collected from the potting soil 9 days after they had been introduced to the medium as prepupae.

To assess the effect of the larval rearing substrate on the SCP and LTime at 5 °C of prepupae, two substrates were selected in a second experiment: catering waste and biogas digestate. These substrates were selected given their potential in organic waste management programs with BSF and their disparity in terms of composition (Chapter 5). The digestate was selected as a model for a nutritionally poor waste stream whereas the catering waste was chosen as a nutrient rich waste stream. The catering waste consisted of a homogenized mixture of cooked vegetables (mainly potatoes, 25% on wet weight basis) and fish (50% on wet weight basis), thus providing a diet that was rich in digestible starch and protein. The biogas digestate, obtained from Trevi NV (Gentbrugge,

Belgium), resulted from anaerobic fermentation of vegetable waste by microorganisms. The digestate was centrifuged into a liquid and a solid fraction, with the latter being used in the experiment. This substrate was deprived of energy rich components (like starch and sugars) and therefore rich in fiber (Chapter 5). The two substrates were tested together with the chicken feed substrate used in the stock colony as a control. For each substrate, 3 replicates of 1000 larvae (7 days old) were reared up to the prepupal stage, during which period the larvae were provided with 1 kg of wet feed every week until the first prepupae were observed.

In a third experiment, the effect of temperature acclimation on both the SCP and LTime at 5 °C of prepupae, reared on the chicken feed diet (i.e. originating from the stock colony), was assessed by keeping them at 16 ± 1 °C (as the temperature in between the rearing temperature of 27 ± 1 °C and the experimental temperature of 5 ± 0.5 °C) for 7 days from the day after the prepupae had settled in the potting soil to the start of the experiment. The values of SCP and LTime at 5 °C of acclimated insects were compared with those of a non-acclimated control, originating from the same rearing batch.

As prepupae of BSF are known to migrate towards a suitable pupation substrate (e.g. soil) (Holmes et al., 2013), in the wild prepupae and pupae are expected to reside in the upper soil layer (i.e. top 10 cm). To evaluate the relevance of our laboratory findings for the overwintering capacity of BSF prepupae in the field, soil temperature data at a depth of 10 cm during the winter months of 1994-2014 in Melle, Belgium, were acquired from the Belgian Royal Meteorological Institute (RMI).

3.2.3 Supercooling point

The SCP was measured using a Picotech TC-08 thermocouple datalogger and a low temperature programmable Haake Phoenix II CP30 alcohol bath. Each thermocouple was led individually through the lid of a 1.5 mL Eppendorf tube. Each Eppendorf tube contained a single insect. The Eppendorf

tubes were placed individually in glass tubes which were immersed in the alcohol bath. The starting temperature was 27 ± 1 °C (rearing temperature). The insects were cooled at 0.5 °C/min until the thermocouple registered the release of exothermal heat, at which point the SCP is reached (Berkvens et al., 2010). Insects were weighed before being subjected to the experiments, using a Sartorius H110 semi-microbalance (± 0.01mg). Emerging adults were sexed. This was done in order to check whether SCP was correlated with fresh body weight and gender. Forty replicates were done per treatment.

3.2.4 Lethal time at 5 °C

Individuals of the different life stages collected from the stock culture at 27 ± 1 °C (experiment 1), prepupae reared on different substrates (experiment 2) and acclimated versus non-acclimated prepupae (experiment 3) were placed per 10 individuals in 10 cm diameter insect breeding dishes (SPL Life Sciences, Korea). The dishes were transferred to incubators at 19 ± 1 °C and 12 ± 1 °C (Weiss ET 2028), and held there for 30 minutes each time to avoid possible mortality due to cold shock, before being finally transferred to an incubator set at 5 ± 0.5 °C (Weiss ET 2028). This short acclimation was not performed on the acclimated prepupae in experiment 3. Larvae (both early and late instars) collected from the stock culture to be used in the lethal time experiments were not provided with food during the experiment as preliminary tests showed that the availability of a feed substrate had no influence on their survival. Throughout their exposure to 5 ± 0.5 °C, the insects were kept in total darkness and relative humidity was not controlled. Three insect breeding dishes (10 individuals per dish) were taken from the incubator at 5 ± 0.5 °C at regular time intervals (i.e. every 24h), and subsequently placed in incubators at 12 ± 1 °C and 19 ± 1 °C, where they were held for 30 minutes each time, and then finally transferred to 27 ± 1 °C. From then onwards, the larvae were provided with chicken feed (i.e. the rearing diet of the stock culture) and kept there for 48 h, after which survival was monitored. Survival of prepupae and pupae was determined by monitoring adult emergence. The lethal time at 5 °C of eggs, both fresh and 3 days old, and adults was not studied in detail as no egg hatching was observed and all of the exposed adults died in less than 3

days during preliminary tests. According to Hatherly et al. (2005, 2008), Hughes et al. (2009) and Allen (2010), a strong positive correlation (R^2=0.9299) exists between maximum field survival during northwestern European winters and survival at 5 °C measured under laboratory conditions for 9 arthropod species:

$$\text{field survival (days)} = 1.4906 * \text{LTime}_{50}\text{ (days)} + 12.444 \quad \text{(Allen, 2010).}$$

3.2.5 Statistical analysis

All data were analyzed using SPSS 22.0 (IBM Corp, 2013). For the SCPs and body weights (only for prepupae reared on different substrates) a Kolmogorov–Smirnov test was conducted to assess normality of the data. In case of normality, one-way ANOVA was used to analyze the data. Subsequently, depending on the outcome of a Levene-test, a Tukey (homoscedasticity) or Tamhane test (heteroscedasticity) was performed to separate the means. In case of not normally distributed data, a non-parametric Kruskal-Wallis test was used. When significant differences were detected, means were compared two by two using Mann-Whitney U tests. An unpaired t-test was used to check for differences in SCP between males and females, whereas correlation of this parameter with the body weight of the different stages prior to testing was investigated using regression analysis. P-values below 0.05 were considered significant. Results from the lethal time experiments were analyzed with probit/logit analysis, which estimated the time necessary to kill 10, 50 and 90% of the population at a temperature of 5 °C. Significant differences were identified by non-overlapping fiducial limits (Hart et al., 2002).

3.3 Results

3.3.1 Supercooling point

There were significant differences in SCPs between the different life stages of BSF (χ^2=90.245, df=4, P<0.001) (Table 7). The average SCP of the early instar larvae was 2.26 °C below that of the older larvae. The SCP of the prepupal stage was significantly lower compared to that of the larval stages (P<0.001). The pupal stage showed the lowest SCP of all tested stages (P<0.001). The SCP of the pupal stage was 2.17 °C, 6.42 °C and 4.16 °C lower than that of the prepupae, late and early instars, respectively. The SCP of the adults (males and females pooled) was similar to that of the early instar larvae (P=0.166), but differed from that of the other life stages tested (P<0.001). There was no significant linear relationship between the SCPs and body weights of the different stages (R^2=0.063, P=0.129, N=38 for early instars; R^2=0.045, P=0.226, N=34 for late instars; R^2=0.044, P=0.197, N=39 for prepupae; R^2=0.023, P=0.354, N=40 for pupae; R^2=0.001, P=0.847, N=40 for adults). Moreover, the SCP was not affected by the gender of the adults (t=0.170, df=38, P=0.866).

Table 7: Body weight and supercooling point (SCP) of different life stages of BSF

Life stage	N[a]	Body weight (mg)[b]	SCP (°C)[b,c]	Range of SCP (°C)
Early instar (L2-L3)	38	52 ± 5	-9.54 ± 0.46c	-7.12 to -15.75
Late instar (L4-L5)	34	207 ± 6	-7.28 ± 0.18d	-5.40 to -11.30
Prepupa	39	207 ± 5	-11.53 ± 0.36b	-6.98 to -15.87
Pupa	40	192 ± 4	-13.70 ± 0.43a	-7.12 to -18.30
Adult (sexes pooled)	40	96 ± 3	-10.00 ± 0.39c	-4.96 to -14.84
Male adult	25	92 ± 3	-10.05 ± 0.54	-4.96 to -14.84
Female adult	15	108 ± 4	-9.91 ± 0.56	-6.95 to -13.61

Number of individuals tested; [b]Means ± SE; [c]Means within a column followed by different letters are significantly different (Mann-Whitney U Test, P<0.05)

Larval rearing substrate had a significant influence on the SCP of the ensuing prepupae (χ^2=21.728; df=2; P<0.05) (Table 8). Prepupae reared on catering waste had the lowest SCP (-14.08 °C) as compared to those that had developed on the control substrate and biogas digestate (-12.36 °C and -11.15 °C, respectively). There was no significant difference between the SCPs of the acclimated prepupae and the control batch (χ^2=1.190; df= 1; P=0.275).

Table 8: Body weight and supercooling point (SCP) of BSF prepupae as affected by diet or acclimation

Treatment	N[a]	Body weight (mg)[b]	SCP (°C)[b]	Range of SCP (°C)
Substrate				
Control	36	220 ± 4a	-12.36 ± 0.49b	-5.19 to -16.81
Digestate	38	84 ± 3b	-11.15 ± 0.43c	-6.11 to -16.22
Catering waste	40	219 ± 4a	-14.08 ± 0.39a	-8.50 to -17.48
Acclimation				
Control	38	Nd[c]	-11.11 ± 0.46a	-6.59 to -16.37
Acclimated	38	Nd	-11.77 ± 0.44a	-6.69 to -17.69

[a]Number of individuals tested; [b]Means ± SE; means within a column and a treatment (substrate or acclimation), followed by different letters are significantly different (Mann-Whitney U Test, P<0.05); [c]Not determined

Over all experiments, none of the exposed individuals survived the supercooling experiments. In the period from 1994-2014, the minimum soil temperature recorded in Melle, Belgium, was -3.3 °C which is well above the SCP of all life stages.

3.3.2 Lethal time at 5 °C

The lethal time values ($LTime_{10, 50, 90}$) at 5 °C of the tested life stages of BSF are presented in Table 9. Significant differences were observed between the instars (Pearson χ^2 test for fitting probit model: χ^2=58.19; df=51; P=0.228). Second to third instars were most vulnerable to low above-zero temperatures with only 50% surviving after 3.92 days. Based on non-overlapping 95% fiducial limits, the $LTime_{50}$ of older larvae was significantly longer compared to that of the early instars. For the

prepupal and pupal stage, half of the individuals were still alive after 7.01 and 6.66 days, respectively. Prepupae survived significantly longer at 5 °C than the previous stages with still 10% survival after 10.90 days. The difference in survival at 5 °C between prepupae and pupae was not significant.

Table 9: Lethal times [±95% fiducial limits] at 5 °C for different life stages of BSF

Life stage	N[a]	Slope + SE	$LTime_{10}$ (days)	$LTime_{50}$ (days)	$LTime_{90}$ (days)	χ^2
Early instar (L2-L3)	12	-3.55 + 0.21	2.39 [2.16-2.63]c	3.92 [3.61-4.24]c	6.41 [5.91-6.99]c	58.19
Late instar (L4-L5)	12	-4.30 + 0.23	3.18 [2.89-3.47]b	5.21 [4.83-5.60]b	8.52 [7.89-9.25]b	
Prepupa	16	-4.94 + 0.25	4.07 [3.73-4.40]a	6.66 [6.25-7.09]a	10.90 [10.20-11.72]a	
Pupa	16	-5.07 + 0.25	4.28 [2.56-4.05]ab	7.01 [6.58-7.45]a	11.46 [10.73-12.32]a	

[a]Number of sampling events (i.e. every 24h, 30 individuals were sampled); Lethal time values within a column; followed by different letters are significantly different (non-overlapping 95% fiducial limits)

Larval rearing substrate had significant effects on the LTime of the ensuing prepupae (Pearson χ^2 test for fitting logit model: χ^2=187.03; df=65; P<0.001) (Table 10). Prepupae that had developed on digestate showed the highest survival at 5 °C. After 14.09 days there still was a survival of 50%, while only 10% of the prepupae reared on the control substrate survived an exposure of 11.07 days. The average $LTime_{50}$ of prepupae reared on catering waste was 9.56 days. Acclimation significantly affected the LTime of prepupae (Pearson χ^2 test for fitting probit model): χ^2=64.35; df=33; P=0.001). The lethal time values ($LTime_{10,\ 50,\ 90}$) at 5°C for acclimated versus non-acclimated prepupae are shown in Table 10.

Table 10: Lethal times [±95% fiducial limits] at 5 °C for BSF prepupae from different treatments

Treatment	N[a]	Slope + SE	$LTime_{10}$ (days)	$LTime_{50}$ (days)	$LTime_{90}$ (days)	χ^2
Substrate						
Control	23	7.74 + 0.40	3.07 [2.48-3.67]c	5.83 [4.99-6.76]c	11.07 [9.49-13.21]c	187.03
Digestate	23	6.05 + 0.34	7.42 [6.20-8.56]a	14.09 [12.59-15.66]a	26.73 [23.67-30.95]a	
Catering waste	23	9.07 + 0.44	5.03 [4.11-5.93]b	9.56 [8.35-10.83]b	18.13 [15.88-21.15]b	
Acclimation						
Control	18	2.06 + 0.14	5.43 [3.04-7.23]b	14.37 [13.08-15.67]b	23.32 [21.50-25.74]b	64.35
Acclimated	18	3.30 + 0.18	14.11 [12.32-15.62]a	23.06 [21.54-24.89]a	32.01 [29.56-35.36]a	

[a]Number of sampling events (i.e. every 24h, 30 individuals were sampled); Lethal time values within a column and a treatment (substrate or acclimation) followed by different letters are significantly different (non-overlapping 95% fiducial limits)

3.4 Discussion

The present study provides a first insight into the capacity of BSF to survive the winters of northwestern Europe. Our experiments indicated significant differences in the SCPs of the insect's life stages. The difference in SCP of young versus older larvae may be related to their body size. Smaller-sized insects generally have a lower SCP (Sømme, 1982; Johnston and Lee, 1990). As an alternative explanation, differences in gut content of the larvae may be responsible for the observed differences in SCPs: the lesser the gut content, the fewer potential ice forming particles are present and thus, the lower the SCP (Koch et al., 2004). Since the stadium duration of the combined second and third instars is shorter than that of the fourth and fifth instars (Kim et al., 2010), a higher proportion of the young larvae had probably just molted. During the molting process the gut of larvae and prepupae of BSF is partly voided (Sheppard et al., 1994; personal observations). Thus, fewer ice nucleating particles present in the gut of the tested batch of second-third instars may have made them less susceptible to freezing. The differences in SCP between the larval stages on the one hand and the

prepupal and pupal stages on the other could be related to the same principle. The latter non-feeding stages likely had little gut content, making them less susceptible to freezing. This pattern of lower SCPs in non-feeding versus feeding stages has been documented in various insects (Salt, 1953; Leather et al., 1996; Duman, 2001; Koch et al., 2004). The difference in SCP between the prepupal and the pupal stage could be due to the presence of a puparium in the latter. This protecting structure reduces direct contact with moisture, resulting in a prevention or delay of freezing (Miller, 2003).

All individuals, irrespective of their developmental stage, eventually died when their SCP was reached. According to Bale (1993) and Lee (2010), this indicates that BSF may be a freeze-intolerant species. Bale (1993) pointed out that the majority of freeze-intolerant species may die before the SCP is reached as a result of cumulative chilling injury; the resulting damage to the cell membranes may lead to metabolic imbalances and ultimately result in death (Lee, 2010). Lethal time experiments at 5 °C indicate that prepupae and pupae were the most cold tolerant life stages. Since no individual of any tested life stage survived for more than a few weeks at 5 °C, it can be suggested that BSF is susceptible to chilling injury.

One parameter that could influence the cold hardiness of an insect is the diet on which it is reared (Maes et al., 2012; Maes et al., 2015). Diet can significantly affect the physiology of insects and consequently alter their responses to climatic conditions (Grenier and De Clercq, 2003). A diet rich in certain nutrients may be able to provide the insect with components that promote its cold hardiness (Specty et al., 2003; Maes et al., 2012; Maes et al., 2015). Molecules like the amino acid proline and the disacharide trehalose could help to preserve the cell structure by binding to cell membranes and consequently preventing the binding of water molecules (Doucet et al., 2009). Individuals used in our second experiment were reared on diverse food sources. From the supercooling experiments with the resulting prepupae, one might deduce a positive relationship between the nutritional value of the rearing substrate and the cold hardiness of the insect: prepupae reared on catering waste had a

lower SCP (-14.08 °C) than those reared on chicken feed (-12.36 °C), whereas the SCP of prepupae obtained on digestate was even higher (-11.15 °C). Since prepupae were used in this experiment, the presence of ice nucleating particles present in the gut was probably negligible. However, the insect hemolymph could contain ice nucleating proteins and there might be differences in hemolymph composition between prepupae reared on different substrates (Duman, 2001). The results of the LTime experiments do not show a positive correlation with the nutritional value of the rearing substrate since, contrarily, prepupae reared on the poorest substrate were the most cold hardy. Likewise, Maes et al. (2012; 2015) reported a positive relationship between supercooling ability and nutritional value of the diet in two insect predators, whereas lethal times appeared to be independent of the diet. In order to draw reliable conclusions concerning the winter survival of BSF, additional information should be taken into account and several caveats are to be considered to interpret the results of our study.

Insects used in the first experiment were taken from a stock culture maintained under constant, optimal conditions and they were allowed only a short acclimation to decreasing temperature. In temperate regions insects in the field are subjected to varying temperatures due to seasonal variation. Here, many species become acclimatized to winter conditions due to gradually decreasing temperatures during the autumn months (Hatherly et al., 2005; Maes et al., 2015). The outcome of our LTime experiment suggests that there is a significant effect of acclimation on the cold tolerance of BSF prepupae. Moreover, given that the acclimation treatment in our experiment was rather short (i.e. 7 days) and done only at one temperature (16 °C being in between the optimal temperature of 27 °C and the LTime-temperature of 5 °C), true autumn conditions may improve the cold hardiness of BSF in temperate regions to a greater extent than was found in our laboratory experiment (Hart et al., 2002; Maes et al., 2012; Maes et al., 2015). As prepupae of BSF need a suitable substrate for pupation (Holmes et al., 2013), a significant part of the insect's life cycle in the wild might take place in the upper soil layer. According to the Belgian RMI data from 1994-2014, the mean soil

temperature during winter (December–February) at a depth of 10 cm ranged from 1.9 to 5.3 °C. Since the $LTime_{50}$ of acclimated prepupae at 5 °C was about 23 days, this would imply that prepupae settled in the soil are rather unlikely to survive the whole winter. Applying the relationship between $LTime_{50}$ at 5 °C and field survival established by Allen (2010) (see section 3.2.4) to the data for the non-acclimated prepupae from our study, it can be predicted that BSF would not survive longer than 34 days in the field during northwestern European winters. According to the same relationship, however, field survival of the acclimated prepupae would be over 46 days. When applying the classification proposed by Allen (2010), acclimated prepupae are situated in between the low risk and the medium risk group for establishment. Taking all of our data into consideration, it would thus be rather unlikely for BSF to permanently inhabit the cooler temperate climate regions of northwestern Europe. For instance, compared to the harlequin ladybird *H.axyridis*, that has successfully invaded western Europe and other temperate areas (Berkvens et al., 2010; Brown et al., 2011), the SCP of BSF is rather high, while the $LTime_{50}$ is substantially shorter (*H. axyridis*: SCP= -17.5 °C; $LTime_{50}$ at 0 °C= 18 to 24 weeks (Berkvens et al., 2010)).

Another crucial factor that warrants further investigation is the diapause ability of the species or of its local populations. Insects in temperate areas usually have better cold hardiness in a diapausing state (Bale and Hayward, 2010). Diapause in insects can occur in different developmental stages, depending on the species, and can be triggered by various stimuli. In tropical insects, diapause induction is linked to changes in temperature, moisture, and more often to biotic factors like population density and food availability (Denlinger, 1986). Whereas in temperate regions day length appears to be the most important trigger of diapause, the effect of photoperiod in tropical insects is minimal because of the relatively constant day lengths in regions close to the equator (Bale and Hayward, 2010). Since BSF is widely distributed in the geographic range from 45° N to 40° S, its populations are subjected to variable day lengths. Therefore, it is not unlikely that progressive shortening of day length might trigger diapause in at least some geographic populations of BSF. Unfortunately, there is little or no information on diapause responses in BSF. So far, evidence of

diapause in the family of Stratiomyidae is restricted to only one species, the oriental soldier fly, *Ptecticus flavifemoratus* Rozkosny & Kovac, originating from Malaysia (Rozkosny and Kovac, 1998).

Finally, it deserves emphasis that certain environments could prove to be suitable for certain life stages of BSF to bridge the winter months. Given that populations of BSF in the USA and elsewhere are closely associated with animal production facilities (Newton et al., 2005), it might be possible for the species to avoid low temperatures by overwintering indoors, like in pig or poultry stables where relatively high temperatures are maintained year round. Further, Rohacek and Hora (2013) reported that a larval population of the black soldier fly was found in livestock manure during the spring of 2010 in the Czech Republic at a latitude of 49° N. Although the authors believed that this was probably only a transient population, this observation indicates the potential for BSF larvae or pupae to survive during winter by inhabiting specific hibernacula like manure, organic waste or compost heaps where temperature remains higher than in the surrounding environment due to bacterial activity.

In conclusion, SCPs and lethal times observed in this laboratory study suggest that BSF is unlikely to survive winter in northwestern Europe. However, in order to reliably conclude on the potential of BSF to establish in northwestern Europe, further investigation on overwintering mechanisms in this species is warranted, with a focus on diapausing ability, indoor overwintering and migratory behaviors.

Chapter 4

Potential chemical hazards associated with waste streams used for black soldier fly rearing

4.1 Introduction

Verbeke et al. (2015) investigated the attitude of both farmers and consumers towards the use of insects in animal feed. Although insects were perceived by most responders to be an acceptable feed ingredient, some concerns have to be taken into account. An important concern of the general public was possible feed safety risks, originating from potential contamination of the insects with chemicals, pathogens and toxins. The EFSA opinion, published in 2015, on the use of insects in feed, stated that feed safety should be guaranteed and that much more research is needed. This is especially the case when the insects, like BSF larvae, are reared on waste streams. Low value waste streams such as manure, crop residues, decomposing fruits and vegetables, and biogas digestate might indeed contain substantial amounts of contaminants. The risks associated with the substrate applied for rearing could be either biological (e.g. pathogenic bacteria, viruses, parasites and fungi) or chemical (e.g. heavy metals, pesticides, mycotoxins, veterinary drugs, dioxins and PCBs) (EFSA, 2015). In this chapter, the presence of chemical contaminants (i.e. heavy metals and pesticides) in BSF larvae (i.e. fifth instars) and prepupae reared on prepared substrates was investigated. In contrast to pathogens and mycotoxins, which can also emerge during storage, the contamination of heavy metals and pesticides is solely determined by the rearing substrate. Therefore, given that our research focused on the effects of rearing substrates on BSF (Chapters 3,4 and 5), the substrate dependent contamination of BSF larvae with heavy metals and pesticides was investigated. Substrates like manure and biogas digestate might contain substantial amounts of heavy metals, while vegetable waste streams might be contaminated with pesticides. In addition, pesticides were chosen because these have been less addressed in previous research.

In Chapter 5, an increased ash content is shown in prepupae reared on biogas digestate which could lead to potential heavy metal contamination of the insect material if the substrate was contaminated. In the first part of the current chapter, bioaccumulation factors for BSF fifth instars and prepupae reared on digestate were tested for arsenic (As), lead (Pb) and cadmium (Cd). Since it

has been shown that larvae of the BSF tend to accumulate Cd (Diener et al., 2015; van der Fels-Klerx et al., 2016), for this particular heavy metal a spiking of the substrate fed to the larvae was conducted in order to test the potential bioaccumulation at different concentrations. The background Cd concentration of the substrate was almost equal to the EU maximum for feed ingredients (2 mg/kg on a 88% DM basis), which is potentially hazardous if we consider the insects to be eaten (EFSA, 2015). In addition, two intermediate concentrations and the maximum concentration allowed in digestate (2, 4 and 6 mg/kg Cd respectively, expressed on as is basis) were investigated. To assess whether the Cd had actually been accumulated in the insect body or was just present in the gut, at the end of the larval development both fifth instars and prepupae were harvested (larvae empty their gut when entering the prepupal stage (May, 1961)).

Whereas several studies have been published on the potential contamination of BSF with heavy metals (Charlton et al., 2015, Diener et al., 2015, van der Fels-Klerx et al., 2016), to our knowledge, only Purschke et al. (2017) investigated the impact of a limited spectrum of pesticides (only 3 active substances) on the composition of BSF fifth instars. In the latter study, the tested pesticides were neither accumulated in the larval tissue nor was the insect's development significantly affected. However, Houbraken et al. (2016) showed the possibility of certain active substances to accumulate in the biomass of *T. molitor* larvae. Therefore, in the present study, the potential of chemical contamination was assessed for 12 active substances. The investigated active substances are commonly applied in fruit and vegetable cultivation and in preliminary experiments, a dose of 0.5 mg/kg was tested for every substance, which is the residue level that could be expected when good agricultural practices are followed (FAO, 2017b). However, given that at this concentration almost no residues could be detected or quantified in the larvae, a 10-fold higher dose was chosen for the final experiment (5 mg/kg). For most active substances this concentration is either close to or above the maximum residue levels (MRLs) allowed on fruit and vegetables for human consumption (FAO, 2017b). Another argument for testing this dose was that, in future, insects might potentially be used to valorize batches that were rejected for human consumption due to exceeding MRLs. Both short-

term (24h) and long-term (2 weeks) exposure were evaluated and differences in contamination levels between fifth instars and prepupae of BSF were assessed.

4.2 Materials and methods

4.2.1 Heavy metal contamination in BSF larvae

The biogas digestate that was used as a larval substrate was analyzed for As, Pb and Cd by ICP-OES (ISO 11885:2007). The preparation included incineration at 450 °C until the ash was grey to red-brown followed by dissolving the ash in diluted nitric acid (7 M) (EN 13652:2001). In addition to the control treatment (0.5 mg/kg Cd), the substrate was spiked for Cd, by using cadmium chloride dissolved in distilled water, resulting in a total concentration of 2, 4 and 6 mg/kg Cd, expressed on wet basis since the substrate was provided to the insects on as is basis. These spiked substrates were also analyzed. Two thousand four hundred BSF larvae of 14 days old, reared on chicken feed, were randomly divided over the 4 treatments (0.5 mg/kg (i.e. unspiked control), 2 mg/kg Cd, 4 mg/kg Cd and 6 mg/kg Cd) and each treatment was replicated 3 times. Each replicate contained 200 larvae, reared for 7 days in a climate chamber at 27 ± 1 °C and 65 ± 5% RH. The larvae were weighed at the start and replicates were balanced, so that the total weight of the 200 larvae at the start of each treatment was between 19.9 and 20.0 g for every replicate. At the time of harvesting, the substrates were sieved, fifth instars and prepupae (which were already present) were separated and weighed and the mean number of prepupae was calculated. The collected fifth instars and prepupae were frozen and stored at -20 °C pending chemical analysis. Prior to analysis, the samples were freeze-dried until constant weight. The As, Pb and Cd levels in the fifth instars and the prepupae of the control treatment were determined by ICP-OES (ISO 11885:2007). For the other treatments, only Cd levels were determined. The BAFs (bioaccumulation factors) for As, Pb and Cd were calculated on a DM basis (i.e. % in sample on DM / % in substrate on DM).

4.2.2 Pesticide contamination in BSF larvae

A second experiment was set up to test bioaccumulation of pesticides. In this experiment, the substrates from Chapter 3: biogas digestate, catering waste and chicken feed were again used as substrates. A pesticide cocktail containing 12 active ingredients was mixed in each substrate in order to reach a concentration of 5 mg/kg on a wet basis for every active substance. The active substances (97.00-99.99% pure) were dissolved in acetone and this solution was dripped on the substrates with a pipette. The spiked substrates were kept under a hood overnight in order to evaporate all the acetone. The properties of the pesticides used are given in Table 11. The tested pesticides were both herbicides and fungicides which are commonly applied on fruit and vegetables and, therefore, possible risks for BSF rearing. The treatments for every substrate were control, short-term (24 h) and long-term (2 weeks) exposure to the pesticide cocktail (5 mg/kg), with 4 replicates per treatment. The insects were harvested in the fifth larval instar and the prepupal stage.

Table 11: Properties of the pesticides used for the contamination of the substrates (Tomlin, 2006)

Name of pesticide	Type of pesticide	Log(K_{ow}) (/)	Water solubility (g/l)	MRL feed ($mg.kg^{-1}$)
2,4-D	Herbicide	2.81	23.18 (pH 7, 25 °C)	0.01[a]
Azoxystrobin	Fungicide	2.5	6.00 (20 °C)	100.00[a]
Bentazone	Fungicide	-0.46	0.57 (20 °C, pH 7)	0.30[b]
Clopyralid	Fungicide	-2.63	9.00 (20 °C)	/
Cymoxanil	Herbicide	4.7	0.80 (pH 7, 25 °C)	/
Difenoconazole	Fungicide	4.4	$1.5x10^{-2}$ (25 °C)	/
Fenpropimorph	Fungicide	4.1	$4.32x10^{-4}$ (20 °C, pH 7)	5.00[b]
Linuron	Herbicide	3	0.08 (25 °C)	/
Metalaxyl	Fungicide	1.71	26.00 (25 °C)	/
Pendimethalin	Herbicide	5.18	$3.30x10^{-4}$ (20 °C, pH 7)	/
Pyraclostrobin	Fungicide	3.99	1.90 (20 °C)	/
Tebuconazole	Fungicide	3.7	$2.9x10^{-2}$ (20 °C, pH 7)	40.00[b]

MRLs according to Codex Alimentarius (FAO, 2017b): [a]MRL for soybean fodder, [b]MRL for wheat fodder

For every replicate 100 6-day old larvae were weighed and placed in 10 cm diameter containers (Insect Breeding Dish, SPL Life Sciences, South-Korea). The total weight for all larvae at the start of the experiment was 2.3-2.4 g for every replicate. In the control and short-term toxicity treatments larvae of each replicate received 50 g of unspiked substrate. For the long-term treatment, 50 g of spiked substrate was given to the larvae and the containers were stored for one week in a climate chamber at 27 °C and 65% RH. Thereafter, insects in all treatments were offered again 50 g of fresh diet, added to the old diet, and were stored for another week in the climate chamber. Then, larvae from all containers were separated from the old substrate and were presented with 50 g of fresh feed. This time the replicates of the short-term treatment group also received spiked feed. After 24

hours, 30 fifth instars of each treatment group were sampled and placed in the freezer (-20 °C), while the rest was left in the substrate to develop into prepupae. The rearing procedure was similar for the three tested substrates. However, since the larvae fed the digestate grew very slow because of its poor nutritional quality, only fifth instars and no prepupae were sampled. In order to determine the pesticide concentration of the frozen insect samples, LC–MS/MS analyses were performed on a triple quadrupole system with ESI (Waters ACQUITY UPLC, Xevo TQD mass spectrometer). The extraction procedure used was based on the standard QuEChERS-method for the extraction of pesticides (Houbraken et al., 2016). The limits of detection (LOD) and quantification (LOQ) for the twelve active substances are given in Table 12.

Table 12: Limit of detection (LOD) and limit of quantification (LOQ) of the 12 active ingredients in BSF

Name of pesticide	LOQ (mg/kg)	LOD (mg/kg)
2,4-D	0.005	0.0015
Azoxystrobin	0.001	0.0003
Bentazone	0.001	0.0003
Clopyralid	0.005	0.0015
Cymoxanil	0.001	0.0003
Difenoconazole	0.001	0.0003
Fenpropimorph	0.002	0.0006
Linuron	0.001	0.0003
Metalaxyl	0.001	0.0003
Pendimethalin	0.001	0.0003
Pyraclostrobin	0.001	0.0003
Tebuconazole	0.001	0.0003

4.2.3 Statistical analysis

All data were analyzed using SPSS 22.0 (IBM Corp, 2013). One-way ANOVA was used to compare the Cd levels among the fifth instars and prepupae reared at different concentrations (0.5 mg/kg (i.e. unspiked control), 2 mg/kg, 4 mg/kg and 6 mg/kg). The variances were analyzed using Levene tests and depending on the outcome, post hoc Tuckey (equal variances) or Tamhane (unequal variances) tests were applied. Student's t-tests were applied to compare the Cd, Pb and As concentrations of fifth instars and prepupae in the unspiked control treatment and Cd levels of fifth instars and prepupae for every Cd treatment. All tests had a significance value of 0.05.

Regarding the pesticide experiment, for each substrate, the contents of all active substances present in the fifth instars were compared with those of the prepupae within the same treatment group (control, short-term or long-term) using Student's t-tests. The same tests were applied to compare the fifth instars or prepupae of one treatment with the respective stages in another treatment. If a certain value was beneath the limit of detection (<LOD) or limit of quantification (<LOQ), one-sample t-tests comparing to ½ LOD or ½ LOQ were used. The weights of the fifth instars within each substrate group were compared among treatments using one-way ANOVA. Levene tests were applied to analyze the variances and depending on the outcome, Tuckey (equal variances) or Tamhane (unequal variances) post hoc tests were used. All tests had a significance value of 0.05.

4.3 Results

4.3.1 Heavy metal contamination in BSF larvae

The spiking with Cd did not affect the survival of BSF fifth instars as in all treatments 99-100% survived. However, the rate of development to the prepupal stage decreased with increasing Cd concentrations in the substrate (Table 13). The unspiked control contained 0.5 mg/kg Cd, 4.8 mg/kg Pb and 2.7 mg/kg As on wet basis. The DM content of the substrate being 208 g/kg, the blank thus

contained 2 mg/kg Cd, 18 mg/kg Pb and 11 mg/kg As on an 88% DM basis. Weights and DM contents of fifth instars and prepupae are presented in Table 13, while the respective heavy metal contents are shown in Table 14. In the latter table, the values for Cd, Pb and As are expressed on an 88% DM basis in order to compare them with threshold values for whole feeds and feed ingredients (EFSA, 2015). If the Cd levels in the wet digestate were 2 mg/kg or higher, the levels of both fifth instars and prepupae kept increasing accordingly with the concentration in the substrate (ANOVA across concentrations for fifth instars: $F=253.948$, $df=3$, $P<0.001$; prepupae: $F=205.225$, $df=3$, $P<0.001$). The BAFs in Table 15 show that only Cd accumulated starting from a concentration of 2 mg/kg in a wet substrate (corresponding to 10 mg/kg on DM basis). This accumulation was only witnessed in fifth instars since the values for prepupae are all around 1 (e.g. at a Cd concentration of 4 mg/kg in the substrate, the BAF of the fifth instars was 1.98 while that of the prepupae was only 0.91 ($t=9.381$, $df=4$, $P=0.001$)).

Table 13: Fresh weight and DM content (mean ± SD; weights expressed per individual in mg) of BSF fifth instars and prepupae reared on digestate spiked with different concentrations of Cd (mg/kg as is)

	Fifth instars		Prepupae		% developed prepupae
	Weight	% DM	Weight	% DM	
0.5 mg/kg Cd	90.0 ± 3.2aA	27.1 ± 1.0aB	88.2 ± 1.0aA	34.0 ± 0.2aA	66.4 ± 5.0a
2.0 mg/kg Cd	92.0 ± 1.6aA	26.4 ± 0.3aB	84.8 ± 2.5bB	34.0 ± 0.4aA	60.5 ± 2.0ab
4.0 mg/kg Cd	95.0 ± 4.3aA	26.7 ± 0.3aB	83.5 ± 2.0bB	33.8 ± 0.4aA	58.1 ± 3.8ab
6.0 mg/kg Cd	95.0 ± 2.4aA	27.0 ± 0.2aB	85.5 ± 2.5abB	33.8 ± 0.1aA	51.5 ± 7.4b

Means ± SD; means within a column followed by different lowercase letters are significantly different ($P<0.05$); means within a stage (fifth instars or prepupae) followed by different uppercase letters (weight or % DM) are significantly different ($P<0.05$);

Table 14: Concentrations of Cd, Pb and As in BSF fifth instars and prepupae (mg/kg on 88% DM basis) reared on biogas digestate spiked with different Cd concentrations (mg/kg as is)

	Fifth instars			Prepupae		
	Cd	Pb	As	Cd	Pb	As
0.5 mg/kg Cd	1.9 ± 0.2dA	1.1 ± 0.1A	<0.37	1.3 ± 0.2cB	0.4 ± 0.1B	<0.37
2.0 mg/kg Cd	15.5 ± 0.5cA			8.9 ± 0.3bB		
4.0 mg/kg Cd	31.0 ± 2.2bA			14.3 ± 2.1abB		
6.0 mg/kg Cd	47.7 ± 3.6aA			23.3 ± 0.4aB		

Means ± SD; means within a column followed by different lowercase letters are significantly different (P<0.05); means within a stage (fifth instars or prepupae) followed by different uppercase letters are significantly different (P<0.05)

Table 15: Bioaccumulation factors (% in sample on DM/% in substrate on DM) of BSF fifth instars and prepupae reared on biogas digestate spiked with different Cd concentrations (mg/kg as is)

	Fifth instars			Prepupae		
	Cd	Pb	As	Cd	Pb	As
0.5 mg/kg Cd	0.98 ± 0.09bA	0.05 ± 0.00A	<0.03	0.64 ± 0.11bB	0.02 ± 0.00B	<0.03
2.0 mg/kg Cd	1.74 ± 0.05aA			1.00 ± 0.07aB		
4.0 mg/kg Cd	1.98 ± 0.14aA			0.91 ± 0.13abB		
6.0 mg/kg Cd	1.91 ± 0.15aA			0.94 ± 0.02abB		

Means ± SD; means within a column followed by different lowercase letters are significantly different (P<0.05); means within a stage (fifth instars or prepupae) followed by different uppercase letters are significantly different (P<0.05)

4.3.2 Pesticide contamination in BSF larvae

The survival and final weight data of fifth instars reared on different substrates and subjected to different pesticide exposure regimes is displayed in Table 16. Long-term exposure to a cocktail of pesticides at a concentration of 5 mg/kg did not affect the survival of the fifth instars. However, fifth instars reared on catering waste with a 2 week exposure to the pesticides were significantly heavier than fifth instars reared on the same substrate without pesticides or exposed only for a 24 h period.

Table 16: Fresh weights and survival of 100 BSF larvae reared on different substrates and according to different exposure regimes to a cocktail of 12 active substances

	Control		Short-term		Long-term	
	Survival (%)	Weight (g)	Survival (%)	Weight (g)	Survival (%)	Weight (g)
Chicken feed	99 ± 1	19.4 ± 0.4a	99 ± 1	20.1 ± 0.3a	98 ± 1	18.9 ± 0,2a
Catering waste	98 ± 2	16.2 ± 0.9b	99 ± 1	16.6 ± 0.4b	99 ± 1	21.9 ± 0.7a
Digestate	93 ± 3	4.8 ± 0.4a	97 ± 3	4.9 ± 0.4a	96 ± 1	4.4 ± 0.3a

Means ± SD; means within a column followed by different letters are significantly different ($P<0.05$); there were no significant differences ($P<0.05$) between the means of the survival

The concentrations of active substances present in fifth instars and prepupae are shown in Tables 17, 18 and 19 for chicken feed, catering waste and digestate, respectively. In these tables, the values for the control treatments are not shown since these were all <LOD. The active substances 2,4-D, clopyralid and pyraclostrobin were not detected in fifth instars fed on any of the substrates. For most active substances, the concentrations in the fifth instars were significantly higher than those in the prepupae. When fifth instars of the short-term treatments are compared to those of the corresponding long-term treatments, only a few active substances showed differences. However, these differences are not consistent across substrates. For example, fenpropimorph and pendimethalin are the active substances found in the highest concentrations for fifth instars across all substrates. However, for fifth instars reared on digestate the concentrations found in the short-term treatment group are significantly lower than those in the long-term treatment (fenpropimorph: $t=-2.833$, $df=6$, $P=0.030$; pendimethalin: $t=-2.546$, $df=6$, $P=0.044$) whereas for fifth instars reared on catering waste the opposite is found (fenpropimorph: $t=3.958$, $df=6$, $P=0.007$; pendimethalin: $t=3.113$, $df=6$, $P=0.042$).

Table 17: Pesticide concentrations of BSF fifth instars and prepupae (in mg/kg) reared on chicken feed and exposed for 24 h (short-term) or 2 weeks (long-term) to a cocktail of pesticides at a concentration of 5 mg/kg for each component

	Short-term		Long-term	
	Fifth instars	Prepupae	Fifth instars	Prepupae
2,4-D	<LOD	<LOD	<LOD	<LOD
Azoxystrobin	0.104 ± 0.034aA	0.079 ± 0.009aA	0.129 ± 0.010aA	0.118 ± 0.044aA
Bentazone	<LOD	<LOD	<LOD	<LOD
Clopyralid	<LOD	<LOD	<LOD	<LOD
Cymoxanil	<LOD	<LOD	<LOD	<LOD
Difenoconazole	0.224 ± 0.074aA	<LODb	0.228 ± 0.036aA	<LODb
Fenpropimorph	1.376 ± 0.467aA	<LODb	1.502 ± 0.164aA	<LODb
Linuron	0.105 ± 0.044aA	0.086 ± 0.006aA	0.134 ± 0.025aA	0.108 ± 0.049aA
Metalaxyl	<LOQ	<LOQ	<LOQ	<LOQ
Pendimethalin	0.747 ± 0.226aA	0.037 ± 0.025bA	0.865 ± 0.084aA	0.060 ± 0.009bA
Pyraclostrobin	<LOD	<LOD	<LOD	<LOD
Tebuconazole	0.213 ± 0.059aA	0.065 ± 0.015bA	0.243 ± 0.036aA	0.066 ± 0.009bA

Means ± SD; means within a row and regime (short-term or long-term) followed by different lowercase letters are significantly different ($P<0.05$); means within a row and stage (fifth instars or prepupae) followed by different uppercase letters are significantly different ($P<0.05$); LOD: limit of detection; LOQ: limit of quantification

Table 18: Pesticide concentrations of BSF fifth instars and prepupae (in mg/kg) reared on catering waste and exposed for 24 h (short-term) or 2 weeks (long-term) to a cocktail of pesticides at a concentration of 5 mg/kg for each component

	Short-term		Long-term	
	Fifth instars	Prepupae	Fifth instars	Prepupae
2,4-D	<LOD	<LOD	<LOD	<LOD
Azoxystrobin	0.317 ± 0.083aA	0.125 ± 0.068bA	0.386 ± 0.050aA	0.115 ± 0.041bA
Bentazone	<LOQ	<LOQ	0.069 ± 0.019a	<LODa
Clopyralid	<LOD	<LOD	<LOD	<LOD
Cymoxanil	0.182 ± 0.030aA	<LODb	0.216 ± 0.034aA	<LODb
Difenoconazole	0.383 ± 0.058aA	0.086 ± 0.033bA	0.293 ± 0.016aA	0.054 ± 0.024bA
Fenpropimorph	1.410 ± 0.259aA	0.325 ± 0.145bA	0.893 ± 0.035aB	0.163 ± 0.065bA
Linuron	0.324 ± 0.065aB	0.132 ± 0.060bA	0.487 ± 0.087aA	0.122 ± 0.046bA
Metalaxyl	0.247 ± 0.040aA	0.106 ± 0.061bA	0.314 ± 0.044aA	0.081 ± 0.017bA
Pendimethalin	1.337 ± 0.218aA	<LODb	0.983 ± 0.066aB	<LODb
Pyraclostrobin	<LOD	<LOD	<LOD	<LOD
Tebuconazole	0.473 ± 0.064aA	0.161 ± 0.069bA	0.528 ± 0.053aA	0.154 ± 0.074bA

Means ± SD; means within a row and regime (short-term or long-term) followed by different lowercase letters are significantly different ($P<0.05$); means within a row and stage (fifth instars or prepupae) followed by different uppercase letters are significantly different ($P<0.05$); LOD: limit of detection; LOQ: limit of quantification

Table 19: Pesticide concentrations of fifth instars (in mg/kg) reared on biogas digestate and exposed for 24 h (short-term) or 2 weeks (long-term) to a cocktail of pesticides at a concentration of 5 mg/kg for each component

	Short-term	Long-term
2,4-D	<LOD	<LOD
Azoxystrobin	0.492 ± 0.172a	0.650 ± 0.054a
Bentazone	<LOQ	<LOQ
Clopyralid	<LOD	<LOD
Cymoxanil	<LOD	<LOD
Difenoconazole	1.180 ± 0.236a	1.455 ± 0.036a
Fenpropimorph	3.491 ± 0.661b	4.467 ± 0.194a
Linuron	0.408 ± 0.190a	0.541 ± 0.069a
Metalaxyl	0.145 ± 0.174a	0.268 ± 0.016a
Pendimethanil	3.008 ± 0.553b	3.753 ± 0.194a
Pyraclostrobin	<LOD	<LOD
Tebuconazole	0.835 ± 0.222a	1.112 ± 0.048a

Means ± SD; means within a row followed by different letters are significantly different ($P<0.05$); LOD: limit of detection; LOQ: limit of quantification

4.4 Discussion

If insects were to be considered as farm animals in the EU, biogas digestate could not be used as a substrate since Pb and As levels are expected to exceed the maximum limits (EFSA, 2015). Moreover, if manure had been fermented, the biogas digestate would contain probably even more heavy metals than the batch tested in our research. From Table 14, it can be deduced that only fifth instars and prepupae reared on the blank (i.e. non-spiked) substrate contained Cd levels below the 2 mg/kg Cd threshold for feed ingredients. In the present study, significant bioaccumulation of Cd was only

observed in fifth instars, but not in prepupae, starting from a substrate concentration of 2 mg/kg as is. The BAFs calculated in the present study are much lower than those obtained by Tschirner and Simon (2015), van der Fels-Klerx et al. (2016) and Purschke et al. (2017). In these studies, BAF values of 6.1 to 9.5 were observed. However, Diener et al. (2015) observed a maximum BAF for Cd of 2.8. Diener et al. (2015) was the only study where both fifth instars and prepupae were investigated. In contrast to our results where prepupae contained substantially less Cd than fifth instars, Diener et al. (2015) obtained comparable BAFs for both life stages of BSF. However, in the latter study Cd concentration in adults was significantly lower than in prepupae. The authors assumed that the reason for this was that the insects defecate before pupation or shortly after adult emergence. They hypothesized that defecation had not occurred during the 1-3 day period in which the insects had entered the prepupal stage, explaining the high concentrations in the prepupae. Prepupae from our study were about the same age as those in Diener et al. (2015). However, based on our results, defecation is a plausible explanation for the relatively low Cd content of the prepupae compared to fifth instars in our study. Moreover, from dissections of prepupae that were less than 2 days in this stage, we observed that at this point the prepupae had no gut content (Chapter 7). In the present study, As did not accumulate which is in compliance with the literature (van der Fels-Klerx et al., 2016; Purschke et al., 2017). Pb, on the other hand, accumulated in BSF in the studies by Tschirner and Simon (2015), van der Fels-Klerx et al. (2016) and Purschke et al. (2017) while this was not the case in the present study and Diener et al. (2015).

In terms of feed safety, there is a consensus among studies that Cd can be a risk factor. According to Craig et al. (1999), Cd^{2+} ions are able to pass through Ca^{2+} channels and enter the cells. Because larvae of the BSF are, in general, high in calcium (Chapter 5), they might be more prone to Cd accumulation compared to other insect species. Moreover, according to van der Fels-Klerx et al. (2016), Diptera species could be classified as macroconcentrators of Cd in which the Cd storage capacity is increased due to increased metallothionein levels. Consequently, substrates used for the production of BSF larvae should contain less Cd than the 2 mg/kg maximum limit for feed ingredients

in order to be safe (EFSA, 2015). In addition, the levels of other metals which possibly induce metallothionein expression (e.g. zinc) and, therefore, might increase the Cd accumulation, should be controlled. Prepupae from the present study were safer to use in animal feed than fifth instars. However, given that the BAFs for prepupae are around 1, a BSF rearing substrate should still contain no more than 2 mg/kg Cd to produce feed grade prepupae with Cd levels below this limit. Differences in the accumulation of Cd might be due to the form in which the Cd was present in the substrate. In this study, cadmium chloride dissolved in distilled water was used whereas the other studies used 2% HNO_3 solutions.

In contrast to the observed Cd accumulation, none of the tested active substances from the pesticide experiment accumulated over a two-week exposure period. Moreover, larval development was unaffected, even after a long-term exposure to a pesticide cocktail with 12 active substances at a concentration of 5 mg/kg. This result could in part be expected given that none of the tested pesticides were insecticides. In a preliminary experiment, BSF larvae were exposed to different concentrations of the commonly applied neonicotinoid imidacloprid during a period of 48 h. As long as the concentration of the substrate was 1 mg/kg or lower, the larvae were unaffected. However, at a concentration of 10 mg/kg 15% of the larvae died within 48 h while another 30% showed very little activity, indicating that their death was imminent. In contrast, in the present study, larvae reared on catering waste and exposed for 2 weeks to fungicides and herbicides, grew substantially bigger than the pesticide free control larvae reared on the same substrate. It is speculated that some fungicides may have eliminated certain organisms present in the substrate which were likely to compete with the larvae for nutrients. In addition, this could also mean that less energy was allocated by the insect to the production of antimicrobial peptides while on the other hand more could be invested in the production of body mass (Otvos, 2000). However, it should be noted that certain fungicides might have a negative influence on the gut microbiota of insects. This was shown for the honey bee (*Apis mellifera* L.) by Pettis et al. (2013) who found an increased probability of infection with the gut parasite *Nosema ceranae* in bees that consumed pollen with a high fungicide load.

The pesticides that were the most abundant in BSF fifth instars across substrates were the herbicide pendimethalin and the fungicide fenpropimorph. These active substances are both characterized by a high log(K_{ow}) and a very low water solubility (Table 11). According to Houbraken et al. (2016), in *T. molitor* larvae pesticides with higher log(K_{ow}) values were taken up more easily. In contrast, the excretion rate after 24 h of starvation was lower with higher log(K_{ow}) values. In the present study, however, prepupae contained substantially less pendimethalin and fenpropimorph than the corresponding fifth instars, which suggests a high excretion rate for these active substances with high log(K_{ow}) values. In addition, different metabolic processes might be involved for these active substances (e.g. biotransformation and degradation) (Houbraken et al., 2016). None of the pesticides detected in fifth instars and prepupae of the present study reached values above the MRL for feed ingredients (Table 11). Only fifth instars reared on digestate which were long-term exposed to 5 mg/kg fenpropimorph showed a value close to the MRL (i.e. 4.5 versus 5 mg/kg for MRL).

Differences between fifth instars reared on different substrates could be observed. Fifth instars reared on chicken feed contained traces of only 6 active substances (Table 17) while those reared on digestate (Table 19) and catering waste (Table 18) contained 7 and 8 active substances, respectively. In fifth instars reared on digestate, substantially higher doses of active substances were found compared to fifth instars reared on the other substrates. A possible explanation for this can be the difference in chemical composition (Chapter 5). Fifth instars reared on digestate were substantially lower in fat and given that there is no bioaccumulation, most of the pesticides were situated in the gut. The percentage of the total body mass represented by gut content was probably higher for these fifth instars and therefore the doses of the active substances present were relatively higher too. In addition, other substrate specific factors (e.g. pH, water binding capacity,…) might also play a role.

In conclusion, it can be assumed that pesticide contamination of BSF larvae reared on substrates which are currently allowed as feedstuff is highly unlikely. Moreover, the 5 mg/kg concentration used in the present experiment was about 10 times higher than what could be expected from good

agricultural practices (GAP) (FAO, 2017b). However, when in the future the European legislation (Chapter 1) would change for insects and other substrates would be allowed, monitoring these substrates for the presence of active substances with high log(K_{ow}) values is recommended. Concerning heavy metals, monitoring substrates for Cd will be necessary whereas the risks for Pb and As are lower. However, besides the highly poisoneous Cd, Pb and As, other essential trace elements associated with specific substrates might accumulate in the larval biomass. For example, manure, a natural BSF substrate, could contain substantial levels of zinc (Zn) and copper (Cu). About Cu, to our knowledge, nothing has been published, however, Diener et al. (2015) investigated the potential bioaccumulation for Zn. They observed that Zn did accumulate, however, in contrast to Cd, the BAF for Zn decreased with increasing concentration in the substrate, which suggests active regulation of Zn within the body.

Chapter 5

Nutritional composition of black soldier fly prepupae reared on organic side streams

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5.1 Introduction

BSF larvae contain high amounts of energy, protein and essential amino acids, fat and fatty acids, and minerals (Makkar et al., 2014). Therefore, they have already been formulated as a component of complete diets for poultry, swine and for several commercial fish species (Newton et al., 1977; Sealey et al., 2011; Kroeckel et al., 2012; De Marco et al., 2015; Devic et al., 2017; Magalhães et al., 2017). They were found to support good growth and, therefore, it was generally concluded that BSF larvae can be a suitable protein source for animal feed.

Previously, it was demonstrated that the fat and ash content of BSF larvae were extremely variable depending on the type of manure they were fed on (Newton, et al., 1977; Sheppard et al., 2002; Newton et al., 2005). For proper application in least cost formulation software, it is of paramount importance to understand the factors that contribute to the variation in nutritional value (Chapter 7). Moreover, BSF is a particularly interesting species for the purpose of upgrading low value side streams of which the composition differs substantially. However, so far, an in-depth analysis of the nutritional value of BSF larvae reared on commercially available vegetable side streams has not been reported.

In this study, larvae of BSF were grown on three different vegetable waste substrates and a chicken feed diet, as a high quality reference substrate. The nutritional composition of the resulting prepupae was determined. As compared to the previous larval stages, the prepupae might offer two advantages: 1/ the prepupa empties its digestive tract, reducing the risk to carry pathogenic microorganisms and chemical contaminants (Chapter 4), and 2/ the prepupal migrating behavior offers opportunities for harvesting in a scaled-up rearing system (Chapter 7). In order to evaluate the nutritional value for monogastric farm animals, the proximate and nutrient composition of the prepupae were investigated and relations with substrate composition were established.

5.2 Materials and methods

5.2.1 Rearing and harvesting

Four different substrates were tested for their effects on the growth performance and composition of BSF larvae: chicken feed, vegetable waste, biogas digestate and catering waste. The chicken feed was a layer hen feed containing 155 g/kg crude protein, on as is basis (Legkorrel TOTAL 77, Aveve Veevoeding, Belgium) which was also used to rear the stock colony (Chapter 2). Fresh vegetable waste was obtained from Ardo NV (Ardooie, Belgium), a company processing fruit and vegetables. The vegetable waste was a mixture of carrots, peas, salsify and celery. This waste is anaerobically fermented for energy production on the same site by Trevi NV (Belgium). The digestate resulting from this biogas fermentation was centrifuged into a liquid and a solid fraction; the latter was used as a substrate in this experiment (Chapters 3 and 4). Catering waste was obtained from a student restaurant at Ghent University and contained leftover cooked potatoes, rice, pasta and vegetables from one day (different composition than in Chapters 3 and 4). In accordance to EC Regulation No 1069/09 (EU, 2009a), none of the substrates, tested in this chapter, contained animal products (Chapter 1). Both the restaurant waste and vegetable waste substrates were thoroughly homogenized using a blender. Besides homogenization of the quality, this procedure also improved the structure of the substrates in terms of habitat for the larvae and better accessibility of nutrients. The structure of the biogas digestate was deemed optimal for the larvae and, therefore, this substrate was not blended. Water was added to the chicken feed (70 mL/100 g of substrate) in order to guarantee an optimal moisture content for growth of the larvae. This was not necessary for the other substrates, since their moisture content was deemed sufficient for optimal larval growth. All side streams were kept at -20 °C and, every feeding event, portions were thawed prior to being fed to the larvae.

About 1000 of 6-8 day old BSF larvae were placed in quadruplicate onto 600 g fresh (i.e. wet) material of each substrate in plastic buckets. The buckets (5.5 L) were screened with fine-mesh cotton gauze and covered with a lid provided with a single ventilation hole. The buckets were placed in a climatic chamber set at a temperature of 27 ± 1 °C and a relative humidity of 65 ± 5%. The larvae were subjected to a feeding regime of 600 g fresh material every 3 days, until they reached the prepupal stage. All larvae and prepupae were harvested six days after the first prepupae had appeared, which was about 2-3 weeks after the start of the experiment. After sieving the substrate, prepupae were collected manually using forceps. The remaining larvae were placed back in the same bucket and provided with fresh substrate. All prepupae were collected in three or four times with six-day intervals following the above procedure. The collected prepupae were washed with tap water and frozen at -20 °C overnight. Then, they were vacuum packed and stored at -20 °C. Samples of fresh substrate were also collected, vacuum packed and stored at -20 °C pending chemical analysis. Total prepupal biomass was recorded per bucket and single harvest. Prior to analysis, prepupae and substrate material were freeze-dried until constant weight. While freeze-drying may result in less complete moisture removal compared to oven drying, this difference is minimal and freeze-drying guarantees a better preservation of nutrients. Since no significant differences were observed in prepupal yield (wet weight) and DM content between the buckets of a particular substrate, the freeze-dried prepupal biomass of the buckets was pooled per substrate. This pooling was done to obtain a sufficient amount of material to perform the numerous analyses.

5.2.2 Proximate analyses

The proximate analyses consisted of analytical determinations of water (moisture), crude protein, crude fat (ether extract), crude ash and crude fiber (Greenfield and Southgate, 2003). Moisture content was determined by difference after freeze-drying. Crude ash was determined by incineration at 550 °C for 4 h in a combustion oven (ISO 5984:2002). Total nitrogen content was determined by the Dumas method (ISO 16634-1:2008). Crude protein content in substrates and prepupae were

calculated by multiplying total N with 6.25. According to Finke (2007) this factor is acceptable for estimating the true protein content of most insect species. However, since the exoskeleton of insects contains chitin, a nitrogen-containing polysaccharide, this may lead to an overestimation of the crude protein content (Diener et al., 2009). Therefore, the chitin content was analyzed using the procedure described in Liu et al. (2012). In addition, the nitrogen content of the chitin fraction was determined and by subtracting the chitin nitrogen from the total nitrogen in the prepupae and multiplying this value with 6.25, protein values were corrected. Ether extract (EE), a measure for crude fat, was analyzed gravimetrically after extraction with diethyl ether with a Soxhlet system (ISO 6492:1999). Before Soxhlet extraction, hydrolysis with HCl 3 M at 100 °C for 60 minutes was performed. All these analyses were done in duplicate.

The content of soluble, insoluble and total dietary fiber in the substrates was determined using the Megazyme total dietary fiber assay procedure k-tdfr 05/12 (Megazyme, 2012). This method is a simplified modification of the AACC total dietary fiber (TDF) method, 32-05.01, and the AACC soluble/insoluble dietary fiber method (for oat products), 32-21.0. These analyses were performed in single.

The non-fiber carbohydrate fraction was estimated by subtracting the sum of the other components from 100.

5.2.3 Amino acid, fatty acid and mineral composition

Amino acid, fatty acid and mineral composition was determined on freeze-dried prepupae and substrate material (single analyses). Amino acid composition of protein bound amino acids was determined by HPLC performed on oxidized and hydrolyzed samples, following the procedure in 2009/152/EC. In addition, tryptophan was determined separately, since this amino acid is destroyed during acid hydrolysis (EU, 2009b). Fatty acid composition was assessed by gas chromatography (GC) following the procedure described by Raes et al. (2001) on the Soxhlet extracted fraction. The

mineral composition was determined by ICP-OES (ISO 11885:2007)). The preparation included incineration at 450 °C until the ash was grey to red-brown followed by dissolving the ash in diluted nitric acid (7 M) (EN 13652:2001). The extraction of iron was performed separately using aqua regia (ISO 15587-1:2002).

5.2.4 Statistical analysis

All data were analyzed using SPSS 22.0 (IBM Corp, 2013). One-way ANOVA was used to analyze the data of the harvested prepupal biomass (wet weight) and moisture content. A post hoc Tukey (homoscedasticity) or Tamhane test (heteroscedasticity), based on the outcome of a Levene-test, was performed to separate the means. To compare the mean development periods from the inoculation of the buckets until emergence of the first prepupae, a non-parametric Kruskal-Wallis test was used. When significant differences were detected, means were compared using Mann-Whitney U tests. Correlations of the composition of the substrates with that of the prepupae were tested using regression analysis. P-values below 0.05 were considered statistically significant.

5.3 Results

5.3.1 Larval development and prepupal yield

Larvae reared on chicken feed developed at the highest rate (Table 20). After 12 days the first prepupae were observed in buckets containing chicken feed, whereas on vegetable waste and biogas digestate it took 15 days for the first prepupae to emerge. Larvae reared on catering waste developed the slowest with prepupae emerging not before 18 days. In addition, on the latter substrate it took approximately 4 weeks for all larvae to develop into prepupae, which was about 1 week longer than on the other substrates. The amount of substrate (on DM basis) supplied to the larvae per bucket prior to the first harvest was 930 g, 1259 g, 534 g and 1019 g for chicken feed, catering waste, vegetable waste and digestate, respectively.

5.3.2 Chemical composition of prepupae and substrates

The DM content of the offered substrates was comparable for 3 of the 4 substrates with values between 243 and 262 g/kg (Table 21). Only the vegetable waste was substantially higher in moisture with a DM value of 127 g/kg. However, contents of protein, ash and fiber were highly variable among the substrates. The EE contents of the substrates were rather low (21–62 g/kg DM) with the exception of catering waste (139 g/kg DM). The chicken feed, vegetable waste and catering waste contained high amounts of non-fiber carbohydrates (425, 449 and 618 g/kg DM, respectively), whereas the digestate was almost completely deprived of non-fiber carbohydrates.

Table 20: Development time (starting from feeding organic waste, days), harvested biomass (g wet weight) and proximate composition of BSF prepupae reared on different substrates

	Chicken feed	Digestate	Vegetable waste	Catering waste
Development time[*]	12.3 ± 0.5a	15.0 ± 0.0b	15.5 ± 1.0b	19.0 ± 0.8c
Harvested biomass[*]	219.8 ± 7.8a	90.8 ± 3.6c	140.3 ± 4.4b	154.1 ± 5.1b
Moisture[*x]	613 ± 8a	614 ± 29a	590 ± 10a	619 ± 9a
Crude protein[0]	412 (0.6)	422 (1.4)	399 (0.2)	431 (0.6)
Chitin[0]	62 (2.8)	56 (1.5)	57 (1.8)	67 (1.3)
Chitin corrected protein[0]	388	401	377	407
Ether extract[0]	336 (0.4)	218 (0.5)	371 (1.1)	386 (2.3)
Crude ash[0]	100 (1.0)	197 (0.3)	96 (0.7)	27 (0.3)

[*]Means ± standard deviation within a row followed by different letters are significantly different (P<0.05); [x] g/kg as is; [0]Means (and coefficients of variation) in g/kg DM

The DM content of prepupal biomass was comparable among the treatments, ranging between 381 and 410 g/kg. This was also the case for the protein content (399–431 g/kg DM) (Table 20). In contrast, EE and ash contents were significantly affected by the rearing substrate. Prepupae reared

on digestate were low in EE (218 g/kg DM) and high in ash (197 g/kg DM) compared to those reared on the unfermented vegetable waste (371 g/kg DM EE and 96 g/kg DM ash). The chitin content was comparable for prepupae among the substrates, ranging between 56 and 67 g/kg DM. The chitin extracts contained between 60–62 g N/kg, which is comparable with commercial chitin used as a standard by Liu et al. (2012) but lower than the content in pure chitin (Diener et al., 2009). The corrected protein values were between 377-407 g/kg DM.

Table 21: Proximate composition of the substrates used to rear BSF larvae

	Chicken feed	Digestate	Vegetable waste	Catering waste
Moisture[a]	742 (0.0)	757 (0.2)	873 (0.3)	738 (0.7)
Crude protein[b]	175 (1.0)	246 (0.3)	86 (0.9)	157 (6.1)
Ether extract[b]	53 (0.8)	62 (0.5)	21 (13.5)	139 (0.5)
Crude ash[b]	115 (0.2)	299 (0.3)	108 (2.3)	45 (0.9)
Soluble fiber[c]	57	5	5	0
Insoluble fiber[c]	175	381	331	41
Total dietary fiber[c]	232	386	336	41
Non-fiber carbohydrates	425	7	449	618

[a]Means (and coefficients of variation) in g/kg as is; [b]Means (and coefficients of variation) in g/kg DM; [c]Single analyses in g/kg DM

The difference in proximate composition between prepupae reared on catering waste and those reared on vegetable waste was substantially smaller than the difference between the respective substrates. Moreover, the development of BSF larvae on vegetable waste was faster than on catering waste and less substrate had to be fed (on DM basis). However, the final harvest of prepupal biomass was higher for insects reared on catering waste.

There was no significant correlation between protein and EE contents of the substrate and those of the prepupae (R^2=0.349; P=0.409 for protein and R^2=0.054; P=0.768 for EE). However, a high

correlation was observed between the ash contents of substrates and prepupae (R^2=0.954; P=0.023). A high correlation was also found between the EE content of the prepupae and the non-fiber carbohydrate content of the substrate (R^2=0.942; P=0.030).

5.3.3 Amino acid, fatty acid and mineral composition

The most prevalent essential amino acids in the prepupal biomass were lysine, valine and arginine, with levels between 20 and 30 g/kg DM (Table 22). Despite substantial differences in amino acid composition of the substrates, differences in amino acid content of prepupae reared on different substrates were small. Lysine levels were between 23.4 and 25.7 g/kg DM and all prepupae contained between 15.4 and 16.8 g/kg DM of threonine. The contents of isoleucine and valine ranged from 17.2 to 19.1 g/kg DM and from 24.1 to 28.2 g/kg DM, respectively. Levels of other (semi) essential amino acids were 7.1 to 8.6 g/kg DM for methionine, 5.4 to 6.7 g/kg DM for tryptophan and 19.9 to 20.3 g/kg DM for arginine.

Table 22: Amino acid profile of the tested substrates (Subst.) and BSF prepupae (Prep.) (g/kg DM)

	Chicken feed		Digestate		Vegetable waste		Catering waste	
	Subst.	Prep.	Subst.	Prep.	Subst.	Prep.	Subst.	Prep.
Alanine	8.6	25.2	12.5	24.3	3.7	24.2	6.6	27.8
Arginine	10.6	20.3	9.6	20.3	5.0	20.0	7.3	19.9
Aspartate	14.4	37.8	21.7	33.6	15.6	35.9	14.5	36.9
Cystine	2.8	2.5	2.2	2.4	0.6	2.1	2.0	2.2
Glutamic acid	33.0	41.9	27.0	39.8	7.8	41.3	33.2	45.8
Glycine	7.7	22.6	10.6	22.6	3.0	22.2	6.0	25.2
Histidine	4.1	13.6	3.6	13.5	1.4	12.4	3.6	13.8
Isoleucine	6.5	17.2	9.8	18.4	2.8	17.3	6.0	19.1
Leucine	13.8	28.6	15.5	29.5	4.6	28.0	11.1	30.6
Lysine	7.1	23.4	10.3	25.7	3.8	22.6	6.9	23.0
Methionine	3.1	7.6	4.1	8.7	1.0	7.6	2.8	7.1
Phenylalanine	8.2	17.0	9.7	18.7	2.9	16.3	6.8	16.4
Proline	10.4	22.5	8.3	22.1	2.9	21.4	10.9	25.1
Serine	7.7	16.6	8.0	15.5	2.8	15.0	6.9	15.9
Threonine	6.4	16.4	9.5	16.8	2.8	15.4	5.5	16.2
Tryptophan	1.5	6.7	1.6	6.2	0.9	5.8	1.8	5.4
Valine	7.9	24.1	11.7	24.9	3.4	24.8	7.3	28.2

The fatty acid composition of the prepupae was largely composed of saturated fatty acids (648–828 g/kg fatty acid methyl esters (= FAME) (Table 23). Whereas prepupae fed digestate contained only 648 g/kg FAME saturated fatty acids, those reared on the other substrates contained 774–828 g/kg FAME. The EE of the former group was also rich in monounsaturated fatty acids as compared to the other prepupae (191 *vs.* 95–120 g/kg FAME). The levels of n-6 polyunsaturated fatty acids (PUFA) of the prepupae ranged between 46 and 120 g/kg FAME, whereas the levels of n-3 PUFA were rather low, ranging from 9 to 23 g/kg FAME. The fatty acid profile of the prepupae was characterized by high levels of C12:0. The EE of prepupae reared on chicken feed, vegetable waste and catering waste contained at least 573 g C12:0 kg^{-1} FAME, whereas prepupae fed digestate contained only 437 g C12:0 kg^{-1} FAME. Prepupae reared on digestate contained a significant amount of branched chain fatty acids.

Table 23: Fatty acid composition of the tested substrates (Subst.) and BSF prepupae (Prep.) (g/kg fatty acid methyl esters)

	Chicken feed		Digestate		Vegetable waste		Catering waste	
	Subst.	Prep.	Subst.	Prep.	Subst.	Prep.	Subst.	Prep.
C10:0	1.4	14.3	8.5	11.7	2.3	16.3	13.3	20.3
C12:0	14.5	573.5	97.5	436.5	21.3	608.9	154.9	575.6
C14:0	3.3	73.4	43.1	68.7	12.8	94.8	59.0	71.4
C16:0	160.0	96.5	236.3	101.2	305.2	87.0	231.2	102.9
C18:0	25.1	13.6	38.5	17.5	31.8	11.1	67.5	9.8
SFA	214.6	774.4	483.2	648.2	406.8	828.0	540.5	782.9
Iso and anteiso	0.5	1.0	80.3	64.6	4.6	7.1	6.0	2.9
C16:1	2.0	19.7	8.8	75.8	15.3	29.3	17.2	33.4
c9C18:1	239.6	75.4	119.3	79.3	66.0	56.6	251.3	79.7
c11C18:1	8.4	2.3	35.7	23.2	28.3	3.3	99.0	1.2
MUFA	255.3	100.1	189.8	190.8	119.6	95.4	289.4	119.9
C18:2n-6	499.9	115.5	163.5	79.0	312.2	45.2	138.3	78.3
n-6 PUFA	501.0	115.9	175.6	80.4	319.3	46.2	142.4	80.0
C18:3n-3	24.3	7.0	17.3	8.3	116.4	13.7	16.3	11.0
C18:4n-3	0.5	0.7	0.8	6.5	4.4	8.7	2.1	0.5
C20:5n-3	0.2	0.6	1.3	1.1	1.3	0.1	0.7	2.3
C22:6n-3	3.2	0.1	35.0	0.2	15.0	0.1	1.4	0.1
n-3 PUFA	28.5	8.6	71.1	16.0	149.7	23.3	21.8	14.3

Calcium levels were very variable ranging between 66 g/kg DM for prepupae reared on digestate and 1 g/kg DM for those fed on catering waste (Table 24). Comparable differences were also observed in the respective substrates (16 *vs*. 1 g/kg DM). However, the calcium content of the prepupae was not always linked to that of their respective substrates (R^2=0.179; P=0.581). For example, the calcium content of prepupae fed chicken feed was equal to that of prepupae reared on vegetable waste (29 g/kg DM), while the calcium levels of the respective substrates were markedly different (29 *vs.* 7 g/kg DM, respectively). The contents of the other minerals were all within a small range. Phosphorus levels ranged between 4.0 and 5.0 g/kg DM while the potassium contents were between 5.9 and 6.8 g/kg DM. The iron content of the prepupae was variable but was not related to that in the substrate (R^2=0.511; P=0.285).

Table 24: Mineral composition of the tested substrates (Subst.) and BSF prepupae (Prep.) (g/kg DM)

	Chicken feed		Digestate		Vegetable waste		Catering waste	
	Subst.	Prep.	Subst.	Prep.	Subst.	Prep.	Subst.	Prep.
Ca	29.49	28.70	15.55	66.15	6.83	28.72	1.41	1.23
Cu	0.03	0.01	0.02	0.01	0.01	0.01	0.00	0.01
Fe	0.29	0.35	23.59	0.43	1.06	0.11	0.42	0.11
K	7.31	6.16	11.3	6.75	10.65	5.94	8.04	5.98
Mg	2.57	2.65	4.98	3.13	1.49	2.46	0.53	2.11
Mn	0.09	0.22	0.19	0.38	0.05	0.24	0.01	0.02
Na	1.62	0.67	6.32	0.89	8.39	0.60	8.12	0.68
P	5.56	4.99	15.35	4.44	2.39	4.04	2.37	4.08
S	0.73	0.20	4.54	0.31	1.11	0.18	0.51	0.11
Zn	0.12	0.16	0.10	0.05	0.07	0.07	0.02	0.07

5.4 Discussion

In this study, the influence of the proximate and nutrient composition of the rearing substrate on that of BSF prepupae was investigated. The substrates used were commonly available vegetable waste streams and a high quality chicken feed as a reference. The total biomass of the harvested prepupae differed substantially among the four tested substrates. The total prepupal biomass was highest for chicken feed, and lowest for biogas fermentation digestate. Our results on digestate are comparable with those obtained by Li et al. (2011) for prepupae reared on dairy manure. The 1200 larvae inoculated in their experiment produced only 70.8 g prepupal biomass compared to the 90.8 g from the initial 1000 larvae in our study. On the other hand, the amount of dairy manure fed to the BSF larvae by Li et al. (2011) was substantially lower than the amount of digestate used in our study (582 *vs.* 1019 g DM). The slow development of larvae reared on catering waste may be due to the high amount of grease in the substrate, which could be observed on top of the substrates in the rearing buckets. According to Barry (2004) grease is difficult to process for BSF larvae, leading to a prolongation of their developmental time. Fresh vegetable waste showed to be the most favorable substrate in terms of substrate biomass fed (533.8 g DM) versus prepupal biomass harvested (140.3 g DM). Since natural populations of BSF are adapted to decompose decaying organic materials (food waste, rotting fruit, plant litter, manure, etc.), this material is probably preferred.

In this study, the state of decomposition of the substrates was not monitored during the experiment. However, keeping these initially fresh organic materials with high moisture content at a temperature of 27 °C and 65% RH, will, even on the short term, have lead to a substantial degree of decay. Consequently, more nutrients would have become readily available for the larvae to digest. However, the interaction between different organisms associated with decay (e.g. microorganisms and fly maggots) is currently not well understood. Weatherbee et al. (2017) characterized microbial communities present on swine carcasses over time. They observed that, besides a change in relative abundance of the microbial taxa over time, also the species composition of the maggot community

(i.e. 3 species of blowfly belonging to the Calliphoridae family) present on the carcass evolved, suggesting a significant interaction between the microbes and insect larvae. Tomberlin et al. (2005) reported that blowflies are the primary colonizers of carrion during the summer in Georgia and South Carolina, US, whereas BSF females started to deposite their eggs after 6 days of decomposition. Concerning the microbiome in the gut of BSF larvae, Boccazzi et al. (2017) showed that the composition of the fungal communities (i.e. different yeast and mold genera) was dependent of the rearing substrate. Interestingly, their results showed that BSF larvae reared on heat treated catering waste (which could be compared to the batch of cooked catering waste from our research) possessed a greater diversity of fungal species compared to those reared on chicken feed.

The values for the protein content of the BSF prepupae are within the range of those reported in the literature (400-440 g/kg DM), whereas the variability in EE and ash contents could also be anticipated based on earlier reports (Makkar et al., 2014). However, the protein content may have been overestimated by about 20-25 g/kg because of the presence of chitin in the prepupae. The chitin contents of the prepupae in this study are slightly lower than the contents reported in the literature, ranging from 75 up to 87 g/kg DM (Diener et al., 2009; Finke, 2013). This might be due to differences in the applied methodology. The presence of chitin in a commercial BSF meal may be of interest since chitin has been reported to negatively influence nutrient digestibility even at low inclusion levels in some fish species and in poultry (Olsen et al., 2006; De Marco et al., 2015). The differences in EE content can likely be explained by a higher synthesis of fatty acids, mainly C12:0, in larvae reared on energy dense substrates. Chicken feed and vegetable waste contained high levels of non-fiber carbohydrates whereas catering waste was rich in both non-fiber carbohydrates and EE. On the contrary, almost no non-fiber carbohydrates were present in the biogas fermentation digestate. This could be anticipated since most of the carbohydrates were likely used by microorganisms transforming them into methane during the fermentation process. From the nature of the rearing substrates used in the present experiment, it could be deduced that most of the non-fiber carbohydrates present were starches, whereas the amounts of easy digestible sugars were probably

much lower. However, the composition and the structure of the starches are, besides their absolute amounts, of equal importance concerning the digestibility. The starch in the catering waste substrate was probably the most easy to digest given the high content of boiled potatoes, rice and pasta (Garcia-Alonso and Goni, 2000). The starch from the vegetable waste was probably less digestible in pure form, however, by blending everything into a puree like mixture, the digestibility could be altered. Given that the chicken feed control was rich in cereals, which had been grinded and pelletized during the production process, a high starch digestibility could be expected from this substrate (Svihus et al., 2005).

The amino acid composition of the BSF prepupae is similar to that reported in the literature for most amino acids, including those with relevance for animal feed, like lysine, isoleucine, threonine, valine and methionine (Newton et al., 1977; Newton et al., 2005; St-Hilaire et al., 2007; Sealey et al., 2011). Moreover, the levels of these essential amino acids in BSF prepupae appeared to be sufficient to comply with requirements for pigs and poultry (CVB, 2012; NRC, 2012). The possible deficiency in methionine + cystine and threonine reported by Makkar et al. (2014) was not reflected in the amino acid profiles of the prepupae in our study. For tryptophan, another essential amino acid for pigs and poultry, only few data are available in the literature. Newton et al. (1977; 2005) suggested a substantial variability in levels of tryptophan (2.0–5.9 g/kg DM) in BSF prepupae reared on different substrates. However, such a variation was much less pronounced in the prepupae harvested in the present study, containing between 5.4 and 6.7 g tryptophan kg^{-1} DM. In addition, this small range was observed for all amino acids of all prepupae in our study, suggesting that rearing substrate had no substantial influence on the amino acid composition of the prepupae. When the values of essential amino acids in BSF prepupae are compared with those of soybean meal with a similar crude protein content (440 g/kg DM), the profiles appear to be largely consistent (NRC, 2012). Moreover, if BSF prepupae would be defatted, which is the case for soybean meal, crude protein levels of over 60% could be reached. Consequently, such defatted prepupal meal would have an amino acid composition superior to that of soybean meal.

The fatty acid profiles of the prepupae are in line with those reported by several authors (St-Hilaire et al., 2007; Sealey et al., 2011; Finke, 2013). When comparing the fatty acid profile of the prepupae with that of the respective substrates they had developed in, it appears that the substrate only partially affects the fatty acid profile of the prepupae. Interestingly, lipids of the harvested prepupae were mainly composed of C12:0, even when the substrate contained this fatty acid only in trace amounts. This suggests that C12:0 in BSF was synthesized from other nutrients present in the substrate, such as carbohydrates (starch and sugars). This conversion of carbohydrates, which are a major component in the diet of various insect species, into lipids stored in their fat body, has been well documented (Venkatesh et al., 1980; Inagaki and Yamashita, 1986; Briegel, 1990). Interestingly, appreciable amounts of branched chain fatty acids were found in prepupae reared on digestate. These fatty acids are mainly synthetized by bacteria and fungi suggesting that they originated from the anaerobic bacteria from the biogas fermentation (Ruess et al., 2002; Vlaeminck et al., 2006).

A further element that can be mentioned in favor of the inclusion of BSF prepupae in poultry and pig feed, given their richness in C12:0, is the faster and more efficient absorption and metabolism of medium chain fatty acids (MCFA) compared to long chain fatty acids (LCFA) and their nutraceutical potential (Mohana Devi and Kim, 2014). Skrivanova et al. (2006) showed that C12:0 had the highest activity against Clostridium perfringens. as compared to other MCFA. Furthermore, it had the lowest impact on the beneficial Lactobacilli. This mechanism could optimize performance and health of pigs and poultry by management of the microbiota in the upper part of the small intestine, which is dominated by gram-positive bacteria. Even though these results have been obtained with specific fatty acid supplementation, the proposed effects can be mimicked with natural sources of these fatty acids such as insect meals. As in-feed antibiotics are banned in the EU since January 2006 (regulation EC/1831/2003) there is an increasing need for reliable in-feed antibiotic alternatives (EMA, 2017). On the other hand, the high fat content of the prepupae could limit their application as a feed ingredient. Therefore, it could be interesting to partially extract the fat from the prepupal meal. Thus, a sufficient amount of C12:0 rich fat could be kept in the feed creating added value compared

to soybean meal, while the extracted part could be useful as a high quality oil product, for example for the production of biodiesel (Li et al., 2011).

Calcium levels in BSF prepupae from our experiments were rather low compared to the levels reported by Makkar et al. (2014) only prepupae reared on digestate contained a value situated between the range reported in the latter study (50–86 g/kg DM). The high ash content of prepupae reared on the digestate compared to those reared on catering waste was mainly due to a much higher level of calcium in the prepupae. However, Finke (2013) reported a more similar value to that of prepupae reared on catering waste (6 g/kg DM). As indicated in 5.2.3., there was no correlation between the calcium content of the substrate and that of the reared prepupae. Given that BSF has a mineralized exoskeleton with most of the calcium present in the form of calcium carbonate (Finke, 2013), other substrate specific factors besides calcium content, such as pH, might be of importance for the construction of the exoskeleton, as is shown for marine arthropods (Taylor et al., 2015). However, this can only be hypothesized since it was not monitored during the present research. The prepupal contents of other minerals with importance for animal feed, such as phosphorus, potassium and magnesium, appear to be unaffected by the rearing substrate. Moreover, phosphorus levels are in compliance with the requirements of pigs and poultry (CVB, 2012; NRC, 2012). On the other hand, a high ash content could also be undesirable for the use of BSF prepupae as an ingredient in a feed formulation. Since calcium levels of prepupae reared on digestate are well above the recommendations for pigs and even layer hens (CVB, 2012; NRC, 2012), using these prepupae could have certain drawbacks. This may be a concern especially in feed formulations for young animals like piglets. High feed calcium levels may increase the stomach pH, increasing the risk of bacterial infection (Lawlor et al., 2005). Moreover, extracting fat from the prepupae, as suggested above, would raise their mineral content even more. However, prepupae reared on energy rich substrates with a low content of ash and fiber, like the catering waste, appear to have a very low ash content making them more suitable as a feed ingredient.

In conclusion, our findings indicate that a rearing system of BSF larvae on vegetable waste streams could deliver a high quality insect resource with potential for being incorporated in animal feed. The quality of this resource would be constant in terms of crude protein content and amino acid profile, irrespective of the type of waste material the larvae were offered. However, fat and ash contents appear to be dependent on the rearing substrate. Larvae reared on energy dense substrates turn into prepupae with a high fat content, which is most rich in MCFA. This fat could provide an added value to the BSF prepupae in comparison to conventional feed resources. Future research should focus on how these prepupae can be incorporated in a feed formulation.

Chapter 6

Gut antimicrobial effects and nutritional value of BSF prepupae for weaned piglets

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6.1 Introduction

Prepupae of the BSF are a potential source of high value protein that could be incorporated in feed for monogastrics. They are also rich in fat, with levels ranging between 15 and 49% on DM basis (Makkar et al., 2014). Notably, the fatty acid profile of BSF prepupae is, in general, high in the medium-chain fatty acid (MCFA) lauric acid (C12:0). The fat of prepupae reared on organic waste streams with high amounts of starch contains up to 60% lauric acid (Chapter 5). MCFAs are well known for their antimicrobial effects on gut microbiota, while lauric acid is particularly active against Gram positive bacteria (Dierick et al., 2002a; Skrivanova et al., 2005). As in-feed antibiotics are banned in the EU since January 2006 (EU, 2003) and anticipating the withdrawal of zinc oxide at pharmacological doses and copper as a growth promoter, there is an increasing need for reliable in-feed alternatives (EMA, 2017). Therefore, the possible antimicrobial effects of BSF fat could provide an important added value when complete larvae/prepupae are used as a protein source in the feed of monogastrics. Exploiting this added value will probably be required if BSF larvae/prepupae are ever to be competitive with soybean meal as a conventional protein source, since soybean offers lower marginal protein costs when included in monogastric feeds (ABN AMRO, 2016).

A niche that could be particularly interesting for BSF containing feeds is the rearing phase of weaner piglets. Weaning at early age (3 to 4 weeks) exposes these piglets to multiple stressors (both nutritional and environmental) which results in a reduced feed intake, gut alterations and reduced digestive capacity, frequently associated with the proliferation of pathogens, such as enteropathogenic *E. coli.* (Aumaître et al., 1995; Thacker, 1999). In order to prevent this, diets are enriched with antimicrobial feed additives. Since early weaned piglets are prone to bacterial infections, in the present study the effects of BSF prepupal fat on porcine gut microbiota were assessed using *in vitro* incubations simulating the digestion in the proximal small intestine of piglets. Next, an *in vivo* trial was conducted to investigate the effects of the inclusion of BSF prepupae on the

gut microbiota and function (i.e. gut health, daily gain, feed intake and digestibility) of weaned piglets. For this purpose, early weaned piglets were reared on diets either containing full-fat or defatted BSF prepupae, and compared to piglets reared on a control feed containing soybean as a conventional protein source, allowing to differentiate the effects of the protein and fat within BSF prepupae.

6.2 Materials and methods

6.2.1 Animal ethics statement

The study was conducted in accordance with the European recommendations for the protection of animals used for agricultural research (EU Directive 91/630/EEG and 98/58/EG).

6.2.2 In vitro assessment of the antimicrobial properties of BSF fat

Fat from freeze dried prepupae reared at Ghent University on chicken feed (Chapter 2) was extracted with diethyl ether (ISO 6492:1999), and the MCFA profile was determined by GC (Dierick et al., 2002a). Different amounts of BSF fat (0.20, 0.50, 1.00, and 1.50 g/100 mL medium) were added to an incubation medium, next to a blank treatment that did not contain added BSF fat. The medium contained a synthetic diet (corn starch, dextrose, casein, soy oil, pectin, mucin, vitamin/mineral premix and bile salts), inert cellulose (Alphacel^{TM}, MP Biomedicals, LLC, 900453), phosphate buffer (pH 5), and fresh microbiota from one donor piglet (Michiels et al., 2009). For the collection of microbiota, the content of the small intestine was quantitatively collected and centrifuged (10 min, 1500 x g). The supernatant (kept at 4 °C), containing a suspension of luminal bacteria, was used as inoculum in the incubation medium, which was incubated at 37 °C for 4 h. Since C12:0 could be present as a free fatty acid and/or as a glyceride bonded fatty acid in the BSF fat, microbial lipase was added or not to the medium (Dierick et al., 2002a). Additional treatments were included, testing free C12:0 as a positive control (0.12, 0.29, 0.58 and 0.87 g/100 mL medium), corresponding to the levels of C12:0 provided by the prepupae in the incubations.

Bacterial counts (viable counts, $\log_{10}$ CFU/mL) of aliquots taken at the end of the incubations were performed using selective media following the ring plate technique (Van Der Heyde and Henderickx, 1963) (Figure 8). Therefore, serial 10-fold dilutions were made using a sterilized peptone solution (1 g peptone + 0.4 g agar + 8.5 g NaCl in 1 L distilled water). The selective media were Eosin Methylene Blue agar (coliforms), Slanetz & Bartley (D-streptococci), Rogosa agar + acetic acid (lactobacilli) and Reinforced Clostridial agar + hemine (total anaerobic bacteria). The experiment was performed in triplicate using inocula from different piglets during different incubations. Inherently, the inocula were not standardised and may differ between replicate incubations. This is taken into account in the statistical evaluation taking replicate as a blocking factor (see section 6.2.4). In addition, it should be noted that the piglets providing the inocula, received the same feed and were kept under similar conditions.

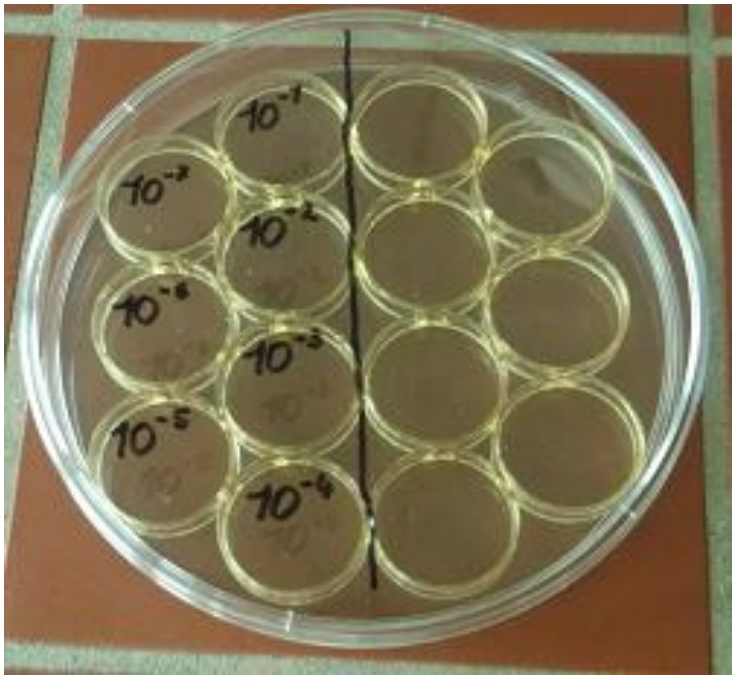

Figure 8: Ring plate with dilution scheme

6.2.3 Inclusion of full-fat and defatted BSF in the diet of weaned piglets

6.2.3.1 Dietary treatments

Batches of full-fat and defatted BSF originating from the same BSF culture were obtained from Hermetia Deutschland GmbH & Co KG (Baruth/Mark, Germany). The prepupae were heat treated at 80 °C for 30 minutes and air dried. Defatted BSF meal was obtained after mechanical extraction (no solvent was used). The analyzed nutrient composition of full-fat and defatted BSF is given in Table 25. Diet formulation was based on analyzed values when possible, however, for apparent ileal digestibility values of amino acids (Kortelainen et al., 2014), the net energy for pigs and digestible phosphorus, literature data and assumptions based on fishmeal and animal meal were applied (Table 26). The net energy for pigs was approximated by using the general formula for net energy reported in CVB (2011) (formula F.V09), using results from proximate analysis and digestibility coefficients for crude protein and ether extract derived from animal resources fish and animal meal.

Table 25: Analyzed chemical composition (%) and gross energy (MJ/kg) of BSF prepupae used in the experiment with weaned piglets

Nutrient	Full-fat BSF[1]	Defatted BSF[1]	Formulation requirements
Dry matter	97.30	96.58	
Gross energy	24.27	18.31	
Crude protein (N x 6.25)	40.88	60.69	17.20
Ether extract	40.99	7.97	5.20 < X < 5.50
Saturated fatty acids	29.58	4.95	
Lauric acid (C12:0)	23.38	2.78	
Mono-unsaturated fatty acids (MUFA)	4.82	1.36	
Poly unsaturated fatty acids (PUFA)	2.41	0.73	
n-6 PUFA	2.15	0.65	
n-3 PUFA	0.26	0.07	>0.10
Ash	4.98	8.07	<5.50
Sodium	0.08	0.14	
Potassium	1.14	1.93	
Chloride	0.27	0.42	
Magnesium	0.33	0.51	
Calcium	0.55	0.95	0.66
Phosphorus	0.93	1.46	
Alanine	2.81	4.37	
Arginine	1.72	2.55	
Aspartic acid	3.03	4.58	
Cystine	0.31	0.47	
Glutamic acid	4.24	6.33	
Glycine	1.94	2.96	
Histidine	1.03	1.53	
Isoleucine	1.47	2.34	
Leucine	2.42	3.76	
Lysine	1.93	2.96	
Methionine + Cystine	0.49	0.72	
Phenylalanine	1.29	2.00	
Proline	2.28	3.36	
Serine	1.57	2.08	
Threonine	1.37	2.01	
Tryptophan	0.45	0.71	
Valine	2.08	3.33	

[1]The batches of full-fat and defatted BSF were obtained from Hermetia Deutschland GmgH & Co KG (An der Birkenpfuhlheide 10, 15837 Baruth/Mar, Deutschland). The larvae were heat treated at 80 °C for 30 minutes, air dried. Defatted BSF was obtained after mechanical extraction (no hexane).

Table 26: Nutrient composition (in %, unless otherwise stated) of BSF prepupae and restrictions used in formulation of diet for weaned piglets

Nutrient	Full-fat BSF	Defatted BSF	Reference	Formulation requirements
Digestible phosphorus	0.70	1.10	Assumption (P digestibility of fishmeal: 75.0%)	0.31
Ca / dig. P	0.79	0.86	Calculated	2.10 < X <2.15
Na + K - Cl (meq/100g)	40.89	43.45	Analyzed and calculated	19.50
Net Energy pigs (MJ/kg)	16.54	8.37	Assumptions based on fishmeal and animal meal (CVB, 2011)	9.94
AID Lysine	1.53	2.35	Analyzed and calculated using digestibility value of Kortelainen et al. (2014)	1.10
AID (Methionine + Cystine) / AID Lysine	0.37	0.36	Analyzed and calculated using digestibility value of Kortelainen et al. (2014)	0.59
AID Isoleucine / AID Lysine	0.88	0.91	Analyzed and calculated using digestibility value of Kortelainen et al. (2014)	0.51
AID Threonine / AID Lysine	0.71	0.68	Analyzed and calculated using digestibility value of Kortelainen et al. (2014)	0.62
AID Tryptophan / AID Lysine	0.22	0.23	Analyzed and assumed ileal digestibility value of 76.5% (fishmeal)	0.21
AID Valine / AID Lysine	1.24	1.29	Analyzed and calculated using digestibility value of Kortelainen et al. (2014)	0.68

The animal trial consisted of four dietary treatments (Table 27). The control diet (CON) was a pre-starter diet for weaners, including a digestibility marker (Celite 545 coarse, source of 4 mol/L HCl insoluble ash), excluding supplementary organic acids and at copper and zinc levels beyond animal requirements. Experimental diets (BSF4, BSF8 and DF-BSF) were formulated by adding different

levels of full-fat and defatted BSF into the control diet, i.e. 4% full-fat BSF (BSF4), 8% full-fat BSF (BSF8) and defatted BSF in an amount supplying a similar level of protein to the diet as BSF8 (DF-BSF). Diets CON, BSF4, BSF8 and DF-BSF were identical with respect to all nutrients with restrictions in the linear programming formulation, thus providing equal amounts of the most important nutrients. Batches of barley, corn, wheat, toasted soybeans, soybean meal 49CP, oat flakes and sweet whey powder were analyzed for crude protein, and herewith amino acid and digestible amino acid levels were corrected. Matrix values for other nutrients and ingredients were provided by DSM Nutritional Products Belgium (updated matrix by January 2016, Deinze, Belgium). Feeds were first prepared as a batch, and subsequently pelletized on a Labor Monoroll Pellet Mill (2 mm die) after steam conditioning at 70 °C during 45 seconds. Feed samples of all diets were collected at the moment of feed preparation and proximate analyses together with analyses of important nutrients were performed (Tables 27 and 28).

Table 27: Ingredients of the diets used in the experiment with weaned piglets

	CON	BSF4	BSF8	DF-BSF
Ingredients, %				
Barley	25.00	25.00	25.00	25.00
Corn	18.73	20.16	21.31	21.52
Wheat	12.00	12.00	12.00	12.00
Soybean meal CP49	11.31	12.21	13.15	10.55
Toasted soybeans	12.00	6.000		3.351
Full-fat BSF		4.000	8.000	
Defatted BSF				5.420
Oat flakes	8.000	8.000	8.000	8.000
Sweet whey powder	4.000	4.000	4.000	4.000
Lactose	1.539	1.539	1.539	1.539
Soybean oil	0.848	0.475	0.337	2.000
Premix Min &ViCON[1]	1.000	1.000	1.000	1.000
Sugar beet pulp	1.000	1.000	1.000	1.000
Monocalciumphosphate	0.959	0.857	0.757	0.741
Limestone	0.929	0.946	0.962	0.952
L-lysine HCl	0.525	0.555	0.583	0.588
Salt	0.240	0.240	0.240	0.240
DL-methionine	0.233	0.243	0.253	0.258
L-threonine	0.225	0.231	0.236	0.243
L-valine	0.142	0.124	0.106	0.103
L-tryptophan	0.073	0.078	0.082	0.081
Sodium bicarbonate	0.244	0.344	0.443	0.417
Celite 545 coarse	1.000	1.000	1.000	1.000

[1]Providing per kg of diet: vit A (retinyl acetate), 15000 IU; vit D3 (cholecalciferol), 2000 IU; vit E (all-rac-alfa-tocopherylacetate), 50.0 mg; vit K3 (menadion), 4.0 mg; vit B1 (thiamine mononitrate), 3.1 mg; vit B2 (riboflavine), 8.0 mg; vit B3 (calcium-D-pantothenate), 20 mg; vit B6 (pyridoxine hydrochloride), 6.0 mg; vit B12 (cyanocobalamine), 50.0 µg; vit PP (niacinamide), 40.0 mg; folic acid, 2.0 mg; biotin, 0.3 mg; betaine anhydrate, 285 mg; endo-1,4-beta-glucanase E3.2.1.4, 250 TGU; endo-1,4-beta-xylanase E3.2.1.8, 560 TXU; Cu (copper(II)sulphate pentahydrate), 15.0 mg; Zn (zincsulphate), 110 mg; Fe (iron(II)sulphate monohydrate), 100 mg; Mn (manganese(II)oxide), 48.0 mg; I (calciumjodate anhydrate), 1.9 mg; Se (sodium selenite), 200 µg; Se (selenomethionine produced by *Saccharomyces cerevisae* NCYC-R397), E306 extract of vegetable oils rich in tocopherols, tocopherols, 228 mg; 100 µg; clinoptioliet, 1.64 g, aromatic compounds, 72 mg;

Table 28: Chemical composition of the diets used in the experiment with weaned piglets

	CON	BSF4	BSF8	DF-BSF
Analyzed chemical composition (unless otherwise stated)				
Dry matter, %	88.40	88.50	88.90	89.20
Crude protein, %	16.80	16.70	16.80	16.80
Ether extract, %	4.90	5.00	5.30	4.80
Lauric acid (total), %	0.01	0.78	1.58	0.18
Lauric acid (free), %	0.01	0.36	0.54	0.08
Lactose, %[1]	4.50	4.50	4.50	4.50
Starch + sugars, %[1]	44.44	44.85	45.06	45.21
Crude ash, %	6.00	6.00	5.90	6.00
Crude fiber, %	3.60	3.70	3.60	3.50
Calcium, %[1]	0.66	0.66	0.66	0.66
Digestible phosphorus, %[1]	0.31	0.31	0.31	0.31
NE Pigs (kcal) , %[1]	2370	2370	2380	2370
Lysine, %	1.24	1.22	1.22	1.23
Threonine, %	0.81	0.79	0.80	0.80
Methionine, %	0.44	0.45	0.45	0.45
Cystine, %	0.29	0.28	0.27	0.27
Valine, %	0.88	0.86	0.84	0.85

[1]Calculated content

6.2.3.2 Animal trial

Fifty-six weaned piglets (males and females, weaned on 21 days of age; 6.178 ± 0.562 kg) were assigned to the 4 treatments. Each treatment was replicated in 7 pens of 2 pigs per pen (n=7 for all measurements). The experiment lasted for 15 days. The piglets originated from 7 litters and were assigned to the treatments according to litter, gender and weight. *In concreto*, the study design contained 7 blocks, referring to the origin (litter) of the piglets. In each block, each treatment was replicated once. All piglets in a single block originated from one litter and were assigned to the pens so that each pen had one male and one female, and pen body weights were similar. Pigs were housed in a piglet unit (2.10 m²/pen) with full slatted floors, conventional ventilation scheme, with an ambient temperature of 30 ± 1 °C and a 24 h light schedule up to day 5 post weaning. From day 6

up to day 15, ambient temperature was linearly adjusted to 28 ± 1 °C. The pigs were weighed at day 0, 5 and at the end of the trial, i.e. day 15. For each period (day 0-5, 5-15 and total period, day 0-15), the performance (i.e. average daily gain (ADG)), average daily feed intake (ADFI) and feed to gain ratio (F:G) were recorded.

6.2.3.3 Sampling and data acquisition

From each pen, one piglet (piglet with weaning weight closest to average weaning weight of that block) was used for tissue sampling. Piglets were stunned by electrocution, and exsanguinated. Immediately thereafter, their abdomen was opened to collect the digesta and intestinal sections. Digesta of the stomach, the first 3 m of the small intestine and of the area stretching from 4 m to 1 m proximal to the ileo-cecal valve, were quantitatively collected. The pH and weight of the fresh digesta were determined in each section, 1 g of digesta was used to count the bacteria by plating (same bacterial groups as for the *in vitro* assessment) and 5 g was sampled and stored at -20 °C for pending MCFA analysis (Dierick et al., 2002a). The rest of the digesta was freeze-dried to determine the DM content. Subsequently, the dry matter was used to determine 4 mol/L HCl insoluble ash as a marker for digestibility. The degree of absorption of MCFA (C6:0 to C10:0) in these sections could then be calculated by the indicator method (Dierick et al., 2002b). Small intestinal segments (5 cm) at 3 m distal to the pylorus and 3 m proximal to the ileo-caecal valve were excised and used for villus height and crypt depth measurements (segments were flushed with saline and immersed in formaline) (Michiels et al., 2010). Digesta of the last meter of the small intestine and the last 15 cm of the rectum were collected and pooled per treatment and then freeze-dried to determine DM content of digesta. The DM was used to determine 4 mol/L HCl insoluble ash as a marker for digestibility and for proximate analyses (gross energy, DM, crude protein and EE). The total ileal and fecal apparent digestibility of gross energy, DM, crude protein and EE was calculated for the ileal and rectum samples, respectively. Digestibility of nutrient (gross energy, DM, crude protein and EE) in % was calculated as [1 - (nutrient in digesta/nutrient in diet) x (4 mol/L HCl insoluble ash in diet/4 mol/L HCl insoluble ash in digesta)] (Kortelainen et al., 2014).

6.2.3.4 Feed and digesta analyses

DM content was determined by oven drying at 103 °C until constant weight (ISO 6496:1999). Determination of gross energy values was done using the bomb calorimeter method (ISO 9831:1998). Crude ash was analyzed by incineration at 550 °C for 4 h in a combustion oven (ISO 5984:2002). Total nitrogen (N) content was determined by the Kjeldahl method (ISO 5983-1:2005). Crude protein content was calculated by multiplying total N with 6.25. Ether extract (EE), a measure for crude fat, was analyzed gravimetrically after extraction with diethyl ether with a Soxhlet system (ISO 6492:1999). Crude fiber content was determined using the method with intermediate filtration (ISO 6865:2000). Amino acid composition of protein bound amino acids was determined by HPLC performed on hydrolyzed and oxidized samples (ISO 13903:2005). Tryptophan was determined using a separate analysis, since this amino acid is destroyed by acid hydrolysis (ISO 13904:2016). Fatty acid composition (without C12:0) was assessed by preparation of methyl esters (ISO/TS 17764-1:2002) followed by GC (ISO/TS 17764-2:2002). For C12:0, both bonded and free, the method described by Dierick et al. (2002a) was applied. The calcium and phosphorus content was determined by ICP-OES (ISO 11885:2007). The samples were prepared by incineration at 450 °C until the ash was grey to red-brown, subsequently followed by dissolving the ash in diluted nitric acid (7 M).

6.2.4. Statistical analyses

The data were analyzed using SPSS 22.0 (IBM Corp, 2013). For the *in vitro* study, a general linear model (GLM) with 2 fixed factors (treatment and concentration) and replicate as blocking factor, excluding the control from the data set, was applied (De Smet et al., 2016). To allow comparing the effect of treatment or concentration versus the control, a GLM was applied with one fixed factor (treatment or concentration) and replicate as blocking factor. In case of significance ($P<0.05$), composite means were compared by the Tukey test.

The performance data, pH values, C12:0 concentrations, bacterial counts and histo-morphological measurements, obtained from the *in vivo* experiment, were statistically analyzed using a GLM with

treatment as fixed factor and block as random factor. In case of significant differences (P<0.05), the Tukey test was used to compare the means.

6.3 Results

6.3.1. In vitro assessment of the antimicrobial properties of BSF fat

The content of C12:0 in prepupal fat was 57.9 g/100 g EE. The levels of other MCFAs (i.e. C6:0 – C10:0) were negligible. All prepupal treatments failed to reduce the counts of coliforms and total anaerobic bacteria, as compared to the blank (P<0.05) (Table 29). However, inhibition of the growth of lactobacilli and D-streptococci was observed. The addition of lipase enhanced the antimicrobial effects of prepupal fat at inclusion levels of 0.50 and 1.00 g/100 mL (corresponding to 0.29 and 0.58 g C12:0/100 mL, respectively) against both lactobacilli and D-streptococci. However, at the highest inclusion level of 1.50 g fat/100 mL (0.87 g C12:0/100 mL) the addition of lipase did not further reduce counts of lactobacilli. The maximum suppression observed for lactobacilli was 1.4 $\log_{10}$ CFU/mL compared to the blank. For D-streptococci about 2.2 log reduction was recorded for prepupal fat + lipase at a concentration of 1.50 g fat/100 mL. The corresponding control treatment (pure C12:0 at a concentration of 0.87 g/100 mL) resulted in a 2.6 log reduction compared to the blank.

Table 29: Effect of treatment and concentration on the microbial counts (log_{10} CFU/mL) in the *in vitro* incubations simulating the upper small intestine

Treatment	Concentration (% C12:0)	No.	Coliforms	Streptococci	Lactobacilli	Total anaerobes
Mean values						
Blank before incubation		3	1.93	5.46	6.33	5.44
Blank after incubation		3	5.44	5.85	6.64	6.32
Prepupal fat	0.12	3	4.90	5.75	6.15	6.22
	0.29	3	5.18	5.33	6.02	6.02
	0.58	3	5.30	5.01	5.64	5.84
	0.87	3	5.06	4.09	5.27	6.14
Prepupal fat + lipase	0.12	3	5.37	5.49	5.98	6.51
	0.29	3	5.23	4.64	5.43	6.25
	0.58	3	5.29	4.03	5.37	6.16
	0.87	3	5.21	3.68	5.23	6.01
Pure C12:0	0.12	3	4.77	5.73	6.21	6.20
	0.29	3	5.27	4.81	5.51	6.01
	0.58	3	4.89	3.64	4.95	5.63
	0.87	3	5.11	3.26	4.52	5.52
Composite mean values						
Prepupal fat		12	5.11	5.05^{b}	5.77^{a}	6.05^{b}
Prepupal fat + lipase		12	5.27	4.46^{a}	5.50^{1a}	6.23^{b}
Pure C12:0		12	5.01	4.36^{a}	5.30^{1a}	5.84^{a}
	0.12	9	5.01	5.66^{z}	6.12^{z}	6.31^{y}
	0.29	9	5.23	4.93^{y}	5.65^{1y}	6.09^{xy}
	0.58	9	5.16	4.23^{1x}	5.32^{1xy}	5.87^{x}
	0.87	9	5.13	3.68^{1x}	5.01^{1x}	5.89^{x}
General linear model (excluding the control)						
RMSE			0.292	0.444	0.224	0.217
P-value treatment			0.105	0.001	<0.001	0.001
P-value concentration			0.475	<0.001	<0.001	0.001
P-value treatment x concentration			0.396	0.210	0.010	0.142

[a,b]Mean values for the treatments with different letters are significantly different at $P<0.05$ following GLM.
[x-z]Mean values for the concentration effect with different letters are significantly different at $P<0.05$ following GLM.
[1]Mean value different from the blank following GLM for testing the effect of treatment and concentration separately.

6.3.2 Inclusion of full-fat and defatted BSF in the diet of weaned piglets

Performance data are displayed in Table 30. None of the parameters show significant differences between treatments. Dietary C12:0 levels were reflected in MCFA contents in the different compartments of the digestive tract (Table 31, $P<0.05$), although cumulative absorption in different compartments did not differ between treatments containing BSF prepupal material. In contrast, pH of contents was not affected by treatment. Concerning bacterial counts, no differences could be observed for the counts of coliforms and total anaerobic bacteria ($P>0.05$; data not displayed). Based on the outcome of the *in vitro* experiment, differences between the diets could be expected for lactobacilli and D-streptococci (Table 31).

Table 30: Effect of dietary treatment on performances of weaned piglets (n=7)[1]

	CON	BSF4	BSF8	DF-BSF	RMSE	P-value
Average daily gain (g/day)						
Day 0-5	27	26	28	41	66.4	0.973
Day 5-15	221	202	199	215	48.1	0.884
Day 0-15	174	143	143	157	41.1	0.668
Average daily feed intake (g/day)						
Day 0-5	121	161	135	143	64.5	0.690
Day 5-15	299	292	250	262	58.2	0.299
Day 0-15	236	248	205	225	47.8	0.450
Feed:gain ratio[2]						
Day 5-15	1.36	1.24	1.30	1.23	0.333	0.122
Day 0-15	1.48	1.49	1.58	1.43	0.446	0.216

[1]CON is the control diet, diets BSF4, BSF8 and DF-BSF contained 4% full-fat BSF, 8% full-fat BSF and defatted BSF in an amount providing similar nitrogen to the diet as BSF8, respectively; [2]F:G for period d0-5 not given.

Table 31: Effect of dietary treatments on pH, lauric acid (C12:0) and bacterial counts in the different compartments of the digestive tract (n=7)[1,2]

	CON	BSF4	BSF8	DF-BSF	RMSE	P-value
Stomach						
pH	3.94	3.75	3.77	3.82	0.577	0.634
C12:0 Content (%)	0.001d	0.094b	0.212a	0.021c	0.1260	<0.001
Cumulative absorption (%)	ND[3]	58.3	57.3	59.9		
Lactobacilli (log_{10} CFU/mL)	8.37	8.31	7.90	8.44	0.499	0.213
D-Streptococci (log_{10} CFU/mL)	8.27	8.21	7.97	8.25	0.376	0.659
Proximal small intestine						
pH	5.56	5.78	5.67	5.83	0.352	0.458
C12:0 Content (%)	0.000b	0.017ab	0.026a	0.009b	0.1265	0.005
Lactobacilli (log10 CFU/mL)	7.73	7.53	7.39	7.82	0.376	0.179
D-Streptococci (log10 CFU/mL)	7.74	7.24	7.27	7.67	0.591	0.299
Distal small intestine						
pH	6.52	6.59	6.64	6.58	0.583	0.982
C12:0 Content (%)	0.000c	0.007ab	0.012a	0.002bc	0.0441	0.001
Lactobacilli (log10 CFU/mL)	8.68	8.57	8.64	8.62	0.655	0.997
D-Streptococci (log10 CFU/mL)	8.65	8.62	8.57	8.38	0.577	0.802

[1]CON is the control diet, diets BSF4, BSF8 and DF-BSF contained 4% full-fat BSF, 8% full-fat BSF and defatted BSF in an amount providing similar nitrogen to the diet as BSF8, respectively; [2]Values with different letters within a row per intestinal compartment are different, *P<0.05*. [3]Not determined.

However, the observed decrease in log_{10} CFU/mL in the stomach and the upper small intestine (SI1) of piglets that were fed diets with increasing C12:0 content was not significant (P>0.05). The histo-morphological measurements of segments at 3 m distal to the pylorus (proximal jejunum) and 3 m proximal to the ileo-cecal valve (distal jejunum) (Table 32) did not indicate significant effects. An overview of the digestibility data can be found in Table 33. Inclusion of defatted BSF resulted in equal or higher ileal digestibility of nutrients as compared to the control, whereas use of full-fat BSF

reduced ileal energy digestibility. Ileal crude protein digestibility was higher with 4% full-fat and defatted BSF prepupae in the diet compared to the control (73.3% and 69.7% for BSF4 and DF-BSF *vs.* 67.4% for CON, respectively).

Table 32: Effect of treatments on villus height (µm), crypt depth (µm) and ratio villus height:crypt depth at different sites of the digestive tract (n=7)[1]

	CON	BSF4	BSF8	DF-BSF	RMSE	P-value
Proximal jejunum						
Villus height	428	427	432	427	24.6	0.949
Crypt depth	108	114	113	110	8.9	0.721
Villus height:crypt depth	4.0	3.8	4.0	3.9	0.32	0.416
Distal jejunum						
Villus height	426	424	428	430	19.1	0.803
Crypt depth	107	106	109	108	5.3	0.887
Villus height:crypt depth	4.0	4.0	4.0	4.1	0.20	0.379

[1]CON is the control diet, diets BSF4, BSF8 and DF-BSF contained 4% full-fat BSF, 8% full-fat BSF and defatted BSF in an amount providing similar nitrogen to the diet as BSF8, respectively;

Table 33: Effect of treatments on apparent ileal and total tract digestibility of nutrients (%) in weaned piglets fed the experimental diets[1,2]

	CON	BSF4	BSF8	DF-BSF
Apparent ileal digestibility				
DM	64.6	63.0	64.9	66.4
Gross energy	71.4	68.1	69.9	71.5
EE	78.3	77.1	80.1	80.3
Crude protein	69.7	73.3	67.4	73.3
Apparent total tract digestibility				
DM	80.6	80.9	81.5	81.4
Gross energy	83.4	84.1	83.4	82.9
EE	78.2	77.3	80.3	77.9
Crude protein	76.8	77.4	77.6	78.3

[1]CON is the control diet, diets BSF4, BSF8 and DF-BSF contained 4% full-fat BSF, 8% full-fat BSF and defatted BSF in an amount providing similar nitrogen to the diet as BSF8, respectively; [2]Measurements were done on pooled samples per treatment

6.4 Discussion

According to the literature, lauric acid-containing fats could be a means to control Gram positive infections (Skrivanova et al., 2005). Despite the fact that the mode of action of MCFAs like C12:0 is not fully understood, it is known that their antimicrobial activity is related to the reduction of pH, as well as to their ability to dissociate (Roth and Kirchgessner, 1998). Undissociated forms of MCFAs can penetrate the lipid membrane of the bacterial cell and subsequently dissociate within the cytoplasm. As the targeted bacteria struggle to maintain a neutral pH, by exporting excess protons, the cellular ATP is consumed resulting in the depletion of energy and ultimately leading to the death of the cell (Ricke, 2003). In our *in vitro* assessment, the high amount of C12:0 in the BSF fat extracts resulted in substantial antimicrobial effects against D-streptococci, while no significant effects were recorded for coliforms. Skrivanova et al. (2006) observed in an assay with free C12:0, that the Gram positive

Clostridium perfringens was suppressed while the Gram negative *E. coli* and *Salmonella* spp. were less affected. This could be anticipated given that Gram positive bacteria appear to be more susceptible to compounds interfering with the ion transport across the cell membrane (Nagaraja, 1995). In fact, most antimicrobial feed additives are substances active against Gram positive bacteria (Brander, 1982). The antimicrobial effects of BSF fat could provide an important added value when complete prepupae are used as a protein source in the feed of monogastrics.

From previous *in vitro* incubations conducted in our laboratory (Dierick et al., 2002b), it was deduced that a concentration causing at least 1 $\log_{10}$ CFU/mL reduction (i.e. equivalent to at least a 10 fold reduction in microbial numbers) could result in meaningful suppression of the gut bacteria under *in vivo* conditions and consequently, have impact on health and performance of piglets. This is due to the fact that, for young animals, a bacterial overload can be negative in terms of competition for nutrients, production of toxic metabolites, deconjugation of bile acids, increased inflammation and increased mucosal turnover (Anderson et al., 1999; Gaskins et al., 2002). Considering the *in vitro* results of the present study this would imply that, under *in vivo* conditions, a dose of at least 0.58 g C12:0/100 mL should be present in the proximal part of the small intestine to reduce lactobacilli and D-streptococci. However, from the concentration of 1.58 g C12:0/100 g feed in diet BSF8, only 0.21 g was left in the stomach while the upper small intestine (SI1) contained 0.03 g C12:0/100 g. Despite these seemingly inadequate concentrations, the bacterial counts of D-streptococci in the SI1 of piglets fed BSF8 showed a 0.5 log difference with those of the CON piglets, who received negligible amounts of C12:0. Moreover, even for piglets fed only 0.78 g C12/100 g feed (BSF4), this 0.5 log reduction compared to CON was observed. These findings suggest an effect of the prepupal fat on the microbial community in the gut of piglets. However, since the differences were not statistically significant, this observation need to be interpreted with caution and verified in further research. Nonetheless, the results suggest that when higher concentrations of the prepupal C12:0 reach the targeted sites, the antimicrobial activity could be enhanced. In the small intestine, the antimicrobial

effect of free MCFAs is restricted because of rapid absorption. Therefore, triacylglycerols of MCFAs may be suitable alternatives to free MCFAs (Dierick et al., 2002a), on the condition that there is sufficient lipolytic activity. If not, exogenous lipase could be added to the feed. From the *in vitro* study, the effect of lipase in the incubation medium on the bacterial counts indicates that a substantial amount of C12:0 is bonded in glycerides. However, the diets in our *in vivo* trial already contained a high amount of free C12:0 (34 – 46% of the total C12:0 content; Table 28). This could be due to endogenous lipase activity of the various feedstuffs applied in the diet preparation (which depends on treatment, cultivar, storage time and conditions, etc.). The release of C12:0 might be advantageous during storage because it could reduce the growth of microbial contaminants (Dierick and Decuypere, 2002). However, the additional lipolytic activity in the stomach of the piglets appears to be strong and comparable to the results obtained by Dierick et al. (2002b). Therefore, most of the C12:0 from the feed was absorbed too early (57 – 60% already in the stomach; Table 31) and could not reach the sites where it would find optimal conditions to inhibit bacterial growth. These targeted sites are situated in the proximal small intestine with pH conditions from 4.0 to 6.0, since the pKa of C12:0 is about 5.3 (Skrivanova et al., 2005). Under these conditions, most of the C12:0 will be undissociated, and consequently it can freely penetrate through the semipermeable peptidoglycan membrane of the Gram positive bacteria into the cytoplasm (Dierick et al., 2002a).

In addition to direct effects on intestinal microbiota, MCFAs could have several other positive effects on gut health. In the case of hypotrophic villi caused by malnutrition, MCFAs have been shown to improve the intestinal morphology and function, through their beneficial effects on crypt cell renewal (Galluser et al., 1993; Jenkins and Thompson, 1993). In this study, however, the histo-morphology of the small intestine did not indicate any differences between piglets subjected to the different diets. Since the histo-morphology of piglets reared on the conventional diet CON was already optimal, no further improvement was to be expected in the gut of the MCFA fed piglets. In addition to MCFAs in the fat, there might be other components in the insect biomass that could

suppress bacterial growth. It is known that insects produce a wide range of antimicrobial peptides (Otvos, 2000; Li et al., 2012). However, no difference in bacterial counts was observed between CON and DF-BSF, where defatted prepupae were an important protein source. Since antimicrobial peptides are mostly produced by insects as a reaction against invading pathogens, they were probably not present in the current batches. Moreover, even if they had been present, the instability of these antimicrobial peptides outside the insect hemolymph would have them being easily degraded in the intestinal lumen (Otvos, 2000).

Besides assessing the possible added value of BSF fat as an alternative antimicrobial agent, the current *in vivo* study focused on the performance of piglets reared on insect-containing diets. Despite the fact that there were no statistical differences between the ADG values of the different treatments, piglets reared on the full-fat insect diets (BSF4 and BSF8) gained slightly less weight compared to CON and DF-BSF reared piglets (respectively 18 and 9% less). In addition, the piglets that received the diet with the highest insect inclusion (BSF8) have consumed the least amount of feed (13% less than CON). This might be due to a reduced palatability because of the presence of a substantial amount of free MCFAs in the feed (Dierick et al., 2002a). Strategies for improving the palatability of diets could be including flavourings (EU Register of Feed Additives, category 2b) and using fresh palatable ingredients such as dairy products. According to Newton et al. (1977), a diet containing 33% of full-fat BSF prepupae was equally palatable to 6-week-old piglets (weaned at 4 weeks of age) as a soybean based diet. To our knowledge, the only study reporting the performance of pigs reared on BSF diets was conducted by Newton et al. (2005). In the latter study, dried BSF prepupae meal was fed to early weaned pigs as a substitute for dried plasma. During the first growing stage, the control diet contained 5% dried plasma and this was compared to diets containing increasing amounts of BSF (i.e. a diet with 2.5% BSF and 2.5% plasma and a diet with 5% BSF and 0% plasma). The diet with equal amounts of plasma and BSF resulted in a slightly better performance (+4% gain and +9% feed efficiency) compared to the control. In the present study only the first 15

days were assessed. The immediate post-weaning period is most critical, and generally significantly determines performances in following stages. As performance was not affected by BSF inclusion in our study, it can be suggested that adverse effects during later life are not to be expected.

In Newton et al. (1977), the apparent fecal digestibility of macronutrients (DM, crude protein and EE) for piglets reared on a 33% BSF diet is compared with conventionally (i.e. with soybean) reared piglets. In contrast to our study, the DM digestibility in Newton et al. (1977) was substantially lower for the BSF diet (78 *vs.* 85% for the conventional diet). The values for protein (76 *vs.* 77%, resp.) were almost the same and correspond to the values obtained in our study (78% for an 8% BSF diet and 77% for a 0% BSF diet). Contrarily, the apparent ileal digestibility, calculated in our study, was higher for the conventional diet compared to the 8% BSF diet. However, the highest ileal protein digestibility values were recorded for the diets containing 4% full-fat (BSF4) and defatted (DF-BSF) prepupal meal. This indicates that providing a limited amount of BSF protein might have a positive effect on the protein digestibility of the diet. However, this effect became negative when a higher amount of BSF protein was provided. The ether extract digestibility assessed by Newton et al. (1977) was much higher for the BSF diet (84%) compared to the control (73%). Since the BSF diet in the latter study contained 33% of full-fat prepupae, the main part of the fat consisted of lauric acid. This MCFA is more easily absorbed than the long chain fatty acids present in soybean (Dierick et al., 2002a). In our experiment, the maximum lauric acid inclusion (i.e. 30% of the total fat) was present in the 8% BSF diet. However, no substantial differences were observed in ether extract digestibility between the different treatments. Concerning the digestibility, it should be stated that the data originated from pooled samples and, therefore, only indications could be provided since no statistical tests could be applied.

So far, most studies evaluating BSF as a feed ingredient were performed in poultry and fish (St-Hilaire et al., 2007; De Marco et al., 2015; Borgogno et al., 2016; Cullere et al., 2016). Our trial with piglets

showed that piglet feed may contain a considerable amount of either full-fat or defatted BSF prepupae without causing adverse effects on performance. However, more research is warranted in order to draw reliable conclusions. The piglet feed in the present study contained up to 8% BSF, replacing toasted soybeans by 100% compared to a control diet. However, the current prices for soybean products are much lower than those of BSF products (ABN AMRO, 2016). As the results from our *in vitro* study indicate that the prepupal fat has potential as an antimicrobial agent, future research should further focus on exploring the added value of whole BSF prepupae compared to conventional protein sources. An additional aspect that needs to be addressed is the optimization of the insect rearing systems. Further upscaling, mechanization and automatization would allow decreasing the production cost which may lead to lower market prices for these insect materials. This would ultimately improve the competitiveness and the economic perspective of insect meals as sustainable feedstuffs (ABN AMRO, 2016).

Chapter 7

General discussion, conclusions and future perspectives

7.1 Alternative feed ingredients

In this dissertation, BSF prepupae were investigated as a potential alternative feed ingredient for monogastric farm animals. The main reasons for the need for alternative feed ingredients in Europe are :

- the dependency on the import of protein resources (i.e. soybean products). This could make the livestock sector in the EU vulnerable to possible price volatility and trade distortions (van Krimpen et al., 2013).
- the increasing demand for feed resources in the world increases competition between Europe and emerging economies (e.g. China) and consequently feed prices may rise (Fefac, 2016).
- feed costs represent 60-70% of total animal production costs. Therefore, the profitability of the livestock sector is strongly affected by price changes of feed resources (van Huis., 2013).
- the negative environmental impact associated with soybean culture. There are indications that insect production would require less land and water than soybean cultivation and in the case of BSF, organic side streams could be processed, resulting in a reduced nutrient load for the environment (van Huis and Oonincx, 2017). However, comparing the environmental impact of industrial BSF production to soybean culture is currently not possible. To our knowledge, the insect producing sector is still in the upscaling phase and, therefore, reliable environmental impact studies could not be completed yet, despite efforts by several researchers (Oonincx, 2015; Roffeis et al., 2015; Smetana et al., 2016).

The compound feed production in the EU for 2016 was 50 million metric tons for pigs and 54 million metric tons for poultry. Even if only 1% of the piglet feed in Belgium and the Netherlands would consist of BSF, there would be a demand for 17,000 metric tons of insect material (Fefac, 2017). Given that the estimated world production of BSF in 2016 was only 14,000 metric tons, it is clear that

the production capacity needs to be enhanced substantially in order to become meaningful (ABN AMRO, 2016). In addition, soybean products could be further replaced in the future by a combination of BSF and other protein rich ingredients such as grain legumes (e.g. lupines and chick peas), leaf proteins (e.g. grass and sugar beet leaves) and aquatic organisms (e.g. microalgae, seaweed and duckweed). Enhancing the production of soybean on European soil could also be an option (van Krimpen et al., 2013). According to van Krimpen et al. (2013), homegrown European soybean has the best potential to replace imported soybean in the long term, under the condition that the production capacity is enhanced to at least 5 metric tons/ha. Given that the maximum yield in the US currently is less than 4 metric tons/ha (Langemeier, 2016), this will be a significant challenge for European producers. In addition, given that most of the arable land in Europe is already occupied, the cultivation of soybean would have to compete with other crops (IndexMundi, 2017).

As previously stated, insects like BSF could be used to improve the valorization of organic side streams. Insects could be sinks for components like nitrogen and phosphorus which otherwise would be emitted to the environment. In addition, the insects use these side streams to build up their protein and fat rich body mass. As a result, added value is created twice, both in an ecological and economic way. From an ecological point of view, potentially harmful side streams are processed by the insects whereas the need for importing nutrients is reduced (Veldkamp et al., 2012; van Huis et al., 2013). Introducing exotic insect species in Europe for this purpose might, however, entail certain ecological risks (Chapter 3). Our study showed that in the case of BSF, establishment in temperate regions with cold winters is rather unlikely. In addition, established populations of the species in southern Europe displayed no invasiveness, despite being present for decades. As to the economics, the costs associated with side streams (e.g. transport, processing,…) are compensated by generating a valuable product. In addition, the need for overseas feedstuffs is reduced (Veldkamp et al., 2012; van Huis et al., 2013).

There are many types of side streams, some of which are more suitable than others to optimally rear insects in a safe way. In this chapter, potential substrates for large scale BSF rearing are explored and the optimal stage for harvesting is discussed. This information could be useful for the industrial production of BSF. Further, the state of the art and future perspectives concerning legislation and economics as related to the mass production of insects for feed are discussed. In addition to economics (i.e. price), the other important parameter for least cost compound feed formulation, being nutritional value of BSF prepupae, is discussed.

7.2 Choice of substrate

Throughout the research chapters of the thesis (Chapters 3, 4 and 5) different substrates have been tested for the rearing of BSF. As concluded in Chapter 5, a high non-fiber carbohydrate substrate gives the larvae the opportunity to develop fast and to synthesize the necessary body fat. The substrate that resulted in the largest growth and prepupal yield, disregarding the chicken feed control, was catering waste. However, our study indicates that a good substrate for BSF should not contain too much fat. In Chapters 3 and 4, there was no difference in the development time and the prepupal yield of larvae reared on catering waste versus those from the control. This batch of catering waste contained less fat than the one used in Chapter 5, where a substantially longer development and a lower prepupal yield were recorded compared to the control. This variable response of BSF to catering waste was also described by Barry (2004) who investigated different batches of this type of substrate. Consequently, for the purpose of large scale rearing of BSF using catering waste, it would be desirable that the provided batches of the rearing substrate are somewhat similar in terms of composition and structure in order to obtain a consistent larval production. Alternatively, quality control of the rearing substrate would allow to balance the substrate for the nutritional needs of the larvae by adding specific ingredients and nutrients. However, no clear data are available yet on the nutritional requirements of the growing larvae.

From Chapter 5, it could be deduced that on a vegetable waste substrate, containing less starch and fat and more fiber than the catering waste, a similar amount of prepupal biomass was obtained. The problem, however, is that the vegetable waste had a much higher water content than the other substrates (only 12% DM compared to 25% of the other substrates). This might have some practical implications for commercial rearing systems of BSF. It means that, despite the lower feed conversion ratio (kg feed/kg increase in body weight) expressed on DM basis for BSF reared on the vegetable waste compared to the drier catering waste (data not published), a considerable mass of water is provided by the fresh vegetable waste in the rearing facility. Therefore, the dimensions of these facilities should be very large in order to produce a sufficient amount of larvae. In Chapter 5, a reduction of only 9% of the wet vegetable waste was observed, indicating that a substantial amount of the substrate fed to the larvae was not consumed. It should be stated that the feeding regime in this experiment could be considered as excessive in order to guarantee ad libitum feeding. However, given that the insects are living in their food substrate, there always will be a need for offering more substrate than they can consume. In Diener et al. (2009) optimal feeding rates were determined for BSF reared on chicken feed. The optimal feeding rate corresponded to 60% of residual material. This material contained feces but also a substantial amount of unconsumed feed. This is confirmed by a release on the website of Co-Prot (www.co-prot.com), a BSF producing company that discontinued its activities in 2015:

"*First, to produce 1 ton of dry insect meal, one will need about 15-20 tons of fresh (wet) organic waste of high quality. So to produce a 40 foot container of insect meal, one will need 400 tons of fresh waste. That requires a huge logistical operation of waste collection sorting and transport. This practically means that primary operation of such a company is waste management, and secondary activity is protein production*."

Drying might be an option to remove part of the water, however, this will consume energy and the process might damage the structure of the material. Moisture content is not the only important

parameter for optimal growth of BSF larvae. The structure of the substrate is actually more crucial since the larvae prefer a semi-solid puree like substrate in which water and nutrients are easily accessible (own observations and personal communication of Dennis Oonincx). The larvae are not able to degrade fiber rich materials and have difficulties to process solid food, therefore, substrate preparations (e.g. cooking, mashing, grinding, …) might be necessary. In addition, the degree of decomposition of the rearing substrate plays an important role since BSF larvae are detritivores by nature (Chapter 5). The inability of BSF larvae to digest fiber has important consequences towards the suitability of the substrate. High fiber substrates like biogas digestate and dairy manure might be suitable for BSF development, but even at high feeding rates, the prepupal yields are poor. In addition, it is very unlikely that these substrates will ever be allowed to rear insects intended for the food chain (EFSA, 2015). From the findings concerning the chemical safety reported in Chapter 4, it is highly advisable that these substrates are screened thoroughly for possible contaminants (mainly heavy metals).

For the mass rearing of BSF, using highly nutritional substrates such as catering waste is advised in order to obtain adequate numbers of fertile flies which are successful in mating and producing viable offspring (Chapter 2). Prepupae reared on catering waste are also more cold tolerant than those reared on chicken feed (Chapter 3). This might be an interesting trait for the purpose of storing and transporting live prepupae at low temperatures. For the moment, catering waste is not allowed as a substrate for insects based on EU Regulation No. 1069/2009 (Chapter 1). However, the EU is taking action to tackle the problem of wasting food since around 88 million metric tons of food are wasted annually in the EU, with associated costs estimated at € 143 billion (EU, 2017). Allowing catering waste to be fed to insects, intended as a feed source for pigs and poultry, could be a part of the solution.

7.3 Optimal harvesting stage

In Chapters 5 and 6, BSF larvae harvested in their prepupal stage were used for the experiments. The reason for this was that these prepupae have little or no gut content and consequently, contamination with intestinal material was substantially lower. This has been described in the literature (May, 1961) and was confirmed by the differences in contamination level between fifth instar larvae and prepupae observed in the present study (Chapter 4). Moreover, from dissections performed during this research, it has been shown that the gut of BSF prepupae disintegrates already during the first 24 h in this stage (Figure 9). Consequently, no gut system could be found after this point in time (Figure 10). On the other hand, when fifth instar larvae were dissected, a very long gut system, folded multiple times from the head to the anus and with a straight length of about 5 times the larval body size, could be observed (Figure 11). When the gut is filled, its weight is about 25% of the total larval wet weight (own observations).

Figure 9: Gut system of a BSF prepupa, less than 24 h in this stage

Figure 10: Dissected BSF prepupa, longer than 24 h in this stage

Figure 11: Gut system of a fifth instar BSF larva

Commercial companies (Millibeter, Envirovlight, Protix,… personal communications) are not willing to wait for the prepupal stage and harvest the larvae in the fourth to fifth instar. Besides the shortening of the life cycle, the lower body weight of the prepupae is an important reason for this. However, from observations in our research, it can be deduced that the weight difference between larvae and prepupae is mainly due to the absence of gut content in the prepupae. In addition, prepupae have a DM content of about 5-10% higher than fifth instar larvae which could reduce potential drying costs. The difference in nutritional composition between larvae and prepupae is minimal (own observations). In our colony, prepupae were slightly higher in protein (38% *vs.* 36% on DM basis), lower in fat (33% *vs.* 35%) and had a higher DM content (33% *vs.* 40%) than fifth instar larvae. Prepupae contained 6% chitin while fifth instars contained 5% on DM basis.

Harvesting BSF fifth instars requires sieving from the substrate while the migration properties of prepupae could be used to develop a self-harvesting system (Newton et al., 2005; Alvarez, 2012). This system can easily be applied on a small laboratory scale (observations from our own colony and personal communication with Dennis Oonincx) since only a limited number has to be collected in order to maintain the colony or to perform experiments. However, in order to collect all prepupae to record the total yield in Chapter 5, sieving was necessary. In addition, the rate of development into prepupae can be quite variable (depending on substrate, larval density, temperature and other unknown factors) and thus unpredictable (own observations), making this stage less interesting to harvest in automated rearing systems. On the other hand, Newton et al. (2005) developed a pilot installation using self-harvesting where the migration of the prepupae is facilitated by ramps. In Europe, the largest producer of BSF is the German company Hermetia Gruppe (www.hermetia.de). Since this company is selling BSF in the prepupal stage rather than larvae, it is possible that the self-harvesting principle is applied here. However, due to confidentiality, this could not be verified.

In conclusion, it could be stated that waiting for the prepupal stage is only necessary when substrates are used which are likely to be contaminated and/or in systems where self-harvesting is optimized.

Contamination of larvae could also be prevented by allowing the larvae to empty their guts through fasting. This practice is generally applied in commercial mealworm rearing (van Huis et al., 2013).

7.4 Use of BSF in animal feeds

Whether or not BSF will ever become a common feed ingredient for monogastric farm animals which could be incorporated in least cost formulation will largely depend on two factors:

- quality (i.e. consistency of nutritional composition and value)
- economic viability of insect rearing systems which would lead to competitive prices

7.4.1 Quality

Nutritional quality (i.e. the ability to match the animal requirements) is the first parameter of importance when using a feedstuff in feed formulation. From Chapter 5, it is clear that in the case of BSF prepupae, the quality in terms of nutritional composition is dependent of the applied rearing substrate. Protein content and amino acid composition of prepupae were less affected by rearing substrate than fat and ash content and composition. The batch of prepupae used in the piglet feed in Chapter 6 contained more than 400 g fat/kg DM and less than 50 g ash/kg DM (Table 25) suggesting that these prepupae were reared on a diet comparable to the catering waste (Chapter 5) in terms of value for the larvae. From Tables 27 and 28 it could be observed that by raising the full-fat BSF prepupae content from 4 to 8%, the total fat content in the compound feed raised from 5.0 to 5.3%. Therefore, the 5.5% upper limit for fat in the formulation (Table 25) would be exceeded when more than 10% full-fat BSF would be incorporated. Omitting all soybean oil from the formulation could make it possible to incorporate more full-fat prepupae. However, given that BSF fat is very low in n-3 PUFAs this is not advised. Prepupae reared on digestate might be incorporated in the formulation at higher levels without exceeding the fat limit. However, the high calcium content of these prepupae, which might be interesting for laying hens, would restrict their use in piglet feed (Chapter 5).

The apparent ileal digestibility coefficients (AIDCs) of amino acids are important parameters for formulation. In our study, these values were not determined for BSF in piglet feed and, to our knowledge, so far, only Kortelainen et al. (2014) reported these values. In Table 34 an overview is provided of important AIDCs of BSF compared to soybean with emphasis on formulation for pigs and broilers.

Table 34: Apparant ileal digestibility coefficients (%) for amino acids applied in formulation software

	Pigs			Broilers	
	BSF[1] (19% fat, 63% protein)	Toasted full-fat soybeans[2] (20% fat, 38% protein	Soybean meal[2] (1.5% fat, 48% protein)	BSF[3] (18% fat, 55% protein)	Soybean meal[4] (1.5 % fat, 49% protein)
Cystine	43	70	79	44	-
Isoleucine	92	75	87	83	87
Lysine	79	79	87	80	91
Methionine	89	75	88	83	92
Threonine	79	71	80	73	82
Valine	92	73	83	90	86

[1]Kortelainen et al. (2014); [2]NRC (2012); [3]Schiavone et al. (2017) ; [4]Huang et al. (2007)

The AIDCs for lysine in BSF are, both for pigs (79%) and broilers (80%), substantially lower than the respective coefficients for soybean meal (87% and 90%) (Table 34). Moreover, the AIDCs for the sulfur-containing cystine are very low for BSF (43% for pigs and 44% for broilers). Concerning pigs, this could be compensated by the other sulfur-containing amino acid methionine of which BSF (89%) has an AIDC comparable to soybean meal (88%). However, from Table 26 it could be observed that the combined levels of methionine and cystine in BSF are insufficient given that the sum of the apparent ileal digestible (AID) methionine and the AID cystine divided by the AID lysine is beneath the 0.59 requirement for AID (methionine + cystine)/AID lysine. In contrast, for the other amino acids this ratio is well above the suggested requirements. Moreover, for valine and isoleucine (both 92%) the AIDCs are higher than those of soybean meal (83% and 87%, respectively).

For the 8% BSF containing diet in Chapter 6, toasted soybeans, a common ingredient in piglet feed, were for 100% replaced by full-fat BSF. Replacing toasted soybeans could be particularly interesting given that the AIDCs for most amino acids are lower than the AIDCs of BSF (Table 34). However, given that for both broilers (De Marco et al., 2015; Schiavone et al., 2017) and pigs (Kortelainen et al., 2014) the number of studies reporting the AIDCs for amino acids of BSF products is limited, more research is warranted. Moreover, the results obtained by De Marco et al. (2015) differed substantially from those by Schiavone et al. (2017), while two different BSF batches tested by Kortelainen et al. (2014) had different AIDCs. These findings suggest that the AIDCs for amino acids of BSF products could be dependent of certain factors such as composition and treatment (e.g. drying, fat extraction method,…).

7.4.2 Economics

For the moment the prices of BSF products (full-fat larvae/prepupae or defatted meal) are far too high in comparison to traditional protein sources for these products to be selected in compound feed formulations based on least-cost linear programming (Table 35). The only product of which the price is more or less comparable to BSF meal is soy protein concentrate. This product is made for specific animal feed products, mainly intended for young animals (chicks and piglets) and fish (Nordic Soya, 2017). Soy protein concentrate has a protein content comparable with defatted BSF meal (Table 35). However, both products are probably not entirely interchangeable because of differences in the composition and nutritional value. Defatted BSF meal still contains about 8% fat (Chapter 6) while soy protein concentrate contains only about 1.5% fat (Sodrugestvo, 2017). In addition, the protein digestibility of low fat BSF meal, reported by Schiavone et al. (2017) for broiler chicks, is lower than that of soy protein concentrate (Huang et al., 2007). BSF digestibility might be enhanced by extrusion which is performed for soy protein concentrate under pressure, moist conditions (20-30%) and high temperature (120-180 °C) (U.S. Soybean Export Council, 2008). Whether this technique could be useful for BSF needs investigation. A study conducted by Ottoboni et al. (2017) showed the feasibility

of extrusion of different wheat/BSF mixtures. However, the *in vitro* protein digestibility of these mixtures was not improved by the extrusion process. Furthermore, including the extrusion process would increase the prices of BSF meal even more.

Table 35: Prices of raw and processed protein sources for animal feed

Protein source	Price of full-fat dry product (€/kg)	Price of defatted meal (€/kg)	Protein content of defatted meal (%)	Reference
BSF larvae/prepupae	3.80	4.00	63	Hermetia Gruppe (June 2017)
Fishmeal	0.98-1.15	/	65	IndexMundi (January-June 2017)
Soybean	0.30-0.36	0.29-0.34	49	IndexMundi (January-June 2017)
Soy protein concentrate	/	3.67	62	ABN AMRO (December 2016)

As indicated in the discussion of Chapter 6, the weaner diets for piglets might be an interesting niche market for full-fat BSF products. The added value provided by the antimicrobial properties of the BSF fat could justify higher prices for BSF compared to soybean. However, from Table 35 it can be observed that full-fat BSF is over 10 times more expensive than soybean meal and, therefore, the added value of the BSF fat will be insufficient. In addition, the true value of the antimicrobial effects of BSF in the gastro-intestinal system of piglets could still not be fully assessed since the results of the *in vivo* study (chapter 6) were inconclusive.

Compared to soybean meal, fishmeal is currently about 3 times as expensive and thus, BSF might be more a substitute for fishmeal. In addition, both in the EU and North America, the aquaculture market has recently been opened for BSF products (Chapter 1). The aquaculture sector is

continuously growing and using increasing amounts of fishmeal. Given that fishmeal predominantly originates from unsustainable fishery activities, the need for alternatives is imminent (van Huis et al., 2013). In case of BSF, the potential for becoming an alternative feed ingredient for aquaculture systems has been demonstrated by multiple authors (Sealey et al., 2011; Kroeckel et al., 2012; Devic et al., 2017; Magalhães et al., 2017; Zhou et al., 2017)

A substantial increase in the production of BSF through upscaling, mechanization and automation will be necessary to reduce BSF prices. The latter two factors will reduce the currently high labor costs, which are mostly associated with harvesting (ABN AMRO, 2016). The substrate used for BSF rearing will also have an important influence on the price. The lower the economic value, and consequently the price of the substrate, the better; however, as discussed before, these substrates must be of sufficient nutritional quality in order to guarantee efficient BSF production. In the case of catering waste, the substrate considered the most interesting in our study, most of the material is now allocated to biogas fermentation, composting, incineration or ends up at landfills. These applications, however, are not preferred by most sectors since gate fees are raised for disposal whereas the generation of financial return is limited compared to valorization as a feedstuff (EU, 2017b).

According to Dortmans et al. (2017), the economic viability of BSF rearing will also depend on the potential revenue from waste processing (tipping fees), sales revenue from harvested whole larvae and products derived from larvae like protein meal and larval oil, and the market value of the waste residue as soil amendment or its potential use in a biogas plant. Given that the optimal rearing of BSF requires temperatures of at least 25 °C, year round production in areas with cold winters will come with high energy costs. To reduce these costs, heat generated by friction of dense larval populations might be applied (Alvarez, 2012).

7.4.3 Application in least cost formulation

In order to give an example of the application of BSF in least cost formulation (BESTMIX, adifo software; Maldegem, Belgium), the control diet and nutritional constraints given in Tables 27 and 28

(Chapter 6) were used as starting point. First, the control diet from Chapter 6 was optimized for minimum price using commodity prices for feedstuffs and feed ingredients of September 2017 (source Vanden Avenne, Izegem, Belgium) (Table 36). Initially, in Chapter 6 the amount of toasted soybeans was fixed at 12%, whereas the cereals barley, wheat and oat flakes were fixed at 25, 12 and 8%, respectively, in the diet. In the first optimization, the amount of toasted soybeans was not kept fixed because it is assumed that BSF sources will replace mainly soybean products. Hence, the optimized control diet in Table 36 contains less toasted soybeans while amounts of other ingredients that were not fixed also slightly changed. The control diet in Table 36 is thus the cheapest diet using the ingredients offered for formulation. Next, the software calculates the entry price of the BSF sources, in case it is offered as potential ingredient. It was estimated that the maximum price for full-fat BSF, with feeding value as in Chapter 6, in order to be incorporated in the feed (i.e. the entry price) is € 563.36/metric ton (Table 36). As soon as the price of full-fat BSF would drop beneath this level, about 5.2% of this ingredient would be incorporated in the feed. In addition, toasted soybeans would be omitted from the feed. In the case of defatted BSF, the entry price is € 476.22/metric ton and about 4% would be incorporated. It is clear that these entry prices are much lower than the BSF prices presented in Table 34. Therefore, as indicated in section 7.4.2, BSF products still have a long way to go to become competitive with conventional feedstuffs.

Table 36: Least cost formulation of piglet feeds with or without BSF products at the price levels of September 2017 (fixed crude protein levels of 17.2%)

	Price (€/metric ton)	Control	Including full-fat BSF	Including defatted BSF
Ingredients (%)				
BARLEY	154	25.000	25.000	25.000
CORN	186	20.064	21.972	22.259
WHEAT	163	12.000	12.000	12.000
SOYBEAN MEAL CP49	300	14.059	15.702	14.703
TOASTED SOYBEANS	398	8.392	0.000	4.528
FULL-FAT BSF		0.000	5.170	
DEFATTED BSF		0.000		3.985
OAT FLAKES	350	8.000	8.000	8.000
SWEET WHEYPOWDER	820	4.000	4.000	4.000
LACTOSE	850	1.539	1.539	1.539
SOYBEAN OIL	780	1.408	1.025	2.500
PREMIX TRACE MIN &VIT	1.500	1.000	1.000	1.000
SUGAR BEET PULP	167	1.000	1.000	1.000
MONOCALCIUM PHOSPHATE	1400	0.957	0.857	0.796
LIME	700	0.930	0.951	0.947
L-LYSINE HCl	1400	0.517	0.553	0.559
SALT	100	0.240	0.240	0.240
DL-METHIONINE	2500	0.228	0.243	0.244
L-THREONINE	1500	0.221	0.227	0.231
L-VALINE	6330	0.134	0.109	0.102
L-TRYPTOPHAN	8880	0.072	0.078	0.078
SODIUM BICARBONATE	500	0.241	0.370	0.366
Price Feed (€/metric ton)		324.97	324.97	324.97
Entry Price BSF (€/metric ton)			563.36	476.22

In the previous scenario (Table 36), the crude protein level of the diets was fixed at 17.2% which was also the case for all the diets applied in Chapter 6. In order to further optimize the price in the least cost formulation, protein levels could be allowed to fluctuate in the diet. Consequently, the more expensive synthetic amino acids would be less interesting since more essential amino acids could be delivered by cheaper crude protein sources (e.g. BSF products). In Table 37, the control diet from Table 36 is recalculated by least cost formulation by allowing the crude protein to fluctuate above a minimum value of 16%, and maintaining levels of the digestible essential amino acids lysine, methione+cystine, threonine, tryptophane, and valine. In this scenario, the total feed price decreases to € 317.44/metric ton, with a crude protein level of 18.64%. When allowing higher crude protein levels, the entry price for full-fat BSF increases to € 627.15/metric ton. This means that allowing higher crude protein levels is more favorable for BSF sources, though in this scenario the amount of full-fat BSF that would be incorporated is only 1.5%. In the case of defatted BSF, about 1% of BSF would be incorporated at an entry price of € 423.40/metric ton which is less favorable compared to the fixed protein scenario. It could be observed that in the second scenario, soybean meal is favored above other protein sources.

Table 37: Least cost formulation of piglet feeds with or without BSF products at the price levels of September 2017 (fluctuating crude protein levels)

	Control	Including full-fat BSF	Including defatted BSF
Ingredients (%)			
BARLEY	25.000	25.000	25.000
CORN	16.566	16.538	16.376
WHEAT	12.000	12.000	12.000
SOYBEAN MEAL CP49	22.149	23.613	21.366
TOASTED SOYBEANS	3.382	0.000	3.439
FULL-FAT BSF	0.000	1.515	
DEFATTED BSF	0.000		0.979
OAT FLAKES	8.000	8.000	8.000
SWEET WHEYPOWDER	4.000	4.000	4.000
LACTOSE	1.539	1.539	1.539
SOYBEAN OIL	2.500	2.500	2.500
PREMIX TRACE MIN &VIT	1.000	1.000	1.000
SUGAR BEET PULP	1.000	1.000	1.000
MONOCALCIUM PHOSPHATE	0.905	0.859	0.860
LIME	0.924	0.951	0.928
L-LYSINE HCl	0.517	0.357	0.365
SALT	0.240	0.240	0.240
DL-METHIONINE	0.228	0.175	0.180
L-THREONINE	0.152	0.144	0.149
L-VALINE	0.046	0.026	0.102
L-TRYPTOPHAN	0.049	0.047	0.032
SODIUM BICARBONATE	0.000	0.000	0.000
Protein (%)	18.64	18.87	18.84
Price Feed (€/metric ton)	317.44	317.44	317.44
Entry Price BSF (€/metric ton)		627.15	423.40

It should be stated that the above simulated scenarios provide only simplified examples of least cost formulation with BSF products. In addition, a lot of factors are uncertain such as the true nutritional value of BSF products which was partially based on literature and values for fishmeal and animal meal during our research (Table 26), and can be variable as shown in Chapter 5. Concerning the second scenario (Table 37), it should be noted that most nutritionists formulating weaner diets in Western Europe would recommend crude protein levels between 16.5 and 17.5% (Orffa, personal communication), and complying with animal requirements for digestible essential amino acids. Higher crude protein levels might influence microbial profiles in the gastrointestinal tract of weaned piglets, which might negatively affect the gut health (Heo et al., 2012). Analogue to minerals like calcium (Chapter 5), exceeding crude protein levels might increase gastric pH, allowing proliferation of deleterious microorganisms (Bolduan et al., 1988; Lawlor et al., 2005). In addition, as the digestive system of weaner piglets is not yet fully developed to sufficiently digest and absorb dietary proteins, feeding a high protein diet might cause protein maldigestion (Högberg and Lindberg, 2004). As a result, increasing amounts of undigested crude protein are present in the large intestine where microbial fermentation of this undigested dietary protein can provoke post-weaning diarrhoea by contributing to an increased production of indole, branched-chain fatty acids, ammonia, biogenic amines and phenols (Bolduan et al., 1988; Pluske et al., 2002).

7.4.4 Legislation

In Chapter 1, section 1.1.2.3, the legislative status of insects for animal feed in the EU was explained. In what follows, the current situation for BSF worldwide is described. In the EU, BSF is one of the species which are allowed as a feedstuff for aquaculture since the summer of 2017. In North America, the Canadian Food Inspection Agency approved whole dried BSF larvae as a feed ingredient for poultry broilers in 2016. In addition, feeding BSF to Salmonid fish is allowed both in the US (2016) and Canada (2017) (Enterra, 2017). However, in the US, the Association of American Feed Control Officers (AAFCO) approved dried BSF larvae under the condition that they were reared exclusively on

feed grade materials, which is comparable to the situation in the EU. In addition, the dried larvae must contain at least 34% crude protein and 32% fat on an as-fed basis (AAFCO, 2016). From a global perspective, the Codex Alimentarius, a United Nations list containing what is considered "food or feed", does not contain insects, except as impurities that contaminate food (FAO, 2017b). Consequently, insects are prevented to become a legally well-accepted feedstuff worldwide (Wang and Shelomi, 2017).

7.5 Conclusions

In the following, the hypotheses from Chapter 1 (section 1.2) are evaluated:

- H1: larger prepupae will develop into heavier flies with higher fecundity than their lighter counterparts.

  ➔ this was indeed the case, the heaviest females were especially more successful in mating
- H2: the (sub)tropical BSF is not able to survive northwestern European winters.

  ➔ it could be assumed that it is indeed unlikely for BSF to survive our winter conditions outdoors.
- H3: the cold tolerance of BSF will depend on developmental stage, rearing substrate and acclimation.

  ➔ all three parameters had a substantial influence on the cold tolerance of BSF.
- H4: cadmium and pesticides with high log(K_{ow}) value are most likely to accumulate.

  ➔ cadmium did accumulate, whereas this was not the case for any of the pesticides. However, pendimethalin and fenpropimorph have high log(K_{ow}) values and were indeed the most abundant active substances in the larvae.

- H5: lower concentrations of heavy metals and pesticides are present in the post-feeding prepupae compared to fifth instar larvae.

  ➔ this was the case for all chemicals tested, suggesting that most of them were situated in the gut.

- H6: the composition of the substrate will substantially affect the growth and composition of the resulting prepupae.

  ➔ this was indeed the case as expected because of the diverse nature of the substrates.

- H7: the effect of substrate on the composition of the prepupae will differ according to the nutrient considered.

  ➔ differences for minerals and fat and fatty acids were much more pronounced than those for protein and amino acids.

- H8: BSF fat will inhibit the growth of gram positive bacteria given their richness in lauric acid.

  ➔ BSF fat extracts did inhibit the growth of lactobacilli and, even more important, D-streptococci.

- H9: diets containing BSF fat will have an inhibitory effect on gram positive bacteria in the proximate small intestine of piglets.

  ➔ our results only provide indications which were not statistically significant.

- H10: piglets reared on diets containing BSF will show no differences in performance compared to the control.

  ➔ there were no substantial differences between the BSF diets and the control.

  Since there were no significant differences in gut health, consequently, no differences could be expected for performance given that all the diets were formulated iso-nutritionally.

The findings from this research, combined with all the valuable information generated by the high number of studies published during the past few years, confirm that BSF larvae/prepupae are indeed an interesting alternative feed ingredient for monogastric farm animals in terms of nutritional

composition and value. In addition, the number of publications about the feed safety aspects of BSF larvae reared on side streams is increasing. Consequently, under the impulse of IPIFF, the European legislative hurdles for industrial BSF production as a feedstuff are gradually being removed and are expected to be completely resolved by the early 2020's. However, the biggest challenge for the BSF producers will be to produce adequate volumes at reasonable cost in order to place their products on the market at prices which are competitive with conventional feed ingredients like soybean meal.

7.6 Future perspectives

The number of publications addressing the potential of BSF as an alternative feed ingredient is expanding rapidly. However, more research is still warranted:

- most publications focus on the nutritional value of BSF for aquaculture. This information is currently the most interesting given that the aquaculture market has recently opened up for BSF protein products. However, in order to support the permission to use BSF for terrestrial monogastrics, more research is warranted. In addition, the more data are provided, the faster the legislation might change. While the number of studies for poultry is growing, to our knowledge, only Newton et al. (1977), Kortelainen et al. (2014) and our research investigated the nutritional value for pigs.
- as previously mentioned, multiple companies are trying to upscale, mechanize and automatize their BSF production. Consequently, the research becomes more practice oriented and ins increasingly done at R&D departments of companies. However, independent researchers might still have a role to play in the future and contribute to the improvement of BSF rearing. For example, the complex behavior of the adult flies and possible triggers for the migratory behavior of prepupae are still understudied.
- in order to speed up the legal permission to place BSF protein products on the market for poultry and pigs, more studies about the possible cross contamination from the rearing

substrate is warranted. Possible contamination of BSF with heavy metals, pesticides and mycotoxins has been assessed and current insights suggest that only heavy metals (i.e. cadmium) might be a risk factor. However, more research is warranted for the latter two contaminants and according to EFSA (2015) other potential chemicals hazards are PCB's, dioxins, veterinary drugs and hormones. In addition, information is lacking about the potential transmission of pathogens (e.g. bacteria and viruses).

- given that large scale BSF rearing, to our knowledge, is currently still under development, accurate Life Cycle Assessments mapping the sustainability of the production process in comparison to traditional systems could not yet be performed. However, with data from developing companies researchers could simulate possible scenarios and subsequently provide feedback to these companies on how to develop more sustainable systems.

Summary

European livestock production highly depends on the import of protein resources, which puts this sector in a vulnerable position towards competing (emerging) economies. In addition, cultivation of crops allocated to livestock, like soybean, puts pressure on land availability and biodiversity, particularly in fragile tropical areas. Therefore, the need for alternative protein sources for livestock is high. Such an alternative protein source could be provided by insects. Species belonging to this very diverse class of arthropods can be sources of energy, protein and essential amino acids, fat and fatty acids and micronutrients (e.g. copper, iron, zinc). One of the species with a high potential for large-scale production is the black soldier fly (BSF) (*Hermetia illucens* L.), originating from the Americas. In the present dissertation, BSF was thoroughly investigated through assessing the rearing aspects, its potential as a feedstuff for monogastric farm animals and possible risks regarding feed safety and introduction of alien species in Europe.

Mating and reproduction of BSF adults are complicated aspects of BSF rearing which are currently understudied. The reproductive capacity can be expressed as the fecundity, which is the number of eggs oviposited per female, and can be influenced by various factors. Therefore, possible differences in fecundity between BSF females, originating from prepupae divided into 3 different classes based on their weight, were assessed. It was observed that 46% of the females belonging to the lightest class (i.e. females originating from the lightest class of prepupae which developed into the lightest females, this was also the case for the other weight classes) were able to produce hatching eggs. This number increased to 68% for intermediate sized females and reached 88% for the heaviest females. The higher number of hatched egg clusters deposited by the heaviest females indicated that these females mated more successfully than their lighter counterparts. Individual egg weights did not differ statistically, whereas the number of eggs per cluster was highest for intermediate females, but not statistically different from the heaviest females, and lowest for the lightest females. Moreover, less larvae hatched from egg clusters deposited by the lightest females compared to those from the other weight classes. These results suggest that fecundity might be positively correlated with female weight. In addition, a positive correlation existed between the weight of prepupae of the first

generation and those of the second generation. The findings of this experiment might have interesting implications towards the development of selective breeding programs for BSF. However, in order to draw reliable conclusions, multiple generations would need to be assessed.

Industrial production in regions where the BSF is not native, like northwestern Europe, could lead to permanent establishment, which might entail environmental risks. In temperate climates, establishment depends on the insect's ability to overwinter. Therefore, the insect's cold hardiness was assessed by determining the supercooling point (SCP), the temperature at which body liquids freeze, and lower lethal time at 5 °C ($LTime_{10,50,90}$), the time point at which 50% of the population is expected to die, for different life stages. As diet or acclimation can influence cold hardiness, prepupae reared on different substrates and acclimated prepupae were tested in separate experiments. The SCP ranged from -7.3 °C for late instar larvae to -13.7 °C for pupae. Prepupae reared on catering waste had a lower SCP compared to a control diet composed of chicken feed (-14.1 °C *vs.* -12.4 °C, respectively) whereas the SCP was unaffected by acclimation. Based on the LTime, prepupae and pupae were the most cold hardy life stages. Acclimated prepupae were most cold tolerant with an $LTime_{50}$ of 23 days. Based on an empirical relationship between $LTime_{50}$ and field survival of various arthropods, it was predicted that BSF prepupae would survive about 47 days in the field during northwestern European winters. The results from this laboratory study suggest that BSF is rather unlikely to overwinter in northwestern Europe. However, caution is warranted given that diet and acclimation can influence the insect's cold hardiness and the insect might survive in the field in a diapausing state or in protected hibernacula.

In 2015, EFSA published an opinion regarding feed safety aspects of the application of insects in feed. Possible risks associated with substrates used for BSF rearing could be either biological (e.g. pathogenic bacteria, viruses, fungi and parasites) or chemical (e.g. heavy metals, mycotoxins, pesticides, veterinary drugs, PCBs and dioxins). In our research, the presence of chemical contaminants (i.e. heavy metals and pesticides) in BSF larvae reared on contaminated substrates was

investigated. In the first part, bioaccumulation factors for BSF fifth instars and prepupae reared on biogas digestate were tested for arsenic (As), lead (Pb) and cadmium (Cd). In addition, for Cd a spiking of the substrate was conducted in order to test the potential bioaccumulation at different concentrations (i.e. 0.5 mg/kg, 2 mg/kg, 4 mg/kg and 6 mg/kg Cd). In the second part, the potential of pesticide contamination of BSF reared on chicken feed, biogas digestate and catering waste (all substrates were spiked) was assessed for 12 active substances (2,4-D, azoxystrobin, bentazone, clopyralid, cymoxanil, difenoconazole, fenpropimorph, linuron, metalaxyl, pendimethalin, pyraclostrobin and tebuconazole). These pesticides are commonly applied in fruit and vegetable cultivation and the applied dose was 5 mg/kg for each active substance. Both short-term (24h) and long-term (2 weeks) exposure were evaluated and differences between fifth instars and prepupae were assessed. Concerning heavy metals, significant bioaccumulation of Cd was observed in fifth instars, but not in prepupae, starting from a substrate concentration of 2 mg/kg. In contrast, Pb and As did not accumulate while none of the tested active substances from the pesticide experiment accumulated. In addition, none of the active substances detected in fifth instars and prepupae reached values above the MRL for feed ingredients. The fungicide fenpropimorph and the herbicide pendimethalin were the most abundant pesticides in BSF fifth instars across substrates. These active substances are both characterized by high log(K_{ow}) values (4.1 and 5.2, respectively) and a very low water solubility. Prepupae, on the other hand, contained substantially less of these pesticides than the corresponding fifth instars, indicating a high excretion rate for these pesticides. In addition, BSF fifth instars reared on different substrates displayed differences in the number and concentration of detected active substances. Fifth instars reared on biogas digestate contained the highest concentrations of pesticides. A possible explanation could be that, because these pesticides were mainly found in the gut, the proportion of gut content relative to the total body mass was higher in these larvae compared to the fatter larvae reared on substrates with a higher nutritional value. However, other substrate specific factors (e.g. pH, water binding capacity,…) might also play a role.

BSF larvae are able to convert a wide range of organic side streams into high quality biomass, which can be processed into animal feed. In our research, BSF larvae were grown on four different substrates: chicken feed, vegetable waste, biogas digestate, and catering waste. The fresh or moistened substrates were inoculated with 6-8 day old larvae and placed in incubators at 27 °C. At the end of larval development, prepupae were collected. Samples of prepupae and the tested substrates were freeze-dried and proximate, amino acid, fatty acid and mineral analyses were performed. Relatively small differences were observed in protein content (399 – 431 g/kg DM) and amino acid profiles of the prepupae across rearing substrates, whereas ether extract (EE) and ash contents differed substantially. Prepupae reared on digestate were low in EE and high in ash (218 and 197 g/kg DM respectively) compared to those reared on vegetable waste (371 and 96 g/kg DM respectively), chicken feed (336 and 100 g/kg DM respectively) and catering waste (386 and 27 g/kg DM respectively). The difference in nutrient composition between prepupae reared on catering waste and those reared on vegetable waste was substantially smaller than the difference between their respective substrates (159 *vs.* 92 g protein/kg DM, 139 *vs.* 21 g EE/kg DM and 41 *vs.* 336 g fiber/kg DM, for catering waste and vegetable waste substrates, respectively). The prepupal fatty acid profiles were characterised by high levels of lauric acid (C12:0) in all treatments. The prepupae reared on chicken feed, vegetable waste and catering waste contained 600 g C12:0/kg fatty acid methyl esters (FAME), whereas prepupae fed digestate contained 440 g C12:0/kg FAME. The differences in fatty acid composition can likely be explained by a higher synthesis of fatty acids in prepupae reared on energy dense substrates. In conclusion, protein content and quality were high and comparable for prepupae reared on different substrates, suggesting that BSF could be an interesting alternative protein source for monogastrics. However, differences in EE and ash content as a function of substrate have to be considered when incorporating BSF in animal feed.

Subsequently in the last research part, gut antimicrobial effects and nutritional value of BSF prepupae for weaned piglets were evaluated. Since prepupal fat has high amounts of the antimicrobial C12:0, the effects of BSF fat on the porcine gut microbiota were assessed *in vitro* by

simulating digestion in the upper small intestine of piglets. Different amounts of BSF fat were added to an incubation medium, which contained a synthetic diet, a phosphate buffer (pH 5) and a microbial inoculum from one donor piglet. The medium was incubated at 37 °C for 4 h. Using selective media, coliforms, D-streptococci, lactobacilli and total anaerobic bacteria were counted on aliquots taken at the end of the incubations. Next, weaned piglets were reared on diets including full-fat (4 and 8%) and defatted (5.4%, providing equal amounts of protein as 8% full-fat BSF) BSF prepupae and compared to a control diet (i.e. with soybean as a source of protein and fat). Besides the effects on gut microbiota, selected gut health parameters were investigated, performance was recorded and digestibility of the diets was calculated. *In vitro*, the prepupal fat at 0.58 g C12:0/100 mL suppressed the growth of lactobacilli, but the most substantial antibacterial effects were recorded against D-streptococci. At the highest inclusion level (equivalent to 0.87 g C12:0/100 mL), around 2 log reductions of D-streptococci were observed. From the animal trial, only 0.5 log reductions were observed for D-streptococci in the gut of piglets fed full-fat BSF containing diets. No differences were recorded for daily gain, feed intake and feed to gain ratio among treatments. The apparent fecal digestibility of the control feed did not differ significantly to that of the insect-containing feed (protein digestibility between 77 and 78% for all treatments). Whereas the ileal protein digestibility of the 8% full-fat BSF diet (67.4%) was slightly lower than that of the control (69.7%), the values for the 4% full-fat and the defatted BSF diets were higher (73.3%). In conclusion, our trial with piglets showed that a substantial amount of soybean products (meal and/or toasted beans) can be replaced by BSF without adverse effects on performance. However, given that the current price of BSF prepupae is substantially higher than that of soybean, future research should focus on exploring the potential added value of BSF compared to conventional protein sources.

Finally, a general discussion of the findings is presented and future perspectives are given. It is concluded that BSF larvae/prepupae reared on various side streams could be an interesting alternative feed ingredient for piglets in terms of nutritional composition and feeding value. However, the substrate used for BSF rearing could affect its biology (e.g. development into adult,

fecundity, cold tolerance,…), yield and composition, with implications for its nutritional value and/or feed safety. The biggest challenge for the BSF industry will be to sell its products at market prices which are competitive with conventional feed ingredients.

Samenvatting

De veehouderij in Europa is grotendeels afhankelijk van de invoer van eiwitrijke grondstoffen, wat de sector in een zwakke positie plaatst ten opzichte van (opkomende) concurrerende economieën. Bovendien bedreigt de teelt van gewassen ten behoeve van de veeteelt, zoals soja, de landbeschikbaarheid en de biodiversiteit, vooral in kwetsbare tropische gebieden. Daarom is er een hoge nood aan alternatieve eiwitbronnen zoals insecten. Soorten die behoren tot deze zeer diverse klasse der Arthropoden zouden bronnen van energie, eiwit en essentiële aminozuren, vet en vetzuren en micronutriënten (bv. koper, ijzer en zink) kunnen zijn. Eén van de veelbelovende soorten voor productie op grote schaal is de zwarte wapenvlieg (BSF) (*Hermetia illucens* L.), afkomstig uit Amerika. In deze dissertatie werd BSF grondig onderzocht op gebied van: kweekaspecten, het potentieel als grondstof voor voeders van monogastrische landbouwhuisdieren en de mogelijke risico's voor de voedselveiligheid en de introductie van exotische soorten.

Paring en ovipositie van BSF adulten zijn gecompliceerde kweekaspecten die momenteel nog te weinig bestudeerd zijn. De voortplantingscapaciteit kan uitgedrukt worden als de fecunditeit, welke het aantal eitjes afgelegd per wijfje is, en beïnvloed wordt door verschillende factoren. In deze studie werden mogelijke verschillen in fecunditeit tussen BSF wijfjes, afkomstig van prepoppen verdeeld over 3 verschillende klassen gebaseerd op hun gewicht, geëvalueerd. Uit de resultaten bleek dat 46% van de wijfjes afkomstig van de lichtste klasse van prepoppen (dewelke ook uitgroeiden tot de lichtste wijfjes, analoog voor de andere klassen) in staat waren om uitkomende ei-clusters te leggen. Dit aantal verhoogde tot 68% voor de intermediaire klasse en bereikte 88% voor de zwaarste klasse. Het hoger aantal uitgekomen ei-clusters bij de zwaarste klasse duidde erop dat deze wijfjes succesvoller waren bij de paring dan hun lichtere soortgenoten. De individuele ei-gewichten verschilden niet statistisch terwijl het aantal eitjes per cluster het hoogst was voor intermediaire wijfjes, doch niet statistisch verschillende van de zwaarste wijfjes, en het laagst voor de lichtste wijfjes. Bovendien kwamen er minder larven uit de ei-clusters gelegd door de lichtste wijfjes in vergelijking met de andere gewichtsklassen. Deze resultaten suggereren dat fecunditeit mogelijks positief gecorreleerd is met het gewicht van de wijfjes. Bovendien bestond er een positieve correlatie

tussen het gewicht van de prepoppen van de 1ste generatie en deze van de 2de generatie. De bevindingen uit dit experiment zouden interessante implicaties kunnen hebben naar de ontwikkeling van selectieve veredelingsprogramma's toe.

Industriële productie in de regio's waar BSF niet inheems is, zoals noordwest Europa, zou kunnen leiden tot permanente vestiging van deze exotisch soort, wat risico's voor het milieu zou kunnen inhouden. In gematigde klimaatzones hangt de al dan niet vestiging af van het vermogen van het insect om de winter te overleven. De koude-hardheid werd onderzocht door middel van de bepaling van het onderkoelingspunt (OKP), de temperatuur waarbij lichaamsvloeistoffen bevriezen, en de onderste letale tijd bij 5 °C ($LTijd_{10,50,90}$), het moment waarop 10, 50 of 90% van de populatie verwacht wordt te sterven, van verschillende levensstadia. Aangezien dieet en acclimatisatie invloed kunnen hebben op de koude-hardheid werden prepoppen gekweekt op verschillende substraten en geacclimatiseerde prepoppen getest in gescheiden experimenten. Het OKP varieerde van -7,3 °C voor larven behorende tot het 4de en 5de stadium tot -13,7 °C voor poppen. Prepoppen gekweekt op cateringafval hadden een lager OKP vergeleken met een controle dieet bestaande uit kippenvoer (-14,1 °C *vs*. -12,4 °C, respectievelijk) terwijl het OKP niet beïnvloed werd door acclimatisatie. Gebaseerd op de LTijd bleek dat prepoppen en poppen de meest koude-tolerante stadia waren. Geacclimatiseerde prepoppen waren het meeste koude-tolerant met een $LTiid_{50}$ van 23 dagen. Gebaseerd op een empirische relatie tussen de $LTijd_{50}$ en de overleving in het veld voor verschillende arthropoden werd voorspeld dat BSF prepoppen ongeveer 47 dagen zouden overleven in het veld tijdens noordwest Europese winters. De resultaten van deze laboratoriumstudie suggereren dat de kans klein is dat BSF ooit overwintert in noordwest Europa. Voorzichtigheid is echter geboden gegeven dat dieet en acclimatisatie een invloed kunnen hebben op de koude-hardheid en bovendien zou het insect kunnen overleven in een staat van diapause of in beschermde hibernacula.

In 2015 werd door EFSA een opinie gepubliceerd over de voedselveiligheidsaspecten van het gebruik van insecten in voeders. Mogelijke risico's geassocieerd met bepaalde substraten die gebruikt

worden voor de BSF kweek kunnen enerzijds biologisch zijn van aard (bv. pathogene bacteriën, virussen, schimmels en parasieten) of anderzijds chemisch (bv. zware metalen, mycotoxines, pesticiden, geneesmiddelen, PCB's en dioxines). In ons onderzoek werd de aanwezigheid van chemische contaminanten (zijnde zware metalen en pesticiden) in BSF larven gekweekt op gecontamineerd substraten onderzocht. In het eerste deel werden bioaccumulatiefactoren voor 5de stadium larven en prepoppen gekweekt op biogas digestaat getest voor arsenicum (As), lood (Pb) en cadmium (Cd). Voor Cd werd een spiking van het substraat uitgevoerd bij verschillende concentraties (0,5 mg/kg, 2 mg/kg, 4 mg/kg en 6 mg/kg Cd). In het 2de deel werd het potentieel van pesticidecontaminatie van BSF gekweekt op kippenvoer, biogas digestaat en cateringafval (telkens gespiked) met 12 actieve stoffen (2,4-D, azoxystrobin, bentazone, clopyralid, cymoxanil, difenoconazole, fenpropimorph, linuron, metalaxyl, pendimethalin, pyraclostrobin and tebuconazole) getest. Deze pesticiden worden algemeen toegepast in fruit- en groenteteelt en de toegepaste dosis was 5 mg/kg voor elke actieve stof. Blootstelling zowel op korte (24h) als op lange (2 weken) termijn werden geëvalueerd en verschillen tussen 5de stadium larven en prepoppen werden onderzocht. Betreffende de zware metalen werd er significante bioaccumulatie van Cd geobserveerd in 5de stadium larven, maar niet in prepoppen, vanaf een substraatconcentratie van 2 mg/kg. Pb en As accumuleerden daarentegen niet terwijl ook geen enkele actieve stof van het pesticiden experiment accumuleerde. Bovendien werd voor geen enkele actieve stof, gedetecteerd in 5de stadium larven en prepoppen, de MRL voor voederingrediënten overschreden. Het fungicide fenpropimorph en de herbicide pendimethalin werden het meest teruggevonden in 5de stadium larven over de verschillende substraten heen. Deze actieve stoffen worden beide gekarakteriseerd door hoge log(K_{ow}) waarden (respectievelijk 4,1 en 5,2) en zeer lage wateroplosbaarheid. Prepoppen bevatten daarentegen aanzienlijk minder van deze pesticiden ten opzichte van de corresponderende 5de stadium larven wat wijst op een hoge graad van excretie voor deze pesticiden. Er werden ook verschillen waargenomen voor het aantal gedetecteerde pesticiden en hun concentratie tussen 5de stadium larven gekweekt op de verschillende substraten. 5de stadium larven gekweekt op biogas

digestaat bevatten de hoogste concentraties aan actieve stoffen. Een mogelijke verklaring hiervoor zou kunnen zijn dat, aangezien deze pesticiden voornamelijk in de darm werden teruggevonden, de proportie aan darminhoud relatief ten opzichte van de totale lichaamsmassa hoger was in deze larven in vergelijking met de vettere larven gekweekt op substraten met een hogere voedingswaarde. Andere substraat specifieke factoren (zoals pH, waterbindend vermogen,…) zouden hierin echter ook een rol kunnen spelen.

BSF larven zijn in staat om een brede waaier aan organische zijstromen om te zetten in biomassa van hoge kwaliteit dewelke kan worden aangewend in diervoeder. In ons onderzoek werden BSF larven gekweekt op 4 verschillende substraten: kippenvoer, groenteafval, biogas digestaat en cateringafval. De verse of bevochtigde substraten werden geïnoculeerd met 6-8 dagen oude larven en geplaatst in incubators bij 27 °C. Aan het einde van de larvale ontwikkeling werden prepoppen verzameld. Stalen van deze prepoppen en de geteste substraten werden gevriesdroogd en weende, aminozuur-, vetzuur- en mineralenanalyses werden uitgevoerd. Relatief kleine verschillen werden geobserveerd in eiwitgehalte (399 – 431 g/kg droge stof (DS)) en aminozuurprofielen van prepoppen gekweekt op de verschillende substraten terwijl het ether extract (EE) en as gehalten duidelijk verschilden. Prepoppen gekweekt op digestaat waren laag in EE en hoog in as (respectievelijk 218 en 197 g/kg DS) vergeleken met deze gekweekt op groenteafval (respectievelijk 371 en 96 g/kg DS), kippenvoer (respectievelijk 336 en 100 g/kg DS) en cateringafval (respectievelijk 386 en 27 g/kg DS). Het verschil in nutriëntensamenstelling tussen prepoppen gekweekt op cateringafval en deze gekweekt op groenteafval was merkelijk kleiner dan de verschillen tussen de respectievelijke substraten (159 *vs.* 92 g eiwit/kg DS, 139 *vs.* 21 g EE/kg DS en 41 *vs.* 336 g vezel/kg DS, respectievelijk voor cateringafval- en groenteafvalsubstraten). De vetzuurprofielen van de prepoppen werden gekarakteriseerd door hoge hoeveelheden aan laurinezuur (C12:0) in alle behandelingen. De prepoppen gekweekt op kippenvoer, groenteafval en cateringafval bevatten ongeveer 600 g C12:0/kg vetzuurmethylesters terwijl deze gekweekt op digestaat 440 g C12:0/kg vetzuurmethylesters bevatten. De verschillen in vetzuursamenstelling zouden kunnen verklaard worden door een hogere mate van vetzuursynthese

in de prepoppen gekweekt op energiedichte substraten. Er kan geconcludeerd worden dat eiwitgehalte en –kwaliteit vergelijkbaar waren voor prepoppen gekweekt op verschillende substraten wat suggereert dat BSF een interessante alternatieve eiwitbron zou kunnen zijn. Hoe dan ook, voor de incorporatie van BSF in diervoeders dient rekening gehouden te worden met mogelijke verschillen in EE en as in functie van het substraat.

Vervolgens werden in het laatste deel van dit onderzoek antimicrobiële effecten in het gastro-intestinaal stelsel en de voedingswaarde van BSF prepoppen voor gespeende biggen geëvalueerd. Aangezien het vet van de prepoppen rijk is aan het antimicrobiële C12:0, werden de effecten van BSF vet op microbiota in het gastro-intestinaal stelsel van biggen *in vitro* getest door middel van simulatie van de vertering in het eerste deel van de dunne darm. Verschillende hoeveelheden BSF vet werden toegevoegd aan een incubatiemedium bestaande uit een synthetisch dieet, een fosfaatbuffer (pH 5) en een microbieel inoculum afkomstig van één donorbig. Het medium werd geïncubeerd bij 37 °C gedurende 4 u. Gebruikmakende van selectieve media werden coliformen, D-streptococcen, lactobacillen en het totale aantal anaerobe bacteriën geteld op uitgeplate stalen genomen aan het einde van de incubaties. Vervolgens werden gespeende biggen gevoederd met diëten met volvette (4 en 8%) en ontvette (5,4%, waardoor evenveel eiwit werd aangebracht als bij 8% volvette) BSF prepoppen en vergeleken met een controledieet (i.e. met soja als eiwit- en vetbron). Naast de effecten op darmmicrobiota werden ook andere darmgezondheidsparameters onderzocht, werden de prestaties opgevolgd en de verteerbaarheid van de diëten berekend. Het prepopvet onderdrukte *in vitro*, bij een concentratie van 0,58 g C12:0/100 mL, de groei van lactobacillen, maar de grootste antimicrobiële effecten werden waargenomen tegen D-streptococcen. Bij de hoogste dosis (equivalent aan 0,87 g C12:0/100 mL) werden reducties van ongeveer 2 log eenheden geobserveerd. Bij de dierproef werden echter maximale reducties van 0,5 log eenheden geobserveerd voor D-streptococcen in het gastro-intestinaal stelsel van biggen gevoederd met volvette BSF. Er werden geen verschillen opgetekend tussen de behandelingen voor de dagelijkse gewichtsaanzet, voederinname en de verhouding van voederinname over gewichtsaanzet. De schijnbare fecale

verteerbaarheid van het controledieet verschilde niet significant van deze van de insecten bevattende diëten (eiwitverteerbaarheid tussen 77 en 78% voor alle behandelingen). Terwijl de ileale eiwitverteerbaarheid van het 8% volvette BSF dieet (67,4%) iets lager was dan deze van de controle (69,7%), waren de waarden voor het 4% volvette en het ontvette BSF dieet hoger (73,3%). Onze studie toonde aan dat een aanzienlijk deel van de sojaproducten (schroot en/of geroosterde bonen) vervangen zouden kunnen worden door BSF zonder negatieve effecten op de dierprestaties. Aangezien de huidige prijs voor BSF prepoppen aanzienlijk hoger is dan deze voor soja afgeleide producten, zal er in de toekomst nog onderzoek moeten gebeuren naar de toegevoegde waarde van BSF ten opzichte van conventionele eiwitbronnen.

Tenslotte werd een algemene discussie gepresenteerd over de bevindingen en werden toekomstperspectieven meegegeven. Het kan geconcludeerd worden dat BSF larven/prepoppen gekweekt op verschillende zijstromen een interessant alternatief ingrediënt voor biggen zouden kunnen zijn in termen van nutritionele samenstelling en voederwaarde. Men dient echter wel rekening te houden met het gegeven dat het gebruikte substraat voor BSF kweek invloed kan hebben op de biologie (vb. ontwikkeling tot adult, fecunditeit, koude-tolerantie,…), de opbrengst en de samenstelling met implicaties naar de voedingswaarde en/of de voedselveiligheid toe. De grootste uitdaging voor de BSF industrie zal zijn om hun producten te verkopen aan marktprijzen dewelke kunnen concurreren met conventionele voederingrediënten.

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Curriculum vitae

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Publications

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